EVIDENCE FOR A SIGNIFICANT ROLE OF PARAMAGNETIC IONS IN THE OBSERVED NMR RELAXATION RATES OF LIVING TISSUES

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From the earliest days of nuclear magnetic resonance investigation, the ability of paramagnetic ions in aqueous solutions to cause the rapid relaxation of water protons has been recognized.[1-3] When biologists began to study the NMR relaxation of water protons in living tissues, they raised the question if the shortened spin-lattice relaxation time, or T_1, and the spin-spin relaxation time, or T_2, are significantly shortened by the presence of paramagnetic ions in the living tissues. The concensus of opinion from those early inquiries, expressed or implied, was that paramagnetic ions play little or no part in the shortened T_1 and T_2 seen in virtually all living cells. However, several authors did mention that the existing knowledge could not be considered adequate enough to discount paramagnetic ion contribution to T_1 and T_2 altogether. Among these may be mentioned Hollis et al[4], Ranade et al[5], and Lewa and Baczkowski.[6] Nevertheless virtually all agreed that the variable water contents were a major cause of the differences observed among different tissues, normal or neoplastic.[7-9]

In 1980 Ling and Tucker[10] showed that large differences exist among the T_1's of water protons in different living tissues even after their water contents had all been normalized to a uniform 80%. Ling and Tucker suggested that paramagnetic ion content might be an underlying cause for the difference in NMR relaxation rates of water protons of different tissues (see also Ling,[11,12] and Ling and Murphy.[13])

The present communication describes results of an attempt to find out if there is significant contribution of the "free" paramagnetic ion contents to the diverse T_1's of water proton among normal frog tissues.

MATERIALS AND METHODS

Leopard frogs (Rana pipiens pipiens, Schreber) from Vermont provided all living tissues for the present study. As a rule, before sacrifice, the frogs received an injection of enough heparin sodium to prevent blood coagulation (1 unit per gram of frog weight). After pithing, the frog was decapitated and hung upside down until most of its blood had drained off before dissection began. To make NMR measurement, theisolated tissues were first blotted on filter paper wetted with frog Ringer phosphate solution, and then introduced into 5 mm wide NMR tubes. For blood cell samples, a somewhat different procedure was used: the blood was taken from the heart and the blood cells separated from plasma by centrifugation. To obtain the longitudinal relaxation time of tissues normalized to 80% water content, I used the procedure described by Ling and Tucker:[10] equilibration in normal Ringer solution, in Ringer solutions made hypotonic by withholding varying portions of their NaCl, and in Ringer solutions made hypertonic by adding varying amounts of sucrose.

Trichloroacetic acid (TCA) was from Fisher Scientific Company, Philadelphia, PA (Lot 724342: Fe, 0.0003%. heavy metal (as Pb), 0.0003%). Bovine hemoglobin (Lot 63F-9321), whale myoglobin (Lot 61F-7036), cytochrome C (Lot 92F-0361), and hemocyanin (Lot 60F-

9550) were all from Sigma Chemical Company, St. Louis, MO.

For making the TCA extracts of tissues, 10% TCA was added to fresh tissue samples at 3 times the fresh tissue volume. The tissues were then ground in small homogenizers, and heated for 20 min. in a boiling water bath. After thorough mixing, the homogenates were transferred to capped 0.5 ml plastic centrifuge tubes and spun on an Eppendorf (Model 5412) microcentrifuge for 3 min. Aliquots of the clear supernatant solution wen than placed in NMR tubes and their T_1 determined using 180°–τ–90° pulses on a Spin Lock Coherent Spectrometer, Model CPS-2, operating at 17.1 MHz. Readings were taken at time intervals apart equal to or in excess of 10 X T_1.

To remove free paramagnetic contamination in hemoglobin, myoglobin, cytochrome C, and hemocyanin, these proteins were dissolved in 100 mM disodium ethylenediaminetetraacetic acid (EDTA) and dialyzed against several changes of similar solution overnight (4°C). This was followed by dialysis against many more changes of distilled water at 4°C for 3 days. This purification procedure does not remove bound paramagnetic ion from the proteins. Indeed even boiling for 20 min. in 100 mM EDTA (and 20% TCA) does not liberate bound iron from hemoglobin, myoglobin, and cytochrome C (unpublished work).

RESULTS

The accuracy of the TCA extraction procedure. One main requirement of this study was a method that would effectively extract "free" paramagnetic materials from the tissues but would not liberate bound (paramagnetic) iron from hemoglobin, myoglobin, or cytochrome C that may be present in the tissues. The extractive I chose was 10% trichloroacetic acid, which has long been used to extract non-hemoglobin iron from blood,[14] a fact already indicating that iron in hemoglobin cannot be liberated by TCA to any significant degree. However, to verify, I prepared solutions of bovine hemoglobin, whale myoglobin, cytochrome C, and hemocyanin and freed them of paramagnetic impurities. To one volume of each of these purified protein solutions was then added 3 volumes of 10% TCA, and they wen heated for 20 min. in a boiling water bath. After cooling and vigorous mixing, the samples were spun down in a centrifuge and the T_1 of the clear supernatant solution determined. The results are shown in Table 1. The differences between the protein extraction and TCA alone are insignificant with the exception of hemoglobin. Now an 8.27% hemoglobin solution contains (82.7/68,000) X 4 $=4.9 \times 10^{-3}$ M or 4.9 mM of Fe. 0.01 mM Fe^{3+} dissolved in the same TCA solution yields a T_1 of 1740 msec. roughly equal to that of the TCA extract of hemoglobin shown in Table 1. We can therefore conclude that the hot TCA procedure liberates no more than 0.01/4.9 = 0.002 or 0.2% of the iron in hemoglobin which showed the greatest iron liberation. It may also be relevant to point out that with the sole exception of hemoglobin in erythrocytes, the contents of other bound iron-(and bound copper-) containing proteins are very low. Thus heart muscle and

TABLE 1. T_1 of water proton of the TCA extracts of proteins which contain bound iron (hemoglobin, myoglobin, and cytochrome C) and bound copper (hemocyanin).

	Concentration (w/v)	T_1 (msec)
Hemoglobin	8.27%	1728±14
Myoglobin	25.2%	2225±66
Cytochrome C	0.6%	2298±53
Hemocyanin	1.43%	2258±54
TCA (alone)		2250±43

kidney contain the highest concentration of cytochrome C; yet their levels are respectively only 0.45 and 0.35 m g per gram of wet tissue. Similarly skeletal muscle and heart contain the highest amount of myoglobin; yet their levels are only 0.80 and 0.91 m g per gram of wet tissue.[15] However, hemoglobin exists at a much higher level in blood cells (i.e., 35% of its fresh weight).[16] This corresponds to $4 \times 350/68{,}000 = 0.02$ M Fe. After taking into consideration the dilution factor (4), one finds only $2 \times 10^{-2} \times 10^{-3}/4 = 5 \times 10^{-6}$ M or 5 micromolar of free Fe liberated from the hemoglobin. This amount of free Fe does not produce a significant change in a TCA extract of the erythrocytes.

The correlation between T_1 of the fresh living tissues normalized ro a water content of 80% and the T_1 *of the TCA extract. The* T_1 of tissues normalized to a water content of 80% can be obtained from the data like those shown in Figure 1 by reading the T_1 from the smoothed curve corresponding to the H_2O content of 80%. Figure 2 plou the normalized T_1 of the fresh tissues against the T_1 of the TCA extracts. Each point in the graph represents the average of 4 determinations. A statistical analysis of the data gave a correlation coefficient of +0.58. For the total sample number of 19, this correlation coefficient is significant at the 1% level in a two-tailed ten (see Snedecor and Cochran[17]).

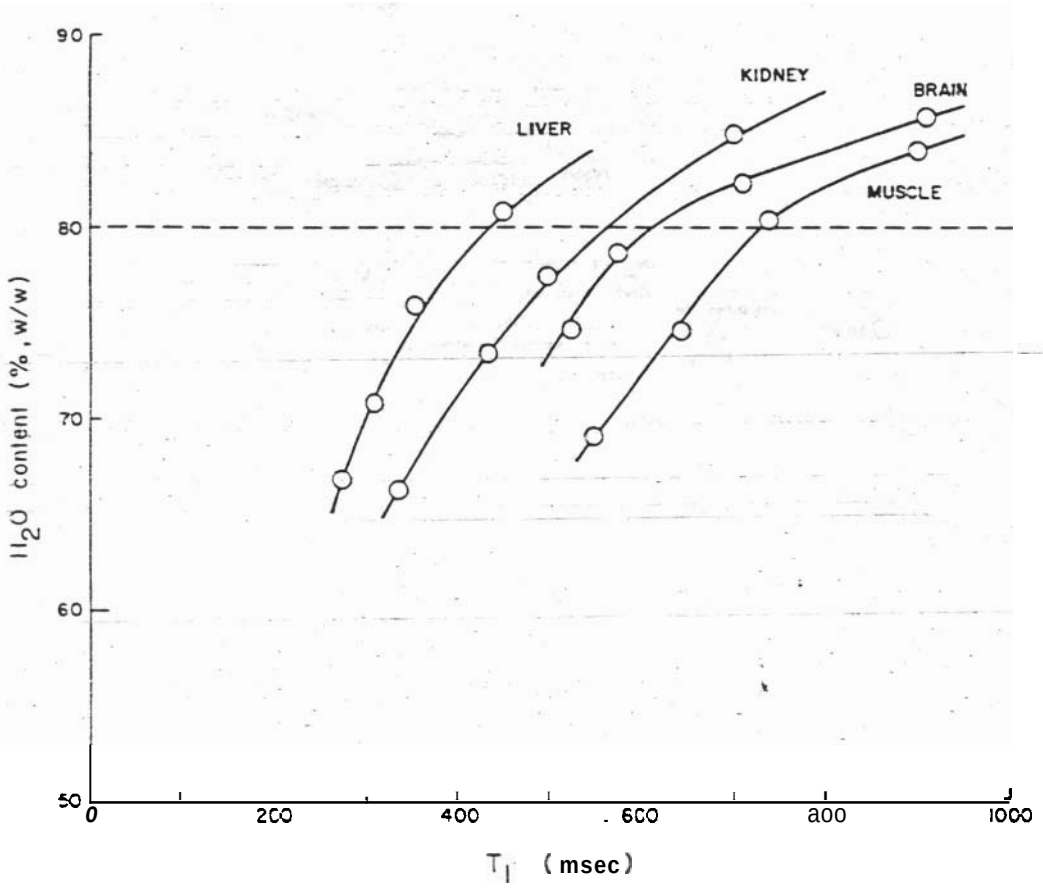

FIGURE 1. Relation between water contents of frog tissues and their spin-lattice relaxation times (T_1). Data are presented to illustrate methods used to obtain the T_1 normalized to a uniform water content of 80%.

DISCUSSION

A correlation coefficient of +0.58 signifies that about 30% of the T_1 values of the fresh tissues and the T_1 values of the 10% TCA extract are correlated.[17] It is entirely possible that by improving the extraction media and other procedural details, a higher positive correlation coefficient may be obtainable in the future. It is, nevertheless, not unexpected that the estimated correlation coefficient is not higher, because the T_1 value appears to have a compound origin of which the paramagnetic ion content is only one contributing factor. Other factors considered are the polarization of the bulk phase water[12, 13, 17], minor phase water associated with the diamagnetic proteins,[22] and spin diffusion between water protons and proteins.[23]

In retrospect, one may also understand why in the past many investigators who have looked into the matter, came to the conclusion that paramagnetic ions play an insignificant role in the NMR relaxation; most of

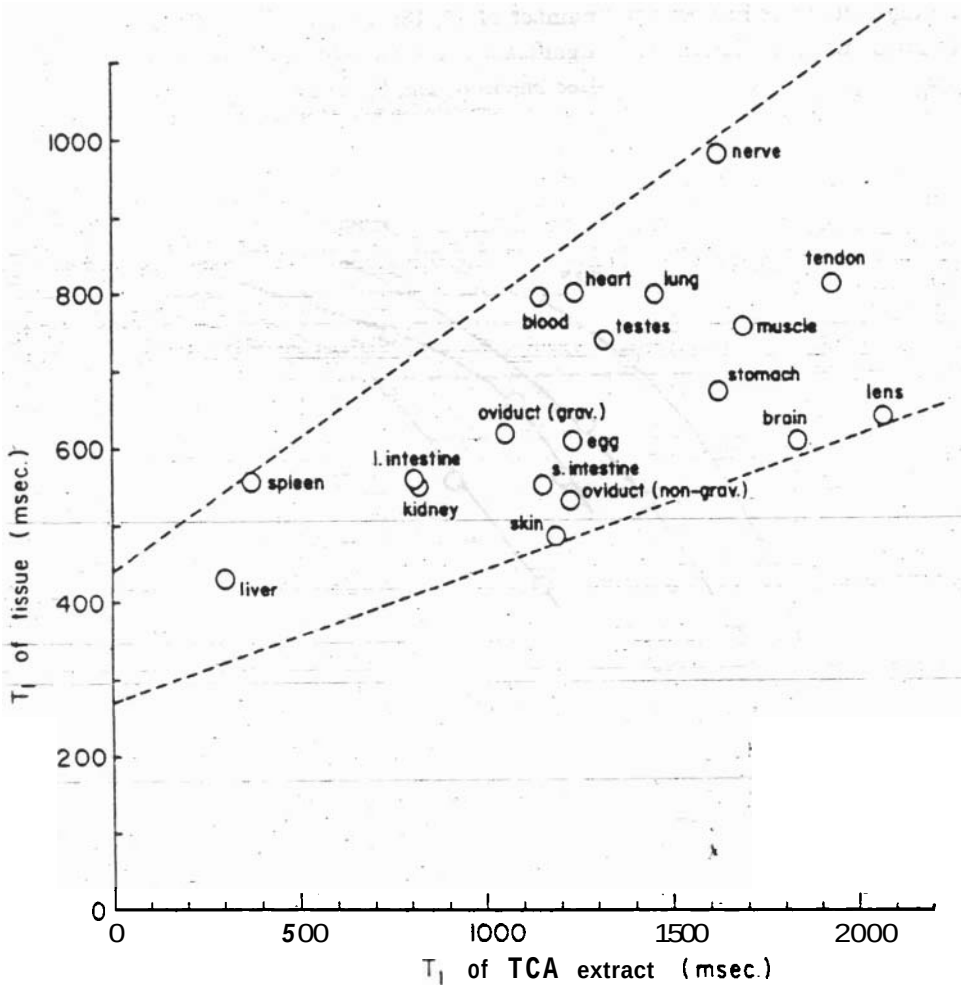

FIGURE 2. Relation between spin lattice relaxation time (T_1) of water protons of fresh tissues normalized to a uniform 80% water content and the T_1 of hot trichloracetic acid extracts of the tissues. "Blood" refers to separated blood cells, mostly erythrocytes. Each point is the average of four determinations. Correlation coefficient measured is +0.58.

these studies were on muscle tissue which as the data of figure 2 shows contains relatively little TCA extractible paramagnetic materials.

With the recognition that paramagnetic-ion contents play an important role in the different NMR relaxation rates among normal tissues and between normal tissues and cancer cells,[24] it seems reasonable to consider that the observed increase of T_1 of blood plasma after the inoculation of the mouse with ascites cancer cells[25] may be completely or partly due to the decrease of plasma iron levek, already established following the inoculation of mice with live cancer cells or with extracts of the cancer cells (e.g., toxohormone).[26-29]

SUMMARY

The T_1 of water proton in hot 10% TCA extracts of 19 frog tissues correlates with the T_1 of the fresh frog tissues normalized to a uniform 80%, with a correlation coefficient +0.58, significant at the 1% level in a two-tailed test. The data confirm earlier suggestions that 'fret" paramagnetic-ion content differences in different living tissues plays a significant role in the different spin-latrice relaxation rates observed among living tissues.

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