WATER PROTON MAGNETIC RESONANCE IN GLYCEROL/PROTEIN THREE COMPONENT SYSTEMS

by

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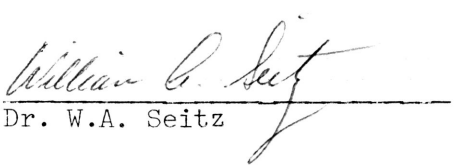
Approved by:



Dr. W.A. Seitz

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Abstract

Nuclear magnetic relaxation times, T_1 and T_2, were measured for a water/glycerol/albumin system as a function of water content. T_1 relaxation times decreased from 95 msec at a hydration of 52 g H_2O/100 g dry solid to 62 msec at 8 g/100g but then sharply increased to 348 msec at the lowest hydration level (6g/100g). T_2 relaxation times were much shorter and less consistent but generally mirrored the T_1 results. The data supports the suggestion that glycerol can substitute for water at macromolecular surfaces under extremely dry conditions. Implications of this result to work done on the brine shrimp, Artemia, are also discussed.

List of Illustrations

WATER PROTON MAGNETIC RESONANCE IN GLYCEROL/PROTEIN THREE COMPONENT SYSTEMS

Introduction

The importance of water in living systems is unquestioned. Water is the most abundant compound on the earth and is the principal constituent of all organisms. It comprises approximately 60 to 90% of the total weight in most biological tissues. Even though some organisms are able to survive varying levels of desiccation, growth and development are not seen until there is sufficient water present.

Despite the fact that water is vital to living systems, the degree to which water is actively involved is still a subject of much debate. In particular, it remains unclear to what extent water's unique characteristics, (such as hydrogen bonding , small size, large dipole moment, high heat capacity & surface tension), contribute to the structure & function of biological macromolecules. To make matters worse, there is a growing amount of evidence that intracellular water is significantly different from pure bulk H_2O. The structure which water assumes intracellularly may be a key factor determining its behavior. The term water structure primarily refers to the allignment of the molecules in various patterns near interfaces (Poechel, 1979). Physical properties of intracellular water have not been clearly defined due primarilly to the extremely complex nature of biological cells. Therefore, to basically describe the physical state of cellular water, several fundamental assumptions are commonly made. The consensus view is that water acts chiefly as a passive solvent. This conception is

essentially only a slightly modified version of the "bag of enzymes" notion popular in the eary days of biochemistry (Clegg, 1979). One purpose of our investigation has been to look closely at this assumption for a simple model biological system.

Water and water-macomolecular interactions in metabolism are important aspects of research. The behavior of water in muscles (Nakano and Yasui, 1976), lysozyme crystals (Jentoft and Bryant, 1974), and various globular proteins (Kuntz and Brassfield, 1971), has been investigated and, as one would expect, several theories have evolved concerning water's role. The properties of water near solid interfaces or "vicinal water" has been observed to be quite different than that of bulk water (Drost-Hansen, 1973). These differences appear to be related to structural dissimilarities between two types of water. Hydrogen bonding, between water molecules, and to surfaces can account qualitatively for a variety of structures. It has even been suggested that the vicinal water fraction can exist in a significant number of layers that extend several hundred angstroms from the interface surface (D-H, 1969). The degree of orientation and mobility of such water molecules could have considerable bearing on the observed physical properties. Ordering, for example, has a strong tendency to reduce the mobility of water molecules and produces longer rotational and/or translational correlation lengths (Hazlewood, 1979).

Pulsed nuclear magnetic resonance is often used as a tool to study the physical state of cellular water. Hazlewood (1973) utilized NMR as a method of studying the dehydration of rat skeletal muscle. Other studies concerning the role of structured water in metabolism have been accomplished by Seitz and others.

Nuclear magnetic resonance provides a nondestructive measurement of the motional freedom of intracellular water. It can be tuned to detect resonance absorption by only a few species of protons, namely as in this case, water protons. When placed in a magnetic field, the spin magnetic moment of the hydrogen nuclei precess

about the direction of the field and the net magnetic moment of each nucleus can be aligned either parallel or antiparallel to the field direction. When excited with RF frequency electromagnetic radiation, the low energy parallel states can be "flipped" to the antiparallel states which upon removal of the RF field relax back to the low energy states with characterestic time constants T_1 and T_2. (see NMR theory explanation). The NMR relaxation times of water protons are related to molecular correlation and therefore reflect the motional freedom of the molecules. The spin-lattice relaxation time, T_1, varies with limitations on mobility due to restrictions imposed by the surrounding lattice (environment). The spin-spin relaxation time, T_2, gives information about spin transfer interactions with other like nuclei. Long relaxation times tend to indicate greater mobility. While short relaxation times tend to indicate a higher degree of structure.

In general, nuclear magnetic resonance T_1 and T_2 in biological systems are considerably reduced (sometimes by orders of magnitude) from the values in bulk water. As expected the trend is toward an increase in water structure (lower T_1 and T_2) with a decrease in water content. Recent observations on the cyst of the brine shrimp, Artemia, have complicated the picture in low hydration regimes where the relaxation times unexpectedly increase dramatically (Seitz, 1977). Speculation about the cause for this apparent decrease in water structure at low hydrations is complicated by the tremendous diversity of other molecules present in brine shrimp cysts. Nevertheless, it has been suggested that this effect is a result of the replacement of water by glycerol at the macromolecular surfaces. This interpretation is based on first, the unusually high glycerol content of brine shrimp, and second, on the molecular structure of glycerol which can participate in hydrogen bonding in a manner similar to water.

At very low water contents, it has been proposed that the hydroxyl (O-H) groups of polyfunctional alcohols, glycerol for example, can substitute for the hydration water of macromolecules (Webb and Dumasia, 1968). This substitution tends to inhibit the biological activity of molecules. However, it is thought to preserve the molecule's structure. Therefore, upon rehydration, the molecules are unharmed and a larger percentage of them will survive.

This project studies the structure of water in a model biochemical system consisting of water, glycerol and albumin. Our purpose is to isolate the possible glycerol substitution effect from the other variables which complicate the analysis in naturally occuring biological systems. As the later sections will show, the phenomenon appears to be even more dramatic here than what was observed earlier in Artemia. This represents the first observation (to our knowledge) of this effect in any biochemical model system.

Materials & Methods

Bovine albumin powder No A-9647, Lot 79C-0153 was a product of Sigma Chemical Co., St. Louis, MO. Glycerol used was MCB lab grade 9J15. Small weighing boats were made from squares of aluminum foil. A total of 10 boats were made. To each of the boats, 0.3 g bovine albumin powder was added. A glycerol/water solution was prepared consisting of 3% glycerol by weight. 1.2 ml of this solution was added to the albumin in each of the weight boats until the albumin slowly dissolved. Stirring the mixture may have increased solubility yet was avoided because of possible weight loss.

Once the albumin had completely dissolved, theweight boats were put into a desiccator and allowed to dehydrate for a period of time, usually 5-7 days (see Fig. 1). At various points within the time frame, a mechanical vacuum pump was connected to the desiccator. Caution was exercised in turning on the pump, for if it was allowed to begin immediately following placement of the samples within the desiccator, the vacuum produced caused the system to boil. Therefore the pump was not started until the sample remained in the desiccator for at least 24 hours. Even at this time, the systems had to be carefully observed while the pump was in operation.

The water content of a system can be adjusted over a large hydration range simply by adjusting the water activity of the environment. This was achieved in practice by placing the dried glycerol/albumin system within sealed humidity chambers. Humidiy chambers were constructed using sealed glass jars. To the bottom of each jar, an inverted plastic cap was attached using silicone glue. The weight boats were placed atop the cap. (see Fig. 2)

Different relative humidities were obtained using varying concentrations of

Figure 1: Desiccation times for samples

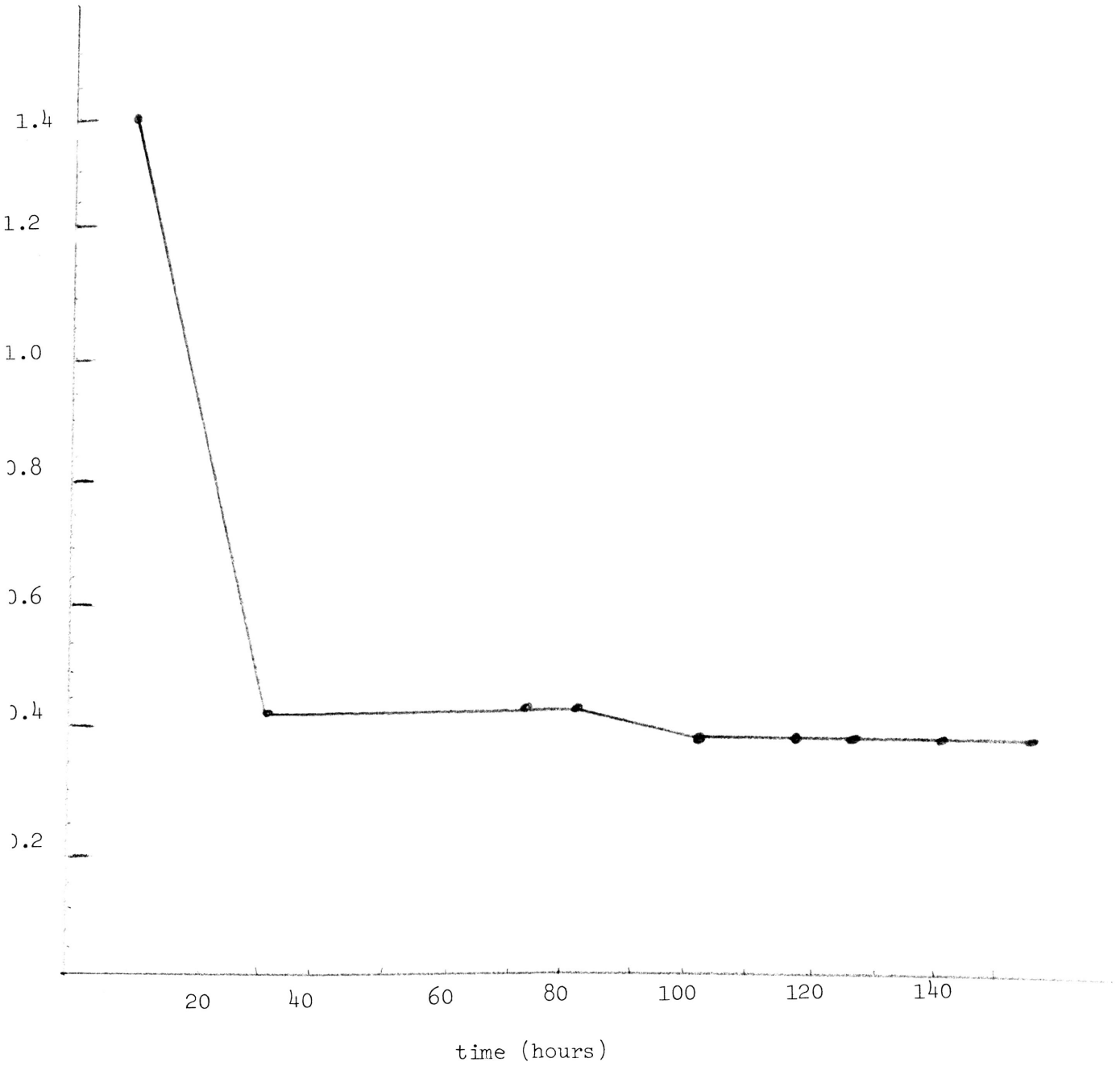
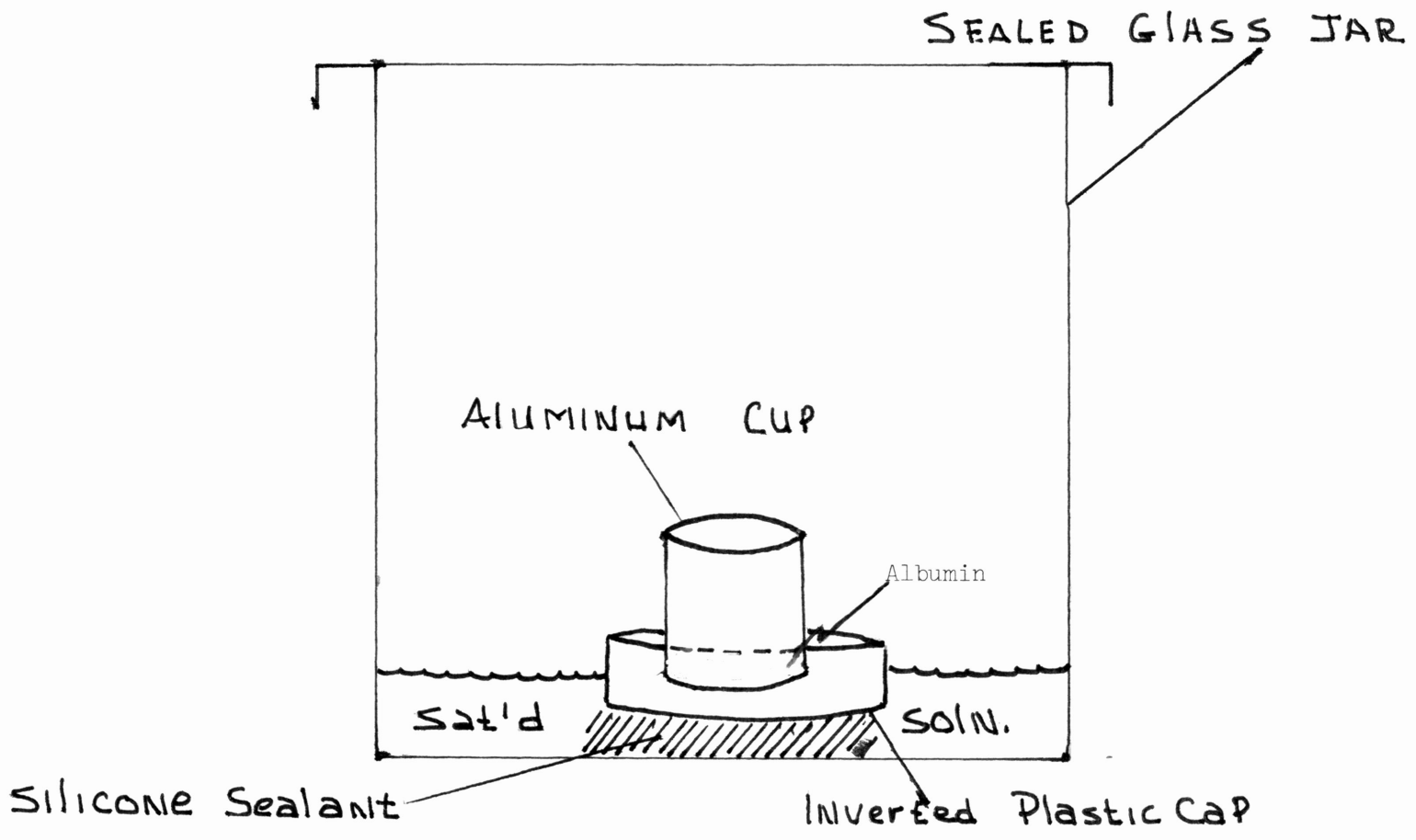


FIGURE 2

HUMIDITY CHAMBER



sulfuric acid. The CRC Handbook of Chemistry and Physics gave information concerning the correct amounts of H_2SO_4 and H_2O to use in preparing the solutions. Percentage of H_2SO_4 used was the percentage by weight. A total of 8 H_2SO_4 mixtures were prepared, each with a different relative humidity. Relative humidities were not measured directly because they did not enter the analysis explicitly.

Percentage H_2SO_4	Relative humidity
50%	37%
45.5%	50%
40%	60%
34%	70%
28%	80%
21.5%	90%
15%	94%
8%	97%

Table 1: Sulfuric Acid Content of Humidity Chambers

A total of 9 humidity chambers were established. A small amount (enough to cover the bottom of the jar) of each H_2SO_4 mixture was poured into the designated humidity chamber. Care was taken not to get the H_2SO_4 inside of the inverted cap. Distilled water was put into the chamber labled 100% relative humidity.

The dried albumin/glycerol was transferred to the humidity chamers. Each system was allowed to hydrate at different relative humidities. They remained within the chambers until equilibrium was reached, which normally took 5-7 days.

This proceedure was carried out on three sets of samples. The first set was basically experimental and gave information about the lengths of hydration and desiccation thereby saving time in the next series of samples.

One set of samples was composed only of albumin and H_2O. They were prepared as a control in order to isolate the effects due to the presence of glycerol. The control was prepared using exactly the same proceedure as with glycerol including the addition of water followed by desiccation.

The two higher relative humidities, 94% and 97%, were established late in the experiment so as to examine the systems at higher hydrations. 100% relative humidity was not of any use, because all of the systems within that chamber had tendencies to mold.

Equipment

All pulsed NMR measurements were taken on a Bruker SXP spectrometer equipped with a model WP-200 superconducting magnet (see Fig. 3). The magnet utilizes liquid He to reduce internal temperatures significantly, creating an essentially resistance-free system. The static magnetic field (H_o) used in in all proton experiments was approximately 7 Kgauss with a long-term stability (greater than 15 minutes) of 1 part in 10^5. A general radio type 1165 Coherent Decade Frequency Synthezer supplied precise frequencies which were derived from a 10 MHz master oscillator crystal. The drift of the frequency synthesizer was less than 1 part/10^9 per day.

The Bruker system consisted essentially of a pulse sequence generator, a transmitter, a probe, and a receiver. The pulse sequence generator was composed of a time base (which controlled the repetition rate of the desired series of pulses), an automated program unit and four pulse channels. Controls on each pulse unit allowed the operator to determine the width of the pulse and a delay relative to the preceding pulse. In all cases, only the first two pulse channels were used.

The two parts of the transmitter were a five stage power amplifier (maximum output power = 3 kilowatts) and an r.f. unit. The amplifier unit was interchangeable with several units with different optimal frequency ranges. The unit used in this work was a 20-30 MHz unit. The height of the r.f. pulses was determined by the output power of the transmitter and was adjusted by a "gain" dial. This was the means of varying the H_1 field.

The sample probe was constructed of glass and teflon with a single coil of copper wire which served as both transmitting and receiving coil. The probe was inserted into an aluminum probe holder whose position could be adjusted for maximum field homogeneity. Sample tubes

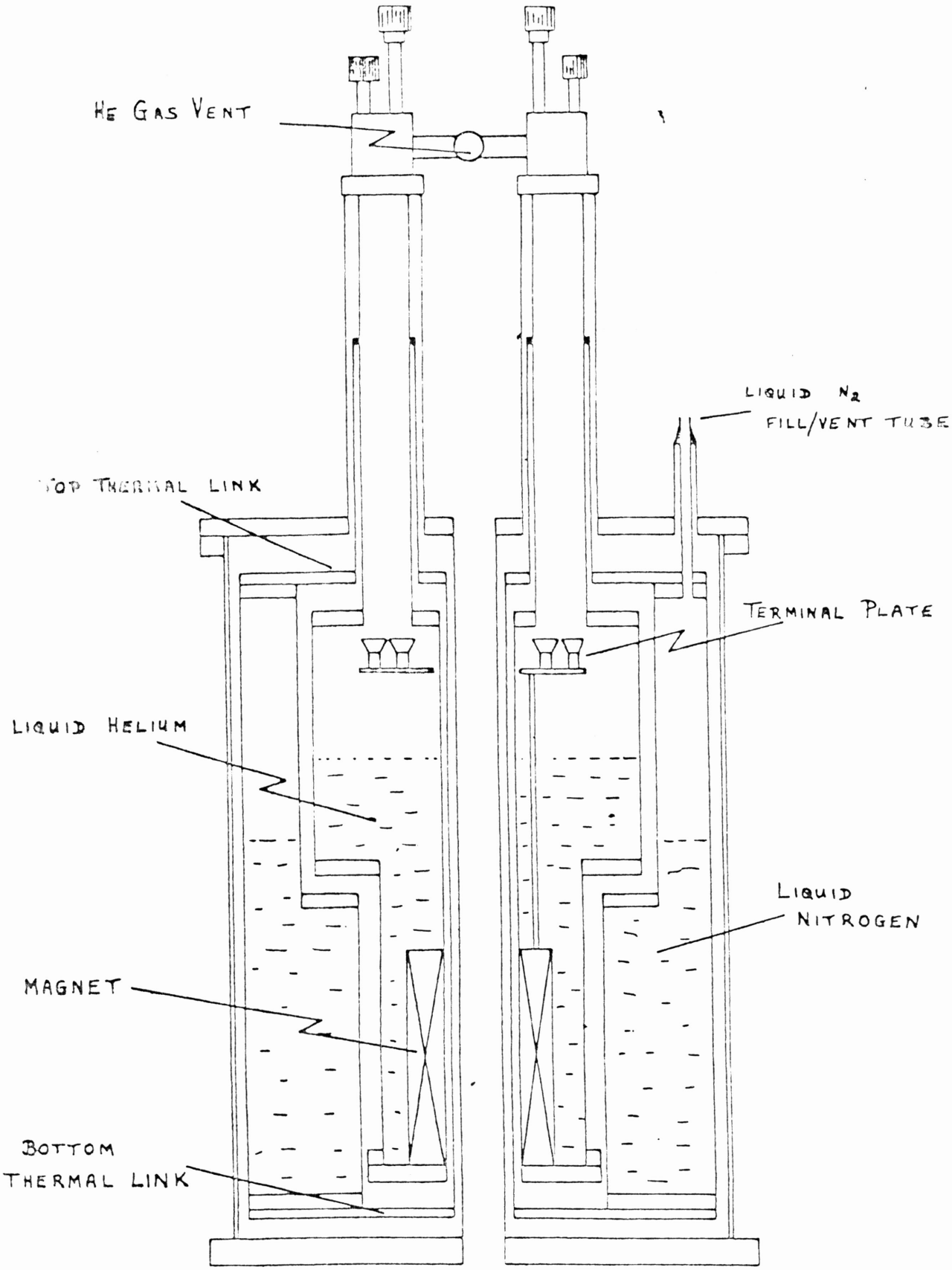


FIGURE 3

NMR Cryomagnetic System

(obtained from the Wilmad Glass Co.) were made of highly polished glass with uniform wall thickness. Five mm O.D. tubes were placed inside 10 mm tubes and centered by means of a Teflon spacer.

Signals from the samples were amplified by the receiver. This system consisted of a preamplifier, a main amplifier, a detector (a choice of either phase sensitive or diode, although the diode detector was used almost exclusively), then a band width limiter and a d.c. amplifier. Total amplification was approximately 110 dB.

The signals from the receiver were simultaneously displayed on a Tektronix 7603 oscilloscope (located on the Bruker console) and sent to a Nicolet 4096 channel signal averager which was equipped with a Tekronix D-10 digital readout oscilloscope and a 5D-71B analog to digital converter. A Model SW-71B wide range sweep control provided variable dwell times (of 10 usec to 9.999 sec) at each channel. A preset number of sweeps (the exact number determined by the size of the signal and the signal to noise ratio) were accumulated in the Nicolet and the peak heights, recorded in digital form. Digital readout vs. input voltage was linear for the Nicolet, but calibration curves were determined to account for the nonlinearity of the spectrometer diode detector. On line data analysis was provided by a Interdata Model 7/16 minicomputer. T_1 and T_2 valves were obtained by least squares fitting to the calibrated data. In most cases the relaxation was well represented by a single component as determined by a successive curve stipping technique (see Seitz, 1977).

NMR THEORY

Before proceeding to a discussion of the results obtained during the course of this project, I will describe briefly some of the theoretical basis for NMR relaxation measurements. NMR is concerned with nuclei which posess a non-zero angular momentum, $\vec{J}$, and magnetic moment, $\vec{u}$, corresponding to a nuclear spin. These quantities are related by the equation

$$\vec{u} = \gamma \vec{J} \qquad (1)$$

where γ is the gyromagnetic ratio characteristic of the particular nucleus (in our case hydrogen). A full quantum mechanical treatment of NMR was beyond the scope of my project so that only a classical treatment will be discussed here. (see C. P. Slichter, 1978)

When a magnetic moment $\vec{u}$ is placed in a magnetic field, $\vec{H}$, a torque is produced which can be equated to the rate of change of the angular momentum, $d\vec{J}/dt = \vec{u} \times \vec{H}$. But since $\vec{u}$ and $\vec{J}$ are related by equation (1), we have the basic equation

$$\frac{d\vec{u}}{dt} = \vec{u} \times \gamma\vec{H} \qquad (2)$$

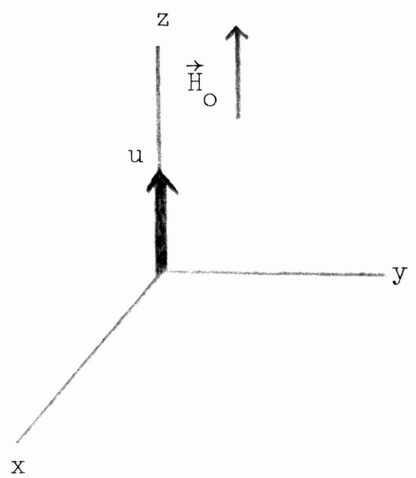
which applies regardless of whether $\vec{H}$ is time dependent or not. It is straightforward to show that if one takes $\vec{H}$ to be a static field of magnitude H_o in the z-direction (ie. $\vec{H}=H_o\hat{z}$), then the solution for the motion of the spin describes a cone with the magnetic moment precessing about the H_o field with an angular frequency γH_o called the Larmour frequency.

The nuclear magnetic resonance phenomenon occurs when one adds to the H_o field, a time dependent field in the x-y plane perpendicular to H_o which also

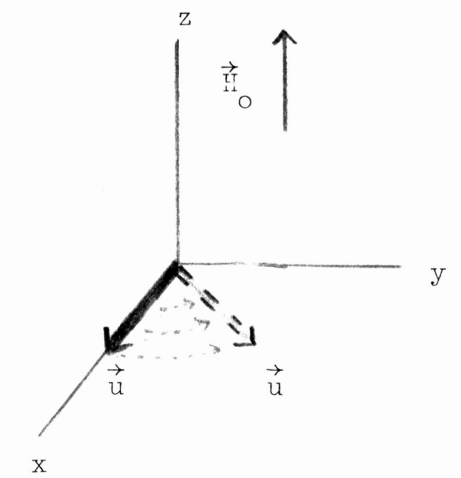
is taken to rotate with $\omega = \gamma H_o$. (Actually this field is an alternating field which is decomposed mathematically into components rotating about the z-axis). If we call this field $\vec{H}_1(t) = H_1(\hat{x}\cos\omega t + \hat{y}\sin\omega t)$ we have the full equation

$$\frac{d\vec{u}}{dt} = \vec{u} \times \left\{ \vec{H}_o + \vec{H}_1(t) \right\} \qquad (3)$$

which must be solved for the motion of the spins. The solution to this equation when resonance occurs ($\omega = \gamma H_o$) for a magnetic moment initially lined up parallel to H_o (along the z-axis) is a precession about the y-axis (the H_1 axis) at a frequency $\omega' = \gamma H_1$. Thus the spin will rotate from being parallel to H_o to perpendicular to antiparallel and back again. If one pulses the $\vec{H}_1(t)$ field only for a short time (t_w), the spin will only have rotated through an angle $\theta = \gamma H_1 t_w$. Choosing the t_w pulse length to correspond to $\theta = 90^o$ (called a 90^o pulse) rotates a spin which is initially parallel to H_o into the x-y plane. This spin then again precesses about H_o with the Larmour frequency (H_1 is off). See the figure below.

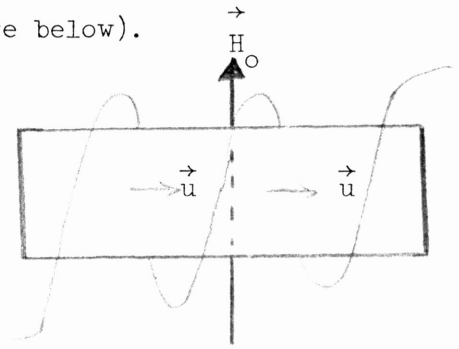


Before 90^o pulse



After 90^o pulse $\vec{u}$ is rotated about the y-axis by 90^o by the H_1 field which when turned off leaves $\vec{u}$ to precess again about the z-axis.

This provides a means of detecting resonance since the net magnetization following a 90° pulse is an alternating field rotating about the z-axis with a frequency γH_o. This can produce an emf in a coil wrapped around the sample. (see figure below).



The signal will be large immediately following the pulse,but then because of interactions with other molecules, the signal will decrease as the nuclei re-allign themselves with the H_o field. This decay (the free induction decay) may last many milliseconds in liquids (our case) or can be very short as in solids which have relaxation times typically 100 microseconds. The time characteristic of this decay is T_1. A similar decay is observed following a 180° pulse with a time constant T_2. Since the T_1 and T_2 relaxations are induced by and vary with the environment, their measurement provides a non-destructive means for observing the degree of interation of water and other molecules with their environments. A short T_1 (or T_2) implies rapid relaxation and hence suggests a very large degree of interaction with the other molecules nearby; whereas a long relaxation time is expected for a molecule which is relatively "free". This is the basis for the experiment which we carried out.

Results and Disucssion

Results:

Measurements of the spin-lattice relaxation times T_1 were made for H_2O/glycerol/albumin samples with hydrations from 52 to 6/100g and for H_2O/albumin samples with hydrations between 15 g/100g and 6 g/100g. Hydrations were obtained gravimetrically following removal from the humidity chambers and checked for constancy after the NMR measurements were complete. Table 2 shows the T_1 results for both the glycerol and control systems. In addition this data is displayed graphically in Figure 4.

The dramatic increase seen in T_1 for water/glycerol/albumin in the low hydration regime is associated, we believe, with the glycerol replacement effect as discussed further below. It is important to notice that the effect is missing in the control. Figure 5 plots some of the calibrated relaxation data on a logarithmic scale with the slope of the curves giving T_1. The curves can be seen to consist primarily of a single relaxing component in all cases but with a significanly longer relaxation time at the lowest hydration. This is to be compared with Figure 6 for the control system showing essentially the same relaxation behavior at all hydrations. Finally Figure 7 gives the data for all samples

Spin-spin relaxation times, T_2, are generally more susceptable to experimental problems associated with paramagnetic impurities and/or field inhomogeneity. In addition, our samples displayed extremely short T_2 relaxation times which could only be obtained from the free induction decay rather than from the more accurate Carr-Purcell sequence method. Because of these difficulties, we obtained reproducible results only for the glycerol system (shown in Figure 8). As for the T_1 curve,

TABLE 2: SPIN-LATTICE RELAXATION TIMES (T_1)

Water/Glycerol/Protein			Water/Protein		
Relative Humidity	Hydration ($g.H_2O$/100 g.solid)	T_1 (msec)	Relative Humidity	Hydration ($g.H_2O$/100g. solid)	T_1 (msec)
97%	52.3	95.4	-	-	-
94%	30.2	84.3	-	-	-
90%	15.4	69.2	90%	13.9	53
80%	11.3	75.9	80%	10.6	44
70%	8.0	62.4	70%	9.4	48.3
60%	8.0	266.	60%	9.0	52.2
50%	7.3	345.	50%	9.2	48.
37%	6.7	348.	37%	5.9	61.4

FIGURE 4

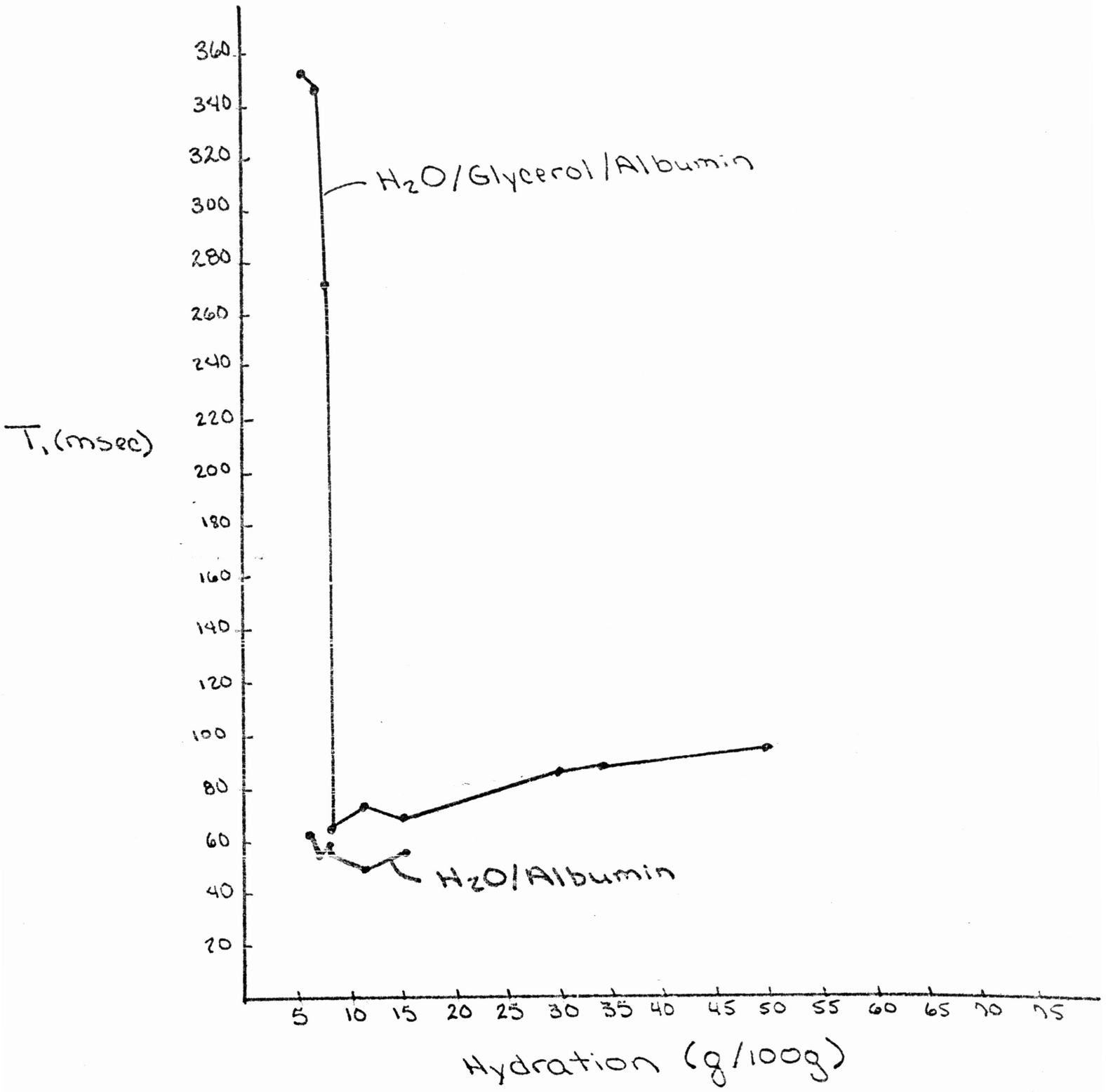

360
340
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
T, (msec)
H2O/Glycerol/Albumin
H2O/Albumin
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
Hydration (g/100g)

Figure 5: Raw calibrated curves at high, medium and low hydrations for water/albumin/glycerol system.

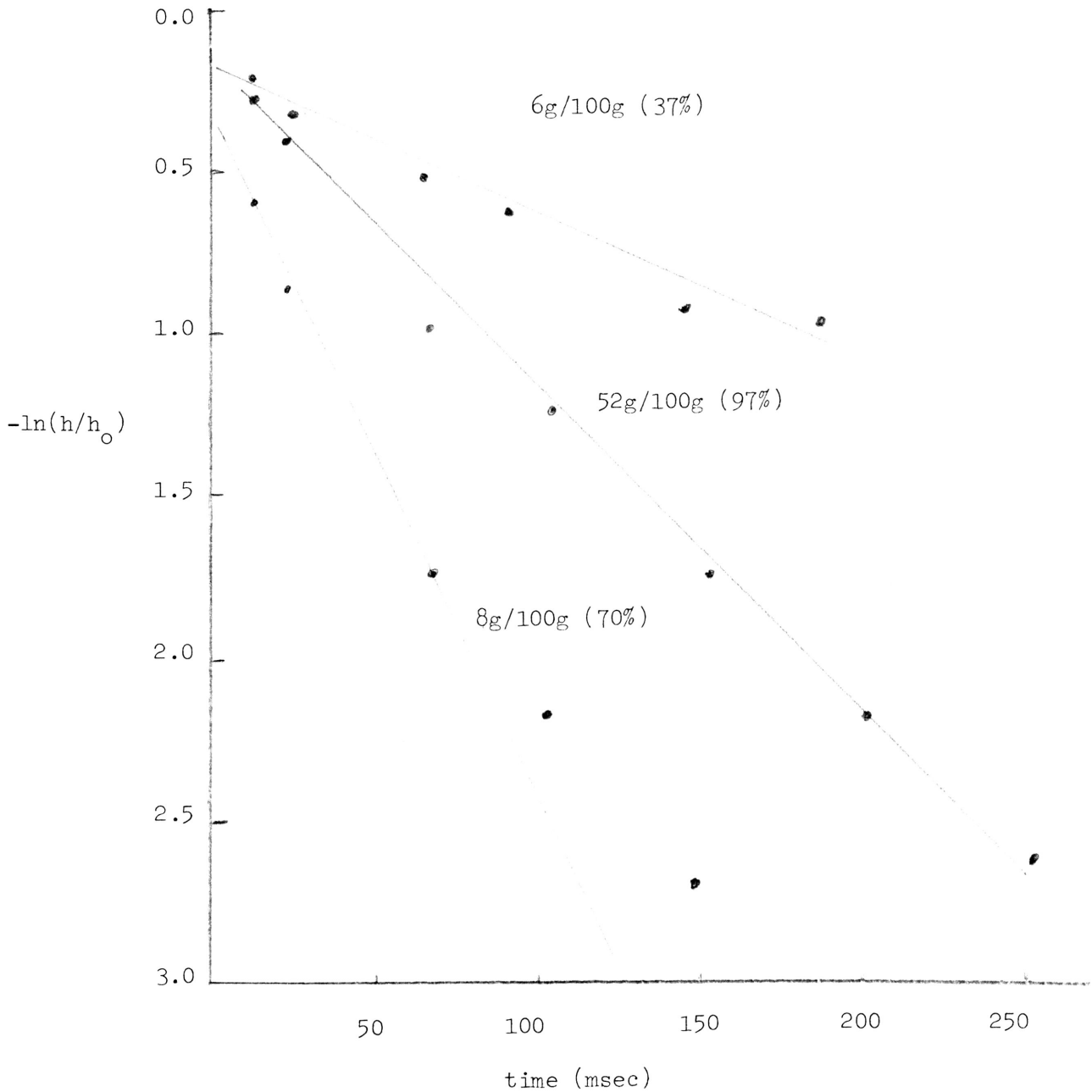


FIGURE 6:

T_1

H_2O/ALBUMIN

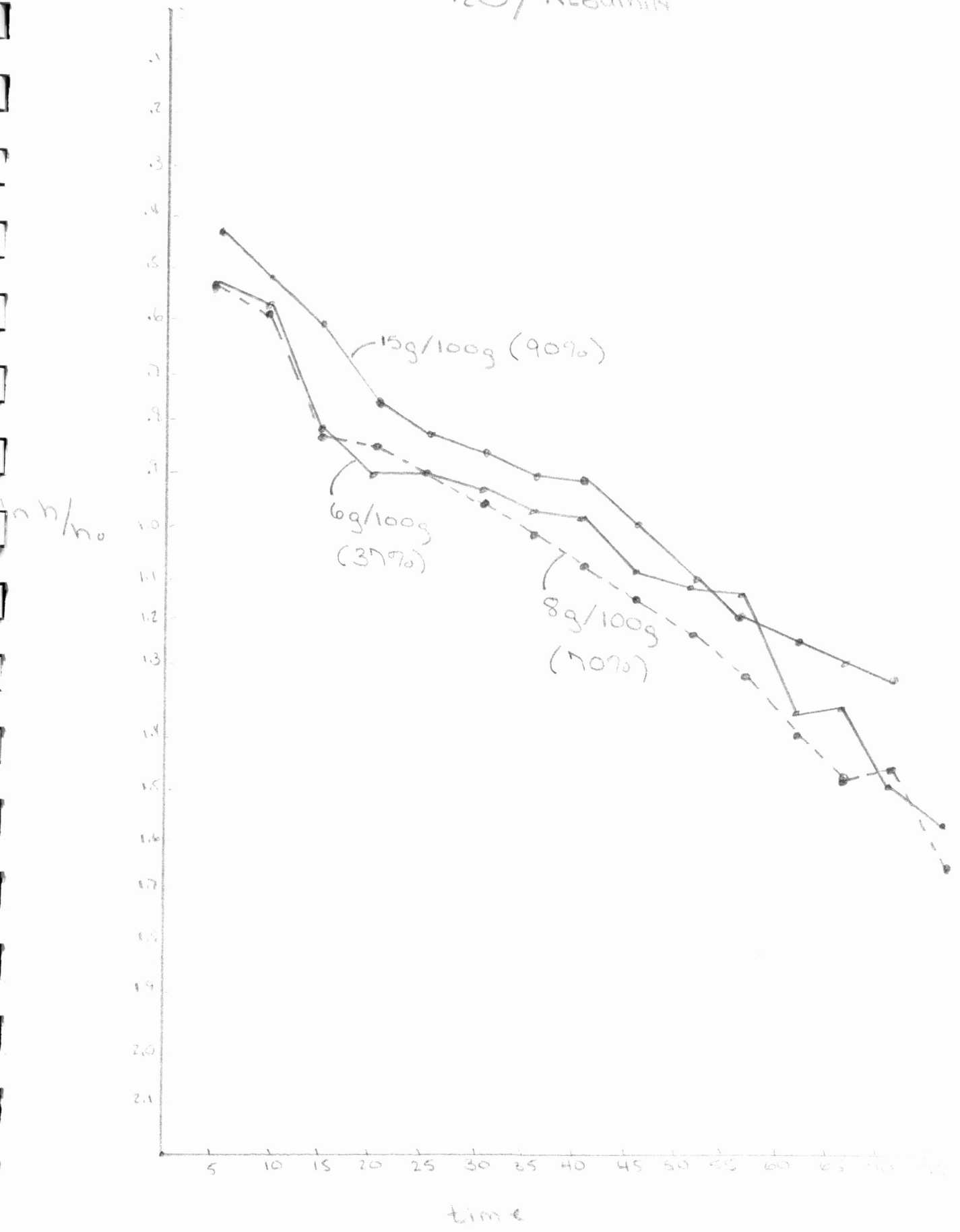


Figure 7: T_1 relaxation times for the water/albumin/glycerol three component systems are obtained as the slopes of the curves below. (Raw data)

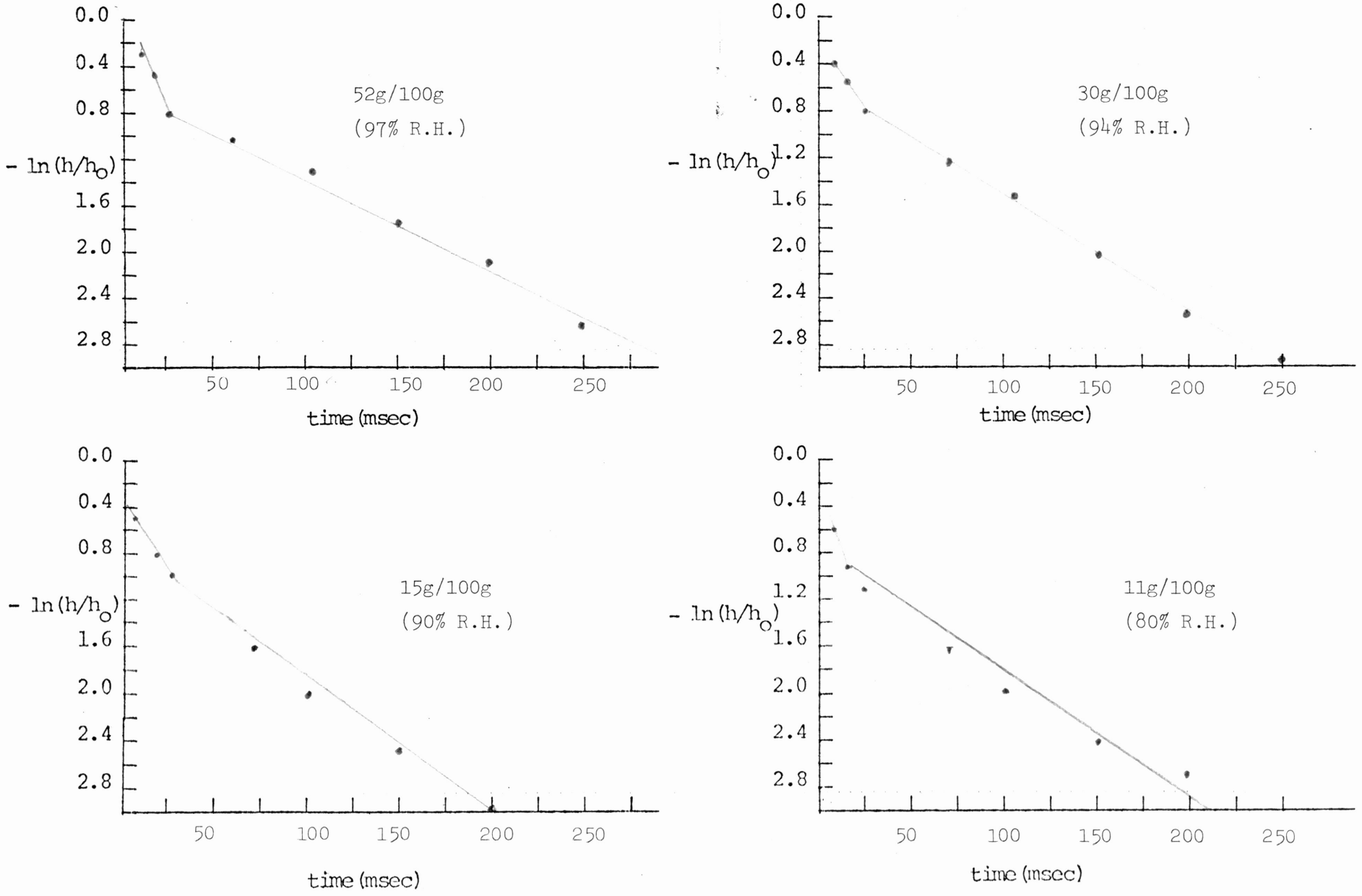


Figure 7 (continued) Raw data for T_1 relaxation in three component system

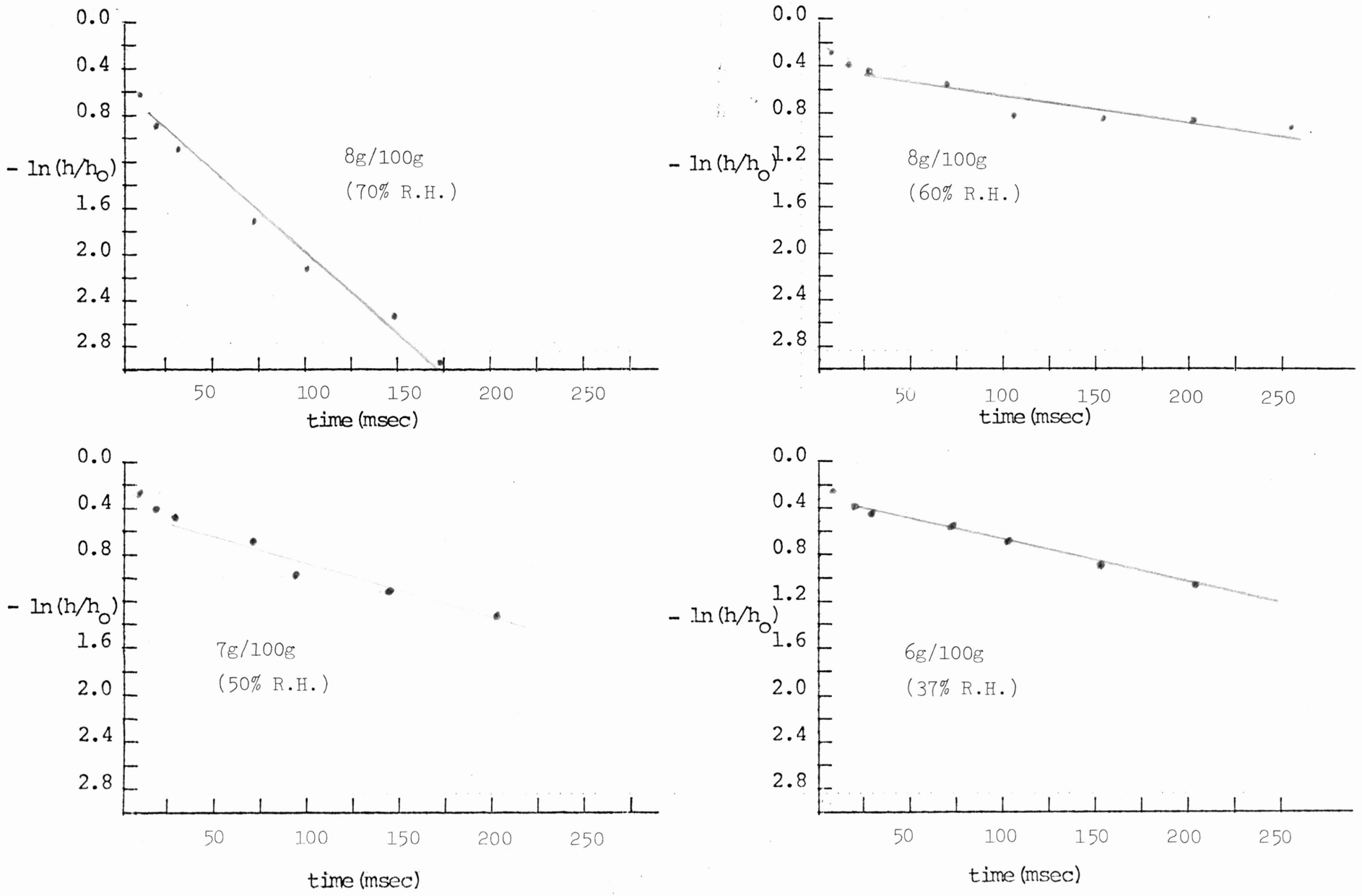


Figure 7 (continued): T_1 raw data for water/albumin without glycerol. (note different time scale)

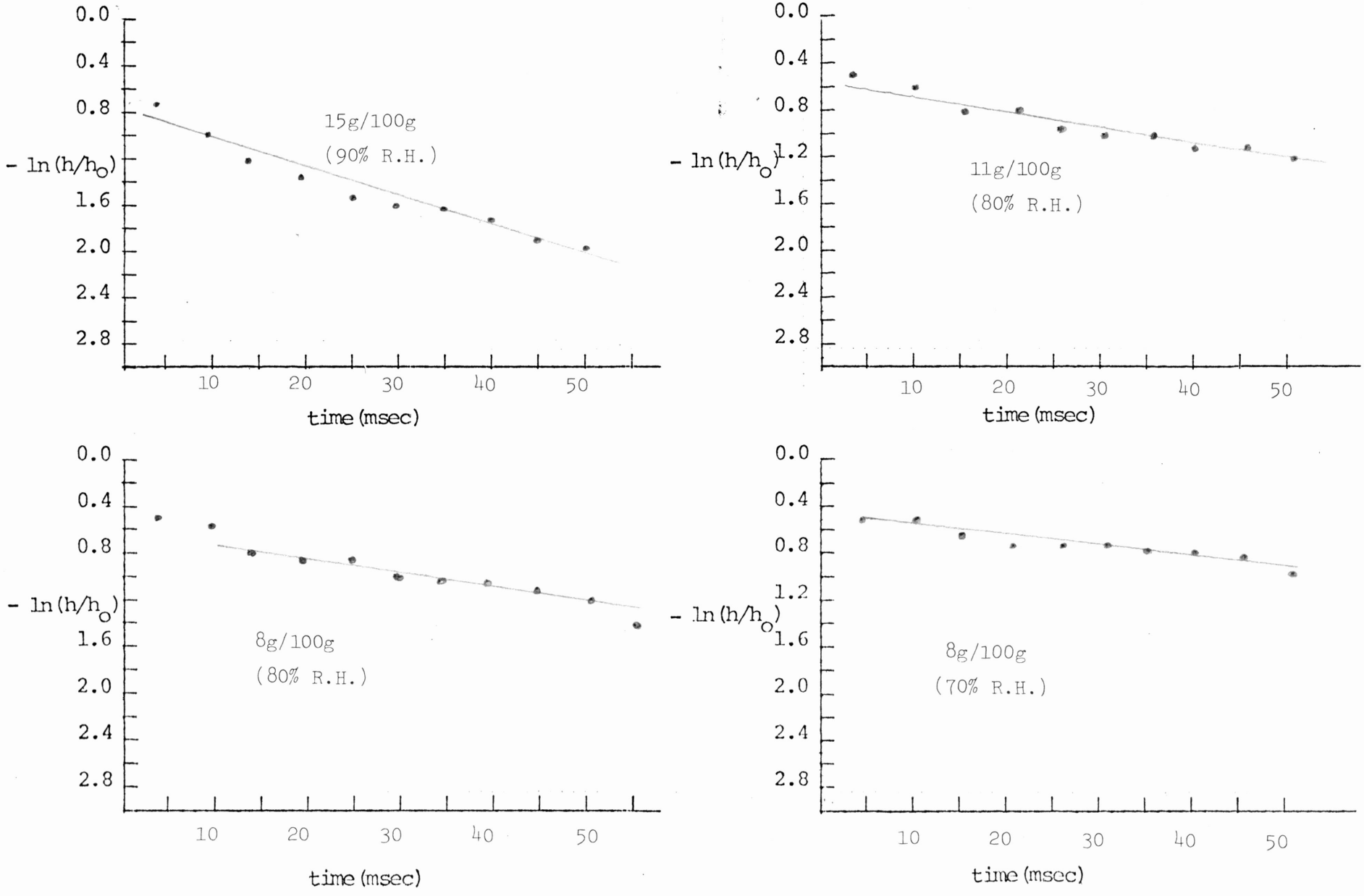
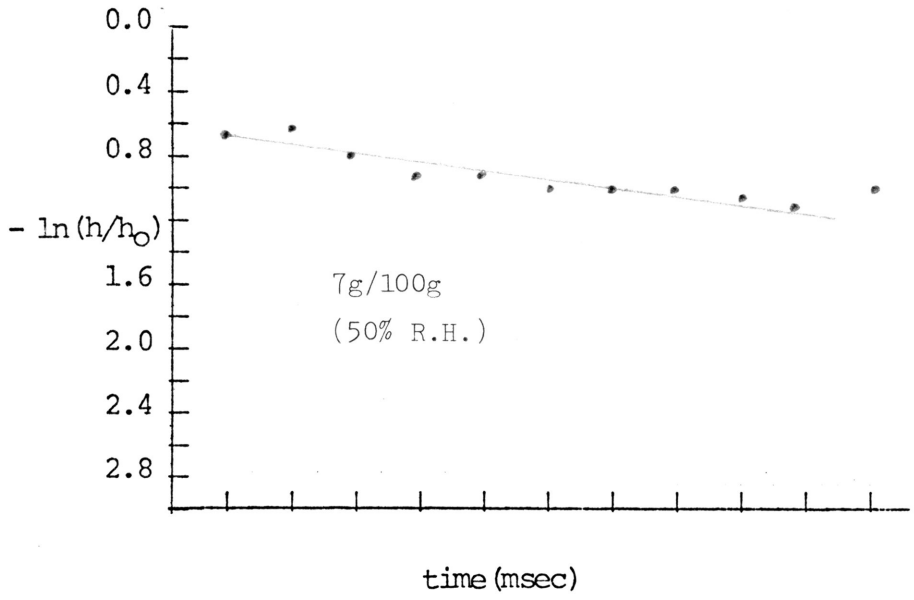
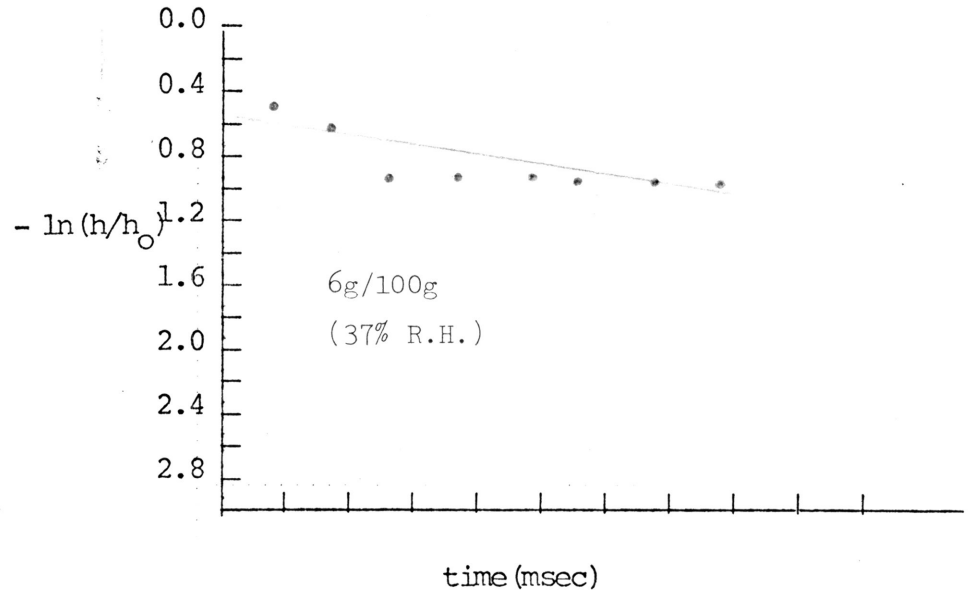


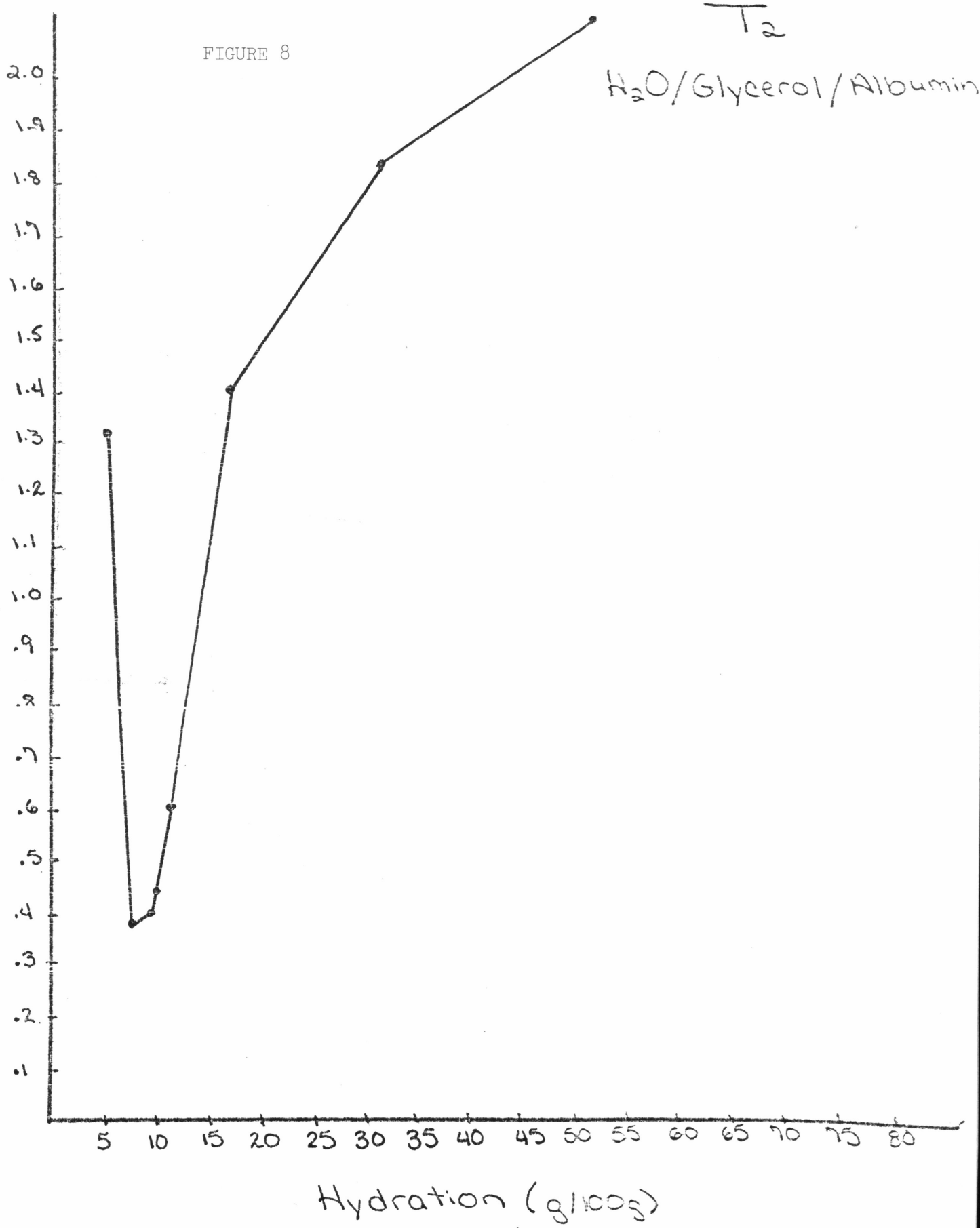
Figure 7 (continued) T_1 raw data for water/albumin without glycerol





T_2 also increases greatly at the lowest water content. Raw curves in Figure 9 show the change occuring at the lowest hydration. These curves do, however, exhibit some interesting features which we will note without a full explanation (until further work is done). First, in the 52g/100g curve a shoulder is noted. This was reproduced on a number of runs using the same sample and appears not to be an instrumental artifact. However, we hesitate in suggesting that the effect is real due to the limited number of samples run. A second important feature can be seen upon comparison of the 52g and 6g curves. The low hydration sample contains two types (fractions) of water - one with a fast relaxation time parallel to the 52g curve and the second with a slow relaxation time which is thought to be the displaced water. This effect is probably present even for the T_1's except contained prior to the first data point in those curves.

FIGURE 8


T_2
H_2O/Glycerol/Albumin
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80
Hydration (g/100g)

Discussion

We begin with a review of the water problem in biology. Over the years biologists, chemists, and physicists have been divided in their views concerning the structure of water in and out of cells. Some of the earliest concepts of water were that it existed as a "bound", inseparable part of the cellular protoplasm (Dujardin, 1835; Nageli, 1855; Butschli, 1894). Water was considered to be a part of the organic complex that forms living matter. Over the years, however, this theory lost its acceptance except in historical reviews.

Ling (1962) revived this viewpoint when he suggested that intracellular water exists as polarized multilayers near cellular surfaces (see Figure 10). The strong dipole moment of the water molecule (1.85×10^{-18} esu) results from an asymmetrical charge distribution with the two positively charged hydrogens and a negatively charged oxygen. This theory of multilayer adsorption suggests that proteins, water, and other living cell components exist in a highly associated state and thereby provide for the functional coherence of the protoplasm (Ling, 1970).

More recently, experimental evidence concerning the physical state of water has been accumulating. Drost-Hansen (1969) proposed that the properties of water near interfaces, or vicinal water, were significantly different than those of bulk water. The vicinal, structured water, however, is found to retain some of the characteristics of liquid water rather than those of crystalling solids (Drost-Hansen, 1973). Then, it could be possible for phase transitions between these two structural water types to occur. Such structural changes appear to be induced by changes in temperature and pressure. The nature of the structure of water near interfaces depends on the specific nature of the surface (such as nonpolar, polar, or ionic) (Drost-Hansen, 1973). It is also his belief that vicinal water may extend several hundred angstroms form the interface itself.

FIGURE 9

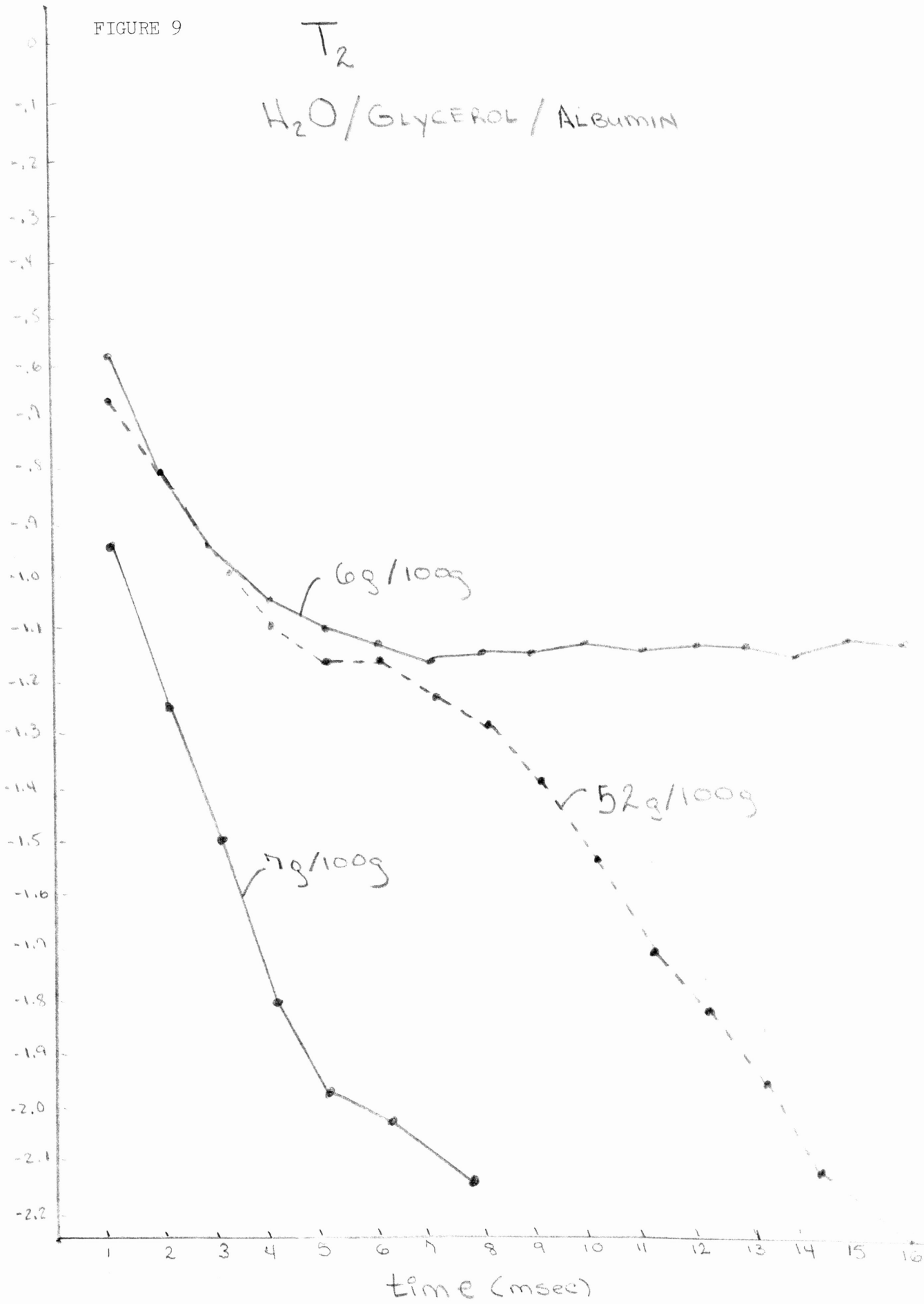
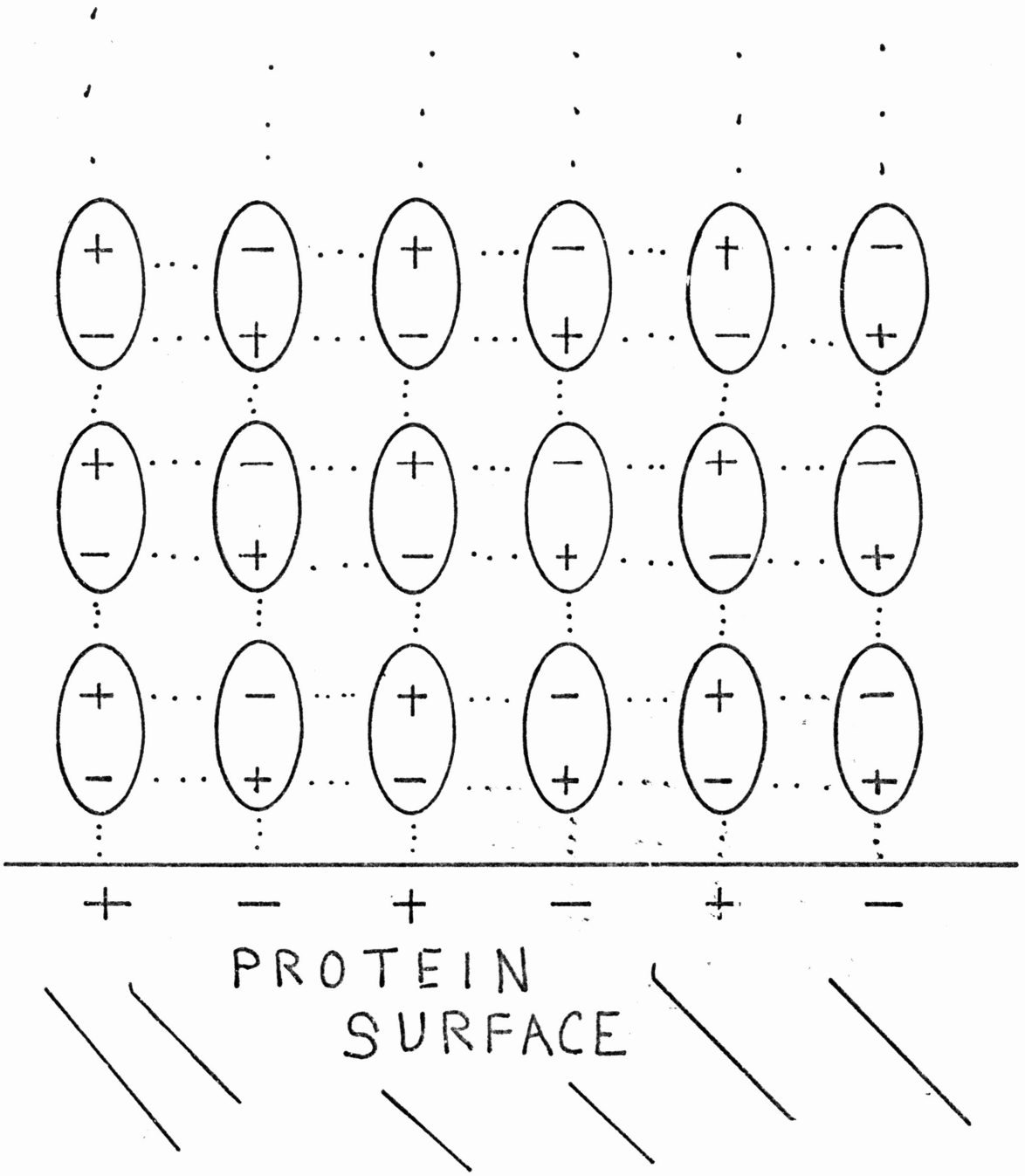

T_2
H_2O/GLYCEROL/ALBUMIN
$n\ h/h_o$
0
-.1
-.2
-.3
-.4
-.5
-.6
-.7
-.8
-.9
-1.0
-1.1
-1.2
-1.3
-1.4
-1.5
-1.6
-1.7
-1.8
-1.9
-2.0
-2.1
-2.2
6g/100g
5.2g/100g
7g/100g
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
time (msec)

Figure 10: Polarized multilayer model for adsorption of water to a protein surface.



A number of theoretical studies concerning the structure of bulk water have also been undertaken by Stillinger using molecular dynamics techniques and by Clementi using Monte-Carlo simulations based on Hartree-Fock intermolecular potentials. The latter displays clearly the hydration layers around ions in solution but does not show any significant degree of order persisting beyond the second layer. This tends to contradict the several hundred angstrom estimates of Drost-Hansen and Ling. Theoretical work of this sort however has not been undertaken for surfaces due to the complexities of the problem and the large computer requirements.

Nuclear magnetic resonance (NMR) studies have recently supplied evidence which tends to support the fundamental association-induction hypothesis of Ling (1962) (at least in a modified form). Using such NMR techniques, the naturally occuring dehydration of rat skeletal muscle associated with maturation was studied (Hazlewood, 1973). As a result of this study, a correlation was seen between hydration, electrolyte composition, and relaxation times of water in the developing muscle. The relaxation times were observed to decrease with age. Similar experiments, conducted on human breast tissue, attempted to correlate relaxaton times with the presence or absence of malignant tumors.

To elucidate further the roles of water structure in metabolism, a more extensive study was required over larger ranges of water content. Such research was performed using the cysts of the brine shrimp, Artemia salina. Artemia cysts, unlike many other organisms, can survive extreme dehydration. The cysts' metabolic activity is suspended, yet when sufficient water levels are again reached, it is gradually re-initiated. Clegg (1974) initially proposed the methods of cyst preparation as a function of water content. Fully hydrated cysts contain very low levels of water, thus providing access to a portion of the hydration curve not normally observed in living cells. Dry cysts are known to contain a significant amount of glycerol (Clegg, 1974) and it has been suggested

that this glycerol helps in desiccation protection of the cysts at lower hydrations. The mechanism proposed for such protection involves hydrogen bonding of the polyfunctional alcohol to the protein surface at critically low water levels. Evidence suggesting such a mechanism came from the Artemia study. At hydrations below 20g/100g, brine shrimp cysts showed a large reproducible increase in T_1 and T_2 (see figure 11). The glycerol replacement theory would accomodate this data if one assumes that the displaced vicinal water is forced into a more disordered liquid state away from surfaces.

The data presented in our work was obtained so as to further test the replacement hypothesis. We regard such a study as important since Artemia are complex organisms containing a large variety of substances (Clegg, 1978), all of which may or may not participate in the T_1 minimum. We chose albumin for its' hydroscopic property and added 4g glycerol per 100 grams albumin to correspond to the Artemia value. We believe that the data in figure 4 not only is consistent with the brine shrimp work, but strongly supports the replacement theory.

Further investigations are clearly warranted and are now underway. Other glycerol levels, other polyfunctional alcohols and other proteins need to be tested. Finally, it is possible that this phenomenon is a general surface effect and may occur in many other non biological systems (for instance in sediments).

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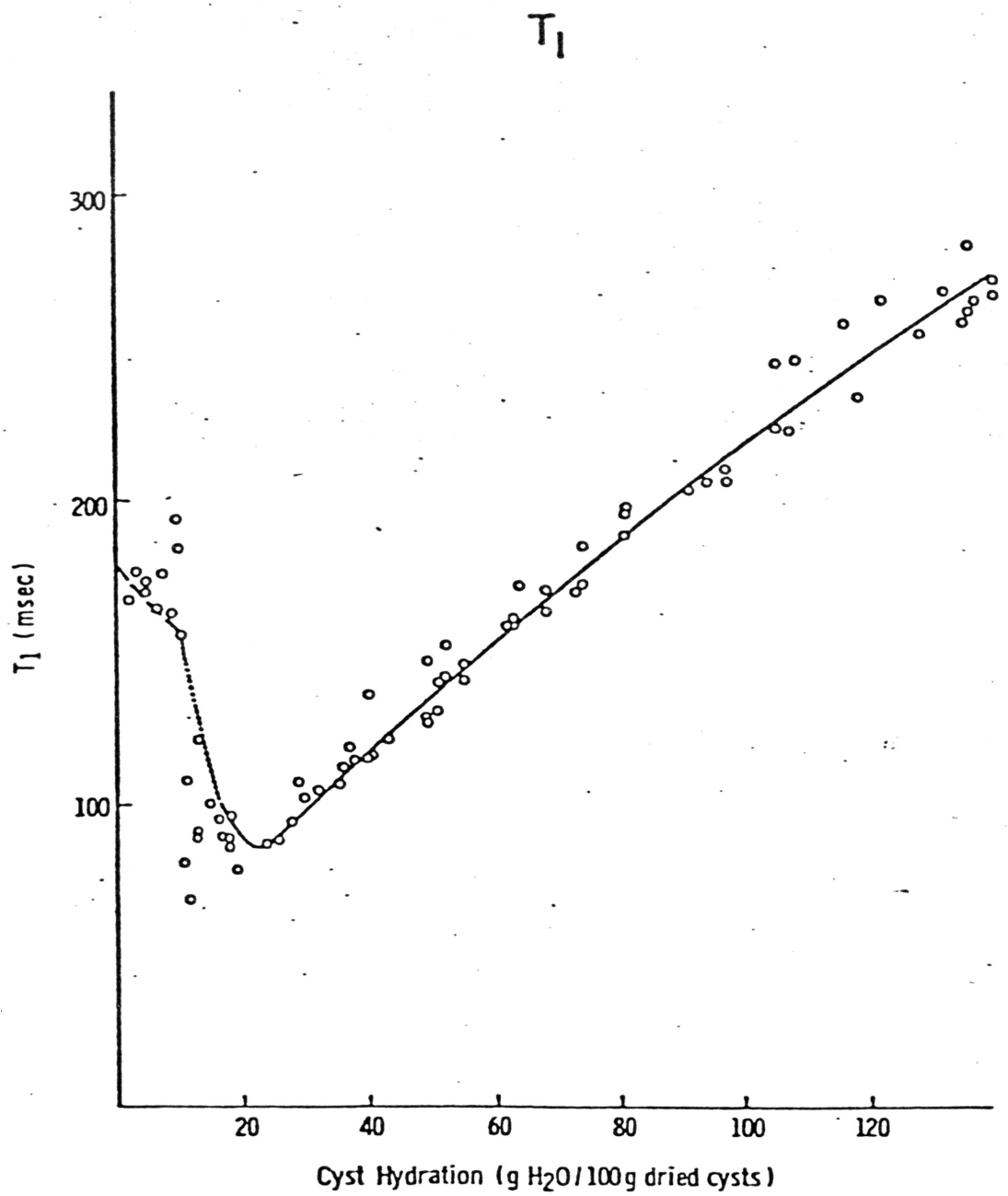
T_1
300
200
100
T_1 (msec)
20
40
60
80
100
120
Cyst Hydration (g H_2O / 100 g dried cysts)

Figure 11

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