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A nuclear magnetic relaxation study of bound water in solutions of disodium cromoglycate

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The amount of water bound to disodium cromoglycate (DSCG) in frozen aqueous solutions was determined from the amplitude of the n.m.r. free induction decay. The hydration number was found to be 15 molecules H_2O/molecule DSCG of which 6 H_2O molecules were less strongly bound than the rest. Proton T_1 and T_2 relaxation times in non-frozen solutions implied a value of 10^{-8} s for the correlation time of the bound water at 291 K. This is consistent with the mobility of DSCG molecules in a smectic mesophase.

Disodium cromoglycate (DSCG) is a synthetic drug used in the prophylaxis of bronchial asthma (Brogden et al 1974). It is believed to stabilize tissue mast cells against degranulation caused by antibody-antigen reactions which take place on their surfaces. The mechanism of action is unknown but work reported by Spataro & Bosmann (1976) suggests that the mast cell membrane is modified so as to prevent an increase in calcium ion permeability which would normally accompany antigen stimulation. As DSCG in the crystalline state has a strong affinity for water (Cox et al 1971) it has been suggested that the drug may alter the hydration sheath of the mast cell membrane thus affecting the permeability to calcium ions. A better knowledge of the hydration of DSCG in solution might provide a basis for understanding some of the effects of DSCG on mast cells and it was with this object in view that we undertook the present work.

Water molecules bound to DSCG will have a lower mobility than those in the bulk solvent and their protons will have different n.m.r. relaxation parameters. Unfortunately because of rapid molecular exchange between the two states only an average relaxation process is observed, so that it is impossible to derive hydration numbers from relaxation time measurements alone. To distinguish bound water from bulk solvent we have made use of the technique of Kuntz et al (1969) in which solutions are first frozen. Most of the water then forms ice but a small non-freezable fraction remains which can be attributed to molecules bound strongly enough to the solute to be incapable of being incorporated into the ice lattice. As this latter fraction is still sufficiently mobile to allow the proton relaxation to be observed (unlike the ice protons which have a transverse relaxation time less than the recovery time of the spectrometer receiver) the residual intensity of the signal can be used to estimate the fraction of water bound. Measurements of the water proton n.m.r. relaxation times T_1 and T_2 in DSCG solutions of different concentrations were also made. By combining these with the hydration numbers obtained from the freezing experiments the correlation time for motion of the bound water was obtained.

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MATERIALS AND METHODS

Materials

DSCG in the form of a white, hydrated crystalline powder was supplied by the Pharmaceutical Division of Fisons Ltd (Loughborough, U.K.). Samples were dried under vacuum at 150 °C for about 15 h and allowed to cool over phosphorus pentoxide before being dissolved in triply distilled water to give concentrations in the range 0–27% w/w. Atomic absorption analysis showed that the triply distilled water contained less than 0·05 μg ml^{-1} of calcium and less than 0·01 μg ml^{-1} of magnesium. The dried material dissolved readily at room temperature to give solutions containing up to 9% w/w. To obtain higher concentrations gentle warming and centrifugation was required to produce homogeneous solutions. After preparation, solutions were transferred to previously steamed out and dried standard n.m.r. tubes. In order to remove dissolved oxygen from the samples used for relaxation time measurements the filled n.m.r. tubes were glass-blown onto a simple vacuum apparatus consisting of a rotary pump, a diffusion pump and a McLeod gauge. They were then evacuated using a freeze-pump-thaw cycle repeated four times. Freezing was achieved by surrounding the tube with a solid carbon dioxide/acetone bath. Finally the tubes were sealed off under vacuum.

Methods

Relaxation time measurements were made at 45 MHz using a home-built pulsed n.m.r. spectrometer. The 90° pulse width was 2 μs and the spectrometer recovery time was about 25 μs. T_1 was measured using a 180°-τ-90° pulse sequence and T_2 by the Gill-Meiboom modification of the Carr-Purcell pulse sequence with a pulse separation of 6 ms (Farrar & Becker 1971). All relaxation rates were governed by single exponential decays. The reproducibility on a given sample was better than 2% for T_1 and 6% for T_2.

In order to achieve a higher sensitivity, the freezing experiments were carried out at 70 MHz on a second home-built multi-frequency spectrometer of similar design to the 45 MHz instrument. The temperature of the sample was controlled by flowing dry nitrogen gas through a copper coil immersed in liquid nitrogen, over a heater and then over the sample in the spectrometer probe. Variation of temperature could be achieved by regulating the heater and the gas flow rate. Sample temperatures were displayed on a digital thermometer connected to a copper-constantan thermocouple which was inserted into the probe. To check that there was no temperature gradient between the inside of the sample and the probe and to find the time required for temperature equilibration a thermocouple was inserted directly into an unsealed sample tube containing ethanol. It was found that 20 min were sufficient for the sample temperature to reach the probe temperature.

Measurements of the intensity of the unfrozen water signal from the frozen solutions were made down to 218 K by observing the initial height of the free induction decay following a single 90° pulse. Pure triply-distilled water gave no detectable signal when frozen since the decay time was within the dead time of the instrument. The fraction of water remaining unfrozen was calculated by comparing the residual signal with the free induction decay obtained from solutions above the freezing point after allowing for the small change in intensity which always accompanies temperature variation due to changes in the Boltzmann factor. The correction was determined by comparing the free induction decays from an ethanol sample over the range 218–273 K.

RESULTS

Fig. 1 shows the temperature dependence of the signal from the most concentrated (25%) and the least concentrated (11%) solutions used in the freezing experiments. Since the DSCG protons constitute

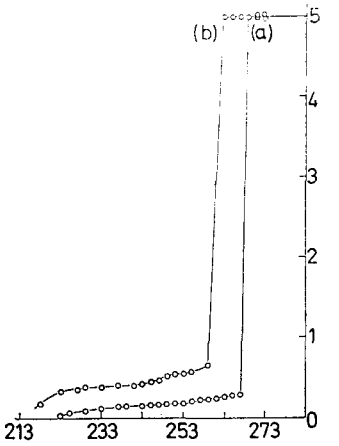


FIG. 1. N.m.r. signal intensities from frozen solutions of DSCG as a function of temperature (a) 10·6% w/w (b) 25·3% w/w. Ordinate: I (arbitrary units). Abscissa: T/K.

less than 1% of the total present even in the 25% solution their contribution to the observed signals can safely be ignored. It can be seen that on freezing the intensity of the water signal is very much reduced, and that there remains a small signal showing a weak temperature dependence which we attribute to bound water.

Fig. 2 shows how the apparent hydration number (h) changes with temperature for four representative samples. Each frozen solution shows the same pattern with h falling from about 15 to 9 over a 15°C range. After which it remains constant for about 10° C and then decreases again down to the lowest temperatures reached.

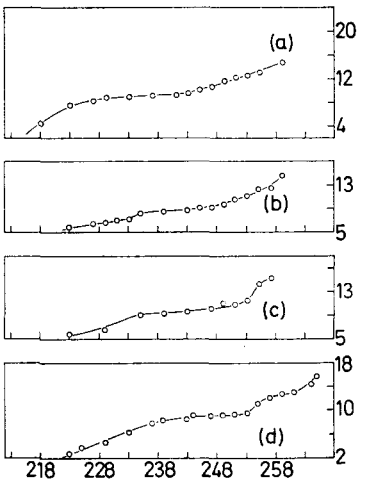


FIG. 2. Hydration numbers for DSCG as a function of temperature (a) 25·3% (b) 21·6% (c) 13·2% (d) 10·7%. Ordinate: h. Abscissa: T/K.

Plots of the fractional intensity remaining immediately after freezing and in the stationary region are shown in Fig. 3. Both sets of data give convincing straight lines which extrapolate back to the origin confirming that we are dealing with a phenomenon involving solute-bound water. From the slopes of the plots in Fig. 3 we obtain the best estimates for the hydration number which are 15 for the initial state following freezing and 9·4 for the stationary state.

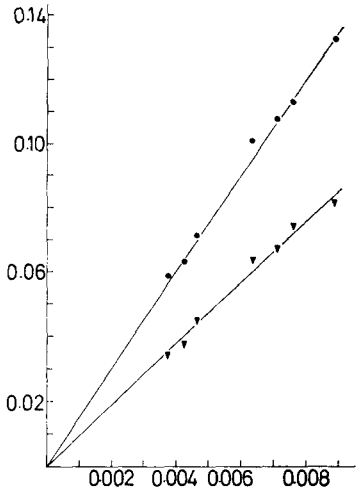


FIG. 3. Fractional intensity of signal from non-freezing water fraction for different concentrations of DSCG: (●) initial intensity after freezing, (▼) intensity in stationary region. Ordinate: I/I_0. Abscissa: mol DSCG/mol H_2O.

Relaxation rates T_1^{-1} and T_2^{-1} for water protons as a function of DSCG concentration at 291·5 K are shown in Fig. 4. In solutions containing up to ca 5% DSCG the two rates are equal, which is typical of non-viscous liquids with isotropic molecular motion. For more concentrated solutions $T_2^{-1} > T_1^{-1}$, the difference becoming greater as DSCG concentration is increased.

DISCUSSION

A free induction decay signal with a relaxation time which is characteristic of a liquid corresponds to a narrow resonance line in the frequency domain and narrow proton resonances have been observed in a variety of frozen aqueous solutions (Ramirez et al 1974). Solutions which form simple eutectics give rise to a signal whose intensity decreases steadily with decreasing temperature until it disappears and this can be taken to represent water in a true liquid phase. Another class of solutions however gives a narrow line whose intensity remains constant down to the lowest temperatures obtainable. Protein solutions show this latter behaviour and by assuming that the signal represents bound water a figure of 0·4 g water g^{-1} protein is typically obtained for globular proteins (Kuntz & Kauzmann 1974). In the case of DSCG solutions the behaviour of the signal from the non-freezing fraction resembles that obtained from protein solutions in that there is an extemely rapid initial fall in intensity followed by a much more gradual reduction as the temperature is lowered. This gradual reduction corresponds to a change in hydration number from 15 to 9·4 and we suggest that it is due to a freezing out of water molecules which are held in a secondary hydration layer, i.e. they are in a lower energy state than those of bulk water but they are not bound directly to the hydrophilic groups of the DSCG molecule. X-Ray analysis of crystals of DSCG at ambient humidities shows that there is a large space between the chromone rings which is sufficient to contain about 6 water molecules (Hamodrakas et al 1974) and it is tempting to identify this as the site of the secondary hydration structure. The levelling off of the hydration number at about 9 is to be expected on two counts. Firstly consideration of the hydrophilic groups present in the structural formula (I) shows that there are 9 potential sites for hydration (carboxylate, ether, hydroxyl and carbonyl groups) apart from the sodium ions.

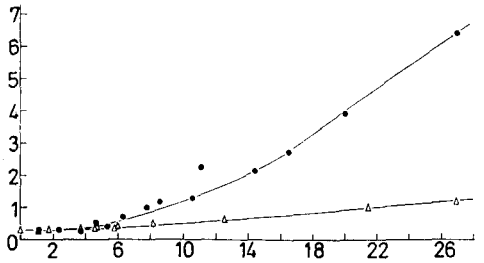
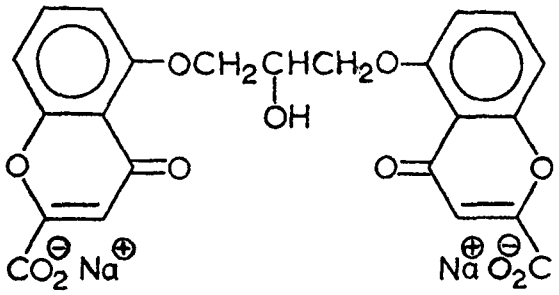


FIG. 4. N.m.r. relaxation rates at 291 K for water protons in DSCG solutions: (●) T_2^{-1}, (△) T_1^{-1}. Ordinate: relaxation rates^{-1}. Abscissa: [DSCG]/wt %.

Secondly crystals can accommodate 9 water mole-



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cules per molecule of DSCG before they collapse to form one of two lyotropic mesophases (Cox et al 1971; Hartshorne & Woodard 1973). These 9 water molecules could be regarded as constituting the primary hydration layer in solution or the water of crystallization in the solid state and as such we would not expect them to form ice however low the temperature. Nevertheless a further decrease in the intensity of the residual signal was observed below about 233 K. The most likely explanation is that the rotational motion for some of the bound water molecules has become so slow at these temperatures that the associated proton transverse relaxation rate is too fast to allow it to be observed.

The second part of the investigation involved measuring the relaxation times T_1 and T_2 which respectively characterize the rate at which the proton spin system comes to thermodynamic equilibrium with its surroundings (spin-lattice relaxation) and the rate at which coherently precessing nuclei become dephased (spin-spin relaxation). Both are determined by the local magnetic fields of the nuclear dipoles. Because of Brownian molecular motion these local fields fluctuate randomly on a time scale characterized by a correlation time (τ_c). This correlation time determines the period over which there is a loss of correlation between successive positions of a molecule. For spin-lattice relaxation the rate is a maximum when τ_c is approximately equal to the reciprocal of the precession frequency of the nuclei (ω_0). For spin-spin relaxation however the rate increases continuously as the molecular motions become slower since the variation in local magnetic fields is not then averaged out so effectively. Eventually a limiting value is reached corresponding to a rigid lattice.

For the two protons in a water molecule which is rotating isotropically the equations for T_1 and T_2 in terms of the rotational correlation time are:

$$\frac{1}{T_1} = A \cdot \left[\frac{\tau_c}{1+\omega_0^2\tau_c^2} + \frac{4\tau_c}{1+4\omega_0^2\tau_c^2} \right] \quad [1]$$

and

$$\frac{1}{T_2} = \frac{A}{2} \cdot \left[3\tau_c + \frac{5\tau_c}{1+\omega_0^2\tau_0^2} + \frac{2\tau_c}{1+4\omega_0^2\tau_c^2} \right] \quad [2]$$

where A is a constant specifying the strength of the intramolecular dipolar interaction (Aleksandrov 1966). For water at room temperature τ_c is of the order 10^{-12} s so that $\omega_0^2\tau^2_c \ll 1$ and equations [1] and [2] reduce to

$$\frac{1}{T_1} = \frac{1}{T_2} = 5A\tau_c.$$

Qualitatively we can understand the effect of DSCG in increasing T_1^{-1} and T_2^{-1} on the basis of an increase in the average value of τ_c brought about by the binding of some water molecules to the solute. A quantitative analysis of the effect can be given if we adopt a simple model in which a small fraction of bound water with a characteristic relaxation behaviour is in rapid exchange with unperturbed bulk water. In these circumstances the observed spin-lattice relaxation time is given by

$$\frac{1}{T_1} = \frac{hc}{1-hc}\left(\frac{1}{T_{1b}}\right) + \frac{1}{T_1^\circ} \quad [3]$$

where h is the hydration number, c is the DSCG concentration (mol mol^{-1} of water), T_{1b} is the relaxation time in the bound state and T_1° is the relaxation time in the bulk (Woessner & Zimmerman 1963). A similar equation applies to T_2.

Figs 5 and 6 show plots of T_1^{-1} and T_2^{-1} against hc/(1–hc) where h = 9·4 and 15. In the dilute solution region the plots are curved indicating that T_{1b} and T_{2b} are concentration dependent. This probably arises from an aggregation process which has been detected with viscometric and light scattering tech-

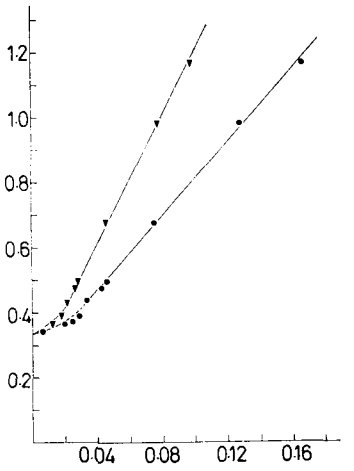

FIG. 5. Spin-lattice relaxation rates at 291 K for water protons vs hc/(1-hc) (abscissa) where c is the DSCG concentration (moles/mole water) and h is taken to be 15 (●) and 9·4 (▼). Ordinate: T_1^{-1} s^{-1}.

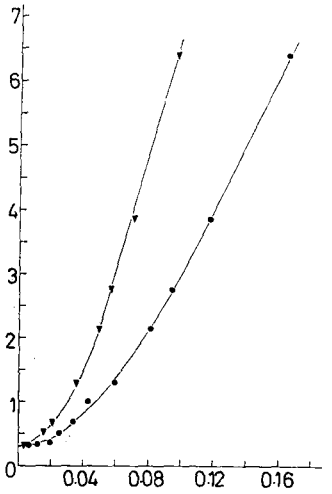

FIG. 6. Spin–spin relaxation rates at 291 K for water protons vs hc/(1-hc) (abscissa) where c is the DSCG concentration (moles/mole water) and h is taken to be 15 (●) and 9·4 (▼). Ordinate: T_2^{-1} s^{-1}.

niques (Champion & Meeten 1973). At concentrations corresponding to the liquid crystalline regions however, the plots become linear and yield T_{1b} = 100 ms, T_{2b} = 11 ms when h = 9·4 and T_{1b} = 185 ms, T_{2b} = 20 ms when h = 15. If either pair of values is substituted into equations [1] and [2] a value of 1×10^{-8} s is obtained for the bound water correlation time. This is four orders of magnitude larger than the value for free water and it indicates that water molecules must be bound to DSCG molecules which are severely restricted in their motion, as would be expected in a mesophase. The mesomorphic phases formed by concentrated DSCG solutions have been studied using X-ray diffraction and polarized light (Cox et al 1971; Hartshorne & Woodard 1973). With increasing concentration of DSCG the 'N' mesophase is formed first with a typical nematic texture between crossed polaroids followed by the 'M' phase which gives an X-ray pattern similar to middle soap phases. These observations have been interpreted in terms of a model for the M phase in which the planar DSCG molecules form cylindrical micelles with the molecular planes normal to the cylinder axis and with the polar groups on the outside. The cylinders are separated by water and on dilution of the solutions their average separation increases. The diameter of the cylinders is estimated to be about 2·6 nm.

The process that determines the correlation time of the DSCG molecules and hence of the bound water molecules in the M phase will be the two-dimensional diffusion around the micelle surface. A rough estimate of the time for diffusion around the micelle can be obtained from $t = c^2/4D$ where c is the circumference of the micelle and D is the self-diffusion coefficient for the lateral translation of the DSCG molecules. Using the self-diffusion coefficient of 1×10^{-10} m^2 s^{-1} found for lateral motion of oleate ions in the lamellar phase of the potassium oleate/water system (Roeder et al 1976) we calculate t $\sim 10^{-7}$ s. In view of the large uncertainties involved, this is sufficiently close to the figure of 10^{-8} s derived for the correlation time from the relaxation time data to suggest that the underlying model is correct.

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