Environment and Ecology 36 (1A) : 209—213, January—March 2018
Website: environmentandecology.com ISSN 0970-0420

# High and Ultra Low Concentrations of Sodium Chloride Initiate their Action on Binding Sites of a Protein

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Received 9 September 2017 ; Accepted 12 October 2017 ; Published on 3 November 2017

**Abstract** There are clinical and experimental evidences which show that ultra low concentrations (ULC) of drugs used in homeopathy produce biological effects. ULCs differ from each other with respect to free water molecules or OH groups and H-bond strength of OH groups. The objective is to see whether ULCs of a drug, *Natrum mur* and its high concentration (θ) initiate their action on the binding sites of a protein, bovine serum albumin (BSA). *Nat-m θ* (500 m M NaCl solution in water) and its 2 potencies 30cH (100μM) and 200 cH (100μM) were prepared in the laboratory. The control was distilled water (55mM). The drug and control solutions were injected separately 20 times at 6 μl/injection every 2 min into a sample cell containing 7μM BSA at 25°C in the isothermal calorimetry (ITC) instrument. While *Nat-m θ* produced endothermic reaction, the potencies and the control produced exothermic reaction. ITC parameters binding constant (K), change in enthalpy (ΔH), entropy (ΔS) and free energy (ΔG) varied markedly from each other among the control and drug solutions. Control, *Nat-m θ*, 30cH and 200cH showed single site, sequential interaction in 3 sites, same in 3 sites and sequential interaction in 4 sites of BSA, respectively. It is concluded that the drug and the control initiate their action on binding sites of BSA. *Nat-m θ* and its potencies produced endothermic and exothermic reaction, respectively on BSA. Repetition of a dose produced increasing saturation.

**Keywords** *Natrum mur*, Potencies, ITC, Binding site, BSA.

## Introduction

Extremely low concentrations of drugs have been used in homeopathy for a couple of centuries. These drugs are prepared by successive dilution with the medium, aqueous ethanol, 1 : 100 followed by mechanical agitation or succussion in several steps. Depending on the number of steps the dilutions are designated as potencies like 6, 12, 30, 200cH. Hahnemann introduced this therapeutic system in 1795. High concentrations of a drug, given to healthy individuals, produce some symptoms, which when found in a patient, are ameliorated by extremely low concentrations or potencies of the same drug. But potencies above 12cH, whose dilution is $10^{24}$, cross the Avogadro number and are, therefore, devoid of original drug molecules. Using FTIR and Raman spec-

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troscopy and also differential scanning calorimetry (DSC) we have demonstrated that the physical basis of potencies of a drug consists of two factors like hydrogen bond strength of the OH groups and also free OH groups or non-hydrogen bonded water molecules [1—5]. It is hypothesized that potencies initiate their action on cell membrane proteins as in oral mucosa or plant leaves on which the drug is applied [6]. In a recent experimental study we have demonstrated that high and ultra low concentrations of mercuric chloride act on α- amylase starch interaction through the binding sites of the enzyme [7]. The objective of the present study is to see whether high and low concentrations of sodium chloride initiate their action directly on the binding sites of a protein like bovine serum albumin (BSA).

## Materials and Methods

Sodium chloride, obtained from SRL, Mumbai, was dissolved in deionized and distilled water to prepare a mother tincture, called *Natrum mur θ* (500 mM NaCl solution). Potencies of this drug have long been used in homeopathy (Boericke, 1927). Besides the mother tincture we prepared two potencies of *Natrum mur*, 30cH and 200cH, in our laboratory in 90% ethanol following the standard procedure. BSA was dissolved in deionized and distilled water to prepare its 7μM solution. Just before experiment, *Nat-m* potencies, 30 cH and 200cH, which were in 90% ethanol, was diluted with deionized and distilled water 1:1000 to minimize ethanol content to a negligible level [8]. Optical density (OD) of these diluted potencies was measured in a UV-VIS spectrophotometer (Shimadzu model UV 2401 PC) and found to be 0.03 at 220.5 nm. Control consisted of deionized distilled water.

Interaction between BSA and *Nat-m* solutions (θ, 30cH, 200cH) and water control were determined by isothermal calorimetry (ITC). The instrument used for all calorimetric measurements is ITC 200, GE Healthcare, Biosciences Ltd, Sweden. The unique advantage with ITC experiment is that it helps in getting accurate data under optimal conditions in a single experiment [9]. ITC is a thermodynamic technique which helps in measuring directly the heat evolved during a chemical reaction, here drug solutions with BSA solution in terms of binding with the protein. The binding reaction may cause release of heat (exothermic) or absorption of heat (endothermic). In the titration experiment a small amount of drug or control solution is injected at 6μl/ injection every 2 min into a measurement cell containing 300μl of 7μM BSA solution. Concentrations of drug solutions and water control were 500mM for *Nat-m θ*, 100 μM for *Nat-m* 200 cH and *Nat-m* 30 cH and 55 mM for water control. *Nat-m θ* and water control were in water only. Potencies like 30cH and 200cH were in 90% ethanol diluted with water 1:1000. In these two cases molar solution relates to ethanol only, which is extremely small in quantity and same in both the potencies. Both the sample and reference cells containing water only were maintained at a constant temperature of 25°C. Once the thermal equilibrium was reached injections of drugs/control were started.

## Results and Discussion

The results are presented in Figures 1–4 and Table 1. Each figure contains two panels A and B. Panel A shows heat rate (μcal/sec) versus time in min. Each peak represents an injection of ligand (control/drug) into the sample cell containing the protein BSA. Panel B shows heat released per mole of ligard during interaction with BSA in relation to the molar ratio, ligand / BSA in the form of non-linear regression. Here the best fit parameters like K (binding constant), ΔH (change in enthalpy), ΔS (change in entropy) and number of binding sites for complex formation are recorded. Gibbs free energy change ΔG is calculated from ΔH and ΔS and T (absolute temperature in Kelvin). $\Delta G = \Delta H - T\Delta S$. All these parameters for each sample are given in Table 1. Figure 1 A, B stands for 7μM BSA + *Nat-m θ* (500 mM NaCl in water), Figure 2 A, B for BSA + *Nat-m* 30cH (100 μM), Figure 3 A, B for BSA + *Nat-m* 200cH (100 μM) and Figure 4 A, B for BSA + water control (55 mM).

An analysis of ITC data by an in-built software Origin 7 indicates single binding site for water control, three sequential binding sites for *Nat-m θ* and *Nat-m* 30cH, and four sequential binding sites for *Nat-m* 200cH (Table 1 and Figs. 1–4 B). Thus the drugs and control show very marked difference in stoichiometry. While *Nat-m θ* produces endothermic reac-

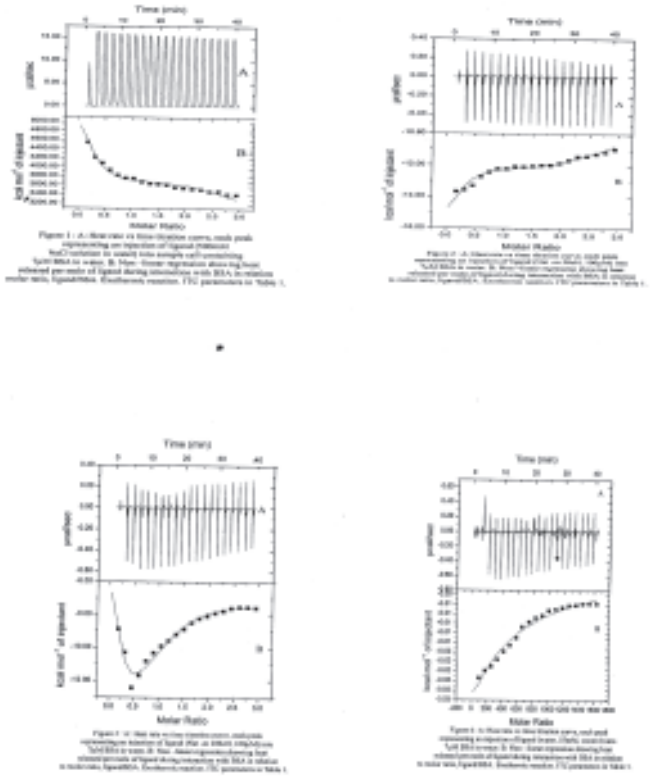

**Fig. 1.** Heat rate vs time titration curve, each peak representing an injection of ligand (500 mM NaCl solution in water) into sample cell containing 7μM BSA in water. B : Non-linear regression showing heat released per mole of ligand during interaction with BSA in relation to molar ratio, ligand / BSA. Exothermic reaction. ITC parameters in Table 1. **Fig. 2.** Heat rate vs time titration curve, each peak representing an injection of ligand (*Nat-m* 30cH, 100 μM) into 7μM BSA in water. **Fig. 3.** Heat rate vs time titration curve, each peak representing an injection of ligand (*Nat-m* 200cH, 100 μM) into 7μM BSA in water. **Fig. 4.** Heat rate vs time titration curve, each peak representing an injection of ligand (water 55 mM, control) into 7μM BSA in water.

tion, all other ligands do exothermic reaction (Fig. and Table 1). In case of sequential binding, one site induces opening up of the next site after saturation. All reactions show a tendency to saturation. Binding constants, change in enthalpy, entropy and Gibbs free energy show marked variation from each other among *Nat-m θ* and potencies, and also between the control and *Nat-m* potencies. Except the mother tincture all other ligands are virtually water.

The binding of ligand molecules to protein molecules is reversible and non-covalent in nature. In case of the mother tincture this binding occurs between sodium chloride molecules and BSA molecules at specific sites which are 3 in number here. Proteins have flexible binding pockets lined with amino acid residues. Proteins' internal motion determines and designs binding properties. Amino acid residues outside the binding site also influence the properties of binding pockets [10]. A binding pocket is a three-dimensional cleft which comprises amino acid residues that are not usually adjacent to each other sequentially but are brought together due to folding (Wikibooks, 2017). In case of *Nat-m θ*, the overall reaction is endothermic, but in potencies it is exothermic. This shows that this drug has a dual action depending on its concentration. Similar results have

**Table 1.** Thermodynamic parameters of interaction between 7μM BSA in water and ligands like *Natrum mur* mother tincture (θ), its potencies (ultra low concentrations) and water control at 25°C. Each ligand was injected into BSA solution 20 times at 6μl / injection every 2 min.

| Ligands to BSA | K $M^{-1}$ Binding constant) | Δ H cal/mol (change in enthalpy) | Δ S Cal/mol/ deg (change in entropy) | Δ G Cal/mol (change in free energy) | No. of binding sites, reaction |
|---|---|---|---|---|---|
| *Nat-mθ* | K1 | H1 | S1 | G1 | 3, sequential, |
| 500 mM | $1.02\times10^{5}$ | $1.197\times10^{7}$ | $4.01\times10^{4}$ | $-1.096\times10^{7}$ | endothermic |
| NaCl | K2 | H2 | S2 | G2 | |
| | $9.72\times10^{4}$ | $-6.69\times10^{4}$ | $-2.24\times10^{4}$ | $-6.138\times10^{6}$ | |
| | K3 | H3 | S3 | G3 | |
| | $1.01\times10^{5}$ | $2.55\times10^{7}$ | $8.55\times10^{4}$ | $2.34\times10^{7}$ | |
| *Nat-m* 30 | K1 | H1 | S1 | G1 | 3,sequential, |
| 100 μM | $1.14\times10^{5}$ | $-3.34\times10^{4}$ | – 88.7 | $-3.11\times10^{5}$ | exothermic |
| | K2 | H2 | S2 | G2 | |
| | $7.49\times10^{4}$ | 2858 | 31.9 | $2.06\times10^{4}$ | |
| | K3 | H3 | S3 | G3 | |
| | $8.45\times10^{4}$ | $-7.32\times10^{4}$ | – 223 | $-6.76\times10^{4}$ | |
| *Nat-m* 200 | K1 | H1 | S1 | G1 | 4, sequential, |
| 100 μM | $9.38\times10^{4}$ | $-1.5\times10^{4}$ | – 29 | $1.47\times10^{4}$ | exothermic |
| | K2 | H2 | S2 | G2 | |
| | $1.10\times10^{5}$ | $-9.96\times10^{4}$ | – 311 | $9.18\times10^{4}$ | |
| | K3 | H3 | S3 | G3 | |
| | $9.17\times10^{4}$ | $2.47\times10^{5}$ | 852 | $2.26\times10^{5}$ | |
| | K4 | H4 | S4 | G4 | |
| | $1.07\times10^{5}$ | $-2.88\times10^{5}$ | – 943 | $-2.64\times10^{5}$ | |
| Water | | | | | |
| (control) | K | H | S | G | Single, |
| 55mM | 91.7 | $-3.85\times10^{4}$ | – 120 | $5.098\times10^{5}$ | exothermic |

been obtained with another drug mercuric chloride [7].

*Nat-m* mother tincture and potencies produce sequential binding with BSA in 3 or more sites as opposed to the water control which shows single binding site (Table 1). *Merc cor* θ and potencies and alcohol showed single binding site with respect to α-amylase [7]. This shows that drugs vary with respect to their binding sites. Potencies of drugs, which are virtually water because of extreme dilution with water (1:1000), still maintain their difference from each other with respect to binding with proteins. Extremely low content of ethanol in diluted potencies cannot be a factor because ethanol also has single binding site.

The binding site in a protein is complementary to the ligand in shape, size, charge, hydrophobic and hydrophilic character. Thus ligand protein binding is specific. Water molecules also bind to many parts of a protein nonspecifically. We have already observed that the physical basis of potency consists of two factors, free OH groups and hydrogen bond strength of the OH groups [1-5]. These factors contribute to the specificity of a potency to binding with binding pockets in a protein. Thus water molecules in a potency bind specifically to proteins. Water molecules play an active role in determining ligand-protein binding as reported recently by the computational modeling group (2017). The results very clearly indicate that homeopathic potencies initiate their action by binding with membrane proteins at specific sites thereby inducing subsequent biochemical events inside the cell.

## Conclusion

Ultra high dilutions of sodium chloride, prepared by

successive dilution with the solvent medium (aqueous ethanol) followed by mechanical agitation or succession, initiate their action on a protein (BSA) at specific binding sites. Sodium chloride at high concentration produces endothermic reaction with the protein, and at ultra low doses do exothermic reaction. While water control acts on a single site, the drug solutions act on multiple sites in a sequential manner. Repetition of the same dose of the drug produces increasing saturation of the binding sites of the protein.

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