NUCLEAR MAGNETIC RESONANCE STUDY OF MAMMALIAN CELL WATER

INFLUENCE OF WATER CONTENT AND IONIC ENVIRONMENT

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ABSTRACT The water proton spin-lattice relaxation time (T_1) in mammalian cells and tissues has been measured as a function of external ion concentration and total cell water content. The results can be interpreted in terms of changes in the fractions of bound and unbound water, and changes in the coordination shells of macromolecules due to alterations in macromolecular configuration caused by changes in salt molarity and the amount of water. It is shown that the direct effect of the ions (Na^+, K^+, Li^+, Cl^-) on structuring cellular water, i.e., into ion coordination shells, is small; the main effect of these ions on cellular water structure is an indirect one, resulting from their capability of changing macromolecular coordination shells.

INTRODUCTION

The state of water in biological systems has been the subject of many investigations, most of which have included nuclear magnetic resonance (NMR) techniques (1–3). However, since living cells are complex and since there are great variations from cell type to cell type in the amount of intracellular water as well as ionic composition and concentration, the NMR results are difficult to compare and interpret.

In this paper, we report on the influence of the total cell water content and the ionic environment on water proton spin-lattice relaxation time (T_1) in mammalian cells and tissues.

MATERIALS AND METHODS

Apparatus

For the NMR measurements an electromagnet with field probe regulation was used. The spectrometer was a 30 MHz coherent Spinlock Electronics spectrometer, model CP2. All T_1 measurements were made using the 180°, τ, 90° pulse sequence (4).

Cell sizing was done with a model Z.B. Coulter Counter (Coulter Electronics Inc., Hialeah, Fla.) attached to a 100-channel particle size distribution analyzer (Coulter Model C100 Channelyzer). The results were digitized and the median cell volume was calculated.

Biological Materials

The cells used were Chinese hamster lung fibroblasts (subline V79–S171). Further details concerning the cells and the methods of culture are described elsewhere (5).

The animals were Swiss albino rats. Both males and females between the ages of 1 and 2 yr were used.

NMR Sample Preparation

Large numbers of V79–S171 cells were grown up in roller bottles (each containing 100 ml of Basal Eagle's Medium) which were incubated at 37°C on a roller apparatus that turned the bottles at 0.5 rpm. In this manner large numbers (10^8) of cells could be obtained. The cells were harvested as follows. The growth medium was removed and the bottle was rinsed with 4 ml of trypsin EDTA. The bottles were then incubated at 37°C for 5 min resulting in the release of the cells from the glass.

About 10^8 cells were suspended in 20 ml of the various salt solutions for 20 min. These solutions were then centrifuged at 56.5 *g* for 1.0 min. The pellet was resuspended in 0.8 ml of the same salt solution and this was transferred to an NMR sample tube. These latter were centrifuged at 226 *g* for 5 min and the supernate was then removed. T_1 was measured immediately after this step. Cell viability, as measured by the trypan blue dye exclusion method, was 98% after the sample preparation. All the chemicals used were reagent grade.

The tissue samples were prepared for NMR measurements by the following technique.

The sample tissue, obtained immediately after cervical dislocation, was excised and cut in small pieces and the surface blood was removed by blotting. The tissue was then packed into NMR tubes and T_1 measured. For the salt treated samples, the tissue was allowed to equilibrate in the various salt solutions for 4 h. The tissue was then removed, blotted and packed into NMR sample tubes and T_1 was immediately measured.

Tissue drying was carried out in a vacuum oven at 80°C. The samples were left in the drying oven for 10 days. At this point they had dried to a constant weight in that no further water could be removed.

Cell Sizing

The cells were suspended in salt solutions at room temperature and allowed to equilibrate for 15 min before sizing. The cell sizing was then carried out in the salt solution with the cells in suspension. It should be noted, however, that the cell sizer aperture current was not derived from a constant current source. Therefore, the different electrolytic characteristics of the salt solutions caused the aperture current to change, resulting in artifactual shifts in the cell size distributions. This was overcome by using paper mulberry pollen particles that remained at constant volume in the various salt solutions. The pollen was sized in salt solutions and the artifactual volume changes (because of electrolytic changes of the solution with varying salt molarities) were then calculated. This result was used to find the actual volume change in the cells subjected to the various NaCl solutions. Serial cell sizing measurements of a cell sample in 0.930 M NaCl showed that the cell size equilibration occured in less than 3 min.

Hetzel et al. (6) have shown that cell viability has not decreased significantly in cells subjected to 0.930 M NaCl at 25°C for 15 min. Raaphorst et al. (7), using the same method as Hetzel et al., have shown that cells subjected to NaCl at molarities ranging from 0.038 to 1.75 at room temperature for 20 min had a survival greater than 95%.

For each figure shown, a different (fresh) animal was used. For each point in the figure, two samples from the same animal (or cell culture) were prepared. Standard errors (SE) were used to express experimental accuracy and are represented as vertical bars in all figures unless smaller than the points as plotted. In all cases, the SE was due to variability in the biological experimental sample rather than in the instrumentation or techniques.

All the solution equilibration procedures and measurements described in this paper were carried out at room temperature.

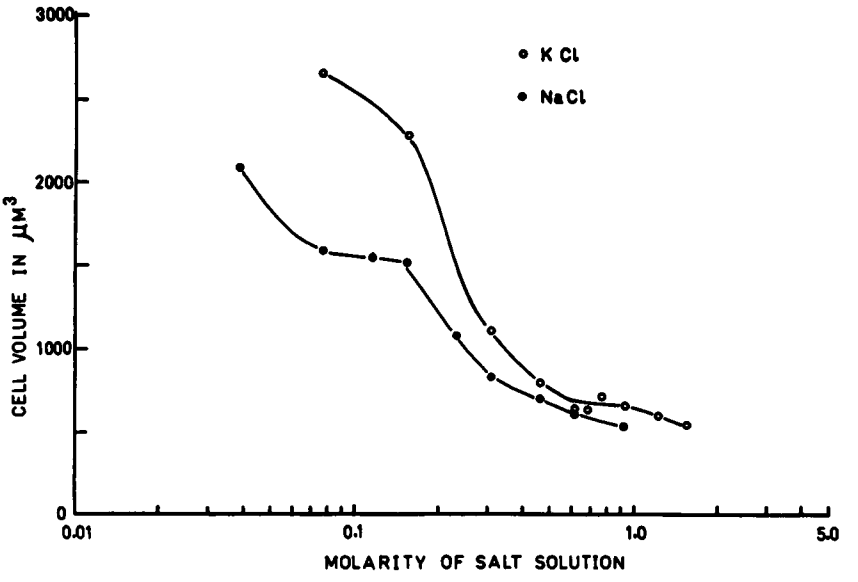

FIGURE 1 Cell volume (in cubic micrometers) vs. molarity of the salt solution for V79 Chinese hamster cells. The open and closed circles represent experiments with KCl and NaCl, respectively, in the external solution.

RESULTS

Fig. 1 shows the volume of the V79 cells as a function of NaCl (closed circles) or KCl (open circles) molarity in the external medium. Generally, cell volume decreases as the external molarity is increased.

Fig. 2 shows the final percent water content of rat liver tissues soaked for 4 h in NaCl (open circles), KCl (closed circles), and LiCl (squares) solutions of various molarities. Again, the percentage of water in the tissues decreases with increasing molarity of the soaking solution which is in agreement with the data in Fig. 1.

The water content of various fresh (untampered) rat tissues is given in Table I. One of the reasons liver was chosen for subsequent experiments was that the results with this tissue were highly reproducible (SE = $\pm 0.1\%$). Also, liver is highly vascularized so that solution equilibration occurs relatively quickly.

Fig. 3 shows water proton T_1 of V79 cells as a function of molarity of NaCl in the external medium. The cells were equilibrated as in Fig. 1. There is a minimum in the curve in the region of 0.155 to 0.3 M NaCl.

Fig. 4 shows water proton T_1 of rat liver as a function of molarity of NaCl. The open circles represent T_1 (right axis) of distilled water with NaCl added, while the closed circles indicate T_1 (left axis) of rat liver tissues soaked as in Fig. 2. This representation also holds for Fig. 5 and 6 where the salt used is KCl and LiCl, respectively.

It can be seen from Figs. 4, 5, and 6, respectively, that NaCl has little effect on T_1 of distilled water (open circles), while KCl causes an increase in T_1 above 1.0 M and LiCl

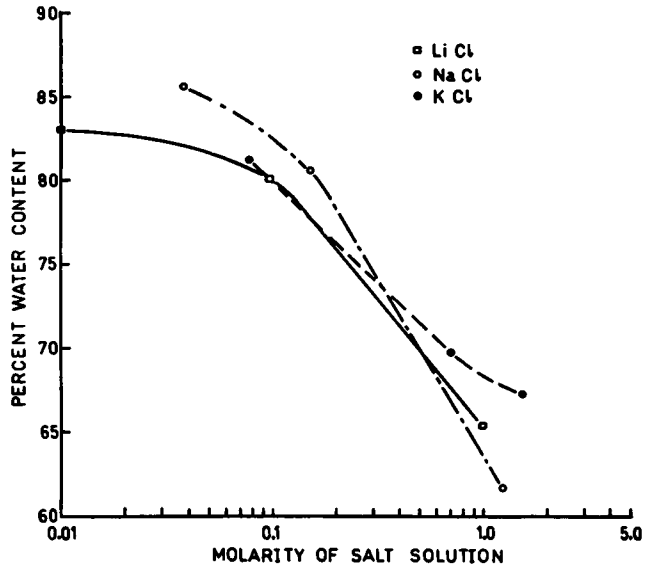

FIGURE 2 Percent water content vs. molarity of salt solution for rat liver tissue. The open and closed circles and open squares represent experiments with NaCl, KCl, and LiCl, respectively, in the external solution.

causes a decrease in T_1 above 0.1 M. These effects are to be understood in terms of the structure breaking (T_1 increases) and structure forming (T_1 decreases) capability of the various ions. A discussion is given in reference 8.

The situation with the rat liver tissues (closed circles) is somewhat more complicated as can be seen from Figs. 4–6. Clearly, there are points of inflection in the T_1 curves

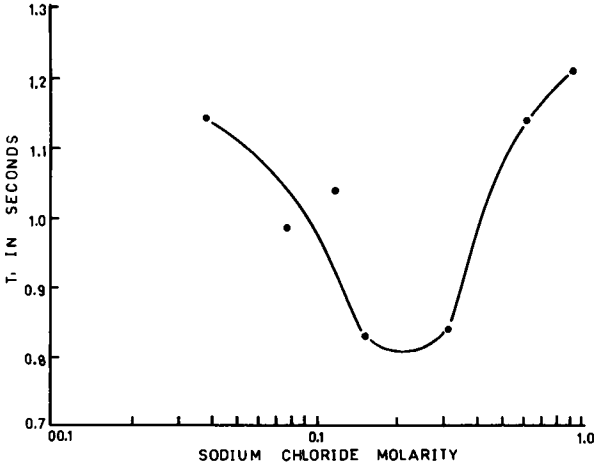

FIGURE 3 The water proton spin-lattice relaxation time, T_1, vs. NaCl molarity for V79 Chinese hamster cells.

TABLE I
WATER CONTENT OF VARIOUS RAT TISSUES

Tissue	% Water (mean ± SE)
Liver	69.9 ± 0.1
Spleen	76.95 ± 0.1
Kidney	75.5 ± 0.5
Muscle	72.85 ± 0.27
Heart	76.1
Blood	85.85

("minima" for simplicity) at 0.155, 0.63, and 0.12 M for NaCl, KCl, and LiCl, respectively. In all cases, there is an increase in T_1 as the molarity is increased above that required for the T_1 minima even though the tissue water content (as well as the cell diameter) is still decreasing with increasing molarity. In the case of LiCl, there is an additional structure to the T_1 vs. molarity curve in that T_1 begins to decrease again at the highest molarities reached in the reported experiment (Fig. 6). The experiments illustrated in Figs. 4–6 were repeated two times and the curves were qualitatively similar with the minima appearing at the same molarities as in the figures shown in each case.

It should be noted that the T_1 of fresh, untampered liver tissue was 305± 6 ms which coincides closely with the T_1 values of the minima in Figs. 4–6.

It should also be mentioned that the equilibration time for tissue is fairly critical in

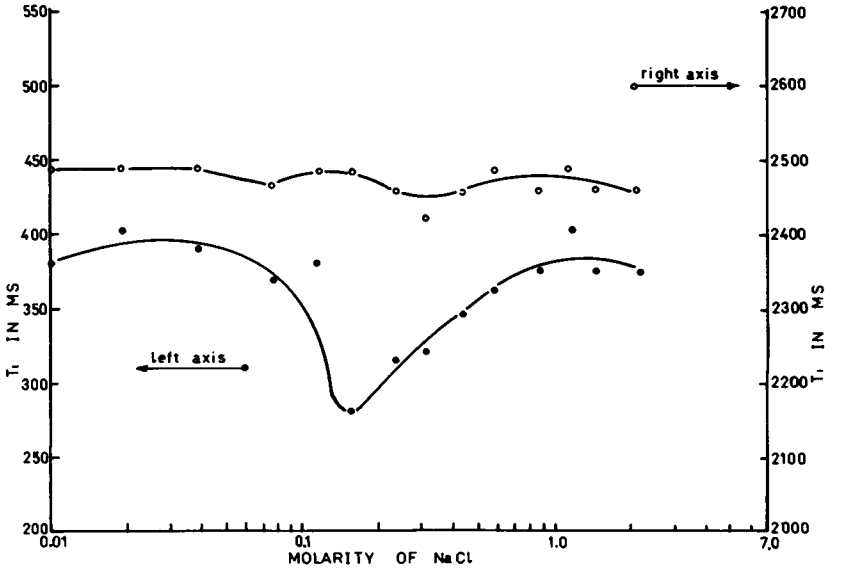

FIGURE 4 The open circles represent T_1 (right axis) of distilled water with NaCl added, while the closed circles indicate T_1 (left axis) of rat liver tissues as a function of molarity of the NaCl soaking solution.

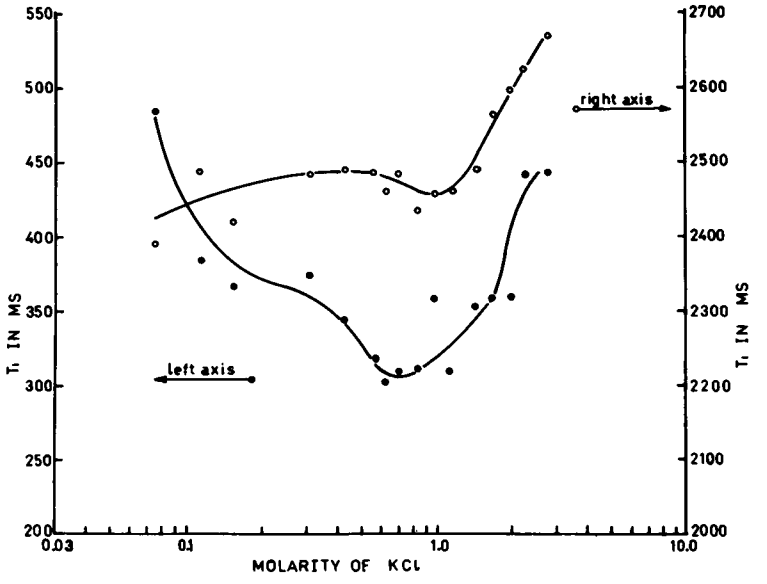

FIGURE 5 The open circles represent T_1 (right axis) of distilled water with KCl added, while the closed circles indicate T_1 (left axis) of rat liver tissues as a function of molarity of the KCl soaking solution.

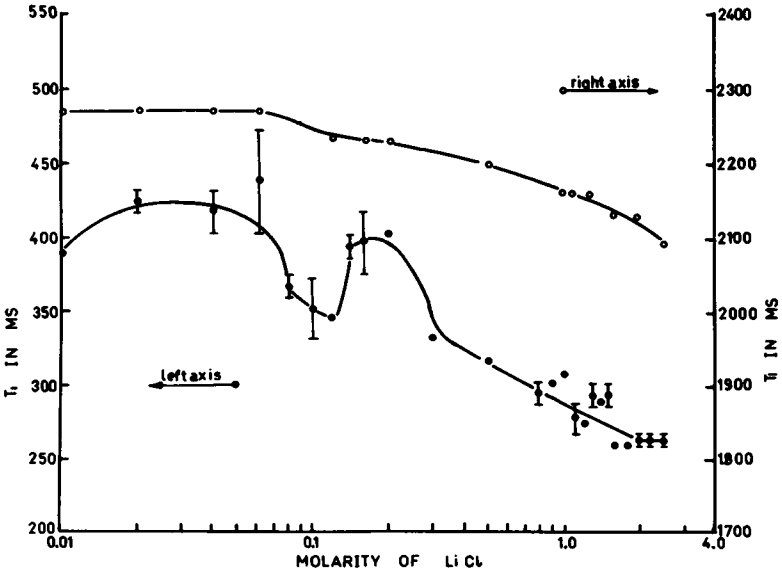

FIGURE 6 The open circles represent T_1 (right axis) of distilled water with LiCl added, while the closed circles indicate T_1 (left axis) of rat liver tissues as a function of molarity of the LiCl soaking solution.

that after a ½ h soaking in NaCl, the tissue T_1 vs. salt concentration curve is structureless. After a 12 h soaking, the T_1 for NaCl decreases continuously as the molarity is increased without any development of a minimum. However, in this case the viability of the cells is probably very low (6). The optimum soaking time was found to be 3–4 h with no significant differences in the T_1 results for these times.

DISCUSSION

Most water in tissues is thought of as being in an almost free state, i.e. not bound into protein coordination shells. In addition there is evidence that some of the water exists in polarized multilayers surrounding cellular macromolecules (9). NMR results imply that only a small fraction, an order of magnitude of 1%, of water is in such an organized state (10, 11). It is this water that is primarily responsible for the water proton spin-lattice relaxation in tissue (12). In addition to the above, a small amount of water is bound into the coordination shells of ions (e.g. K and Na). Futhermore, there should be exchange of water molecules as well as protons between these three compartments (10, 12).

As a result T_1 measures the combined effect of the water proton spin-lattice relaxation in all three (or possibly more) states or compartments. It can be seen that T_1 would be sensitive to any changes in the relative sizes of the different water compartments. For example, any change in the amount of free water molecules would change T_1, since the ratio of the amount of coordination shell water to free water is changed. Thus, a change in T_1 may be the result of (*a*) alterations in the amount of free water inside the cells (13), (*b*) differences in the composition or concentration of "water structure breaking" or "structure making" ions (e.g. K, Na, Li), or (*c*) changes in the volume of macromolecular coordination shells (e.g. as a result of alterations in macromolecular configuration). In addition, it should be pointed out that these effects are not always independent in that the first two may lead to changes in protein configuration (14–16) thus altering the coordination shell volumes.

The hypotonic portions of the T_1 curves (i.e. less than approximately 0.155 M) can be explained on the basis of more free water inside the cell as can be seen in Figs. 1 and 2. This changes the fraction of bound water to unbound (free) water and thus T_1 (12); e.g. a high T_1 value for a tissue indicates more free water in this tissue than in equivalent tissue that has a lower T_1 value. This is in agreement with the data of others (13, 17, 18). However, this explanation is inadequate for the hypertonic regions of the curve (i.e. above 0.155 M) since the cell water content is still decreasing but T_1 begins to grow. The increase in T_1, in the case of KCl, could be attributed, in small part, to the aqueous "structure-breaking" properties of the potassium ion (8). This property can be seen in the control experiment curve (open circles, Fig. 5). However, the effect of any ions on tissue water proton T_1 can be thought of as a small direct effect (i.e. ion coordination shells) plus a large indirect effect (due to alterations of coordination shells of macromolecules). Using the equations and general data in reference 12 and both sets of data in each figure it can be seen that the direct effect of the ions on T_1 is negligible.

That is, using the "distilled water plus salt" curves (open circles, Figs. 4–6), the total relaxation rate of all water protons in the solution is

$$R_s = gR_i + (1 - g)R_f,$$

where R_i and R_f are the respective relaxation rates of water protons in ion coordination shells and in "free" water. The fraction of all water molecules bound in ion coordination shells is given by g. It should be noted that for LiCl and NaCl the molarity changes by a factor of 200 (i.e. g varies by this factor), while T_1 only changes by 8% over this range for LiCl. Similarly for KCl, the molarity changes by a factor of 35 while the T_1 increases by 10% in this range. Thus, R_s is essentially constant over large changes of g. This could theoretically be explained either by having $R_i \approx R_f$ thus making R_s independent of g or by having g be a very small fraction. Certainly, even in a relatively concentrated salt solution like 1 M, the ratio of the number of water molecules to the number of salt molecules is ~55 to 1. Although one can not exactly calculate how many water molecules are bound to the alkali and the chloride ion, it is probable that even in a 1 M solution g is less than 0.5 but still a considerable fraction. Thus, it seems that the other alternative is more likely, i.e. $R_i \approx R_f$.

The relaxation rate of the *tissue* water protons is

$$R_{\text{total}} = b(R_b) + f(R_f) + i(R_i),$$

where b, f, and i are the relative numbers of water molecules in bound (macromolecular), free, and ion coordination shell compartments and $b + f + i = 1$. The order of magnitudes of the respective relaxation rates (second^{-1}) are $R_b \sim 10^3$, $R_f \sim 1$, and $R_i \sim 1$ (12). The magnitude of b is in order of 10^{-2} (12). Since i, the relative number of water molecules in ion coordination shells, is much smaller than f, the relative number of free or majority water molecules, it follows that R_{total} is determined by the first two terms primarily when the ion concentrations are low. The direct relaxation rate of ions, iR_i, is thus negligible. Even at high concentrations of KCl (0.6 M and above), T_1 of tissue changes by 50% while the T_1 of the solution changes by 10% in this same concentration range.

The indirect effect of the ions is their effect on b, the number of water molecules bound to macromolecules. It can be seen that because of the magnitude of R_b, small changes in b can have major effects on R_{total}. Considering the above, the major effect of high KCl concentrations on water proton T_1 must be the reduction of the coordination shells of proteins and other macromolecules.

Alternatively, at high KCl molarities the water content of the cell decreases; thus the protein concentration increases leading to higher collision frequencies and therefore increases in the rotational correlation times of the proteins. This effect may also contribute to the increase in T_1 (by affecting R_b) independently of any changes in b. However, if the above effect is of primary importance the T_1 for the LiCl curve should also increase at high molarities since the aforementioned effect is caused by the (osmotic) shrinking of the cells.

Since the Na ion is not a water structure breaker (reference 8; see also Fig. 4), the increase in T_1 at hypertonic NaCl concentrations is due to both the Na and Cl ion's ability to destabilize macromolecular structures (19, 20) and thus to decrease the volume of the effective coordination shell.

Some of the fine structure in the LiCl curve may be due to the macromolecular destabilizing properties of the lithium ion (19, 20) combined with the aqueous "structure-making" properties as indicated in the control curve (open circles, Fig. 6). However, calculating the importance of the latter property (as in the case of KCl) we find that it is negligible. Thus, at high molarities (above 0.3 M) the prime factors are the decrease in the free water content (Fig. 2) and changes in macromolecular coordination shells.

Thus as one increases the salt concentration, the common features of the T_1 vs. molarity curves are as follows. As low concentrations of ions (below 1 molar) are increased, the T_1 decreases to a minimum value. This decrease can be explained on the basis of loss of free water, thus increasing b, the relative percentage of bound water. The effect on T_1, in this case, may be largely due to osmotic factors rather than effects of specific ions. However, opposing this phenomenon is the effect of total water loss and the ions on the macromolecular coordination shells which are beginning to decrease in volume (i.e. decreasing b). Thus the rise in T_1. The latter effect is ion-specific. It is interesting to note that the minimum of T_1 occurs at 0.12, 0.16, and 0.63 M for LiCl, NaCl, and KCl, respectively. It is known that of the ions of these salts, Li^+ has the largest and K^+ the smallest coordination shell. Thus, it would seem reasonable that Li^+ would considerably disturb the coordination shells of macromolecules at the lowest molarity. The ranking of stabilizing and destabilizing solutes for macromolecules, as compiled by Von Hippel and Wong (19) and Meryman (20), lists lithium as a destabilizer, sodium as a weaker destabilizer and potassium as a weak stabilizer. Even these properties may be related to the ability of these ions to influence macromolecular coordination shells since structured water may be important in maintaining the configuration of macromolecules.

The results of Hazlewood et al. (21) suggest that the effect of possible paramagnetic impurities on the relaxation times of fresh, untampered muscle water is insignificant. Although it is possible that the addition of the salts, in our case, may change the binding of naturally occurring paramagnetic ions to macromolecules in the cells and thus affect the water T_1's, it should be pointed out that this would tend to make T_1 shorter. Since the penetration of the salts into the cell in any significant amounts would only occur under hypertonic conditions (i.e. above approximately 0.15 M for these salts), the above possibility would only help to explain results obtained with LiCl. In the cases of NaCl and KCl, the T_1 is increasing at higher molarities.

It should be pointed out that all our T_1 measurements, including the ones on salt solutions without cells or tissues, were done in air-equilibrated systems. This, of course, will reduce the T_1 with respect to measurements made in air-free solutions (22, 23). However, extreme hypoxia *inside* cells, especially in tissues, is difficult to produce (5) in addition to causing metabolic alterations and changes in viability in many cell systems (24, 25). Since measurements of T_1 *inside* viable, relatively normal cells

were desired, air-free solutions were not considered. It can be seen from the "distilled water plus salt" curves in Figs. 4–6 that at low concentrations (below 0.5 M) the water structure is not strongly disrupted by the electrolyte. Structure makers and structure breakers behave in a similar way at these concentrations, and NMR properties of the solution remain close to that of pure (air-equilibrated) water, in agreement with others using air-free solutions (26).

The qualitative variation in results with tissue as a function of soaking time is also of some interest. Since metabolism (27) and cell cycle movements (5) at 25°C are very slow, changes in pH and oxygen tension are probably very small. If not, both would influence the T_1. However, indications are that cells in multicellular systems are relatively well oxygenated at room temperature (28). If the soaking time is too long, e.g. 12 h, the exposure of the tissue to a salt solution at 25°C for this length of time will reduce the viability of the individual cells to a very small fraction (6). Significant changes in cell viability, for example, by freezing, boiling, or irradiating the sample, can drastically alter not only the T_1 but also the shape of the magnetization decay curve (29). Whatever the reason for the variation in results as a function of soaking time of tissue, it should be pointed out that the T_1 vs. salt concentration curve after a 4 h soaking time is qualitatively similar to that of V79 cells exposed for 20 min to the salt solution (Fig. 3). In the latter case, we have extensive data to show that there are no changes in viability (7) of the cells. Also any changes in pH and oxygen tension in the 20 min time interval at 25°C would be minimal. In addition, we know that the cell diameter has reached a constant value in this case (see Methods). Consideration of all the above makes us suspect that the qualitative variation in results with tissue as a function of a ½ or 4 h soaking time is probably due to time required for osmotic equilibration in excised tissue or for the ions to penetrate the cells. In support of the above, it should be noted that when experiments like those shown in Fig. 2 are performed as a function of soaking time in NaCl, the water content of the tissues changes drastically between 0 and 1½ h and thereafter relatively slowly (unpublished observations).

We feel that observation of alterations of coordination shells by NMR is a useful method in that it may allow predictions of behavior in many biological systems. For example, it is possible that the rate of diffusion of various substances may be different in structured water than in unstructured water (21). Since ionizing radiation damage involves diffusion of free radicals through macromolecular coordination shells, among other things, the size of the coordination shell may help to predict the damage. In fact, there seems to be a correlation between the T_1 vs. molarity curve for KCl (Fig. 5) and the radiation survival vs. molarity curve for V79 cells (7) despite the fact that in the case of the NMR studies we are looking at water associated with all macromolecules, i.e. mainly protein, whereas in the case of the radiation studies it is only the water near the DNA (and possibly the histones) that is of primary importance.

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