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C F HAZLEWOOD ET AL. MAY 85 N00014-76-C-0100

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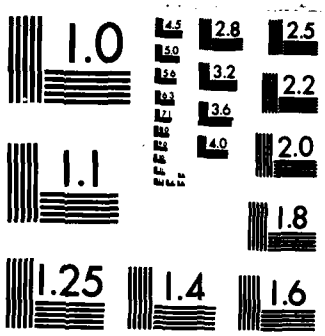

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Final Progress Report for Office of Naval Research Contract No. N00014-76-C-0100

Title: Physical Chemical State of Water in Living Cells

Sub-Project A: Physical Chemical State of Water in Living Cells and Tissues

Sub-Project B: Functional Role of Ion-Water-Protein Interactions in Excitable Cells

Co-Principal Investigator: C.F. Hazlewood, Ph.D.
Professor
Department of Physiology
Baylor College of Medicine
Houston, Texas 77030

Co-Principal Investigator: Donald C. Chang, Ph.D.
Assistant Professor
Department of Physiology
Baylor College of Medicine
Houston, Texas 77030

Co-Investigator: Robert L. Vick, Ph.D.
Professor and Acting Chairman
Department of Physiology
Baylor College of Medicine
Houston, Texas 77030

MAY 1985

Final Progress Report for Office of Naval Research Contract No. N00014-76-C-0100

Title: Physical Chemical State of Water in Living Cells

Sub-Project A: Physical Chemical State of Water in Living Cells and Tissues

Principal Investigator: Carlton F. Hazlewood, Ph.D.
Professor
Department of Physiology
Baylor College of Medicine
Houston, Texas 77030

Co-Investigator: Robert L. Vick, Ph.D.
Professor and Acting Chairman
Department of Physiology
Baylor College of Medicine
Houston, Texas 77030

FINAL PROGRESS REPORT THROUGH 1982

I. Overall Research Program (Specific work tasks) outlined in the proposal (include)

A. Physical properties of water in tissues, cells and subcellular organelles:

1. Tissues and cells: to characterize the T_1, T_2, and D of water protons in brain and striated muscle as a function of development and growth.

2. Cellular organelles: to determine T_1, T_2, and D of water hydrogens in mitochondria isolated from normal and ischemic hearts.

B. Physical properties of water in the brine shrimp Artemia.

1. To determine the T_1, T_2, and D of water hydrogens as a function of hydration and temperature.

2. To determine the T_1, T_2 and D of isotopes of water (^2H and ^{17}O) as a function of hydration and temperature.

C. Effects of hemorrhagic shock on the physical properties of tissue water.

Relaxation times and diffusion coefficients for water protons, hydrogen and isotopes of water were determined. Cont'd Pg. II-1

II. Report of Progress

A. Measurements of the relaxation times (T_1 and T_2) and the diffusion coefficient (D) of water protons. (These measurements are fundamental to each of the work tasks.)

1. Condition of our NMR system.

a. Operation. Unlike the past year, our NMR spectrometer was operational for the bulk of the past year. Minor down time was incurred due to problems with the General Radio Frequency Synthesizer and the periodic deenergizing of the magnet for yearly maintenance. This maintenance consists of re-evacuating the outer liquid vacuum jackets between the helium and liquid nitrogen dewars.

b. New developments in progress.

(1) Hardware. The new Nicolet digital oscilloscope has been purchased and is being interfaced to the spectrometer computer. Once the software development is complete, the Nicolet will provide digitized waveforms for storage on disk, out-put of the waveform to an xy plotter and most important, the ability to resolve frequency signals an order of magnitude faster than our present system allows.

Hardware for the development of a temperature control unit has been ordered. A preliminary design for this unit has been developed. Trials of the first temperature control unit are projected for January 1983.

(2) Pulsed field gradient unit. The diffusion coefficient is an important parameter for characterizing the state of water in biological systems. A nuclear magnetic resonance method of diffusion measurement by the pulsed field gradient technique has been developed. A 90-τ-180 spin-echo sequence is used with one gradient pulse located on each side of the 180 rf pulse. Information on restricted diffusion is obtained by varying the measuring time, which is determined by the separation of the gradient pulse pair. Measuring times greater than 2 msec are possible with the present apparatus. Maximum magnetic field gradients of 110 gauss/cm are applied to cylindrical sample volumes of 1 cm diameter and 0.5 cm in height. This permits the measurement of diffusion coefficients as low as 10^{-7} cm^2/sec. The measurement process is software supported and includes digital data aquisition.

(3) Software. Software is currently being developed to utilize the phase detector for automated data aquisition. This will increase our signal sensitivity by a factor of 2 or more and eliminate some of the data reduction currently done by hand.

2. Data obtained since last years report.

a. Measurements of the diffusion coefficient. Within the frame work of the current program, several contractors are conducting experiments which are complimentary to one another. For example, Dr. G.N. Ling (ONR contract # N00014-71-C-0178) is conducting studies on polyethylene oxide (polyox) water solutions in which he is determining the relaxation times T_1 and T_2 of water protons. From these studies, he is calculating the correlation time (τ_c) of the water protons. In collaboration with Dr. H.E. Rorschach (ONR contract # N00014-79-C-0492) we are, using NMR and quasielastic neutron scattering (QNS) techniques, determining the diffusion coefficient and calculating the τ_c of water protons in various biological and polymer systems. Such studies allow the determination of various physical properties of water by different techniques and, obviously, ways to cross-check our determinations. (A summary of a few of our findings will be given in the summary section of this report.) In conducting our studies, we thought it advantageous to add a simple polymer system such as polyox. Ling has already demonstrated that polyox-water mixtures cause the entropic exclusion of biological important ions such as sodium---thus, a simple polymer system mimics a fundamental biological phenomenon. We have, therefore, integrated the study of this polymer into this work task.

We have measured the diffusion coefficient (by the pulsed gradient technique) of protons in poly ethylene oxide-water system (polyox solution) as a function of concentration

using a pulsed field gradient NMR technique. The range of concentrations studied were from pure water up to 1.2 gm polyox /gm H_2O. Near zero polyox concentration, the slope of the D vs. concentration curve is quite large. As the concentration increases, the slope decreases, and at 1.2 grams of polyox per gram of water the diffusion coefficient (D) is reduced by a factor of about 6 from the value at zero concentration. These data compare well with those obtained by Rorschach utilizing the QNS technique, and between the two technologies we should be able to quantitatively determine the effects of macromolecular obstruction on D.

b. Cellular organelles. This part of work task number 1, has been expanded, because it was recognized that it might be possible to test crutial aspects of the fundamental biological theories that provide the fundamental framework of our concepts of the living state. The following includes: (1) a statement of the rationale for this work; (2) brief mention of the methods used; and, (3) experimental findings.

(1) Rationale. Essentially, all methods and proceedures utilized to determine the exact concentrations and/or activities, of the ions and water within cells and their smaller compartments requires that assumptions be made about the physical state of the ions and water. For example, it is often assumed that the ions (particulary sodium [Na] and potassium [K]) are dissolved in essentially all the cellular water. With this assumption, a typical mammalian cell would have an intracellular concentration of 140-175 milliequilvalents per liter (mEq/L) for K and 10-40 mEq/L for Na. These concentrations are considerably different from those found in extra-cellular fluids which bath these cells (i.e., 5 mEq/L for K and 140 mEq/L for Na). The mechanism more frequently proposed to explain these assumetries in intra - and extracellular ionic concentrations is one that assumes the existence of membrane situated, energy requiring pumps. At the same time, however, alternate hypothesis have been proposed to explain the unequal distribution of ions between the inside and outside of cells. When utilizing these alternate hypothesis, in general, or the association-induction (AI) hypothesis in particular, the assumptions: (1) that the ions are dissolved in most or all of the cellular water; and, (2) that there exist membrane situated energy requiring pumps are relaxed. In their place, assumptions are made that the ions are selectively adsorbed to charged sites in the cellular macromolecules and that the physical properties of cellular water differs from that of ordinary bulk or extracellular water. Therefore, in the case of the conventional view, the membrane is essential for the establishment of cellular ionic gradients; whereas, in the case of the latter view, the cellular surface structures may be important but the bulk of the cytoplasm in responsible for the development of ionic gradients.

The different mechanism proposed, within the context of these divergent views, to explain the unequal distribution of ions between the inside and the outside of the living cells or between organelles and the cytoplasm, offer an excellant opportunity for experimental testing. The conventional view predicting that the integrity of the membrane is required for the development of ionic gradients and the A-I hypothesis predicting that it should be possible to develope ionic gradients without explicit membrane structures. It, therefore, seems reasonable that a biological preparation free of membrane structures would serve as a useful vehicle to test these views. The subcellular organelles seemed to be likely candidates; and, the cellular nucleus, isolated by detergent treatment, was chosen for our first studies.

The choice of the nucleus as the organelle of study was prompted by our earlier studies of chromatin structures in which the polarization microscope was used. In these studies, it was found that interphase nuclei exposed to a total monovalent ionic concentration 160-220 mEq/L (i.e., the calculated monovalent cation concentration in the cytoplasm of the lymphocyte) induced birefringence. Exposure of the nuclei to total cation concentrations of 70 mEq/L or less did not result in nuclear birefringence. These findings led to the conclusion that the chromatin structures would be altered in the intact cell if the cations were freely dissolved in the nuclear and/or cytoplasmic water.

(2) Methods. - Thymus lymphocytes were isolated in calf serum, centrifuged (500xg) and resuspended in a solution containing 0.01 M Tris-HCl buffer (pH 7.2); 0.25 M sucrose, 3mM $CaCl_2$ and 3 mM Mg Cl_2 (here after referred to as the TSCM solution). All procedures were conducted at 25° C. The thymus lymphocytes in TSCM solution were then treated with 0.2% Triton X-100 or 0.2% Brij 58 detergents for 15, 20, or 30 minutes. The cells, so treated, were then centrifuged at 45,000 xg. The nuclear pellet of each was then analysed for water, sodium, and potassium concentration.

(3) Experimental findings. It should be noted that electronmicrographs were made of the various preparations. All membranes were absent within 5 minutes of detergent treatment. In the following table the data on the potassium to sodium ratio is given.

TABLE

K^+/Na^+ Ratios in detergent isolated nuclei of thymus lymphocytes

	TSCM*	Triton	Brij
5 minutes	2.98±0.10 (8)	0.69±0.03 (16)	2.36±0.04 (16)
0 minutes	3.10±0.10 (8)	0.85±0.04 (12)	1.71±0.06 (12)
0 minutes	3.18±0.06 (8)	0.63±0.05 (11)	0.96±0.12 (12)

NOTE: All values are given as mean ± S.E.M.* The column labeled TSCM contains data on intact lymphocytes (nucleus + cytoplasm) exposed to the solution without detergents. With Triton and Brij treatment, only membraneless nuclear material remained. The number samples analysed are in parenthesis.

The selectivity of K^+ over Na^+ was maintained in TSCM treated lymphocytes and in Brij treated lymphocytes. The intactness of the nuclear membrane was not required to maintain the potassium selectivity. The treatment of the lymphocytes with Triton and prolonged treatment with the milder detergent (Brij) led to a reversal of the selectivity. According to Schliwa et al (Proc. Nat. Acad. Sci. 78:4320, 1981), Triton treatment leads to a loss of proteins found in the ground substance (i.e., the microtrabecular structure) within 5 minutes; but, for the same results, requires 30 minutes treatment with Brij. It appears from these studies that soluble nucleoproteins are dynamically involved in ionic selectivity.

B. Physical properties of water in the brine shrimp Artemia.

1. T_1, T_2 and D of water hydrogens as a function of hydration.

See attached reprints. Archives Biochem. Biophys. 210:517, 1981, Crybiol. 19: 306, 1982.

2. T_1, T_2 and D of isotopes of water as a function of hydration.

a. Rationale. The studies of ^2H and ^{17}O relaxation times complement our studies on proton resonance in the following ways: (1) Both deuterium and ^{17}O nuclei have quadrupole moments hence the dominant relaxation of deuterons in the water molecule is provided by the quadrupole interactions. Similar is the case for ^{17}O nuclei in $H_2{}^{17}O$ used for hydrating these cysts; (2) Because of the fast relaxation provided by the quadrupolar interactions, the relaxation is intra-molecular rather than intermolecular. Thus the complications of ^1H NMR relaxation do not arise; and, (3)

In view of the dominant quadrupolar relaxation, the effects of paramagnetic ions on the water relaxation are negligible because of the high concentration of the paramagnetic ions required to have an effect on the observed quadrupolar relaxation. Relaxation is therefore controlled by a single intramolecular relaxation.

In view of the above arguments, it is possible to relate the nuclear quadrupolar relaxation rate ($1/T_1$) to the correlation time τ_c for the motion of water. It is therefore anticipated that it will be possible to obtain τ_c for the motion of water molecules in the brine shrimp Artemia. It should then be possible to correlate these results with the correlation times obtained from the neutron scattering studies.

The τ_c values can be obtained either from the T_1/T_2 ratio or the frequency dependence of these relaxation times. It is proposed to obtain these correlation times at each hydration by both methods. This would enable us to determine if there is any dependence of τ_c on hydration.

b. Results. The preliminary deuterium relaxation times at four hydration values for the brine shrimp are shown in the enclosed table. It may be noticed from the table that (1) the relaxation times of ^2H in the hydrated cysts are a function of hydrations as in the case of the proton relaxation; (2) T_1/T_2 ratio increases with the decrease in hydration; and, (3) even at full hydration $T_1/T_2 \simeq 4.2$ thus indicating the reduced mobility of water.

Preliminary Deuterium NMR Results on Brine Shrimp
[ν = 28 MHz]

Hydration gms of D_2O/gm of dried solids	T_1 (msec)	T_2 (msec)	T_1/T_2
1.52	111	26.3	4.22
1.04	78.4	16.7	4.70
0.88	65.2	12.7	5.13
0.72	53.8	10.3	5.22

I. Summary of an evaluation of data obtained by nuclear megnetic resonance, quasielastic neutron scattering and dielectric relaxation techniques.

The results obtained from the use of each of these technologies are sufficiently complementary that, when the data obtained from each (on a specific system) are combined it should substantially improve our understanding of the molecular properties water and its interaction with macromolecules and ions. The QNS technique offers a probe that is capable of a spatial resolution of the order of 1-10 Å and a time resolution of 10^{-7} to 10^{-12} second. circumvented two major problems inherent in NMR studies of biological and physical model sytems. In the specific QNS studies of water in brine shrimp it has been found that the data can be well described by a combination of translational and rotational diffusion (D_r). For example, $D = 0.29\ D_o$, $\tau_c = 3.9\ \tau_o$ (where D_o and τ_o represent the diffusion coefficient and the correlation times of pure water); and $D_r = 6X10^9\ sec^{-1}$. The line width of the QNS spectrum (Γ) plotted as a function of the square of the momentum transfer (Q^2) may be described in the following manner.

$$\Gamma(Q^2) = \Gamma_r + \Gamma_\tau \qquad (1)$$

where Γ_r and Γ_τ are respectively, the rotational and translational contribution to the line width. Assuming there is a statistical distribution of jump-lengths, then

$$\Gamma_\tau\ (Q) = 2Q^2D/(1+Q^2D\tau) \qquad (2)$$

From the data and equations given above it was found that the value of D (by QNS) is consistent with the NMR diffusion measurements and further shows that the reduction in D is not due to barriers or compartments, but due to "stabilization" of the water by the macromolecule. These findings are similar to those for agarose except that they are more pronounced due to the relatively high concentration of solids in the Artemia and the larger reduction in D.

The dielectric dispersion for water in Artemia cysts has been measured by Clegg, et al. Previous measurements on pure water have shown that the dielectric relaxation time is $T_D = 0.93x10^{-11}$ sec at 20C. The "microscopic" relaxation time T_μ is thus $\frac{T_D}{1.5}$, which is related to the rotational diffusion coefficient by $D_r = \frac{1}{2T_\mu} \simeq 8.1x10^{10}\ sec^{-1}$. The value $D_r = 6x10^9\ sec^{-1}$ measured by neutron scattering for the Artemia cysts is thus lower by a factor of $\simeq$ 13, which is consistent with a similar reduction seen in relatively "dry" systems, such as hemoglobin and lysozyme. The value of T_D reported by Clegg et al. is not reduced by such a large factor but we might expect their results to be strongly influenced by exchange. (The values of T_D obtained for "wetter" biological systems, which show little difference from pure water, are not convincing evidence of the absence of an altered water phase. The dielectric properties would be dominated by a small fraction of free water, if rapid exchange occurs, in the same way that the value of T_2 for biological systems is influenced by a small fraction of a rapidly relaxing phase.)

The above values for D and D_r obtained from the neutron spectra are also consistent with the NMR relaxation time T_1. The value of T_1 for cysts at 1.2 g/g water content is ≃ 250 msec, about a factor of 12 less than that of pure water. For pure water, T_1 is determined by the rotational and translational motion:

$$\left(\frac{1}{T_1}\right)_{H_2O} = \left(\frac{1}{T_1}\right)^0_{\text{rot}} + \left(\frac{1}{T_1}\right)^0_{\text{trans}},$$

where $$\left(\frac{1}{T_1}\right)^0_{\text{rot}} \simeq 2\left(\frac{1}{T_1}\right)^0_{\text{trans}}$$

The neutron results show that the rotational contribution to $(1/T_1)$ for Artemia cysts is about 13 times that for pure water, since the value of D_r is reduced by this factor. The translational contribution is increased by a factor of ≃ 4. This gives an expected reduction in T_1 for Artemi of a factor of ≃ 10, which agrees rather well with the observed values of T_1.

It is concluded that the neutron, NMR and dielectric relaxation measurements on Artemia cysts are all consistent with a picture in which the water has strongly reduced translational and rotational diffusion coefficients that are not due to obstructions, compartments or exchange with minor phases, but are instead an intrinsic feature of the intracellular water.

In order to test further these interpretations, we have begun a series of studies of polyox solutions in which we are comparing NMR and QNS data. This study is underway, but the QNS data are still being analyzed and some additional studies are planned. To date the measurements of D in polyox by way of these two technologies compare well, however, a quantitative comparison must await further experiments and data analysis.

FINAL PROGRESS REPORT THROUGH 1984

II. Summary of Progress 1983-84

A. Mechanisms of NMR Relaxation: Hypothesis of Protein Dynamics

1. The relaxation rate T_1^{-1} for water in many biological tissues and polymer solutions is characterized by a weak frequency dependence, $T_1^{-1} \propto \omega^{-\frac{1}{2}}$. We propose a mechanism for the water-polymer interaction that leads to this frequency-dependence in a natural way without the introduction of ad-hoc assumptions of water fractions with arbitrary distributions of correlation times. The mechanism involves the wave-length-dependent correlation time for the fluctuations of the highly-damped excitation modes of a long flexible chain polymer.

2. We have examined the dependence of the NMR longitudinal relaxation rate in Brine Shrimp (Artemia) with respect to: (1.) the proton NMR frequency over the range .01 - 200 MHz and 2.) four hydration levels between 0.12 - 1.3 grams water/gram dry solid. Relaxation rates T_1 for frequencies between .01 and 50 MHz were obtained at the laboratory of Dr. S.H. Koenig (IBM T.J. Watson Research Center) using a field-cycling technique. Additional inversion recovery T_1 measurements extending to 200 MHz were obtained from fixed-field magnets located at Rice University and Baylor College of Medicine.

   The results have been interpreted in terms of a polymer dynamics model based on the fluctuation motion of long chains in solution. This model predicts a frequency-dependent relaxation rate proportional to $\omega^{-\frac{1}{2}}$ at high NMR frequencies, which we observe in the Brine Shrimp between 5 and 200 MHz. The low frequency behavior is influenced by a correlation time, τ_c, associated with the longest polymer chain-length. For $\omega \ll 1/\tau_c$, the relaxation rate is independent of frequency.

   We have fit this model to the Artemia data with three adjustable parameters: an amplitude, a single correlation time (τ_c), and an additive constant. This constant relaxation rate term is due to the fraction of water protons not directly involved with the polymer fluctuations. Based on the fit results, its value is 2. sec^{-1}, about 6 times the rate for bulk water. Best fit values for τ_c vary from 0.1 usec for the lower hydrations to 1. usec at the highest hydration level (1.3 g/g). A general feature of the fit common to all hydrations is close agreement at high frequencies. The fit at low frequencies is less successful, especially at high hydrations (see figure 1).

Investigation of the low-frequency spectrum has led to two possible considerations. We can assume the Artemia is composed of a distribution of polymer chain-lengths (two in the simplest case), or we can consider mechanisms that would contribute to the relaxation rate primarily at low frequencies. Models with ad-hoc distributions of correlation times, or chain-lengths, yield a variety of frequency dependences and little physical insight. An additional relaxation mechanism is that due to translational diffusion within the hydration water. Abragam's model for this mechanism is based on the dipole interaciton of spins undergoing random-walk motion described by the macroscopic diffusion equation. We apply this model to those water protons closely associated with the polymer chain (in the hydration layer). Escayne (references 13-15 in original proposal) recognized that the longer correlation times associated with the diffusional motion of hydration or "bound" water could contribute to a frequency-dependence of the relaxation rate in the low NMR frequency range.

We have incorporated this model into the fitting function (adding two more adjustable parameters) and obtained good agreement over the entire frequency range at each hydration (see figure 2). The best fit value of the correlation time in the translational diffusion term is nearly the same for all four hydrations (.02 usec). The large temperature dependence of T_1^{-1} seen at low frequencies may indicate a thermally-activated process and further support a diffusion mechanism.

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B. Hemorrhagic Shock

1. Hemorrhage adequate to lower mean arterial blood pressure to 50 mm Hg or less results in a reduction of cerebral blood flow (CBF). The reduction in CBF from hemorrhage can be so severe as to make the brain ischemic. Such an ischemia of brain may lead to or cause respiratory and/or circulatory complications associated with hypovolemic shock. Our research efforts for the past two years have been directed toward developing an animal model that closely simulates hypovolemic shock encountered in a combat situation. In our preliminary studies we observed time-dependent reversible changes in the nuclear magnetic resonance (NMR) relaxation tims of water hydrogen nuclei in the brainstems of rabbits in response to nonlethal hemorrhage. Results of our studies indicate the NMR techniques would be valuable in acquiring a basic understanding of the role of the CNS in hypovolemic shock, in developing procedures for early detection, and for following the response to therapy.

FIGURE 1 : POLYMER DYNAMICS MODEL

BRINE SHRIMP : TWO HYDRATIONS

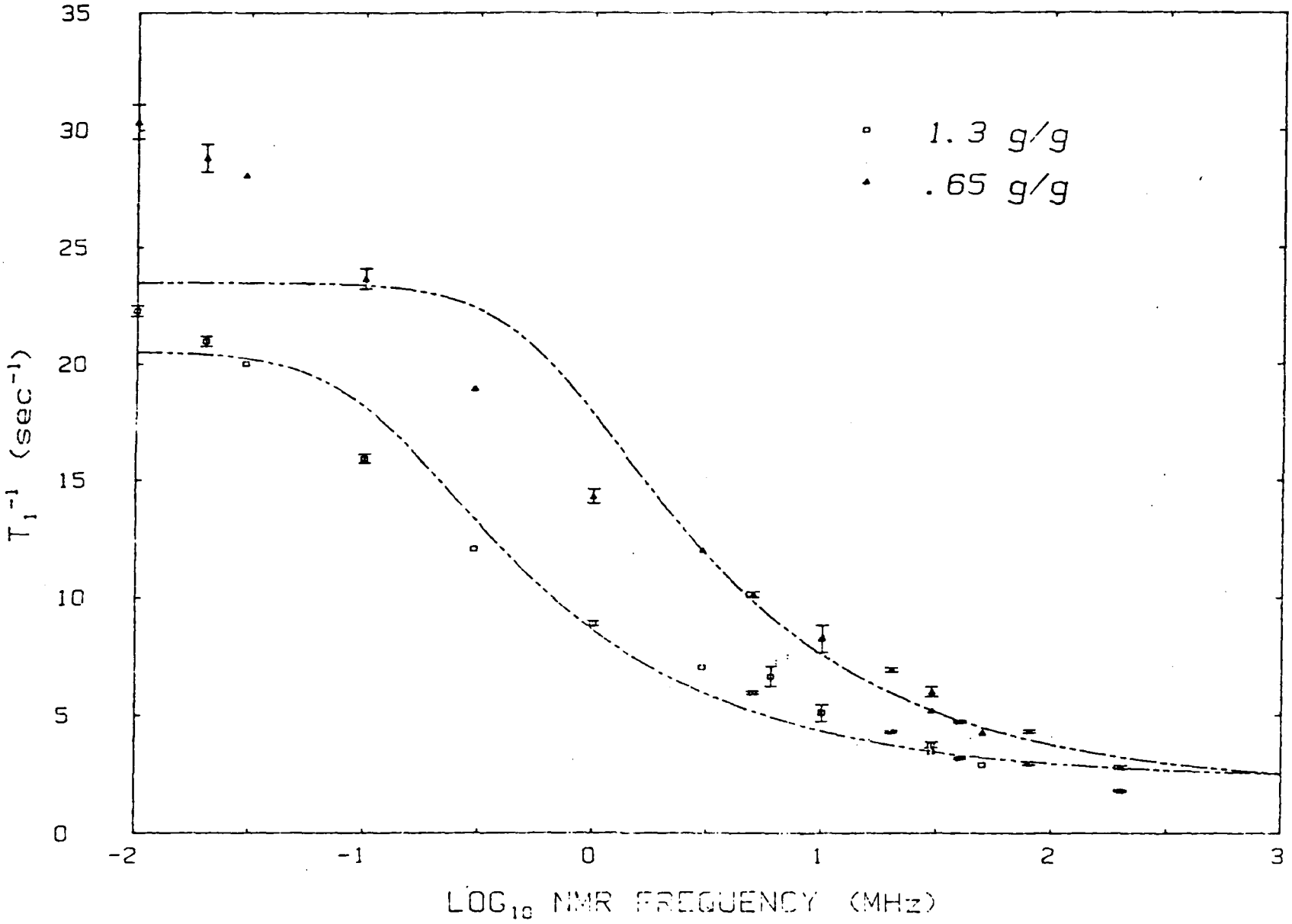
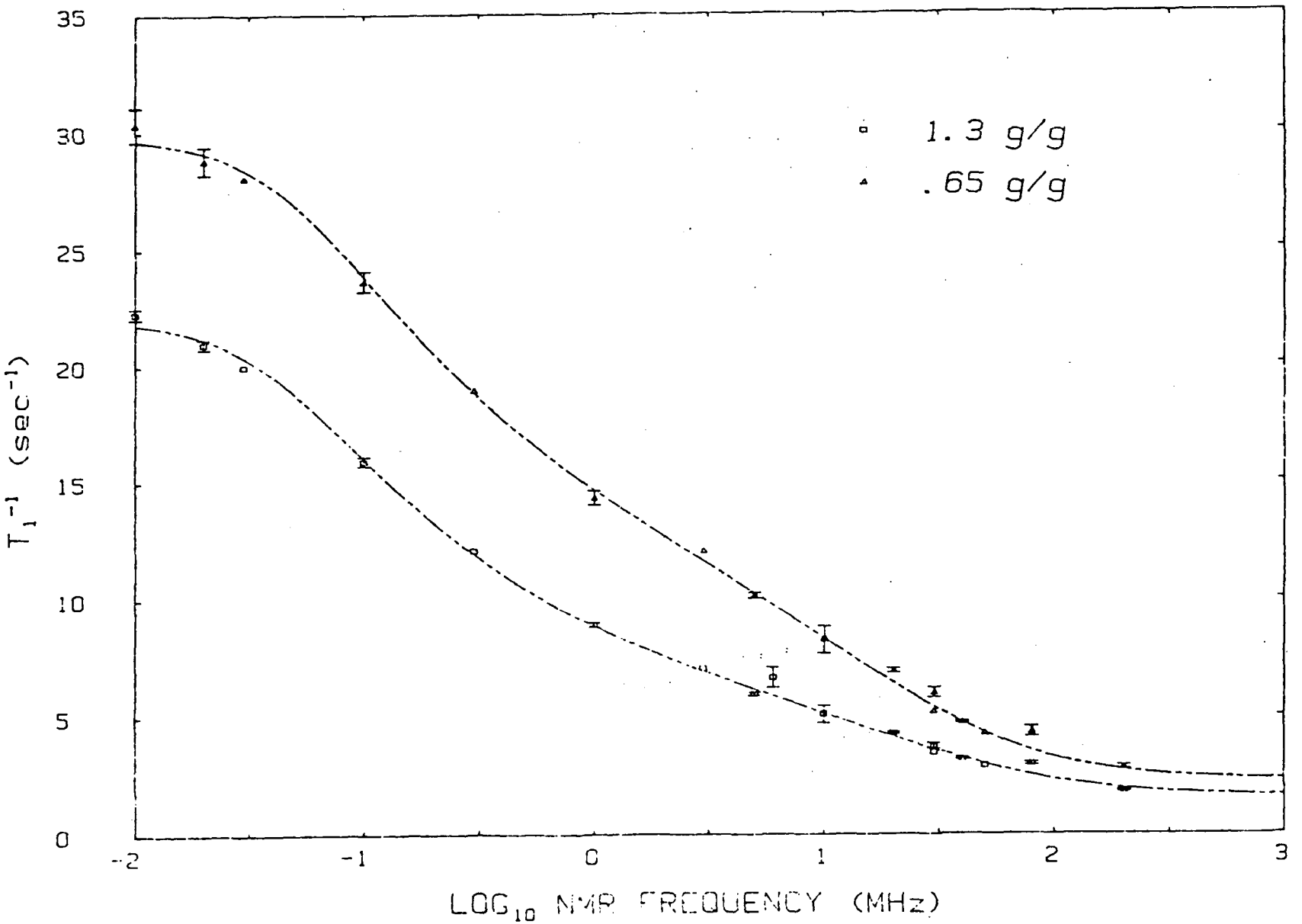


FIGURE 2 : WITH DIFFUSION TERM ADDED

BRINE SHRIMP : TWO HYDRATIONS



FINAL TECHNICAL REPORT

TITLE OF RESEARCH:

Biophysical Study of Cellular Water and Ions in Excitable Cells

ONR CONTRACT NO. N00014-76-C-0100, Subproject B

SUBTITLE:

Functional Role of Ion-Water-Protein Interactions in Excitable Cells

January 31, 1980 through October 31, 1984

BY

Donald C. Chang, Ph.D.

cont'd

FINAL TECHNICAL REPORT

is Summarized. Scientific findings include:

SUMMARY OF SCIENTIFIC FINDINGS

1. Effects of the ionic environment on the gating properties of the excitable ionic channels

A. Effect of internal $Cs^{(+)}$ ions on the early and late conductance in squid axon

Perfusion of Cs^+ ions inside the squid axon can result in a suppression of the late current (or "K^+ current"). This Cs^+ action is time and voltage dependent and varies with the concentration of Cs^+ ions. For an axon perfused with 400 mM K^+ and 100 mM Cs^+ inside, the Cs^+ starts to suppress the late current when the membrane potential (V) reaches approximately 40 mV. At 5°C, the effect starts to take place at roughly 2 msec after the axon is depolarized and saturates at t = 6 msec. In addition to affecting the late conductance, it is found that internal Cs^+ can also partly remove the inactivation of the early conductance (or "Na^+ conductance"). Interestingly enough, this effect is also time, voltage, and concentration dependent. To investigate whether the Cs^+ effects on the early conductance and the late conductance are related, we examined their dependence on each of the experimentally controllable parameters, namely, time, voltage of depolarization, and Cs^+ concentration. We found that the t, V, and concentration dependence of the Cs^+ effects on early conductance and late conductance are very similar. Whenever we observed a suppression of late current by Cs^+ ions, we always found that a component of uninactivated early current is created. This finding suggests that the gating mechanisms of the early conductance pathway and the late conductance pathway may be coupled.

B. The effect of internal sodium ions on the action potential and reversal potential in squid axons

cont'd

In the ionic theory of membrane potential, the overshoot of the action potential is basically the Nernst potential of Na^+ (V_{Na}) with a slight mixture of the K^+ Nernst potential. In principle, the action potential cannot exceed the sodium potential. Tasaki and Luxoro (Science 145:1313-1315, 1964) reported earlier an observation that as the internal Na^+ concentration increases, the action potential is higher than that of V_{Na}. This finding was later dismissed by Chandler and Hodgkin (J. Physiol. 181:594-611, 1965) as an artifact caused by capacitative coupling between the internal electrode and the perfusion cannula. Because of the fundamental importance of this problem, we decided to reinvestigate the effect of internal Na^+ ions on the action potential. Using an experimental design which avoids the capacitative coupling between electrode and cannula, we studied the action potential of squid axon at various internal sodium concentrations. Furthermore, we employed the voltage-clamp technique to measure the reversal potential (V_{rev}), which is theoretically equal to the action potential but has the advantage of being unaffected by leakage current.

In studying over 25 axons with F^- as the major anion, we found that the V_{rev} is smaller than V_{Na} when $[Na]_i$ is less than 50 mM. However, V_{rev}

cont'd

becomes larger than V_{Na} when $[Na]_i$ is equal to or more than 100 mM. For example, the V_{rev} measured at $[Na]_i$ = 400 mM, averaged over 9 axons, is 10.7 ± 1.5 mv, while the V_{Na} calculated based on the Na^+ activity ratio is 4.5 mv. The action potential also is found to be higher than V_{Na} at higher Na^+ concentrations (200 mM or over). The average action potential from four healthy axons is 9.4 ± 1.0 mv, which exceeds V_{Na} by about 5 mv. The cause of this internal Na^+ effect is being investigated.

2. Structure and function of the membrane proteins and the submembrane (cortical) protein network;

A. Structure of the squid axon membrane as seen after freeze-fracture;

A classical excitable membrane is the axolemma of the squid axon. We used freeze-fracture technique to examine the morphology of this membrane to try to identify the membrane protein structures which are thought to be conductance pathways for ions ("channels"). Many large pieces of membrane were seen in the replicas of the intact fixed axon but fractures did not occur through the axolemma. Since there are many layers of Schwann cells, most fracture planes tend to go through the Schwann cell membrane rather than the axolemma. When the Schwann cells are removed, the axolemma is easily recognized at the boundary between the external ice and the axoplasm. However, very little membrane was seen in these replicas of desheathed axons because the axolemma was usually cross-fractured without splitting it over any significant distance. The best results were obtained using axons with Schwann cells chemically detached from the axon but not mechanically removed. In one particularly clear example the fracture plane cut through a stack of Schwann cells and then exposed a large extent of axolemma.

Our first impression of the P-face of the axon membrane is that, unlike the Schwann cell membrane or the membrane of myelinated nerve fibers, the squid axon membrane is marked by many small particles (3 to 4 nm in diameter). It is also clear that there are large P-face particles distributed randomly in the axon membrane. Judging from their size (between 10 and 18 nm) and density (1203 ± 416 per μm^2), some of these large particles are likely candidates for the intramembrane component of the "sodium channels". A peculiar structure was observed in one sample where a particularly large extent of axonal membrane was exposed. Hemispherical blebs having a diameter ranging from 40 to 58 nm were distributed randomly at the axon surface at a density of roughly 80 per μm^2, and the surface of these blebs lacked intramembrane particles. These blebs are tentatively interpreted as contacts, presumably artifactitious, between the axolemma and numerous underlying small vesicles.

B. Possible role of cytoplasmic microtubule structure in the excitation properties of nerve axon;

cont'd

Based on a voltage-clamp study of squid giant axon (Physiol. Chem. Phys. 11:236-288, 1979), it has been proposed that the axon cortex in the resting state resembles a cross-linked polyelectrolyte system in which some ions are absorbed. As the axon is excited, the three-dimensional, macromolecular network of axon cortex begins to unfold or uncoil and break many of the cross-linkages. As a result, the axon cortex behaves less like a cross-linked polyelectrolyte system, whereupon the ion-selectivity approaches that of an

aqueous solution. This change of ion-selectivity at the axon cortex is thought to be one of the key steps in the mechanism for the transition from the resting state to the excited state.

We have designed two experiments to study the functional role of the macromolecular structure at the axon cortex: (1) We examine the structure of microtubules in the axoplasm at different functional states of the axon using an immunofluorescent technique. This method of immunofluorescent staining with a tubulin antibody allows us to study the structural pattern of the microtubule when the axon is at the resting state, at the excited state, and at the non-excitable state; (2) Using voltage-clamp and internal perfusion techniques, we examine the excitation properties (e.g., g_{Na} and g_K) of the axon when the structure of the axoplasmic microtubule is partially destroyed by the use of specific inhibitors, such as colchicine. The preliminary study indicated that the excitability of the axon seems to be correlated with the microtubule structure.

3. Ionic mechanisms involved in the regulation of the resting potential

A. Effects of ionic concentration on the resting potential in squid axons: Evidence supporting a modification of the constant field equation

The resting potential in the squid axon has been measured at various concentrations of Cl, K, Na, and Ca ions. The results of these measurements are compared with the Goldman-Hodgkin-Katz (GHK) equation and a modified constant field equation that was derived by including currents carried by divalent ions and effects of the unstirred layer and the periaxonal space. This modified constant field equation is in the form

$$V = \frac{RT}{F} \ln \left\{ \frac{[K]_o + b[Na]_o}{[K]_i + b[Na]_i} + c \right\}$$

where $b = P_{Na}/P_K$ and c is a constant not sensitive to $[K]_o$ or $[Na]_o$. It is shown that, although the original GHK equation can fit the V versus $[K]_o$ data well, it has difficulty explaining the observed dependence of V on $[Na]_o$ when the axon is bathed in K-free artificial sea water. The use of the modified constant field equation removes this difficulty.

B. Differentiation between the excitable sodium channel and the major pathway for the resting sodium current

It is often assumed that the permeability ratio P_K/P_{Na} of a nerve membrane varies with the potential as the conductance ratio (g_K/g_{Na}) described in the Hodgkin-Huxley model. If this is the case, then the pathways for the resting current must be the same as the excitable channels. This assumption, however, has difficulties in explaining the finding that tetrodotoxin (TTX), which selectively blocks the sodium channel, has very little effect on the resting potential. If the resting sodium current must pass through the Na^+ channel, then blocking this channel should significantly affect the membrane potential.

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One possible explanation of this paradox may be that the total amount of sodium current at the resting state could be very small and thus contribute very little to the membrane potential. To test such interpretations, we studied the resting potential of the squid axon and compared the effects of applying TTX with the replacement of external Na^+ by impermeable ions (choline). Under $[K]_o = 0$ situation, we found that TTX hyperpolarizes the axon for less than 1 mV. On the other hand, removal of external Na^+ gives a hyperpolarization of over 4 mV. Even when the axon was under the treatment of 4×10^{-7} M TTX, which completely blocked the action potential and abolished the voltage-gated sodium conductance, we still observed a hyperpolarization of 3.8 ± 1.1 mV when the external Na^+ was replaced. These observations suggest that the resting Na^+ current is carried mainly through pathways different from the excitable Na^+ channel.

4. Intracellular transport of Na^+ ions inside the nerve cell.

We injected ^{22}Na into the giant axon of squid to study how Na ions are transported within the nerve cell. The purpose of this study is to test an hypothesis that, in addition to the active transport across the cell membrane, the nerve cell may utilize axonal transport to maintain a low concentration of Na^+ inside the axon. We know that not all intracellular ions are free. Studies using ion-selective microelectrodes have indicated that approximately 1/3 of the intracellular Na^+ are osmotically inactive. Some of the axoplasmic Na^+ could be sequestered into vesicles which are moved by axonal transport. To test this scheme, ^{22}Na ions were injected into the axon and allowed to diffuse for 2 to 6 hours, while the axon was kept under oil or natural sea water. Then, the distribution of ^{22}Na along the axon was determined by measuring the profile of radioactivity.

The results suggest that the axoplasmic Na^+ are not irreversibly sequestered into the vesicles involved in axonal transport. The distribution of ^{22}Na follows a smooth bell-shaped curve peaked at the location at which the ^{22}Na was injected. If a significant number of injected ^{22}Na ions were irreversibly sequestered into vesicles and then moved by the fast axonal transport, we would have observed a shift of the ^{22}Na profile toward the direction in which the vesicles move. Such effect was not observed.

The results of our measurements indicate that the transport of Na^+ within the axon is dominated by diffusion. From our data, the diffusion coefficient of Na^+ in the axoplasm is estimated to be $(8.2 \pm 3.5) \times 10^{-6}$ cm^2/s at 4°C, and $(12.7 \pm 3.3) \times 10^{-6}$ cm^2/s at room temperature.

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