Biochem. J. (1986) 237, 281–284 (Printed in Great Britain)

Binding of zinc ions to heparin

Analysis by equilibrium dialysis suggests the occurrence of two, entropy-driven, processes

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Analysis by equilibrium dialysis of the binding of Zn^{2+} to heparin suggested that two interactions, one of high affinity and one of low affinity, occur. The stoichiometry of binding in both cases is about one Zn^{2+} ion bound per average heparin disaccharide unit. Both types of interaction appear to be entropy-driven.

INTRODUCTION

Many possible modulations by metal ions of heparin activities *in vivo* and *in vitro* have been discussed. Results of physico-chemical studies of heparin–cation complexation have frequently been interpreted in terms of simple electrostatic condensation of counterions along a linear, charged, polymer (e.g. Delville & Laszlo, 1983), but there are indications that additional interactions, resulting in more specific cation binding at particular sites, may exist (e.g. Liang & Chakrabarti, 1982). Zn^{2+} has been shown by gel-filtration-chromatography experiments to bind to heparin by mechanisms that have not been investigated; this interaction is of possible physiological significance (Sato & Gyorkey, 1976; Parrish & Fair, 1981). The present paper reports the use of equilibrium dialysis to investigate the stoichiometry and thermodynamics of heparin–Zn^{2+} interaction.

EXPERIMENTAL

Heparin

Heparin was a pharmaceutical-grade preparation derived from porcine intestinal mucosa (Glaxo Operations U.K., Runcorn, Cheshire, U.K.; batch 008). The preparation was treated with chondroitin ABC lyase (EC 4.2.2.4) (Edward *et al.*, 1980), extensively dialysed against

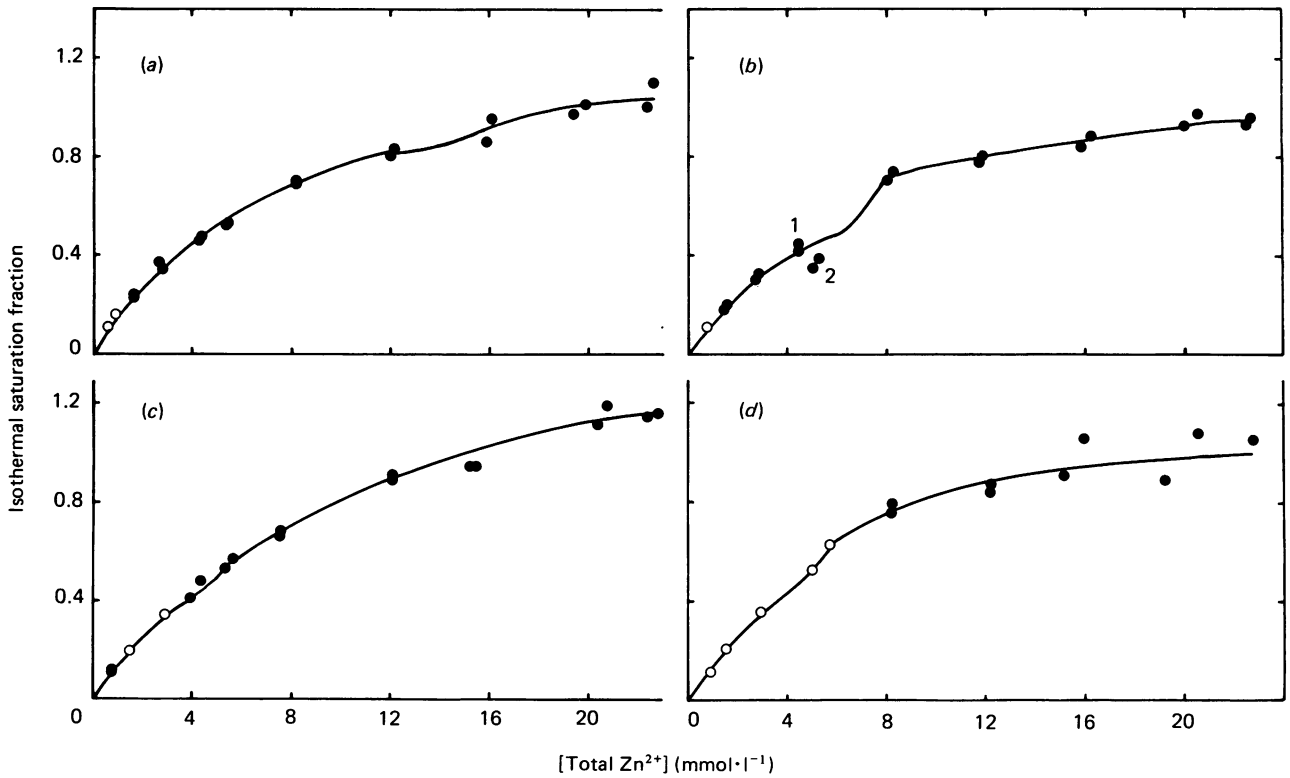

Fig. 1. Isothermal saturation fraction of binding sites on heparin occupied by Zn^{2+} as a function of [total Zn^{2+}]

A binding site was taken to be an average heparin disaccharide unit (see the Experimental section). Open circles indicate two coincident points. Dialysis temperatures were: (*a*) 30 °C, (*b*) 37 °C; (*c*) 45 °C; (*d*) 50 °C.

Abbreviations used: M_n, number-average relative molecular mass; ΔH, enthalpy change; ΔS, entropy change.

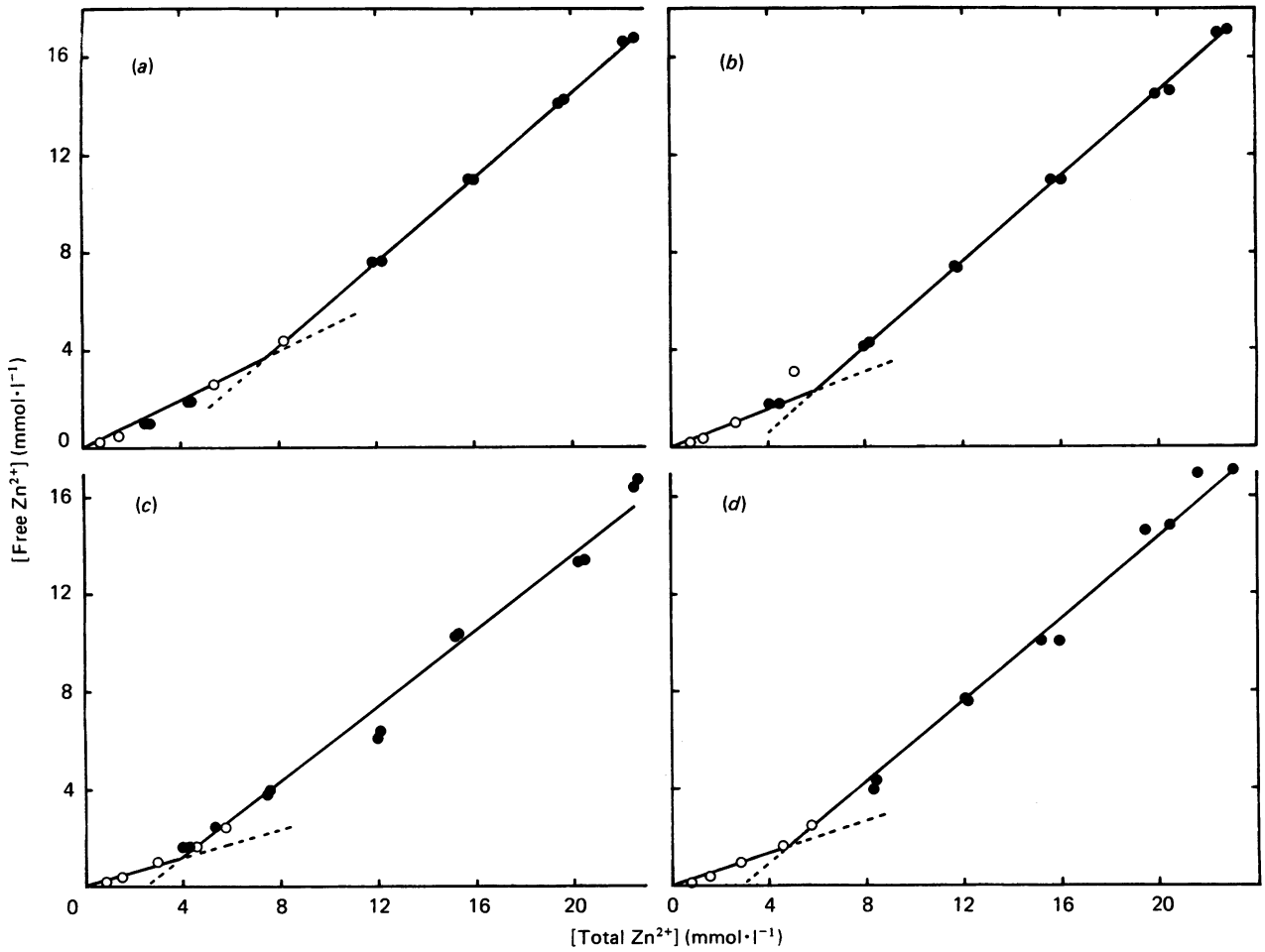

Fig. 2. [Unbound Zn^{2+}] as a function of [total Zn^{2+}]

Open circles indicate two coincident points. Dialysis temperatures were: (*a*) 30 °C; (*b*) 37 °C; (*c*) 45 °C; (*d*) 50 °C.

distilled water and converted into the Na^+ form on Amberlite IR-120 cation-exchange resin. Spark-source m.s. showed a greater-than-99% efficiency of the cation-exchange process. The heparin had a mean M_n of 1.7×10^4. Uronic acid, aminohexose, sulphaminohexose and sulphate contents were determined by the methods of Blumenkrantz & Asboe-Hansen (1973), Blumenkrantz & Asboe-Hansen (1977), Hurst & Settine (1981) and Terho & Hartiala (1971) respectively. Values (each in $\mu mol \cdot mg^{-1}$) were 1.7, 1.6, 1.5 and 4.3 respectively. A concentration of heparin in molar terms was estimated with respect to an average hexadecahydrated (Atkins *et al.* 1974) disaccharide of tetrasodium 2-*O*-sulphatoiduronosyl-*N*-glucosamine 6-*O*-sulphate.

Equilibrium dialysis

A series of solutions was prepared, in each of which the final Na^+-heparin concentration was 5.25 $mmol \cdot l^{-1}$, the final NaCl concentration was 0.15 $mol \cdot l^{-1}$ and the final ^{65}Zn sp. radioactivity was 0.65 $\mu Ci \cdot ml^{-1}$ (carrier-free $^{65}ZnCl_2$ was from Amersham International; original sp. radioactivity 200 Ci/g of Zn); final $ZnCl_2$ concentrations varied between 0.5 and 24 $mmol \cdot l^{-1}$. Portions (1 ml) of solutions were dialysed, at the temperatures stated in the Results and discussion section, in a Dianorm Macro D-2000 equilibrium-dialysis cell (MSE Scientific Instruments, Crawley, Sussex, U.K.; Visking dialysis membrane was used), against 1 ml of solutions identical except that they did not contain heparin. After 2 h, samples were taken from solutions on both sides of the dialysis membrane for measurement of radioactivity. Control experiments, carried out under the experimental conditions used, showed that Zn^{2+} did not bind to the dialysis membrane or cell in the presence or absence of heparin, that heparin did not cross the dialysis membrane and that equilibration of Zn^{2+} across the membrane was complete within 2 h.

RESULTS AND DISCUSSION

Fig. 1 shows the proportions of binding sites on heparin occupied by Zn^{2+} as a function of the total Zn^{2+} concentration present. For the purpose of the plot, a binding site was taken to be an average heparin disaccharide unit (see the Experimental section). The graphs suggest that Zn^{2+} binding occurs by a process that is complete when one Zn^{2+} ion is bound per heparin disaccharide unit. Possible discontinuities in the graphs at the higher concentrations of Zn^{2+} suggested that, at these concentrations, an additional process might be occurring. This possibility was explored by plotting the concentration of unbound Zn^{2+} as a function of the total

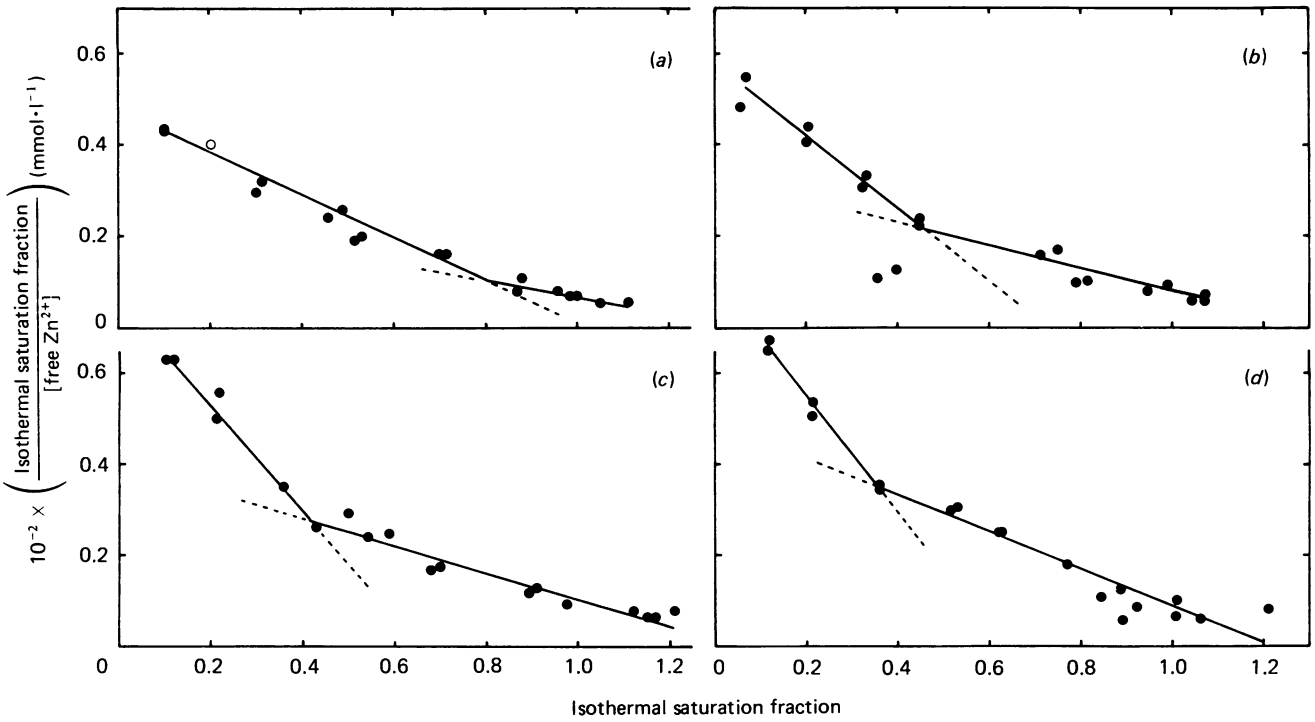

Fig. 3. Scatchard plots: isothermal saturation fraction/[free Zn^{2+}] ratio as a function of the isothermal saturation fraction

The open circle indicates two coincident points. Dialysis temperatures were: (*a*) 30 °C; (*b*) 37 °C; (*c*) 45 °C; (*d*) 50 °C.

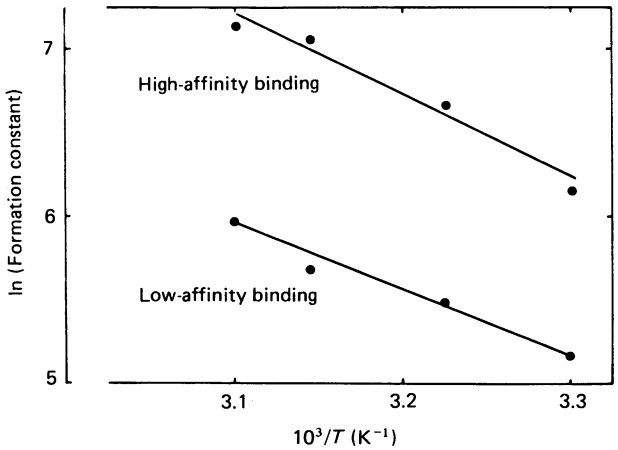

Fig. 4. Van't Hoff isochores [ln (formation constant) as a function of reciprocal temperature]

A stoichiometry of one Zn^{2+} ion bound per average heparin disaccharide unit was assumed from the data in Fig. 3.

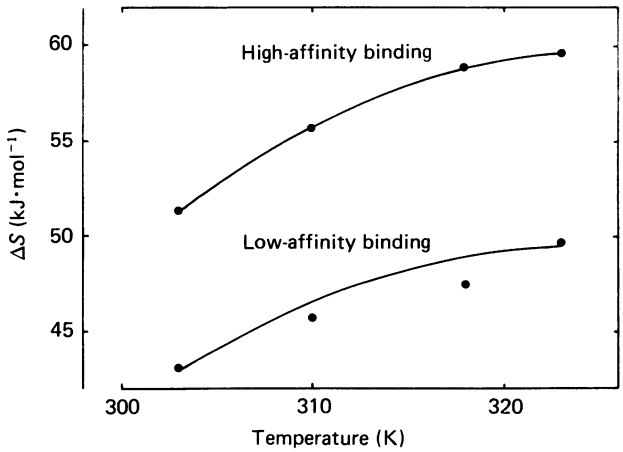

Fig. 5. ΔS as a function of temperature

ΔS values were calculated by using the expression:

$$\Delta S = (\boldsymbol{R} \cdot \ln K_f) + (\Delta H/T)$$

where $\boldsymbol{R}$ is the gas constant and K_f is the formation constant.

Zn^{2+} concentration present (Fig. 2). The distinct discontinuity in the otherwise linear graphs produced suggest that two types of Zn^{2+}–heparin interaction occur, at different total Zn^{2+} concentrations. Scatchard (1949) plots, shown in Fig. 3, permitted the formation constants of the two processes to be tentatively calculated. Formation constants (1·mol^{-1}) for the Zn^{2+}–heparin complex formed at low Zn^{2+} concentrations were: at 30 °C: 471; at 37 °C: 976; at 45 °C: 1172; at 50 °C: 1265. At higher Zn^{2+} concentrations, formation constants (1·mol^{-1}) were: at 30 °C: 175; at 37 °C: 241; at 45 °C: 293; at 50 °C: 393. The Scatchard plots did not allow the stoichiometry of Zn^{2+} binding to be unequivocally assessed, and, for the purpose of plotting Van't Hoff isochores (Fig. 4), a stoichiometry of one Zn^{2+} ion bound per average heparin unit was assumed for both processes. ΔH values for Zn^{2+} binding were estimated from the gradients of the isochores. For both processes, a ΔH value of +40 kJ·mol^{-1} was obtained. ΔS values were calculated and are shown in Fig. 5.

The molecular mechanisms underlying the apparently

entropy-driven interaction of Zn^{2+} with heparin are not clear. However, i.r. spectroscopy of cation–heparin complexes suggests that whereas alkali- and alkaline-earth-metal ions interact predominantly with the carboxylate groups of the polymer, Zn^{2+} and Cu^{2+}, by binding to sulphate half-ester groups on heparin, alter the environment of water molecules associated with the polymer and change the polymer conformation (D. Grant, W. F. Long & F. B. Williamson, unpublished work). Such perturbations, involving the dehydration of the sulphate ester groups, could be responsible for the increase in entropy suggested by these experiments and might, *in vivo*, be concerned with specific modulations of the activities of heparins and heparans.

We thank the Medical Research Council, the Nuffield Foundation and the Cancer Research Campaign for support. N.E.W. held a Science and Engineering Research Council Research Studentship.

Received 10 March 1986/17 April 1986; accepted 22 April 1986

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