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QUASI-ELASTIC SCATTERING STUDIES OF WATER DIFFUSION

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Abstract

Quasi-elastic neutron scattering is a powerful method to study the dynamics of protons in biological systems. The technique has been used both for the study of water diffusion and protein motion. The neutron scattering measurements on water show that the translational and rotational diffusion coefficients in biological systems are reduced from bulk values. We review the measurements on water in frog muscle, cysts of artemia, and phycocyanin. Measurements on dry trypsin and trypsin-D_2O solutions over the temperature range 75-300K show that there is proton motion at the one angstrom level even in the dry or frozen state.

Key Words:

Water, diffusion, protein dynamics, water dynamics, quasi-elastic neutron scattering, Debye-Waller factor, cytoplasmic water, artemia, trypsin, phycocyanin.

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Introduction

The goal of a measurement with the scanning electron microscope (SEM) is to obtain an artifact-free micrograph of the morphology of a biological structure at the 5-10 nm level. To obtain a micrograph requires preparation and manipulation of a sample [6], in which the fixation or removal of water plays an important part. The fixing, drying, freezing and fracturing of a sample can result in severe distortions of the biological structure [7], and it can be expected that the interaction of water with the macromolecules of the cell will play an important role in the quality of the image.

The image contrast is due to the varying electron emission (secondary or back-scattered) from the different regions of the sample, which depends on topography and composition. Water may influence the image in three ways. 1) Water molecules may remain incorporated in the biological structure. This is certainly true for the quick-frozen samples and may also hold for the perfused samples. The secondary electrons from the water could contribute significantly to the image. 2) The association of the water molecules with the protein molecules is an important feature of biological structure. There is a good deal of evidence (principally from NMR studies) that purified crystalline proteins, which are necessary for X-ray and neutron diffraction studies, have structures very similar to the structure in protein solutions [10]. Protein crystals, however, contain typically 40-50% water [5]. Systems of lower water content may suffer changes in structure. Chemical fixatives may introduce cross-linking which modifies the water-protein interaction, leading for example to conformational changes. These interactions are at the molecular level (in the 2-20 Å range) but may well affect the morphology at the SEM scale. (These considerations will become even more important with the application of scanning tunneling microscopy (STM) to biological systems, since the resolution of STM is of the order of two angstroms [17]. 3) The SEM sample preparation involves a re-distribution of water within the sample, although this can be minimized by rapid-freezing techniques. The rearrangement of the water is dependent on the molecular dynamics of the macromolecular structures and the diffusion dynamics of the associated water. Recent work [11] on the molecular dynamics of proteins has shown that the molecules are not static but undergo conformational changes involving motions that in some cases can be as large as an angstrom or more. This motion disappears only slowly with decreasing temperature and is present when the molecule is in vacuum as well as when it is in solution. In addition, the water molecules have their own translational and rotational motion which makes re-arrangements possible even in the frozen state.

In this paper, we will review some of the neutron scattering studies that have been made on the dynamics of protons in water associated with biological structures. We will also review some results on the proton motion in some macromolecular structures (e.g. proteins) with which the water can interact.

Neutron Scattering Considerations

Neutron scattering is a versatile tool for the study of biological systems. This has long been realized in the case of structure determination, where the coherent elastic scattering emphasizes the light atoms and allows powerful H-D contrast variation techniques to be used [22]. Less well developed is the use of energy-sensitive spectroscopy (inelastic and quasi-elastic incoherent scattering) for the study of dynamics of biological systems. This technique is of special importance for biological systems, due to the abundance of hydrogen and to its large incoherent scattering cross-section (~80 barns), which is many times larger than those of other prevalent biological nuclei (C,N,O). In addition, the selective substitution of deuterium for hydrogen permits the examination of selected portions of the solution-protein complex.

A comprehensive review of the application of inelastic and quasi-elastic neutron scattering to biology has been given by Middendorf [13], and the reader is referred to his work for a more detailed treatment. We will summarize in this section the theoretical and experimental considerations needed for an interpretation of the quasi-elastic work described below.

Theoretical Summary

The scattering of neutrons from an ensemble of nuclei is described by the double-differential cross-section:

$$\frac{d^2\sigma}{d\Omega dE} = (\text{const.})\,\frac{k}{k_o}\,S_{inc}(Q,\omega) \qquad (1)$$

where $d^2\sigma$ is the number of neutrons scattered into the solid angle $d\Omega$ in the energy range dE. Each neutron-nucleus interaction results in the transfer to the neutron of momentum $\hbar\mathbf{Q} = \hbar(\mathbf{k} - \mathbf{k}_o)$ and energy $\hbar\omega = E - E_o$, where $\mathbf{k}_o$, E_o and $\mathbf{k}$, E denote incident and scattered quantities, respectively. The "scattering law" $S_{inc}(\mathbf{Q},\omega)$ is thus a function of the momentum and energy transfer. For quasi-elastic scattering, $|\hbar\omega| \ll E_o$ and $k = k_o$. If the scattering centers (the nuclei) are static in the sense that their only motion is a high-frequency thermal motion of small amplitude, then the scattering law may be approximated by:

$$S_{inc}(\mathbf{Q},\omega) = \exp[-Q^2\langle u^2\rangle/3]\,\delta(\omega). \qquad (2)$$

The exponential factor is the "Debye-Waller" factor and arises from the mean square displacement $\langle u^2\rangle$ of the thermal motion. This "thermal cloud" typically has a mean-squared amplitude $\langle u^2\rangle = 0.1$ Å^2 in a room-temperature solid.

If the scattering center undergoes a slow diffusive motion, then the scattered neutron will suffer an energy change that will broaden the delta-function in Eq. (2). In many cases the result is a Lorentzian line

$$S_{inc}(Q,\omega) = \exp[-Q^2\langle u^2\rangle/3]\cdot\frac{\Gamma(Q)/2\pi}{\omega^2 + [\Gamma(Q)/2]^2} \qquad (3)$$

in which the line width $\Gamma(Q)$ can be related to the diffusion dynamics. The theory that relates $\Gamma(Q)$ to the motion was developed originally by Van Hove [26], and a brief summary of the theory as well as some illustrative applications have been given by Springer [21]. In what follows, we present data on $\Gamma(Q)$, which is determined by the slow diffusive motion of protons, and on $\langle u^2\rangle$, which is the mean-squared amplitude of the high-frequency (thermal) motion of the proton. The behavior of $\Gamma(Q)$ for several specific models is given below.

Brownian diffusion. If the water proton obeyed the Langevin equation, then the proton would undergo continuous diffusion with a mean-square displacement during time t given by $\langle r^2\rangle = 6Dt$, where D = translational diffusion coefficient. For this case, the line width in Eq. (3) is given by:

$$\Gamma(Q) = 2DQ^2 \qquad (4)$$

This behavior can be expected for all types of diffusion in the limit of long times and large distances ($\omega \to 0$ and low Q).

Oscillatory jump diffusion. In this model, the proton performs high frequency oscillations at a bond site for a time τ, after which it makes a "jump" to a new position at a root-mean-square distance $\langle \ell^2\rangle^{1/2}$. The width depends on the assumptions made concerning the distribution of jump lengths, but one commonly used model [21] gives:

$$\Gamma(Q) = \frac{2Q^2D}{1 + Q^2D\tau} \qquad (5)$$

with $D = \langle \ell^2\rangle/6\tau$. This model is often used to describe diffusion in liquids.

Lattice diffusion. This model is similar to the oscillatory jump diffusion model, except that the "jump" is made to a discrete number n of adjacent lattice sites located at $\mathbf{r}_m$. This model will be applied to tightly bound protons, which might be expected to diffuse to a limited number of binding sites. The width is given by:

$$\Gamma(Q) = \frac{2}{n\tau}\sum_{m=1}^{n}\left[1 - \exp(-i\mathbf{Q}\cdot\mathbf{r}_m)\right] \qquad (6)$$

Rotational diffusion. The water molecule has two H-atoms, and not only the center-of-mass motion but also the molecular rotation contributes to the line-broadening. This rotational motion can be very complex. In the tightly bound case we may expect hindered rotation, but in the liquid state a simpler model is often used in which the motion is the rotational analogue of Brownian motion. The "scattering law" for this case is given by:

$$S_{inc}^{(r)}(Q,\omega) = j_0^2(Qa)\delta(\omega) + (3/\pi)j_1^2(Qa)\frac{\Gamma_r/2}{\omega^2 + (\Gamma_r/2)^2}$$

+ higher terms (7)

in which 2a = proton separation, j_0 & j_1 = spherical Bessel functions, and $\Gamma_r = 4D_r$, where D_r is the rotational diffusion coefficient. This scattering law can be convolved with Equation (3) to give a scattering law that describes combined translational and rotational motion. The "higher terms" are usually omitted.

Experimental considerations

The analysis of experimental results in terms of the models described above is not without difficulties. Some of these are listed below and are discussed further by Trantham, et. al. [25]. 1) The experimentally obtained neutron spectra for water in biological systems are rarely due to a single "component" of water. To analyze the data, a scattering law for the entire system is constructed from contributions from the models given above. These must be fitted to the experimental spectra by computer, in which the unknown parameters are determined from a least-squares search program. In some cases, the separation of the components can be facilitated by H-D substitution. 2) The neutron spectrometer has a limited resolution which can often be described by a Gaussian resolution function. This function must be convolved with the scattering law to obtain the final fitting function for the spectrum. This introduces no new parameters, since the resolution function is experimentally determined, but it does complicate the search program; it also limits the accuracy of measurements on lines that are narrower than the resolution width. 3) Many biological samples are sufficiently thick that multiple scattering occurs. This can be a serious limitation, and it can lead to errors of 10-20% or larger in values of the experimentally derived parameters. This is particularly serious for the line-width $\Gamma(Q)$ at low Q-values, and it complicates the analysis of the data. 4) The separation of the quasi-elastic spectrum from the inelastic background that falls in the quasi-elastic energy window is not always simple. When the quasi-elastic line is very narrow, the background can be well fitted with a linear function. For broader lines, some modeling of the background is needed (e.g. single-phonon scattering.) For liquids and for biological molecules, however, the harmonic approximation that describes phonons is inadequate, and there is not a good theory for the excitations, although the molecular dynamics theories hold some promise [20]. Lacking a theory, one must rely on measurements and empirical fitting functions.

Experimental Results

In this section, we review some of the experimental results on the motion of protons in biological systems, both water protons and protein-bonded protons. The measured quantities are the diffusion parameters D, τ, and D_r, and the root mean-squared high-frequency amplitude $\langle u^2\rangle^{1/2}$. In most cases, the measurements do not cover the full temperature range for rapid-freezing (70-300K), and this is an area that might be of interest for future work.

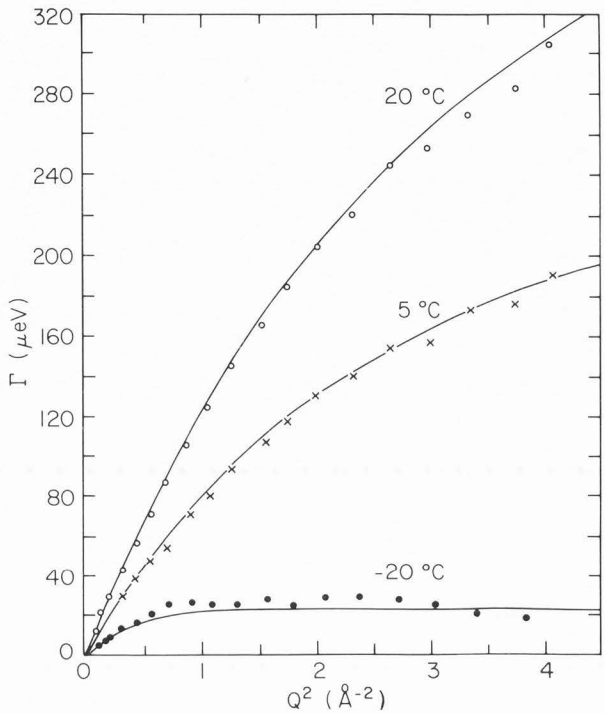

Figure 1. Linewidth $\Gamma(Q)$ vs Q^2 for pure water at three temperatures, 20C, 5C and -20C. The sample at -20C was supercooled. (From Teixeira, et. al. [23])

Water protons

Pure water. We give first some results for pure water for later comparison with the biological cases. The best work on water is that of Teixeira, et. al. [23]. This work was carried out on the IN6 spectrometer at Grenoble, which yields very high quality data. The statistics are sufficiently good that a realistic separation of the translational and rotational contributions to the scattering spectra could be obtained. Spectra were obtained at temperatures from 20C to -20C (supercooled water). The analysis of the spectra was based on a convolution of Eqs. (3) and (7) in which the width $\Gamma(Q)$ of Eq. (5) was used for the translational motion. The rotational and translational motions were assumed to be independent. The results for $\Gamma(Q)$ vs Q^2 are shown in Fig. 1 for three temperatures. The curves are the best fit from Eq. (5). The parameter values for pure water at 20^{O}C are: D = 2.2×10^{-5} cm^2/s, $\tau = 1.25 \times 10^{-12}$s, $D_r = 1.4 \times 10^{11}$ s^{-1} and $\langle u^2\rangle^{1/2} = 0.48$Å

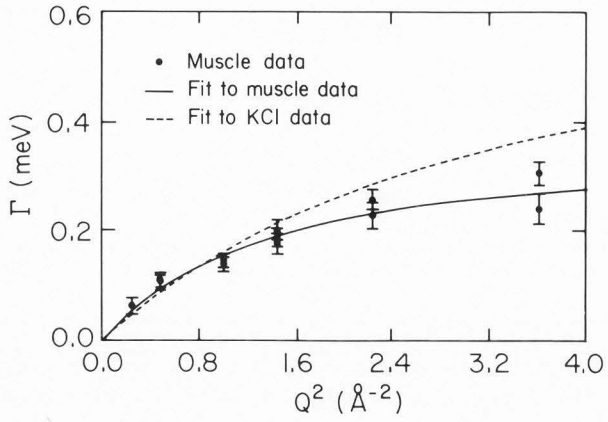

Figure 2. Linewidth Γ (Q) vs. Q^2 for frog muscle at 3C. The hydration is 4.5 gH_2O/g dry solids. Data for an 0.15 m KCl solution that was fitted with the same scattering law is also shown.

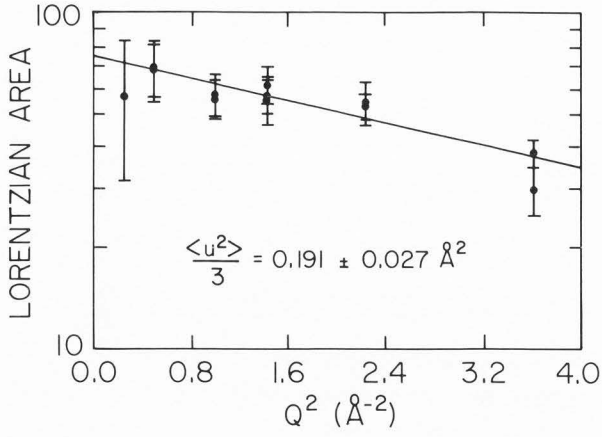

Figure 3. Line area vs. Q^2 for frog muscle. The slope of the line through the data gives the mean square high-frequency amplitude of the proton motion.

Frog muscle. Neutron scattering data were collected on a comparatively "wet" biological system, the sartorius and gracilis major muscles of green leopard frogs [8]. The muscles were carefully excised and maintained at 3 C under sterile conditions. Data were collected on each sample during a 12 to 48 h period. The viability of each muscle sample was verified by a determination of ATP content following the measurements. The data were analyzed in terms of the jump diffusion model, Eqs. (3) and (5). The data were not of sufficient quality to permit the inclusion of a rotational contribution, and this makes a direct comparison with the pure water values difficult. The results for Γ(Q) vs Q^2 are shown in Fig. 2. Data are also shown for a 0.15 molar KCl solution at the same temperature that have been analyzed with the same scattering law.

At low Q, the muscle and KCl data are indistinguishable. The error bars are large, and multiple scattering and the neglect of the rotational term in the analysis with Eqs. (3) and (5) give a broader apparent width for muscle. The data at high Q are more reliable, and a good estimate of τ can be obtained from the asymptotic behaviour at large Q. This also permits an estimate of $D = \langle \ell^2 \rangle / 6\tau$, if we take $\langle \ell^2 \rangle$ the same as in pure water. This analysis gives $D = 1 \times 10^{-5} cm^2/sec$, $\tau = 3 \times 10^{-12}$ s and $\langle u^2 \rangle^{1/2} = 0.75$ Å at 3C (see Fig. 3.). (The experimental values for the KCl solution are $\tau = 1.8 \times 10^{-12}$ s and $\langle u^2 \rangle^{1/2} = 0.75$Å.). These values can be used in a Monte-Carlo calculation to generate data in which rotational diffusion is included. (We used $D_r = 1 \times 10^{10}\ s^{-1}$). If this data is analyzed by Eqs. (3) and (5), we obtain good agreement with the results shown in Fig. 2, which indicates that it is the neglect of the rotational diffusion that is the main source of the broadening at low Q.

Cysts of the brine shrimp (Artemia). The cysts of Artemia consist of an inner mass of ~4000 eucaryotic cells surrounded by a complex shell. Their advantage for neutron scattering is that they can be reversibly dehydrated over a wide range and still retain viability. Trantham et.al. have made a study of this system. [25]. Data were collected on a sample at 293K whose hydration was 1.2g/g, near the maximum hydration which is 1.4g/g. The spectra were analyzed with the inclusion of both translational and rotational motion, and the line width, which in this case is $\Gamma(Q) + \Gamma_r$, vs. Q^2 is shown in Figure 4. The values for D, τ, D_r and $\langle u^2 \rangle^{1/2}$ are given in Table 1

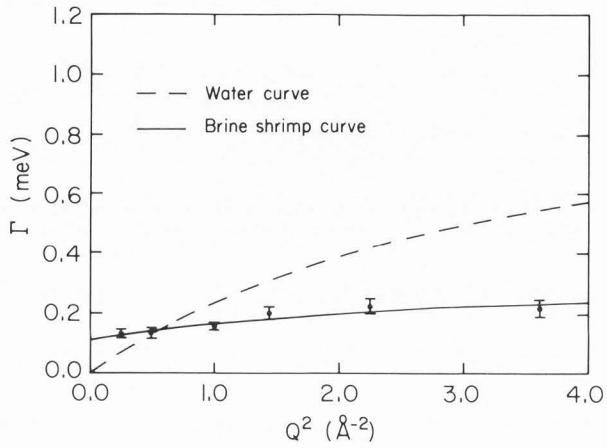

Figure 4. Linewidth Γ (Q) vs. Q^2 for cysts of the brine shrimp Artemia. The hydration level is 1.2 g H_2O/g dry solids. The curve for pure water obtained by the same technique is also shown. (From Trantham, et. al. [25]).

Phycocyanin. This system has been studied by Middendorf, et. al.,[12,14], as part of a long-term project to collect data on a single system over a wide hydration range. It is a light-harvesting protein obtained from blue-green algae which can be prepared in fully deuterated form. This permits the study of very low (submonolayer) hydration levels. The quasi-elastic lines are consequently very narrow and the width Γ (Q) vs. Q can be expected to display solid-like

Table 1. Water parameters for various systems at 20C. Except for $\langle u^2\rangle^{1/2}$, the values are expressed as fractions of the pure water values, (indicated by a prime.)

System	D'	τ'	D_r'	$\langle u^2\rangle^{1/2}$(Å)
Pure H_20*	1	1	1	0.48
Artemia**	0.29	3.9	.043	0.83
Phycocyanin***	0.02	5000	---	---

*For pure water, D = 2.2 x$10^{-5}$$cm^2$/s, τ = 1.25 x 10^{-12}s, D_r = 1.4 x10^{11}s. [23]
**Hydration of 1.2 g/g. [25]
*** Submonolayer hydration. [12,14]

behaviour. The width Γ (Q) vs. Q for several hydration values is given in Figure 5. The oscillatory behaviour is quite different than that seen in more highly hydrated systems. The data have been analyzed in terms of a lattice diffusion model (Eq. (6)) and the estimates of D and τ are given in Table 1.

Protein protons

In the previous section we have presented data on the dynamic properties of water protons. In this section, we review the work on molecular motion in proteins and present the results of some recent work on the motion of protons in the enzymatic protein trypsin.

The refinement of X-ray diffraction data on purified protein crystals has proven to be a powerful method to study molecular motion in proteins [16]. In this method, the position and intensity of a large number (several thousands) of reflections are measured, and the pattern is fitted with a molecular model in which atomic positions and temperature factors are adjustable parameters to be determined by a least-squares fitting [9]. Estimates (accurate to about 10%) can be obtained for the thermal motion associated with each residue. X-ray diffraction studies by Frauenfelder, et. al. on metmyoglobin [4] have shown that the thermal motion of the heavy atoms can be as large as 0.6Å and is consistent with that expected from molecular dynamics calculations [11]. The method has also been applied to the structure of the bound water in acid metmyoglobin by neutron diffraction measurements on deuterated crystals [18]. These measurements give a thermal amplitude as large as 0.5 Å.

Recent molecular dynamics calculations for trypsin [27] have stimulated us to examine the proton motion in this protein by a neutron-scattering measurement of the Debye-Waller factor [1]. We were unable to examine the full quasi-elastic spectrum due to intensity limitations at the High Flux Isotope Reactor at Oak Ridge National Laboratory, and the Debye-Waller factor was determined by a low-resolution measurement of the integrated quasi-elastic intensity. The trypsin was hydrated with D_2O and freeze-dried to replace the exchangeable hydrogens and was examined in dry powdered form and in a 20 weight-percent D_2O solution whose pD was adjusted to 4-5 with DCl. An assay was made of the enzyme activity, and it was unaffected by the preparation and experimental procedures. The results of our measurements for $\langle u^2\rangle$ over the range 75-300K are shown in figure 6. The plot of $\langle u^2\rangle$ vs. T does not pass through the origin, as would be expected from the equipartition theorem. This is due to contributions from the inelastic background, which we

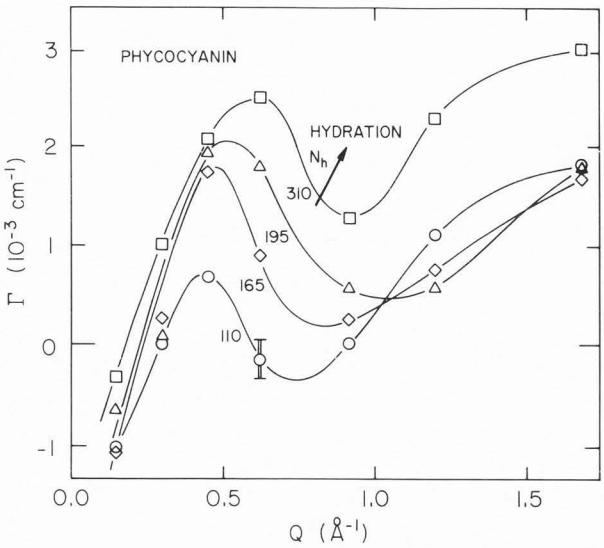

Figure 5. Linewidth Γ (Q) vs. Q for sub-monolayer hydrations of water on deuterated phycocyanin. The hydration level is given by the number N_h of H_2O molecules per 29000 molecular mass subunit of phycocyanin. The negative values of Γ arise from the difference method from which the line broadenings were obtained. (From Middendorf and Randall, [12]).

do not fully understand at this time. If a correction is made so that $\langle u^2\rangle$ = 0 at T = 0, we obtain for T = 300K, $\langle u^2\rangle^{1/2}$ (powder) = 0.47 Å and $\langle u^2\rangle^{1/2}$ (D_20 sol.) = 0.52 Å. These values are in good agreement with the results of the molecular dynamics calculations [27], although these calculations give only the heavy atom motion. Another interesting feature of the trypsin solution not seen in the trypsin powder is the discontinuity in slope at T≅275K. This may indicate important changes in the protein dynamics in the non-frozen state, similar to that seen in metmyoglobin at 210K by Parak, et. al. with Mössbauer techniques [15].

We can also estimate a value of the effective force constant K for the proton motion from the slopes of the data in Figure 6 and the equipartition theorem:

$$K\langle u^2\rangle/2 = 3kT/2 \qquad (8)$$

The values of K are in the range 2-6x10^3 dyne/cm. This is considerably smaller than the bond-stretch force constants expected for water or ice but is comparable to the value for the O-H:O angle-bend motion in ice [3].

In the course of the temperature studies on trypsin-D_20 solutions, we examined the diffraction spectrum of the polycrystalline frozen D_20, both with and without trypsin. The spectra are shown in Figure 7. They are quite similar and are consistent with the known structure of solid D_20 (hexagonal, with 4 molecules per unit cell.) There are, however, some differences. The intensities of some lines are dramatically changed in the trypsin - D_20 solution (e.g. at Q = 3.0 Å^{-1}), and some new lines appear (e. g. at Q = 1.7 Å^{-1}).

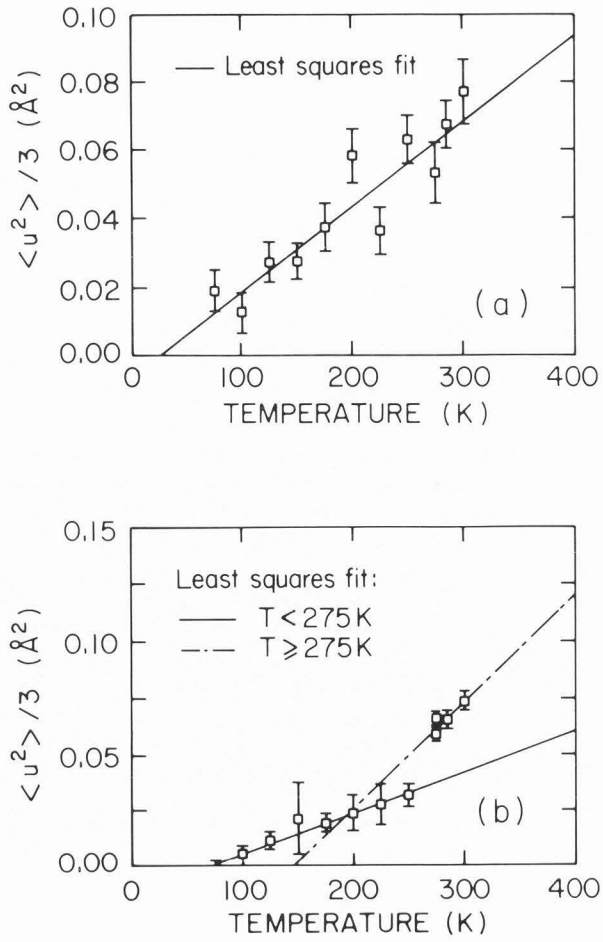

Figure 6. The mean square amplitude $\langle u^2 \rangle$ of the high-frequency proton motion vs. temperature for trypsin: (a) Dry trypsin powder, (b) 20 weight-percent trypsin-D_2O solution.

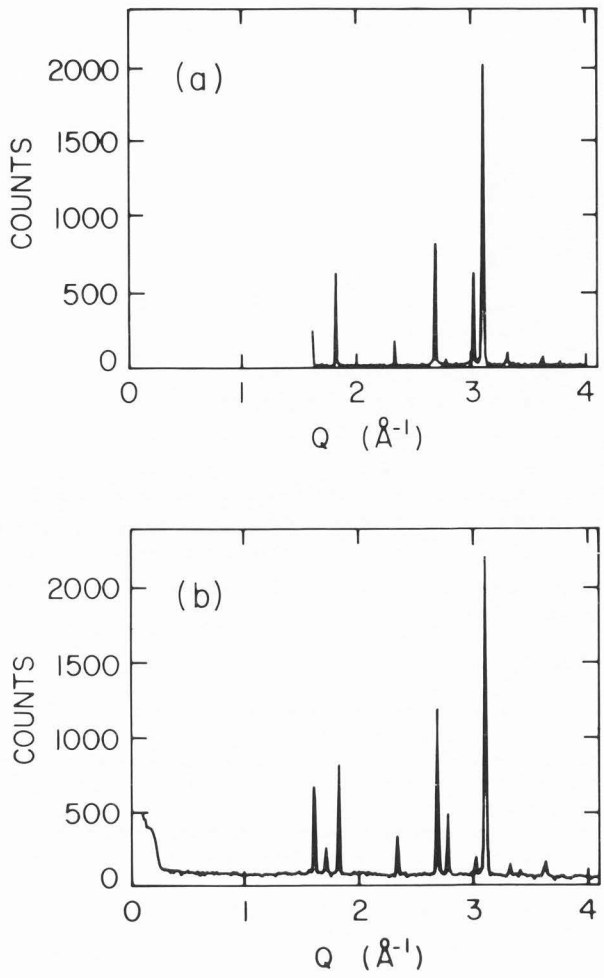

Figure 7. Diffraction spectra for polycrystalline D_2O. (a) pure D_2O. (b) 20 weight-percent trypsin-D_2O solution. (The two largest peaks at Q = 2.7 and 3.1 $Å^{-1}$ are from the aluminum sample chamber).

We have not made a full analysis of this data, but we assume that the distortion of the D_20 structure by the trypsin molecule results in a distortion of the unit cell and a change in the values of the structure factors.

Discussion and Conclusions

In the above section, results were given for the dynamical parameters of water (D,τ, and $\langle u^2 \rangle$), for three systems: a "wet" system, (frog muscle, 4.5 g H_20/g solids), a "dry" system, (cysts of Artemia, 1.2 g H_20/g solids), and a submonolayer hydration of d-phycocyanin. These results show that the water mobility in all systems is reduced relative to pure H_2O, with the largest reductions for the systems of lowest hydration. The principal reason for this reduction in diffusive motion is an increase in the residence time in the jump diffusion model, presumably induced by the presence of the macromolecular structures. (This behaviour is similar to that of many other systems, such as clays and polymers, in which water is in close association with large surface areas [24].) This suggests that the water interacts significantly with the macromolecules and that it may play an important role in determining the conformation of these molecules. It then becomes an important question whether the cell morphology is modified, and at what level, by the removal of water.

The possible high-frequency motion of protons in the water and macromolecules is also important in the consideration of changes that can occur during the freezing process [2,19]. Protein protons display motions ~ 1Å, even down to temperatures well below the freezing point. Neutron scattering data on water protons is not available over this temperature range, but it is not unrealistic to suppose that significant rearrangements can take place in order to accommodate the protein structure to the ice structure, with consequent changes in both (as in the trypsin - D_2O system.)

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Editor's Note: All of the reviewer's concerns were appropriately addressed by text changes, hence there is no Discussion with Reviewers.