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The Effect of Aging and Storage Conditions on Excised Tissues as Monitored by Longitudinal Relaxation Dispersion Profiles*

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Knowledge about the dependence of NMR parameters of excised tissues on time and storage conditions is important for correct interpretation of *in vivo* clinical results based upon *in vitro* measurements which in many cases are easier to perform. Literature data on this topic are scarce and sometimes contradictory. In this study, we investigated the change of the proton longitudinal relaxation rate ($1/T_1$) of freshly excised rat tissues with storage conditions over a wide range of proton Larmor frequencies (0.01–50 MHz). Special interest was paid to long-term storage in the deep frozen state. After deep freezing of tissue samples on dry ice, no significant changes in $1/T_1$ over the whole frequency range could be observed for brain tissue, whereas for muscle and liver tissue characteristic alterations were detected. We conclude that for normal brain tissue this storage procedure is suitable for the prediction of *in vivo* $1/T_1$ results from *in vitro* studies. © 1989 Academic Press, Inc.

INTRODUCTION

The measurement of NMR tissue parameters, mainly proton density, T_1, and T_2, increasingly attracts interest for medical applications, since these parameters are of paramount importance in the characterization of normal and pathological tissues and are hoped to help in improving specificity of the diagnosis by MRI. Due to the difficulties in measuring the above-mentioned parameters, especially T_1, with satisfying accuracy on a MRI system, many investigators have used *in vitro* techniques on small tissue samples. This suggests the question whether the values measured on the tissue samples are the same as they would have been in the living organism. Whereas it seems reasonable that a freshly excised tissue, carefully handled (no chemical or physical action that could change the macro- and microscopic structure and the water content of the sample), should give the same results as it would have given *in vivo*, transport and storage of tissue samples present a problem. Since only in a few cases are measurements directly after excision possible, very often transport from the surgery room to the NMR laboratory will be necessary, and sample storage for some hours or days is unavoidable. Thus, better knowledge about variation of NMR pa-

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rameters of tissues with time and storage conditions is needed. The literature is quite rich in *in vitro* tissue data, but the problem of sample handling and storage has rarely been touched, in some cases not even mentioned. Available data (*1, 2, 3, 9*) do not allow the determination of a "safe" protocol for tissue handling. Efforts are made to standardize tissue sample treatment (*4*), but in this program the effects of long-term storage have not been analyzed.

The present work has been undertaken to study storage effects on $1/T_1$ for selected rat tissues with special attention to brain. Our study is not limited to only one Larmor frequency, but with a range from 200 kHz (10 kHz for some experiments) to 50 MHz it covers the whole frequency range relevant for most clinical applications, and the normally existing difficulty of comparing results obtained at different field strengths can be overcome. Also, the sensitivity to detect changes in the relaxation behavior should be increased relative to studies covering only one frequency. Finally, through the interpretation of the relaxation dispersion profiles, this approach can provide some information concerning possible structure modifications with storage.

MATERIALS AND METHODS

Tissue Preparation and Handling

A total of 14 tissue samples (10 brain, 2 skeletal muscle from the hind leg, 2 liver) was obtained from two female Sprague–Dawley rats, weighing about 250 g. The animals were sacrificed by decapitation, and the samples were dissected in such a way that seven pairs of identical tissues (right/left symmetrical for brain, adjacent for muscle and liver) were obtained. One brain was divided into four parts (front and back, right and left, with the "back" samples including the cerebellum and medulla oblongata), and the other one in six parts (front, back, and cerebellum + medulla oblongata, right and left). The sample weights ranged between approx 200 and 500 mg. The samples were placed in 10-mm-o.d., 75-mm-length disposable glass tubes and the tubes were sealed by rubber plugs. No condensed water on the tube walls or extracellular water at the bottom of the tube was observed on any sample during the whole experiment.

One sample of each pair, denoted "deep frozen" throughout this text, was quickly deep frozen by placing the tube in dry ice (−78°C) for at least 30 min immediately after excision; the other sample of each pair, denoted "fresh," was taken to NMR examination directly or stored at 7°C for less than 1 h.

The deep frozen samples were stored at −20°C for several weeks before NMR measurements. These samples were then allowed to warm at room temperature for about 15 min. For all samples the time delay between placement into the probehead and beginning of the measurement was 5 min, to let the sample warm to 37°C.

After the NMR measurement, three of the deep frozen brain samples were stored at 7°C for about 18 h and the measurement was repeated.

Four of the "fresh" samples (liver, muscle, and two brain samples) were deep frozen on dry ice and kept at −20°C for several weeks before the second NMR measurement. One of these samples (brain) was kept at 7°C for about 18 h, and the measurement was repeated once more.

One of the "fresh" brain samples was stored at 7°C for 18 h and the measurement was repeated. The sample was then deep frozen and remeasured several weeks later.

The last two "fresh" brain samples were stored at 37°C for about 18 h before repetition of NMR. Then these samples were also deep frozen and remeasured several weeks later.

So, up to three successive storage procedures were performed on each sample, together with NMR measurement. For evaluation of the results, all data were compared to the results of the first measurement on the "fresh" sample of each pair.

Several additional experiments were performed on four freshly excised rat tissue samples from four different animals: liver, skeletal muscle (thigh), adipose tissue (renal), and brain (cerebrum). In these cases, the experiment started less than 10 min after excision. For adipose and brain tissue one relaxation dispersion was recorded, respectively, whereas for liver and muscle tissue the measurements were repeated several times after different time delays, keeping the sample temperature constant at 37°C, also between measurements.

NMR Relaxation Measurements

The longitudinal relaxation rates $1/T_1$ of the samples were measured on an IBM Research relaxometer as described by Koenig and Brown (*5*). It uses the NMR field cycling technique (*5–8*) and is capable of measuring $1/T_1$ in a range from about 50 to below 1 s^{-1} at any desired magnetic field strength between 0.00025 and 1.2 T, corresponding to proton Larmor frequencies ranging from 0.01 to 50 MHz. Usually, for the determination of $1/T_1$ at one field strength, 15 individual measurements of the longitudinal magnetization are performed at equal time intervals covering a time of $1.5 * T_1$. The error in $1/T_1$ determination is usually around 1%. The resulting plot of $1/T_1$ versus Larmor frequency is called a "nuclear magnetic relaxation dispersion (NMRD) profile" or simply "relaxation dispersion."

For the long-time storage experiments, only four points of the relaxation dispersion have been recorded. As the collection of data takes about 3 min of experimental time for each point, this limitation helped to speed up experiments and to reduce the possible influence of the warming period during the NMR measurements. The measuring frequencies chosen here were 0.2, 1, 10, and 40 or 50 MHz. For the additional experiments at constant temperature, complete NMRD profiles have been recorded, covering the Larmor frequency range from 0.01 to 50 MHz with 9 to 13 measurement points.

For all the NMRD profile measurements the sample temperature was 37°C.

RESULTS

Relaxation Dispersions of Tissues

The variation of $1/T_1$ over the whole accessible range of field strengths for some species of fresh rat tissues (one sample of each) is shown in Fig. 1. The diagram, which shows results very similar to those published by Koenig and Brown (*9*), demonstrates the large variation of $1/T_1$ with frequency and the partial crossover at high

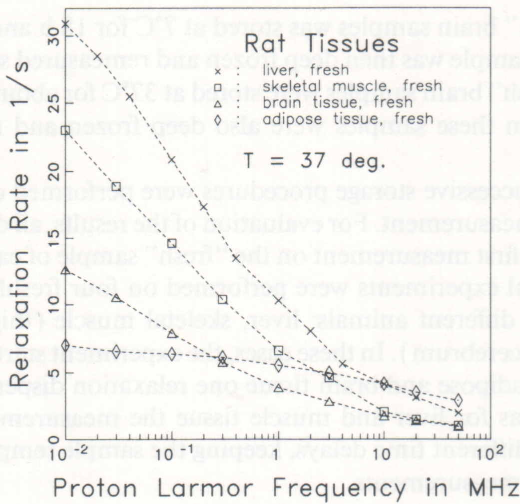

FIG. 1. Typical NMRD profiles for rat tissues at 37°C.

fields, which might have implications for contrast in MRI. For human brain tissue, this topic is discussed by our group in (*10, 11*).

Influence of Storage Conditions

1. Liver. The results of the two liver samples are shown in Fig. 2 which always displays the changes relative to the fresh sample.

It can be seen that neither deep freezing nor storage at low temperatures prevents

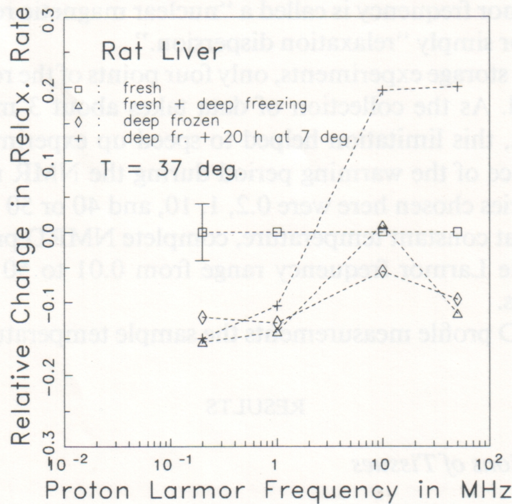

FIG. 2. Changes in the NMRD profile of rat liver after different alternative storage procedures. For the directly "deep frozen" sample all data are calculated relative to the first measurement of the "fresh" sample. The error bar indicates the standard error for each calculated change in relaxation rate and is also valid for Figs. 3 to 6.

characteristic alterations of the relaxation profile. After deep freezing the whole curve is shifted down to lower rates; after subsequent storage at 7°C the rate at high frequencies increases again. The same kind of $1/T_1$ variation has been observed at 20 MHz and 37°C (*12–14*): drop in the rate at the beginning of the experiment and then an increase within several hours up to a value higher than the initial one.

The dispersion profiles recorded at different times after excision for a liver sample kept at 37°C are shown in Fig. 3. Also in Fig. 3, the drop in the rate at high and low fields at the beginning of the experiment is visible. Additionally, a second general drop in the relaxation rate is visible after storage for a long time, first visible 10 h after excision.

Comparison of both graphs indicates that the aging processes of the tissue, as reflected by the NMDR profile, are similar or identical for storage at high or low temperatures, but with different time scales.

2. *Skeletal muscle.* Data for skeletal muscle are presented in the same way as that for liver in Figs. 4 and 5. They show the same behavior as that for the liver samples, yet less pronounced. Here, too, the second general drop in the rate is visible, but only after a very long storage time.

3. *Brain tissues.* The first graph (Fig. 6) shows typical results of the freeze–thaw experiment on one brain sample (frontal).

It is obvious that the "storage effect," if existent, is much less pronounced than that

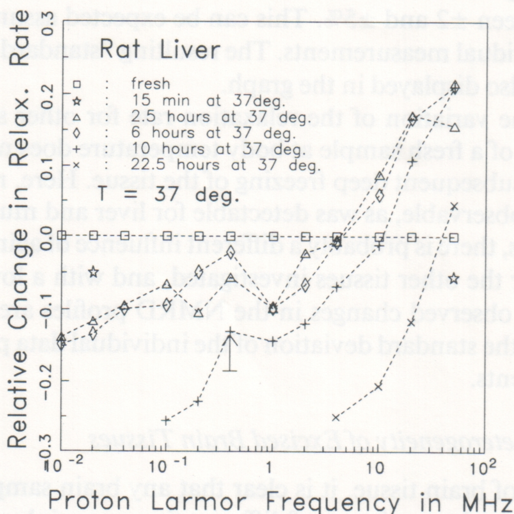

FIG. 3. Changes in the NMRD profile of rat liver with time at a constant temperature of 37°C. "Undulations" in the curves (marked by an arrow) are due to (and prove) $1/T_1$ changes during measurement: 0.4 MHz was one of the last points recorded for the fresh sample, the sample had been in the probehead for about 30 min, and the relaxation rate had dropped a certain amount already. So, the difference to the later measurements is smaller. However, the visibility of the general behavior is not affected by this source of error. For the other tissues investigated, the influence of this effect is negligible, as the relaxation rate does not change so rapidly. The data at 0.02 and 50 MHz for the fresh sample have been recorded in the very beginning of the experiment, and the measurement has been repeated about 15 min later, giving the two points marked with stars.

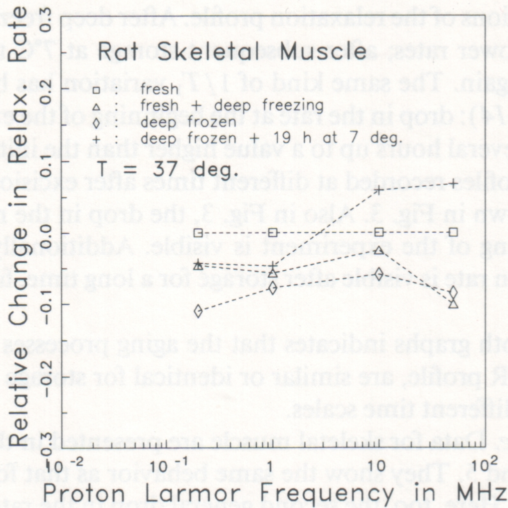

FIG. 4. Changes in the NMRD profile of rat skeletal muscle after different alternative storage procedures. Data for the "deep frozen" sample are relative to the first measurement of the "fresh" sample.

in liver and muscle. The deviations are in the range of the experimental error.[1] This is confirmed by averaging the results of samples which have been treated the same way, as shown in Fig. 7. The standard deviations of the averages ("sample standard deviation") lie between ±2 and ±5%. This can be expected assuming that it reflects the error of the individual measurements. The resulting "standard error of the mean" of about 1 to 2% is also displayed in the graph.

Figure 8 shows the variation of the relaxation rate for other storage procedures. Even longer storage of a fresh sample at body temperature does not have a dramatic effect, nor does the subsequent deep freezing of the tissue. Here, no systematic effect of longer storage is observable, as was detectable for liver and muscle. This indicates that, for brain tissues, there is probably a different influence of aging on the relaxation profile from that for the other tissues investigated, and with a lower amplitude. On the other hand, the observed changes in the NMRD profiles are hardly significant, taking into account the standard deviation of the individual data points and the small number of experiments.

Comments on the Heterogeneity of Excised Brain Tissues

From the nature of brain tissue, it is clear that any brain sample obtained by the method used here contains a mixture of different tissues, mainly gray and white mat-

[1] During the time period in which the experiments described here were performed, the experimental error in $1/T_1$ determination was slightly increased due to an instability in the commercial RF transmitter. Furthermore, in Figs. 2 to 8 we display differences between error-born data, onto which the Gaussian law of error propagation $s = \sqrt{[(s_1)^2 + (s_2)^2]}$ must be applied. Thus, an experimental error of 2 to 3% results in a standard deviation of the difference between two values between about 3.2 and 4.2%. Therefore, the standard deviation of the data displayed in Figs. 2–8 is assumed to be about 4%, as indicated by the error bars in Figs. 2 and 6.

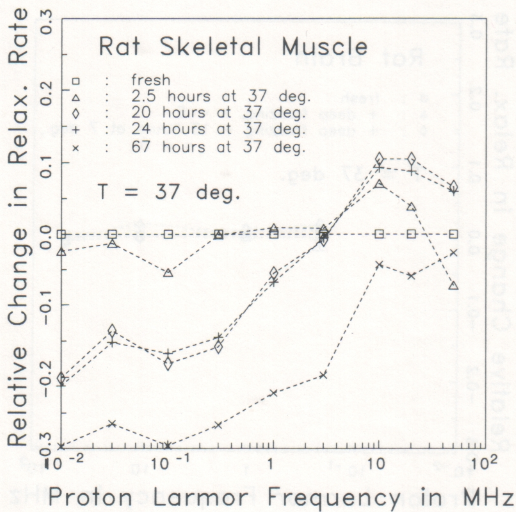

FIG. 5. Changes in the NMRD profile of rat skeletal muscle with time at a constant temperature of 37°C.

ter. This could present problems with respect to the multiexponentiality of the relaxation process and to the comparability of paired left/right brain tissue samples, as the proportions of the different tissues could change.

For human brain samples, the relative difference in $1/T_1$ between gray and white matter is 50% or less, depending on the Larmor frequency, with a maximum around 10 MHz (*10, 11*). Assuming a sample with gray and white matter contributing 50% each to the initial NMR signal and $1/T_1$ (white matter) = $1.5 * 1/T_1$ (gray matter), with the measurement technique applied by us (observation of the magnetization during 1.5 times the estimated T_1), the decay is indistinguishable from a single expo-

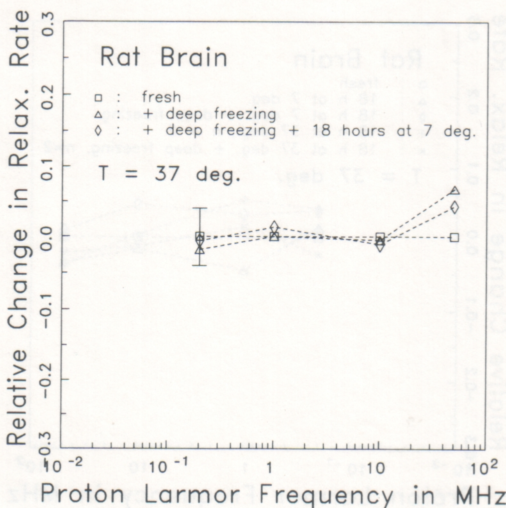

FIG. 6. Changes in the NMRD profile of one brain sample after different storage procedures.

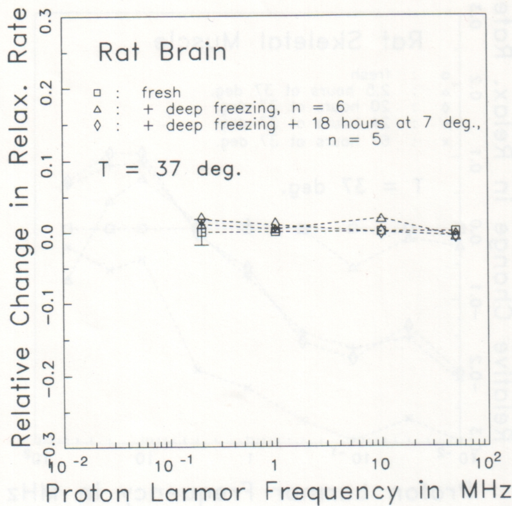

FIG. 7. Same as Fig. 6, averaged over several samples. Both data for "fresh" and "deep frozen" samples are used. The error bar indicates the standard error of the mean.

nential with a T_1 close to the average of the two tissues. This problem will always arise unless the magnetization is followed up for much longer times, where, on the other hand, noise comes into play. So, the relaxation rates of the brain tissues given here are always "averages" of various tissues. But this is of no importance if, as in this case, the scope of the measurements is to investigate the reproducibility of measurements on identical or similar tissue samples.

Another illustrative example of the problem of biexponentiality has been given by Koenig and Brown (*9*).

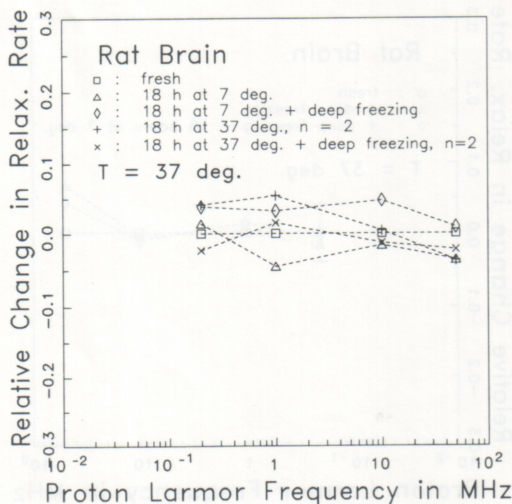

FIG. 8. Changes in the NMRD profile of rat brain tissues after different alternative storage procedures.

Concerning the comparability of dispersion curves stemming from different but paired brain samples, the variations we found between the relaxation rates of paired samples, which had been treated the same way, were small and in the range of the experimental error. A possible explanation is related to the biexponentiality mentioned above: when the proportions of tissue contributions in the given example are changed from 50:50 to 60:40, the resulting $1/T_1$ changes by less than 5%, and we think that the differences in composition between paired samples are in this range or lower. Therefore, a combination of the data from deep frozen samples with those of fresh samples, as performed in Figs. 7 and 8, seems possible.

DISCUSSION

From the results presented, the following conclusions can be drawn:

—Quick deep freezing on dry ice seems to be a satisfactory storage method for normal brain samples, since it does not affect significantly the relaxation rate at any investigated frequency. This indicates that no aging process affecting the longitudinal relaxation has started yet. Since the relaxation rates $1/T_1$ and $1/T_2$ and the water content (or the proton density ρ) of a tissue are interdependent, one can assume that the other principal NMR parameters also remain unchanged, although this has not been proven experimentally in this study.

—For liver and muscle tissues, the method described above is not applicable. Deep freezing and subsequent storage at low temperatures result in a characteristic change in the relaxation profile, giving a flatter slope. The change in the slope might indicate a change in the size distribution of macromolecules involved in the relaxation process (*15*). The change follows the direction of smaller sizes, i.e., disintegration.

—For almost all the measurements on brain tissues, the variation of $1/T_1$ remains in the range $\pm 10\%$. This indicates that, apart from the finding of a "safe" storage procedure, "rough" treatment (e.g., storage at body temperature for longer times) of normal brain tissue samples does not affect the NMR parameters dramatically. The same holds for skeletal muscle treated according to the "safe" storage procedure.

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