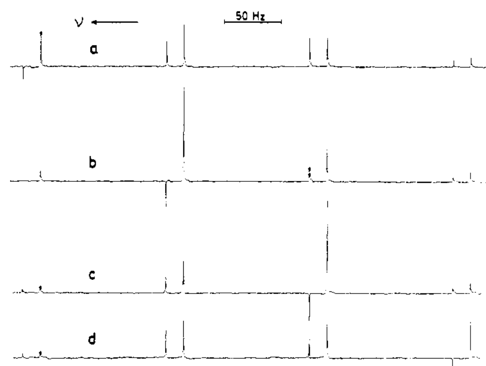

Figure 3. The 25.16-MHz ^{13}C FT SPT NMR spectra of CH_3SiHCl_2 ($^1J_{C-H}$ = +124.3 Hz, $|^2J_{C-H}|$ = 15.0 Hz) obtained after SPT π pulses have been applied to the four lines in the high-frequency quartet of the $^{13}C-^1H$ satellite doublet of quartets ($|^2J_{C-H}|$ = 15.0 Hz, $|^3J_{H-H}|$ = 2.3 Hz) for the proton directly bonded to silicon. Relative to the chemical shift of this proton the irradiation frequencies are: (a) +10.87 Hz, (b) +8.61 Hz, (c) +6.41 Hz, and (d) +4.10 Hz. From the spectra $^1J_{C-H} \times {}^3J_{H-H} > 0$ is obtained, i.e., $^3J_{H-H} > 0$.

This shows $^3K_{H-H} \times {}^1K_{Si-H} > 0$, where $K_{ij} = 4\pi^2 J_{ij}/h\gamma_i\gamma_j$ are the reduced coupling constants. Similarly, from SPT experiments performed in the proton spectrum for the one-bond $^{29}Si-^1H$ satellites, we obtain $^3K_{H-H} \times {}^2K_{Si-H} < 0$. Since $^1K_{Si-H}$ must be considered positive ($^1J_{Si-H} < 0$),[9] a positive sign is obtained for both $^3J_{H-H}$ = +2.8 and $^2J_{Si-H}$ = +7.12 Hz. These results are in agreement with the signs determined for the related couplings in CH_3SiHCl_2 from ^{13}C SPT spectra (Figure 3) and spin tickling experiments.[9]

The present results indicate that the SPT technique may prove useful in ^{29}Si FT NMR studies. This applies especially to systems where the NOE causes reduced intensities in $^{29}Si-\{^1H\}$ spectra.

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Nuclear Magnetic Resonance Relaxation in Frozen Lysozyme Solutions

Sir:

Efforts to understand water in biological systems have been extensive. Nuclear magnetic resonance has been an important tool in this area because it potentially provides both structural and dynamic information.[1,2] Recent work in this laboratory[3] has demonstrated that the NMR relaxation of water in protein crystals breaks into several components, two of which have properties similar to those reported for water adsorbed on surfaces.[4] It was concluded that about 80% of the water protons associated with the protein surface in the crystal does not exchange rapidly with the bulk water while the remainder does. It is important to determine whether the properties of water associated with the protein surface observed in protein crystals are unique to the crystal phase or are characteristic of water-protein interactions in general.

Previous work in concentrated protein solutions has demonstrated that surface water components are too dilute to be resolved using present techniques.[2] The problem of the dominant free water signal may be eliminated by freezing a concentrated protein solution and studying the remaining nuclear magnetic resonance signal.[5]

NMR measurements were made at 30 MHz using a pulsed spectrometer built in this laboratory.[6] Temperature was controlled using a Varian variable temperature controller calibrated against a diode thermometer. Lysozyme used without further purification was purchased from Worthington Biochemical Corporation as the salt-free powder, and solutions were prepared using deionized water. Samples were frozen either in a refrigerator or directly in the NMR probe. T_1 was measured using a 180-90° pulse sequence and T_2 using the Gill-Meiboom[7] modification of the Carr-Purcell[8] pulse sequence. Signals were accumulated on a Varian C-1024 CAT.

The free induction decay amplitude in the frozen solution is a linear function of the protein concentration as shown in Figure 1. The NMR signal observed at 253°K is completely eliminated by exchanging the water with D_2O several times prior to freezing. This demonstrates that the observed signal is associated with the water protons or protein protons which exchange with the solvent. At 253°K the relaxation rates are independent of protein concentration up to 30% by weight.

The temperature dependence of the several relaxation components observed is shown in Figure 2. The proton T_1 relaxation is described by two relaxation times; 81% of the

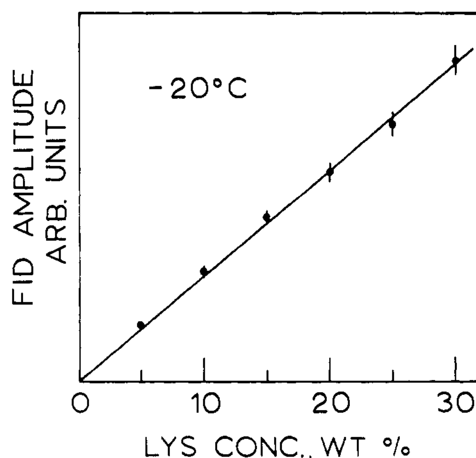

Figure 1. ^1H NMR free induction decay amplitude at 30 MHz as a function of lysozyme concentration in solutions measured at 253°K.

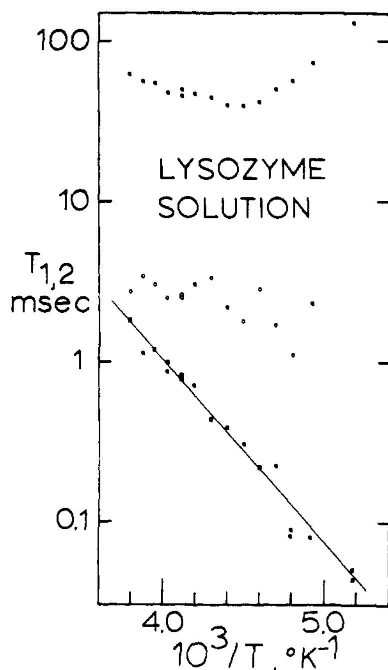

Figure 2. ^1H NMR relaxation times measured in frozen lysozyme solutions at 30 MHz as a function of reciprocal temperature: ●, T_1 of the major relaxation component; O, T_1 of the minor relaxation component, ■, T_2.

proton signal relaxes with a T_1 value in the range of 50 msec and shows a clear but very broad minimum. The proton T_1 of the remaining signal is much shorter and is difficult to measure accurately as shown by the scatter in Figure 2. It is possible that the protons observed relax with two or more values of T_2. Measurements made on protein powders suggest this possibility but also indicate that extraction of the separate relaxation rates would require extremely precise data. Within the experimental errors of the present measurements, all the protons observed relax with a single T_2. The activation energy for the reorientation events causing T_2 relaxation is 5.2 kcal/mol. The relaxation spectrum remains essentially unchanged over the period of a week at low temperatures.

The very broad T_1 minimum and the ratio T_1/T_2 at the T_1 minimum implies that the Bloembergen, Purcell, Pound[9] equations must be modified to account for the observations. A log normal distribution of correlation times has been previously used with success in fitting such data.[4] Although this procedure lacks fundamental justification, it provides a useful parameterization of the data. In the present case T_2 is 360 μsec and T_1 is 39 msec at the T_1 minimum at 227°K. The width, β, of the log normal distribution describing the data is 3.8 and the second moment, σ_0^2, is 2.38 $\times$ 10^{10} sec^{-2}. These values are similar to other values reported in both protein crystals and more complex systems.[10,11] If the second moment is corrected for a slow motion cut off[4] in the distribution, σ_0^2 becomes 2.7 $\times$ 10^{10} sec^{-2} which is close to that for ice of 2.6 $\times$ 10^{10} rad^2 sec^{-2} [12] and similar to the values reported earlier for water adsorbed in protein systems.

Quantitative measurements of the number of protons associated with each group of protons with different relaxation properties is of importance for discussions of protein hydration. We designate as component I the 81% of the observed protons which relax with the longer T_1 values and as component II the remaining protons which relax with the shorter T_1 value. Using the spin intensity measurements to determine the amount of each relaxation component as described previously, we may conclude that the signal intensity of component I corresponds to 0.28 g of water per g of protein or 223 water molecules per protein molecule and component II corresponds to 0.06 g of H_2O per g of protein or 52 water molecules per protein molecule. The sum of components I and II is then 0.34 g of water per g of protein in good agreement with earlier reports.[1]

These data are very similar to that reported for two components of the proton relaxation rates in lysozyme crystals.[3] Therefore the crystal result is not a unique property of protein crystal systems. The general features of these frozen protein solutions are fundamentally different from the well-known liquid components in frozen electrolyte solutions.[13] The long relaxation time component in frozen 5% sodium hydroxide solution at $-20°$, for example, consists of a simple exponential with T_2 equal to 100 msec and T_1 equal to 139 msec. No 3-msec component is apparent and the highly liquid character of the unfrozen part of the sodium hydroxide solution is clear from the small value of T_1:T_2.

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The Three Phase Test for Reaction Intermediates. Evidence for Monomeric Metaphosphate

Sir:

The elusive monomeric metaphosphate ion **1** occupies a central role in the hydrolysis of organophosphate compounds.[1] Alkyl, aryl, and acyl phosphates as well as phosphoramidates and halo phosphonates are all believed to generate **1**, yet incisive experimental evidence for the existence of this ion is rare. We have recently described a new method for detecting reaction intermediates in which an intermediate is generated from a suitable polymer-bound precursor and detected by trapping on a second solid phase.[2] We now report the application of this method to the detection of **1**.