*Biochimica et Biophysica Acta*, 406 (1975) 146–154


BBA 77064

# MOBILITY OF WATER BOUND TO BIOLOGICAL MEMBRANES

## A PROTON NMR RELAXATION STUDY

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(Received December 9th, 1974)
(Revised manuscript received April 1st, 1975)

SUMMARY

Water proton nuclear magnetic resonance relaxation measurements have been obtained for aqueous suspensions of red cell membranes. These data support a model in which water molecules are exchanging rapidly between a bound phase with restricted motions and a free phase with dynamic properties similar to liquid water. From this model and these data, estimates are obtained for the relaxation time for bound phase water. Possible relaxation mechanisms for bound phase water are discussed and some support is found for an intermolecular interaction modulated by translational motions characterized by a diffusion constant of $10^{-9}$ $cm^2/s$.

## INTRODUCTION

The role of water in biologic membrane structure and function is poorly understood. Membrane hydration has been postulated to be involved in stabilizing the lipid bilayer structure within membranes [1, 2], and also to be involved directly in several aspects of cell physiology including nervous conduction [3], anesthetic action [4, 5], and membrane transport [6]. Yet there have been relatively few direct measurements of the amount and state of water bound to biological membranes.

Recently, Schneider and Schneider [7] have measured the extent of adsorption of water on red cell membranes and their circular dichroism spectra as a function of hydration. These measurements have yielded a direct determination of membrane hydration, the enthalphy and entropy of water binding, and the effects of bound water on membrane protein structure. Earlier studies of membrane bound water include reflectivity (leptoscope) measurements of Waugh and Schmitt [8], X-ray studies by Finean and co-workers [1], calorimetric studies of membrane phospholipid and water phase transitions by Ladbrooke and Chapman [2], and dielectric relaxation measurements by Gent et al. [9], and by Clifford et al. [10]. Most of the above work was done on myelin or red cell membranes and gave values for the amount of membrane bound water in the range of 25–70 g $H_2O$/100 g dry membranes or 20–40 wt % of the total hydrated membrane, thus making water a major membrane component.

Information on the state of the membrane bound water has thus far come primarily from the heats and entropies of water vapor binding determined at various degrees of membrane hydration [7]. From such thermodynamic analysis of the adsorption isotherms [7], it is possible to classify the bound water into two portions: (a) tightly bound water ($\approx$ 20 g $H_2O$/100 g dry membranes) with bindings energies ranging from maximum values which are greater than latent heats of sublimation, to values approaching the latent heat of condensation and (b) loosely bound water ($\approx$ 50 g $H_2O$/100 g dry membranes), differing little from bulk liquid, and having binding energies similar to the heat of condensation of vapor into liquid water.

Nuclear magnetic resonance (NMR) methods have been used extensively to study the dynamic properties of water in tissues and protein solutions. These studies have been reviewed recently by Cooke and Kuntz [10] and Kuntz and Kauzman [11]. Clifford et al. [12] used NMR methods to study water in aqueous dispersions of erythrocyte ghosts. Using pulsed techniques, they measured the spin-lattice relaxation time ($T_1$) and the spin-spin relaxation time ($T_2$) in samples containing 92, 50 and 8 wt % water. They found their data to be consistent with a model in which water molecules exist in two phases, one exhibiting restricted motion and identified with water of hydration and another identified as free water with motions similar to ordinary water. In this model water molecules are exchanging rapidly between the two phases so that the observed NMR relaxation times represent a weighted average of relaxation in the two phases. These authors found that bound water represented approximately 0.3 g of $H_2O$ per g dry protein. They were unable to obtain detailed information about the dynamic state of the bound water but did show that its mobility must be much less than ordinary water.

In this study we have measured water proton NMR spin-lattice-relaxation times $T_1$ and $T_{1\rho}$ in both the laboratory and rotating frames, respectively, for samples consisting of aqueous dispersions of red cell membranes. The $T_1$ measurements have been found to be consistent with the previous measurements of Clifford et al. [12]. The $T_{1\rho}$ measurements have been found to be more sensitive to the dynamic state of membrane bound water although their interpretation is model dependent. The results give some support, we believe, to a dynamic model for membrane bound water in which translational motions rather than rotational motions are the dominant factor in $T_{1\rho}$ relaxation.

## METHODS AND MATERIALS

A phase-coherent pulsed NMR spectrometer used in this study has been described previously [13]. All measurements were made at a Larmor frequency of 23.3 MHz. $T_{1\rho}$ measurements were made by subjecting the sample to a $\pi/2$ pulse followed immediately by a second pulse of amplitude, $H_1$, and duration, $t$, which was phase shifted 90 °. $T_{1\rho}$ was obtained by monitoring the amplitude of the RF induction decay following the second pulse. The Larmor frequency in the rotating frame, $\omega_1$, was measured to $\pm$10 % from the length of a $6\pi$ pulse. $T_1$ data were obtained with the $\pi$-$t$-$\pi/2$ technique.

Red cell membranes were prepared from fresh human blood by hypotonic hemolysis in phosphate buffer, pH 7.4, using successive washes of 300, 150, 50, 30 and 25 milliosmolar buffer concentrations as described previously [16]. Buffers and

equipment were sterilized to prevent bacterial contamination. Samples for NMR measurements were prepared with 12.3 and 18.4 wt % solids. This was achieved by partial drying of pelleted membrane suspensions for varying lengths of time up to 6 h in a vacuum (100–200 $\mu$m) at 4 °C. Prior to beginning the vacuum drying step, the salt concentration was reduced about 10-fold in a final wash in order to prevent abnormally high salt concentrations in the partially dried sample. The wt % solids of each sample were determined by removing a portion of the prepared sample, drying it to completion, and determining the weight of water lost. This procedure was repeated on the entire sample after the NMR measurements were completed as a further check.

The membrane samples were transferred to small test tubes (12 mm outside diameter), sealed, and placed in the NMR sample probe for measurements. Sample temperatures were regulated with a Varian Temperature Controller to within $\pm 1/2$ °C.

## RESULTS AND ANALYSIS

Temperature dependent $T_1$ and $T_{1\rho}$ data for samples containing 12.3 and 18.4 wt % solids are plotted in Fig. 1. The uncertainty in these relaxation times is estimated to be $\pm 5$ %. All relaxation decay curves exhibited single exponential behavior in the time domain of our measurements.

The temperature dependence of $T_1$ and $T_{1\rho}$, and the $\omega_1$ dependence of $T_{1\rho}$ is similar to that which we have recently observed in muscle [13]. The results for muscle were consistent with a model in which water is exchanging between a bound (b) phase

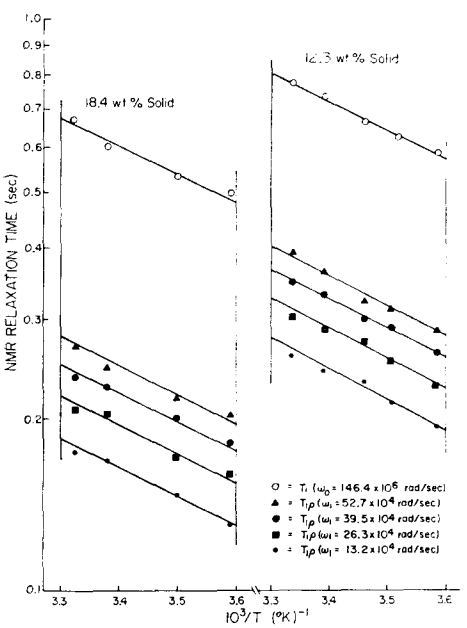


Fig. 1. Water proton NMR $T_{1\rho}$ and $T_1$ relaxation results for aqueous dispersions of erythrocyte membranes. The uncertainty in relaxation measurements is $\pm 5$ %. The uncertainty in temperature is $\pm 0.5$ °C. The uncertainty in $\omega_1$ is $\pm 10$ %.

with restricted motion and the remaining free (f) phase with unrestricted motions; the observed relaxation time, $T_i$, (the index $i$ is either 1 or $1_\rho$) being a weighted average of relaxation in the two phases,

$$\frac{1-x}{T_i} = \frac{1-x-cx}{T_{if}} + \frac{cx}{T_{ib}+R} \tag{1}$$

where $x$ = wt fraction of solids, $1-x$ = wt fraction of water, $c$ = g bound water per g of solids, $cx$ = wt fraction of bound water, $1-x-cx$ = wt fraction of free water, and $R$ = mean residence time of a water molecule in the hydration phase. Eqn 1 is not valid in the slow-exchange condition ($R \gg T_{ib}$) in which case there is effectively no exchange in the NMR time domain. When $R \ll T_{ib}$ we have the fast exchange expression used by Clifford et al. [12], i.e. $R$ can be neglected in Eqn 1.

Clifford et al. [12] argued convincingly that the temperature independence of their spin-spin relaxation ($T_2$) data in the range 0–50 °C was due to an intermediate exchange condition ($R \simeq T_i$). Since our $T_{1\rho}$ data show a temperature dependence similar to $T_1$, we will assume that the fast exchange condition applies ($R \ll T_{ib}$). Eqn 1 can thus be rewritten in the form $Y = mX+b$,

$$\frac{1}{T_i} = \left[c\left(\frac{1}{T_{ib}} - \frac{1}{T_{if}}\right)\right]\frac{x}{1-x} + \frac{1}{T_{if}} \tag{2}$$

Although the range of reciprocal temperatures covered was similar for the 12.3 and 18.4 % solid samples, no data were obtained at exactly the same temperature. To test the validity of Eqn 2 for our system we extrapolated the data to two reciprocal temperatures immediately outside the range covered in the study as shown in Fig. 1. In Fig. 2a we have plotted $1/T_i$ against $x/1-x$ at a reciprocal temperature, $10^3/T = 3.3\ (°K)^{-1}$. The $1/T_i$ values were taken from the extrapolated data in Fig. 1. Fig. 2b is a similar plot for $1/T_i$ values taken from the data extrapolated to $3.6\ (°K)^{-1}$.

Lines drawn through the data in Figs 2a and 2b appear to extrapolate to a common intercept which we identify with $1/T_{if}$; $T_{1\rho}$ is independent of $\omega_1$ and equal to $T_1$ for free water [14]. From the ordinate intercepts of the $T_1$ and $T_{1\rho}$ lines in Figs 2a and 2b we find $1.1 \leqslant T_{if}$ (s) $\leqq 1.5$ and $0.9 \leqq T_{if}$ (s) $\leqq 1.2$ for $10^3/T\ (°K)^{-1}$ equal to 3.3 and 3.6, respectively. While these values are substantially lower than the respective values of $\simeq 2.5$ s and $\simeq 4.0$ s for oxygen-free water, they compare favorably with the respective values of $\simeq 1.1$ and $\simeq 1.8$ s for water under an oxygen atmosphere [17]. Since no attempt was made to remove or regulate dissolved oxygen in our samples, we believe that the $T_{if}$ values obtained from Figs 2a and 2b suggest, at most, only a slight decrease in the average viscosity of free water in our samples as compared to ordinary water at similar temperatures. From the slopes of the lines in Figs 2a and 2b, their ordinate intercepts, and $c$ values (0.28 and 0.32 for $10^3/T = 3.3$ and 3.6, respectively) determined by Clifford et al. [12], we can calculate the corresponding values for $T_{ib}$. The results are listed in Table I. We realize that our identification of $T_{ib}$ would be strengthened by additional data at other wt % solids. However, the common intercepts found in Fig. 2 seems to support the model which we have used.

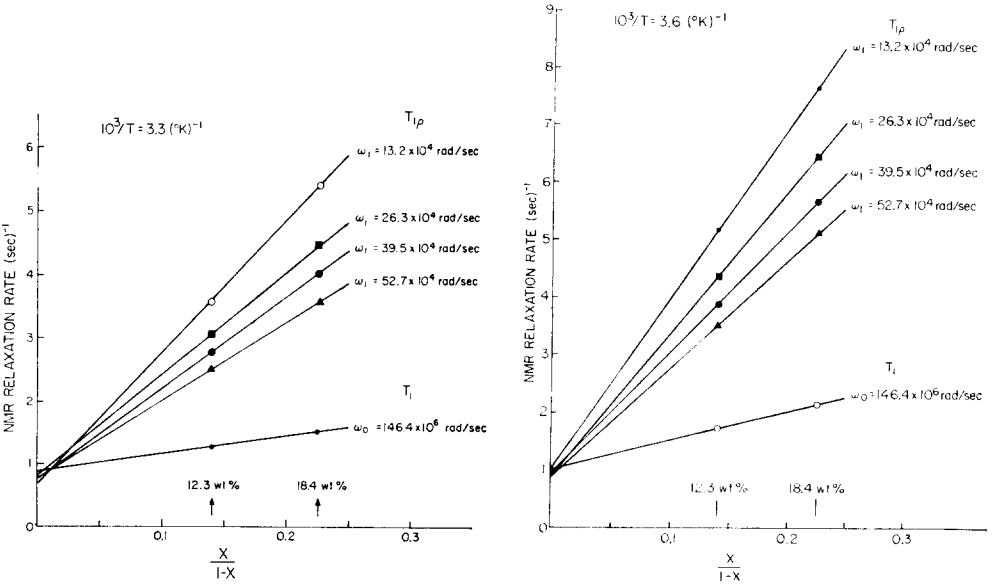


Fig. 2. Lines drawn through the data in Fig. 1 have been used to estimate $T_{1\rho}$ at reciprocal temperatures of $10^3/T = 3.3$ and 3.6 (° K)$^{-1}$. In 2a and 2b these estimates, converted to relaxation rates ($1/T_{1\rho}$), are plotted as a function of $x/1-x$ when $x$ is the wt % solids in the sample.

TABLE I

BOUND PHASE RELAXATION TIMES

Details of the calculation are given in the text.

| | $10^3/T = 3.3$ (°K)$^{-1}$ (ms) | $10^3/T = 3.6$ (°K)$^{-1}$ (ms) | $\omega_1$ (· $10^4$ rad/s) |
|---|---|---|---|
| $T_1$ | 91.9 | 64.0 | |
| $T_{1\rho}$ | 22.2 | 16.6 | 52.7 |
| $T_{1\rho}$ | 19.0 | 15.0 | 39.5 |
| $T_{1\rho}$ | 16.3 | 12.8 | 26.3 |
| $T_{1\rho}$ | 13.3 | 10.7 | 13.3 |

## DISCUSSION

Fig. 1 shows that $T_{1\rho}$ is less than $T_1$ and is a function of $\omega_1$. $T_{1\rho}$ also increases with increasing temperature which is characteristic of the motional narrowing or short correlation time limit, $\omega_1\tau \lesssim 1$ [13]. However, $\tau$ values (correlation times) which produce this behaviour in $T_{1\rho}$ would be expected to give rise to a decrease in $T_1$ with increasing temperature ($\omega_0\tau > 1$) which is opposite to the behavior observed. We have observed similar behavior for muscle water which was found to be consistent with a distribution of rotational correlation times with $T_1$ and $T_{1\rho}$ being sensitive to different $\tau$ values in the distribution. While a similar distribution may

characterize the water bound to red cell membranes, we would like to explore in this discussion an alternative explanation for the behavior of $T_{1\rho}$.

In our previous work [13] and that of others [10, 11] it has generally been assumed that relaxation of bound water protons is dominated by intramolecular dipolar interactions modulated by rotational motions of water molecules rather than intermolecular interactions modulated by translational motions. This is a logical assumption since intramolecular interactions are dominant in liquid/water relaxation; this situation in liquid water is largely the result of the strong coupling of rotational and translational motions of molecules [16]. Evidence of preferential orientation of water molecules near biologic and clay interfaces [17] indicates, however, that translational and rotational motions in bound water may not be so strongly coupled; i.e., they could be characterized by different correlation times. This uncoupling could, we believe, lead to a situation in which intermolecular interactions modulated by translational diffusive motions could dominate NMR $T_{1\rho}$ relaxation in membrane-bound water.

In the short correlation time limit, $\omega_1\tau < \omega_0\tau < 1$, suggested by the temperature dependence of our results in Fig. 1, it is generally expected that $T_1$ and $T_{1\rho}$ will be independent of $\omega_0$ and $\omega_1$, respectively. However, Harmon and Muller [18] have shown that proton spin lattice relaxation can exhibit a dependence upon Larmor frequency even in the short correlation time limit if the intermolecular, rather than intramolecular, interaction dominates the relaxation process.

Burnett and Harmon [19] have found that glycerol $T_{1\rho}$ relaxation is dominated by intermolecular interactions. They have shown that the intermolecular mechanism gives rise to a unique linear dependence of the relaxation rate upon $\omega_1^{\frac{1}{2}}$. A plot of the relaxation rate $(1/T_{1\rho})$ versus $\omega_1^{\frac{1}{2}}$ would be expected to have a slope which is a function of the translational diffusion constant:

$$\frac{d(1/T_{1\rho})}{d(\omega_1^{\frac{1}{2}})} = -\frac{\sqrt{2}\,\gamma^4 h^2 N}{80\pi D^{\frac{1}{2}}} \tag{3}$$

where $\gamma$ is the gyromagnetic ratio for protons, $h$ is Planck's constant, $N$ is the number of protons spins per cm$^3$, and $D$ is the translational diffusion constant. Theory predicts [18, 19] that this relationship should hold when $\omega_1\tau > 10^{-4}$, which for small molecules generally occurs when the translational diffusion constant is less than $10^{-8}$ cm$^2$/s.

In liquid water, $D$ reaches a minimum value of $\approx 10^{-5}$ cm$^2$/s at 0 °C. However, it is quite possible that water bound to red cell membranes might exhibit a diffusion constant of $10^{-8}$ cm$^2$/s or lower. To explore this possibility we have plotted, in Fig. 3, the reciprocal of the bound water $T_{1\rho}$ values listed in Table I against $\omega_1^{\frac{1}{2}}$. We see that the relaxation rate is a linear function of $\omega_1^{\frac{1}{2}}$. If we assume that $N$ in bound water is the same as in free water ($6.75 \cdot 10^{22}$ spins/cm$^3$) [15], we can calculate a diffusion constant using Eqn 3 and the data in Fig. 3. We find $D$ values $\approx 2 \cdot 10^{-9}$ cm$^2$/s similar to those found for glycerol near 277 °K [19]. These results give some support to the idea, suggested previously by Blicharska et al. [20] and Glasel [21], that proton NMR relaxation for bound water may be dominated by an intermolecular mechanism. In the discussion that follows we point out possible circumstances which would be consistent with a bound water diffusion constant near $10^{-9}$ cm$^2$/s.

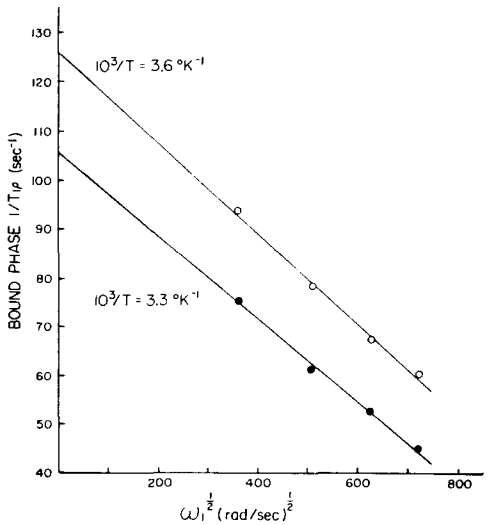


Fig. 3. Bound phase relaxation rates ($1/T_{1\rho}$) calculated from $T_{1\rho}$ values in Table I are plotted against $\omega_1^{\frac{1}{2}}$.

Recent studies have shown that the lipids in membranes are able to diffuse laterally with diffusion constants of the order of $10^{-8}$–$10^{-9}$ $cm^2/s$. [22]. At least some membrane proteins are also able to diffuse laterally; for example, the diffusion constant of rhodopsin in the visual receptor membrane was reported to be $4 \cdot 10^{-9}$ $cm^2/s$ [23]. A value of $1–2 \cdot 10^{-9}$ $cm^2/s$ has also been reported for surface antigens of rat muscle fiber [24]. Recently, a red cell membrane protein was estimated to have a lateral diffusion constant of several orders of magnitude lower than the above values, namely $3 \cdot 10^{-12}$ $cm^2/s$ [25]. However, the latter measurement was primarily for a rather large (mol. wt 100 000) and very tightly bound membrane protein, and thus may not be representative of most other membrane proteins.

We have no evidence of whether NMR-detectable bound water is primarily associated with erythrocyte membrane proteins, lipids, polysaccharides, or all three. The bound water diffusion constants calculated from our $T_{1\rho}$ data are in the general range of the lateral diffusion coefficients most commonly reported for membrane lipids and proteins. It is conceivable that diffusion of membrane-bound water may be modulated by the motion of membrane components. Conversely, the interesting possibility exists that an ice-like network of bound water layers coating the polar surfaces of the membrane may control the movement of hydrated proteins and lipid head groups to the extent that they extend into such a bound-water phase.

There is yet another very interesting comparison to be made with our NMR-estimated value of $\approx 2 \cdot 10^{-9}$ $cm^2/s$ for the self-diffusion constant of membrane-bound water and this is with the well established values for diffusional transport of water across biological membranes. The diffusion constant ($cm^2/s$) for water transport across unit membrane area is determined from the corresponding membrane water permeability coefficient (cm/s) by simply multiplying by the thickness of the transport barrier (cm). For the adult human red cell membrane, water permeability coefficients have been reported to be $3–5 \cdot 10^{-3}$ cm/s [26]. Multiplying this by a membrane thick-

ness of 100 Å = $10^{-6}$ cm yields transport diffusion coefficients of $3–5 \cdot 10^{-9}$ $cm^2/s$. If the thickness of the transport barrier is taken to be less than 100Å, say about 25 Å, through the bound water, the subsequent transport diffusion coefficients would be approximately $1 \cdot 10^{-9}$ $cm^2/s$. These values are remarkably close to our bound water diffusion constant of $2 \cdot 10^{-9}$ $cm^2/s$, while being four orders of magnitude lower than the self-diffusion constant in liquid water ($\approx 10^{-5}$ $cm^2/s$). There is thus the suggestion that membrane bound water may represent the rate-limiting barrier to aqueous transport. Further evidence for such a mechanism comes from the activation energy data of Hays [6] for diffusional water transport across the luminal membrane of the toad bladder, corrected for unstirred layers and other extraneous transport barriers.

In summary, then, we know from our previous studies [7] of membrane water binding that tightly bound water represents a major membrane component ($\sim$ 20–30 g $H_2O$/100 g dry membrane) and that the first few layers of this water have binding energies comparable to and greater than water in ice. The present study suggests that the mobility of the membrane-bound water is four orders of magnitude slower than bulk liquid water, is approximately similar to the lateral mobility of membrane proteins and lipids, and is almost identical to the rate of water transport across the membrane. Other workers have demonstrated that about 20–30 g of membrane-bound water per 100 g dry membrane are required to stabilize the lipid bilayer structure within membranes [1, 2]. We thus suggest that membrane-bound water is of fundamental importance to membrane structure, may control the lateral diffusion of hydrated membrane components in the plane of the membrane, and may be the rate-limiting barrier for aqueous transport across the membrane. Any structural model of the biological membrane may thus be incomplete if it fails to include the bound water components.

ACKNOWLEDGEMENTS

From the Bureau of Medicine and Surgery, Navy Department Research Subtask MR000.01.1085. The opinions and statements contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or of the Naval Service at large. Most of this work was done while A. S. Schneider was associated with the Laboratory of Neurobiology, National Institute of Mental Health, Bethesda, Md. We would like to thank Professor Martin Sonenberg for useful comments on the manuscript.

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