The Effect of Cations on the Calcium Ion Coordination of Heparin

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Received January 4, 1984

The calcium complex formation equilibria of heparin were investigated by potentiometry, using a new calcium ion selective electrode. The measurements were performed in aqueous solutions adjusted to 0.3 constant ionic strength by lithium, sodium, potassium and magnesium chlorides, respectively. The data reflect the effect of the latter cations on the calcium ion coordination of heparin resulting not only in the change of the equilibrium constants, but also in that of the composition of the dominating species in the solution.

Introduction

Heparin belongs to the glycosaminoglycan class of biopolysaccharides. Because of its anti-coagulant and lypolysis stimulating effect it is an important pharmaceutical substance. This macromolecule is composed of repeating tetrasaccharide units, each consisting of two glucose amine and two glucuronic acid moieties. Its molecular weight can vary between 6,000 and 30,000. The product with a molecular weight of approximately 18,000 has the optimal biological activity. Due to its donor groups (see Fig. 1) it is suitable for the coordination of metal ions [2, 3]. Its binding of calcium ions [4] is specially important, because it affects the retarding action which heparin exerts on the rate of thrombin formation.

The interaction of calcium ion and heparin has been studied by several researchers [5–8]. Their results however have proved to be inconsistent with respect to both composition of the complex formed and its stability. Body *et al.* [7], *e.g.*, have demonstrated the binding of one calcium ion per tetrasaccharide unit, while Perlin *et al.* [5, 6] that of one calcium per disaccharide moiety. Most of their investigations were performed by NMR and CD spectroscopy. Potentiometric analysis to determine the composition and formation equilibrium constants of complexes in solution has not yet been used, because heparin poisons the commercial calcium ion selective electrodes.

J. Siemroth *et al.* [9] have produced a calcium ion selective electrode containing bis(4-octoxiphenyl)-

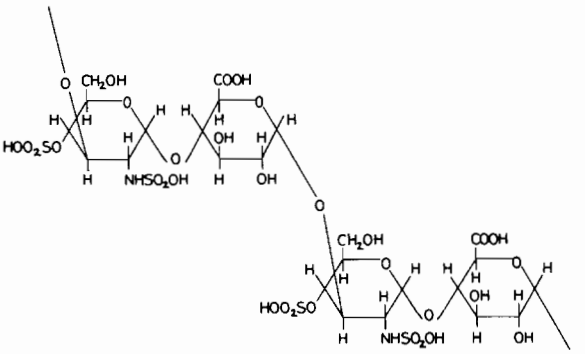

Fig. 1. The tetrasaccharide unit of heparin, consisting of two glucose amine and two glucuronic acid moieties.

phosphate as electroactive substance in a polyvinyl chloride carrier prepared with bis(n-octyl)-phenyl-phosphonate softener. Electrodes made of hydrophobic PVC membranes are not poisoned by biomolecules (peptides, carbohydrates, *etc.*) in solution. According to our preliminary experiments this electrode shows a nernstian function even in concentrated saccharose solutions within the calcium ion concentration range of 10^{-5}–10^{-1} mol dm^{-3}. With appropriate calibration it was found suitable for the measurement of calcium ion activity.

Using Siemroth's electrode, equilibrium analysis of the calcium ion coordination of heparin was performed in solutions adjusted to 0.3 ionic strength with lithium, sodium, potassium and magnesium chlorides, respectively. The parallel measurements in systems containing different base electrolytes were necessary, because – according to previous data – even alkali ions having the smallest affinity in complex formation processes can be coordinated by heparin. According to Dunston [10], *e.g.*, the cations Na^+, K^+, Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+} are bound by heparin with growing strength in the above order. Thus, it might be expected that the calcium ion coordination equilibrium would be influenced by the cations of salts used for keeping the ionic strength constant. The presence of expectably inert tetraalkyl ammonium salts, however, disturbed the nernstian function of the electrode.

0020-1693/84/$3.00

TABLE I. The Composition of Solutions Used in the Calcium Ion Coordination Studies.

Electrolyte present and its concentration, mol dm^{-3}		Total calcium ion concentration range, mol dm^{-3}	Total heparin concentration range, mol dm^{-3}
LiCl	0.3	$4.76 \cdot 10^{-3} - 5.45 \cdot 10^{-2}$	$2.01 \cdot 10^{-2} - 9.14 \cdot 10^{-3}$
NaCl	0.3	$6.98 \cdot 10^{-3} - 5.45 \cdot 10^{-2}$	$1.86 \cdot 10^{-2} - 9.08 \cdot 10^{-3}$
KCl	0.3	$6.10 \cdot 10^{-3} - 5.45 \cdot 10^{-2}$	$1.86 \cdot 10^{-2} - 9.02 \cdot 10^{-3}$
$MgCl_2$	0.1	$4.76 \cdot 10^{-3} - 5.45 \cdot 10^{-2}$	$1.90 \cdot 10^{-2} - 9.08 \cdot 10^{-3}$

In the following our results reflecting the effect of different cations on the calcium ion coordination of heparin are presented.

Experimental

The preparation of the calcium ion selective electrode was carried out according to [9], that of the zinc ion selective electrode according to [14]. The electrode function was checked by calibration in each of the four salt solutions. The E_o' values and the slope of the electrode function were also determined from the data of titrations of solutions of analogous composition, but not containing heparin, before and after the titration of the heparin solutions. The results of these two independent calibrations were identical within the limits of experimental error. The electrodes produced results of good reproducibility over several months. The reproducibility of the data of consecutive titrations has proved to be ±0.5 mV.

In the course of the equilibrium measurements the heparin solution adjusted to constant ionic strength (0.3) was titrated from a Radiometer ABU 12 precision automatic burette by calcium chloride and zinc nitrate standard solutions, respectively, of 0.1 and 0.01 mol dm^{-3} concentration. The total concentrations of the components in the investigated systems are shown in Table I. The electromotive force was measured by an Orion Research 701 A digital ionalyzer. A silver and silver chloride electrode inserted in a Wilhelm-bridge [11] served as reference. All solutions were thermostatted to 25.0 ± 0.1 °C during the measurements.

The computer evaluation of the equilibrium data was carried out according to [12]. The formation constant values thus obtained (Table II) were checked in such a way that by the use of them the original titration curves could be simulated. The deviation between the experimental and calculated data was characterized by the standard deviation which is included in Table II besides the equilibrium constants. It can be seen that the experimental data can be described by these equilibrium constants within the limits of experimental error.

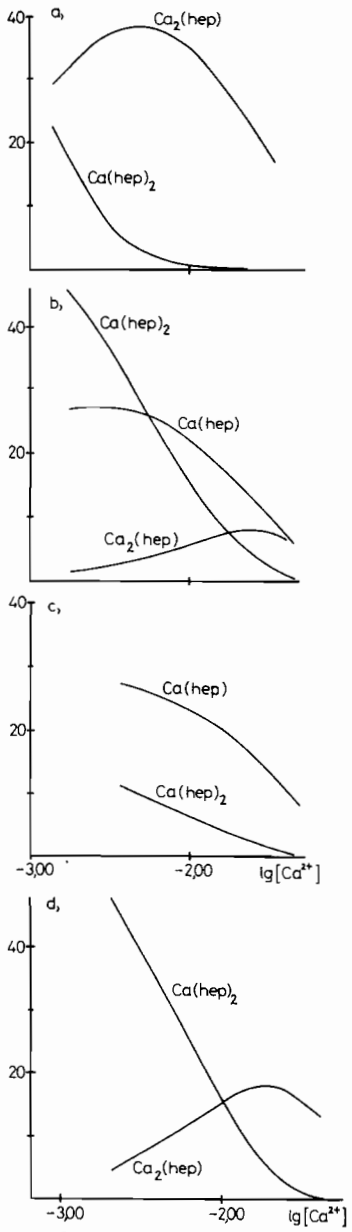

Fig. 2. Distribution curves of the calcium complexes of heparin formed in different electrolyte solutions: a) in 0.3 *M* lithium chloride, b) in 0.3 *M* sodium chloride, c) in 0.3 *M* potassium chloride, d) in 0.1 *M* magnesium chloride.

TABLE II. Logarithms of the Equilibrium Constants of the Calcium Complex Formation of Heparin (Characterizing the Binding of Calcium by the Tetrasaccharide Units of Heparin).

Electrolyte present and and its concentration mol dm^{-3}		log β_n values				Standard deviation, mV
		Ca(hep)*	Ca_2(hep)	Ca_3(hep)	$Ca(hep)_2$	
LiCl	0.3	–	4.90	–	3.79	0.69
NaCl	0.3	2.02	3.43	–	4.23	0.30
KCl	0.3	1.46	–	–	2.85	0.54
$MgCl_2$	0.1	–	3.60	–	3.75	0.40

*hep denotes a tetrasaccharide unit of heparin consisting of two glucose amine and two glucuronic acid moieties.

TABLE III. Logarithms of the Equilibrium Constants of the Zinc Ion Coordination of Heparin (Characterizing the Binding of Zinc Ions by the Tetrasaccharide Units of Heparin).

Ionic strength	log β_n values			Standard deviation, mV
	Zn_2(hep)	Zn_3(hep)	Zn_4(hep)	
0.3 *M* $NaNO_3$	5.82	–	12.07	0.67
0.3 *M* KNO_3	3.75	5.59	–	0.63

To assign the functional groups involved in the equilibrium process, the pH-dependence of calcium ion coordination was studied by Calvin's deprotonation measurements. For this purpose a Radiometer pHM 4 pH-meter and a Radiometer G 202B glass electrode were used. The reference electrode was the same as that applied in the calcium ion measurements.

These latter investigations showed that the calcium ion coordination of heparin in systems of pH $\geqslant$ 5 was a pH-independent process, indicating that carboxylate, aminosulphate and sulphate groups of heparin participated in the process.

Results and Discussion

Data summarized in Table II and the distribution curves calculated from them (Fig. 2) reveal that in the course of calcium ion coordination of heparin, complexes of at least two (in sodium chloride solution of three) different compositions are successively formed over the investigated concentration range. Depending on the cation of the electrolyte used for adjusting the ionic strength of the solution, one calcium ion (in the presence of potassium ions) and two (in the presence of lithium and magnesium ions) are coordinated to each tetrasaccharide unit of heparin. In the presence of sodium ions both species, containing one and two calcium ions, respectively, are formed in the solution. Due to a heparin excess, calcium-bridge formation linking two tetrasaccharide units is also revealed in each system. In the same electrolyte the calcium and heparin concentrations and their ratio determine the composition of the dominating species. Previous investigations [5–7] were performed by different methods in different concentrations. That is the reason why they have shown the presence of species of different composition. The data of Table II, derived from measurements in a broader concentration range, resolve the inconsistencies of the previous results.

The stability constants in Table II assigned to species of the same composition differ from each other by more than one order of magnitude in the different electrolyte solutions. The differences could be explained by the assumption that the cation of the base solution is also coordinated to the heparin molecule, thus, calcium ion coordination is actually a metal ion substitution process. This could be an explanation for the stability decrease caused by the presence of magnesium ions. Alkali cations, however, show a compatible influence and potassium is the cation whose stability-decreasing effect is the highest. Since the donor groups of heparin – the carboxylate and aminosulphate and sulphate groups – have a small affinity to alkali metal ions, one has to assume that the conformation of the macromolecule and steric factors determined by it make possible the relatively strong binding of the alkali cations, particularly that of the potassium ion. In this interaction, besides the mentioned carboxylate, aminosulphate and sulphate groups, carbohydrate-OH groups can also be involved. The different steric

requirements of the cations stabilizes the different shapes of the heparin molecule. The strong cation-dependence of the calcium ion coordination shown in Table II and Fig. 2 includes, apart from the effect of the metal ion substitutions, that of the conformation change. The change of shape and flexibility of the heparin molecule under the influence of cations has been referred to in previous investigations [13]. The data of Table II are in accordance with these investigations.

To make the above conclusions more evident the potentiometric analysis of the zinc ion coordination of heparin has been performed in the same way as that of the calcium ion coordination. The zinc ion activity was measured by a zinc ion selective electrode prepared according to the prescription of Fiedler–Linnersund and Bhatti [14], containing the zinc complex of di(2-ethylhexyl) phosphate as electroactive substance incorporated into the PVC carrier membrane. Because of the chlorocomplex-forming ability of zinc ions, the ionic strength of the solutions was adjusted by sodium nitrate and potassium nitrate, respectively. Stability constants obtained from the experimental data by computer evaluation are summarized in Table III.

It can be seen that the stability of the zinc complexes is higher by several orders of magnitude than that of the corresponding calcium complexes. In the zinc-containing system the maximum number of metal ions coordinated by one tetrasaccharide unit of heparin is also greater (four in sodium nitrate and three in potassium nitrate solution). The equilibrium constants definitely show that the presence of potassium ions strongly decreases the strength of the zinc–heparin interaction.

The potassium ion coordination of heparin could be characterized by the value $\lg \beta_{21} = 2.17$ calculated from the potassium dependence of the zinc complex stability constants assuming that two potassium ions are coordinated by one tetrasaccharide unit of heparin.

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