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RELATIONSHIP BETWEEN EQUILIBRIUM FORMS OF LYSOZYME
CRYSTALS AND PRECIPITANT ANIONS

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Description

Molecular forces, such as electrostatic, hydrophobic, van der Waals and steric forces, are known to be important in determining protein interactions. These forces are affected by the solution conditions and changing the pH, temperature or the ionic strength of the solution can sharply affect protein interactions. Several investigations of protein crystallization have shown that this process is also strongly dependent on solution conditions. As the ionic strength of the solution is increased, the initially soluble protein may either crystallize or form an amorphous precipitate at high ionic strengths [1]. Studies done on the model protein hen egg white lysozyme have shown that different crystal forms can be easily and reproducibly obtained, depending primarily on the anion used to desolubilize the protein [2-4].

More recent systematic investigations of the effect of various salt ions on the solubility of lysozyme also showed that the more chaotropic anions were more effective in crystallizing lysozyme [5-7]. Thus, the more chaotropic thiocyanate ion was shown to be far more effective in crystallizing lysozyme than the less chaotropic chloride ion. There also seems to be a correlation between the crystal form obtained and the relative chaotropicity of the solution resulting from the anion used for lysozyme crystallization. The less chaotropic anions, such chloride, bromide, sulfate, phosphate and acetate produced tetragonal crystals. The more chaotropic iodide, thiocyanate and organic anions produced monoclinic crystals [3,5]. Alcohols, which are also known to be chaotropic agents, can crystallize proteins with methyl pentane diol being a widely used crystallizing agent [8]. Propanol produces monoclinic crystals of lysozyme [9]. These results suggest that decreasing the hydrophobic force with chaotropic agents promotes protein crystallization and may also contribute to the appearance of multiple crystal forms for a protein.

Although these and other results have shown the effect of electrostatic and hydrophobic forces in protein crystallization, attempts at explaining their role in the process have been less successful. The traditional explanation used for the desolubilization of proteins is the salting-out effect [10]. In this theory the solvent ions are assumed to strip water molecules from the hydration layer surrounding the protein, with protein aggregation and desolubilization following due to hydrophobic attraction. The effectiveness of ions for this process is supposed to follow the Hofmeister series, with the less chaotropic ions being more effective in desolubilizing the protein. Colloidal aggregation has also been suggested as the mechanism for protein crystallization [11-13]. Riès-Kautt and Ducruix [6] proposed an "ion-pairing" argument, whereby counterion binding was assumed to neutralize the charged protein molecule after which aggregation leading to crystallization may take place due to the hydrophobic interactions. Thus, all proposed explanations to date have been based on hydrophobicity-driven mechanisms. However, as discussed above,

lysozyme crystallization follows the *reverse* of the Hofmeister series, with *decreases* in the hydrophobic attraction by chaotropic agents *promoting* crystallization. Consequently, it seems unlikely that crystallization proceeds by hydrophobicity-driven mechanisms.

Another difficulty is in quantifying the effect of anions on the solution. So far chaotropicity of anions has only been measured indirectly, by their effect on the desolubilization of proteins (Melander & Horvath). However, in recent years fluorescence spectroscopy has emerged as a sensitive technique for monitoring such molecular processes. Several fluorescent probes that are sensitive to the hydrophobicity/hydrophilicity of aqueous environments are now available. One such probe pyranine (8-hydroxy-1,3,6-pyrenetrisulfonate) has been successfully employed to follow the structure of water as the concentration of sucrose is increased from zero to the saturation point and beyond [14,15]. The increasing sucrose concentration was shown to progressively disrupt the bulk water networks in this manner.

In this study we employ pyranine to probe the effect of various anions on the water structure. Additionally, lysozyme crystallization was carried out at these conditions and the crystal form was determined by X-ray crystallography. The goal of the study was to understand the physico-chemical basis for the effect of changing the anion concentration on the equilibrium form of lysozyme crystals. It will also verify the hypothesis that the anions, by altering the bulk water structure in the crystallizing solutions, alter the surface energy of the between the crystal faces and the solution and, consequently, the equilibrium form of the crystals [16].

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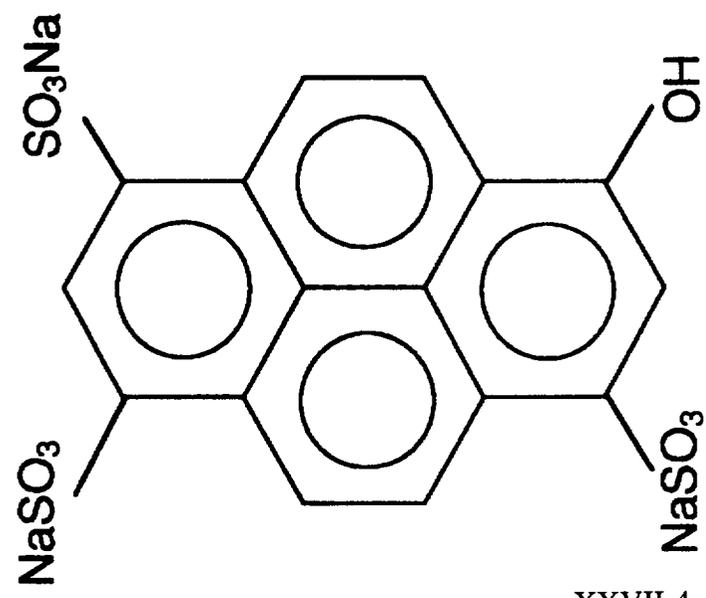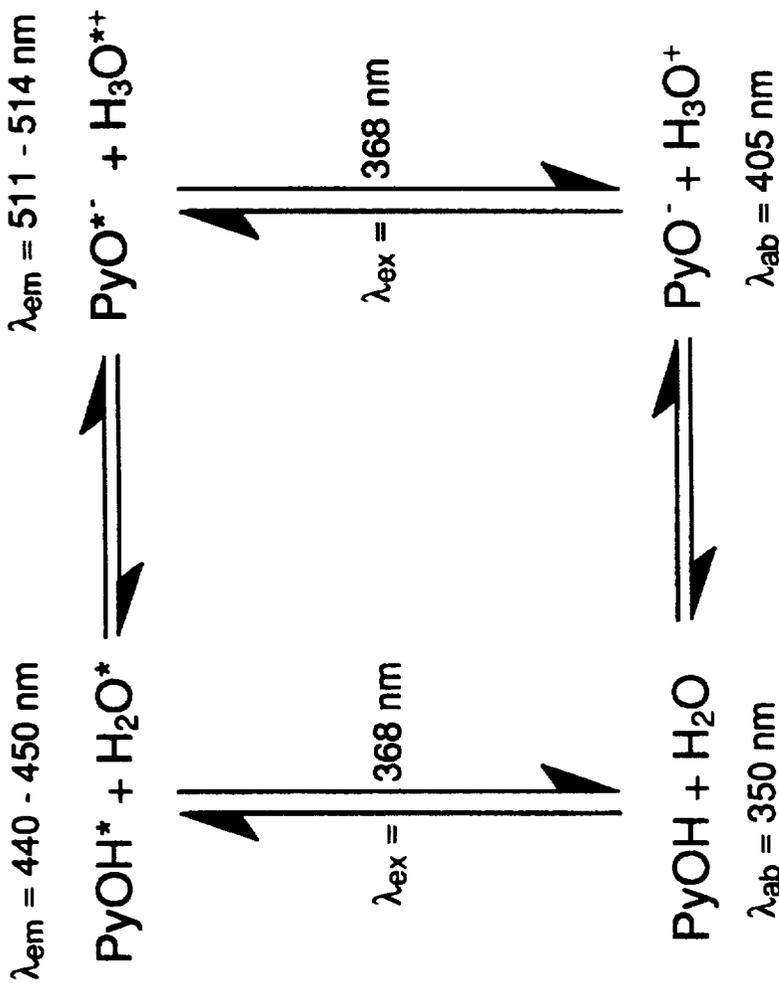[16] M. Kern, in *Morphology of Crystals* (Ed. I. Sunagawa), Terra, Tokyo (1987).

$\lambda_{em} = 511 - 514$ nm

PyO*· + H$_3$O*+

$\lambda_{ex} = 368$ nm

PyO$^-$ + H$_3$O$^+$

$\lambda_{ab} = 405$ nm

$\lambda_{em} = 440 - 450$ nm

PyOH* + H$_2$O*

$\lambda_{ex} = 368$ nm

PyOH + H$_2$O

$\lambda_{ab} = 350$ nm

SO$_3$Na

NaSO$_3$

OH

NaSO$_3$

XXVII-4

Fig. 1: The structure of Pyranine (8-hydroxy-1,3,6-pyrenetrisulfonate). The hydroxyl group is sensitive to the environment and ionizes only in the presence of bulk water networks that can exchange protons with it. The ionization of this group changes the emission wavelength of the pyrene molecule from 440 nm to 511 nm, and this is a sensitive indicator of the aqueous environment. Excessive amounts of additiives to the water causes the prevalence on non-networked water and the pyranine to emit at 440 nm. In netwoked bulk water the emission is at 511 nm.
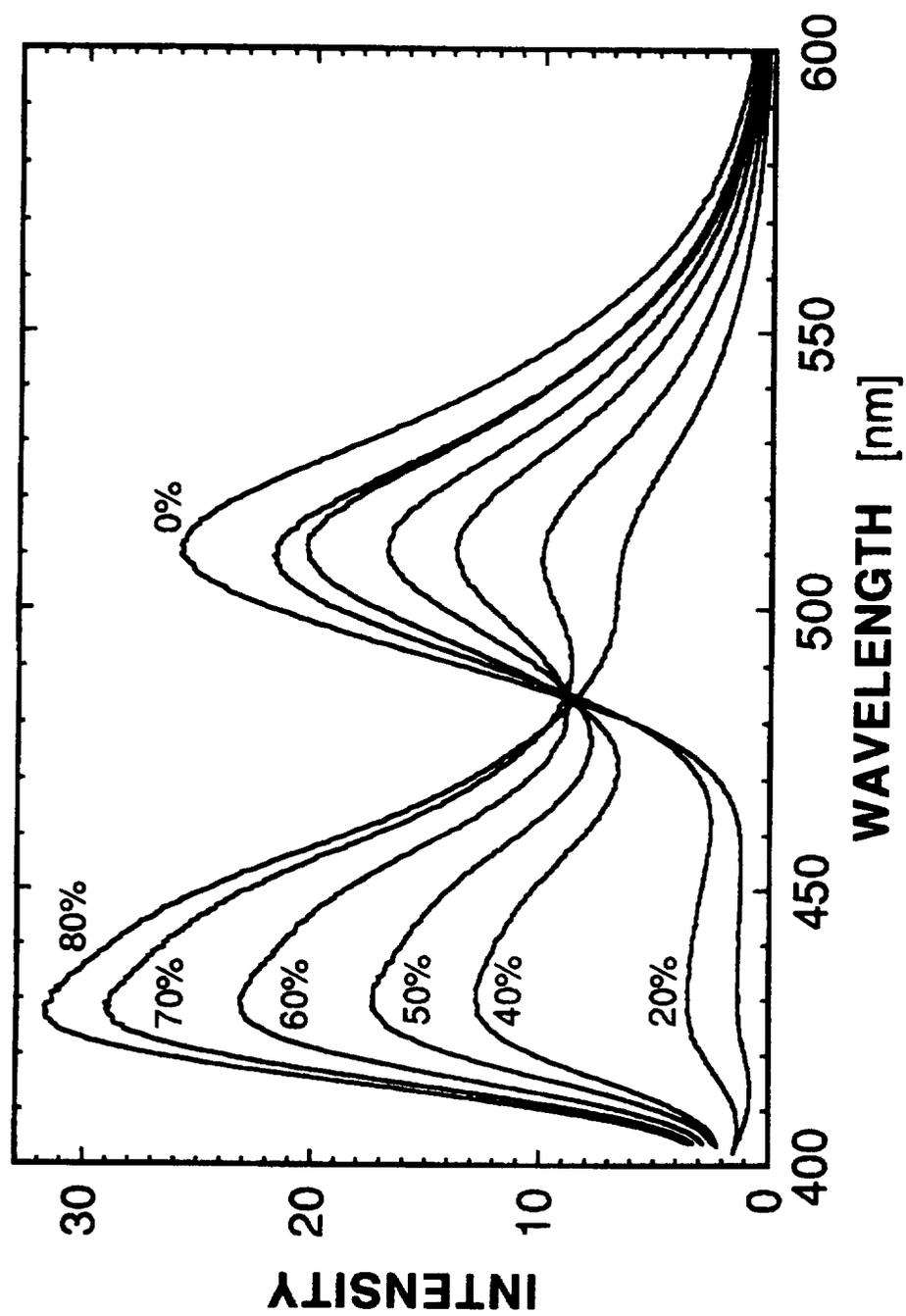
Fig. 2: Fluorescence intensities at various weight percentages of iso-propanol in water. In pure water the pyranine emits almost exclusively at 511 nm. With increasing propanol concentrations the amount of non-networked water and alcohol increases. The amount of pyranine in this phase also increases, resulting in more emission at 440 nm.
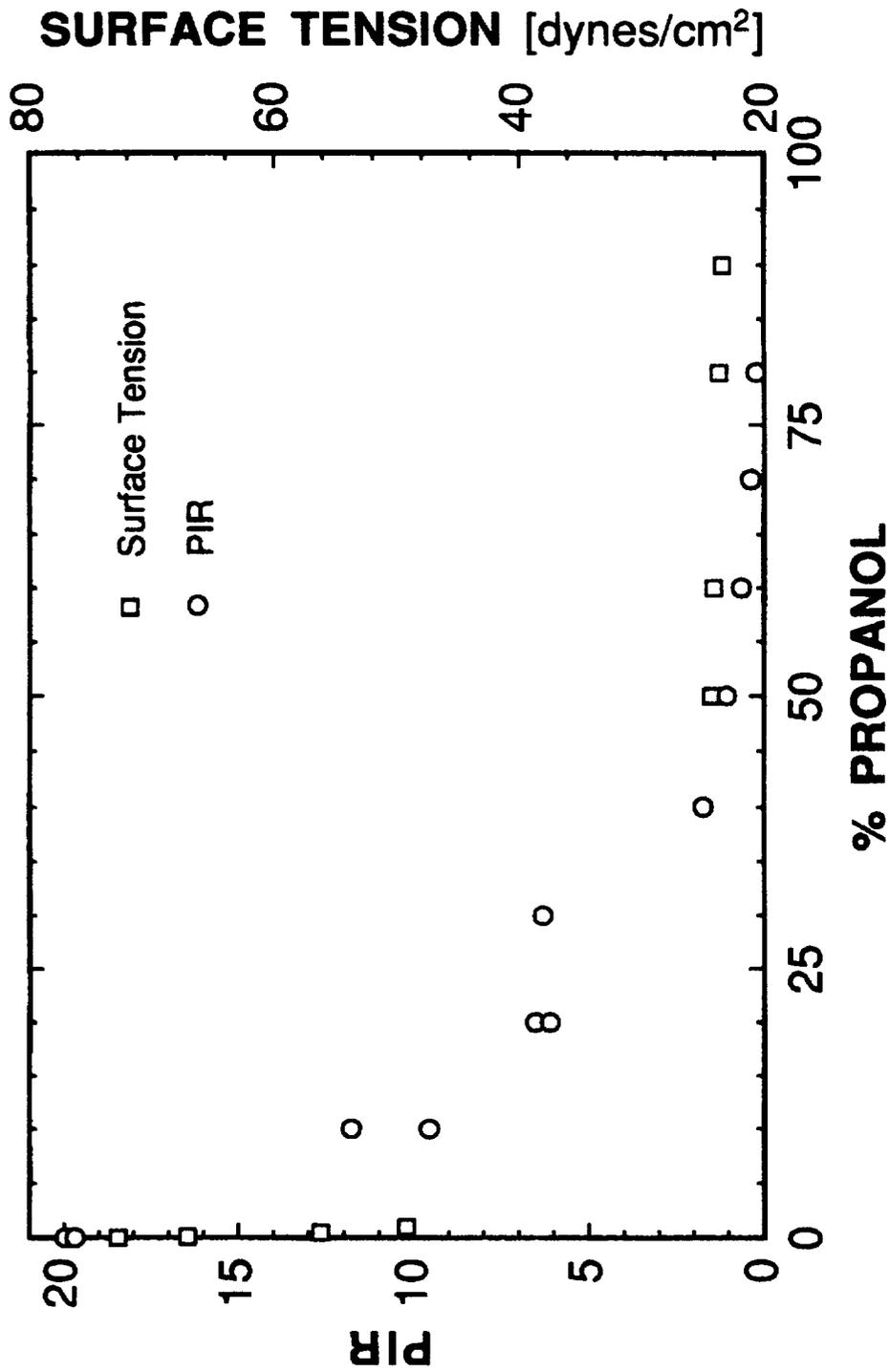
Fig. 3: The peak intensity ratio PIR (ratio of intensity at 511 to that at 440 nm) of pyranine in propanol-water solutions, and the surface tension of the solutions, plotted as a function of propanol concentration. The breakdown of the bulk water networks with increasing propanol concentration is displayed by decreases in the PIR and also results in decreases in the surface tension of the solution.
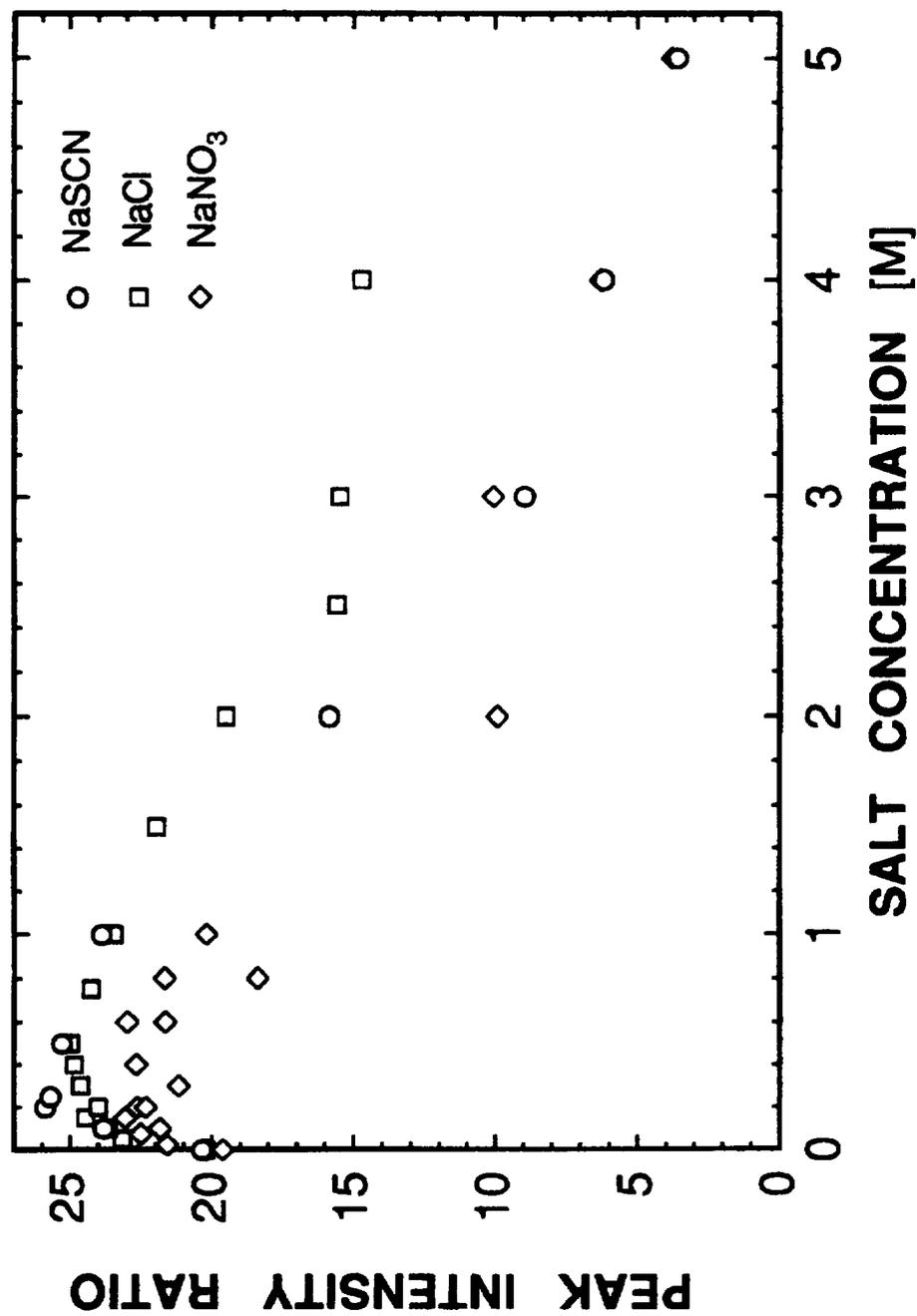
Fig. 4: The PIR values as a function of salt concentration for NaCl, NaNO₃ and NaSCN. Addition of both NaSCN and NaNO₃ results in sharp decreases in the PIR, but addition of NaCl produces comparatively moderate decreases. This is the first direct evidence of the greater chaotropicity of nitrate and thiocyanate ions.
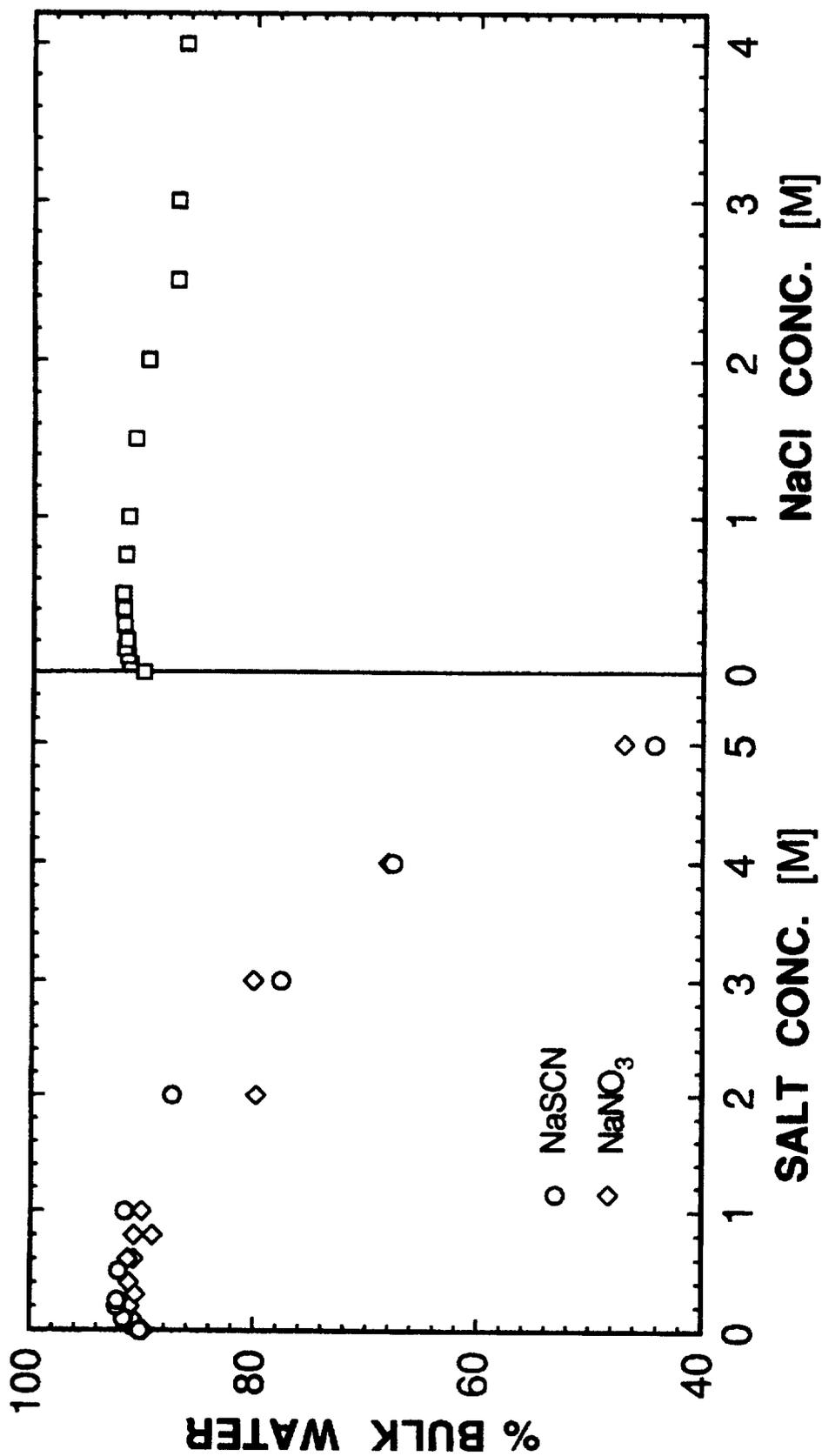
Fig. 5: The fraction of networked bulk water in salt solutions determined from the PIR values of fig. 5 and by assuming a 2:1 distribution of pyranine in networked and non-networked water. The fraction of networked bulk water falls sharply with increasing $NaNO_3$ and NaSCN concentrations, but is little affected by addition of NaCl.
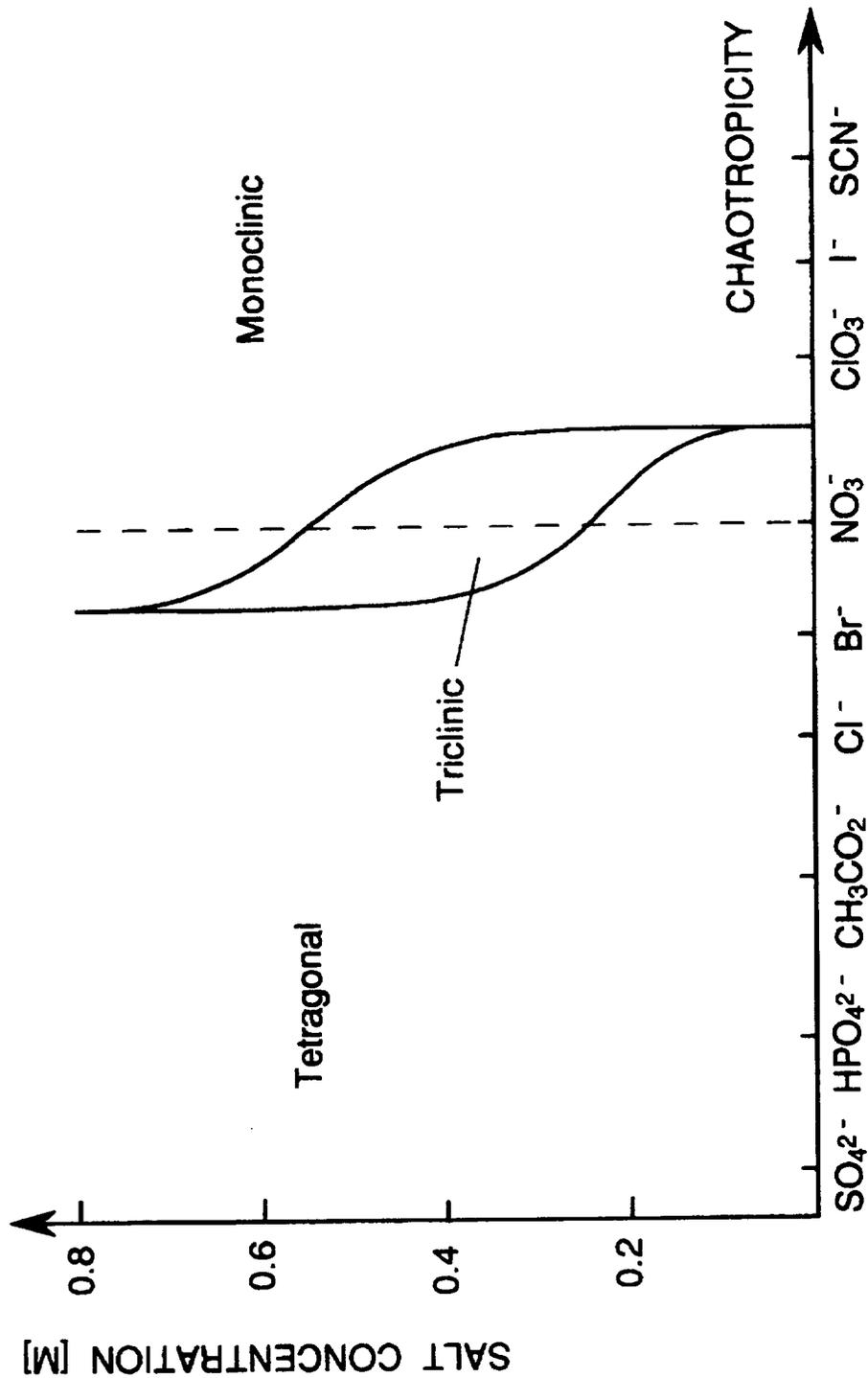
Fig. 6: Suggested phase diagram for the equilibrium forms of lysozyme crystals. (The diagram is incomplete as temperature effects resulting in the formation of orthorhombic crystals have not been included.) It is clear that less disruption of bulk water networks by added salts produces tetragonal crystals and greater disruption monoclinic ones. The triclinic form occupies a small intermediate region and has been seen only with nitrate ions, which also produces the other two forms.