CARBON-13 AND PROTON MAGNETIC RESONANCE OF MOUSE MUSCLE

B. M. FUNG, *Department of Chemistry, University of Oklahoma, Norman, Oklahoma 73019 U.S.A.*

ABSTRACT It is shown that roughly 4 mmol carbon atoms/g mouse muscle can give rise to a "high resolution" ^{13}C NMR spectrum. From the ^{13}C spectrum, it is estimated that the protons from mobile organic molecules or molecular segments amount to 6–8% of total nonrigid protons (organic plus water) in muscle. Their spin-spin relaxation times (T_2) are of the order of 0.4–2 ms. At 37°C, the proton spin-echo decay of mouse muscle changes rapidly with time after death, while that of mouse brain does not.

In a study of broad-line proton nuclear magnetic resonance (NMR) of muscle, it was found that part of the protons that gave rise to a relatively narrow NMR signal (ca. 1 kHz wide) could not be replaced by D_2O and did not disappear upon dehydration (1). The authors assigned this signal to "bound water" in an ordered minor phase. In studies of the transverse relaxation of protons in muscle, it was found that the spin-echo decay of the proton signal was not exponential (2–4). These authors decomposed the decay curve into three fractions, assigning the slow fraction to extracellular water, the intermediate fraction to intracellular water, and the fast fraction either to bound water (2, 3) or to "relatively mobile protons in tissue protein and lipid" (4). In this paper we will show that nonrigid protons in organic molecules, rather than "bound water," are responsible for the fast-relaxing fraction of the proton signal, and that the intermediate and slowly decaying parts are not necessarily caused by two fractions of water (intracellular and extracellular).

Fig. 1 shows the carbon-13 spectra of natural and dehydrated mouse muscle. The ^{13}C spectra were taken with a Varian XL-100 spectrometer (Varian Associates, Palo Alto, Calif.) equipped with Fourier transform accessories manufactured by Nicolet Technology Corp. (Mountain View, Calif.). An external F-19 lock and a proton decoupling band width of 1.2 kHz were used. The dehydrated muscle was prepared by pumping in vacuum for 24 hr and then storing over P_2O_5 for several days.

The ^{13}C spectrum of natural muscle (Fig. 1, A) indicates that an appreciable number of organic molecules or molecular fragments are relatively mobile, undergoing considerable rotational motion. The peaks centered around −30, −130, and −175 ppm (from tetramethylsilane) can be assigned to aliphatic, aromatic, and carbonyl carbons, respectively. The corresponding half-widths ($\Delta\nu$) of the three peaks are about 200, 200, and 500 Hz and the apparent spin-spin relaxation times ($T_2^* = 1/(\pi \cdot \Delta\nu)$) are about 1.6, 1.6, and 0.6 ms, respectively. Since the peaks are composed of overlapping signals, the actual T_2^*'s should be larger. The true spin-spin relaxation time (T_2) is usually

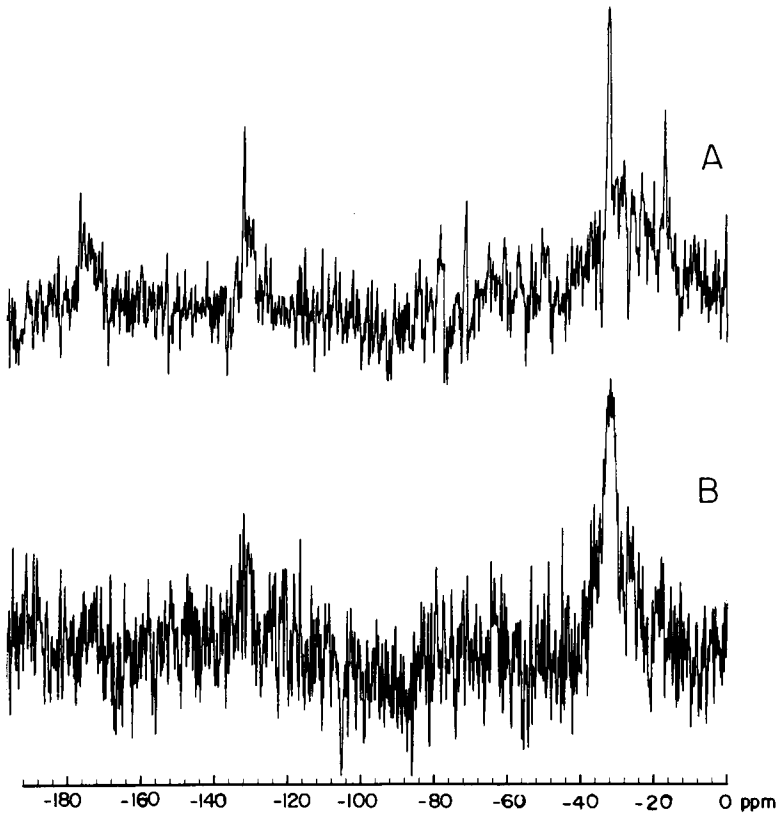

FIGURE 1 ^{13}C spectra of mouse muscle at 25.2 MHz and 27°C. The bottom scale is chemical shift from tetramethylsilane. (A) intact muscle (18,000 scans over 7 h), (B) dry muscle (18,000 scans over 21 h; a longer delay time after each pulse was used because of larger T_1).

longer than T_2^* because of inhomogeneities in the sample and magnetic field. Therefore, we estimate T_2 of the mobile carbons to be 2–4 ms. The relaxation times of protons are usually 2–5 times shorter than the directly attached carbons (5). Therefore, T_2's of the aliphatic and aromatic protons in the mobile organic molecules or molecular segments might be expected to be of the order of 0.4–2 ms, which coincides with the value cited for the fast-decaying segment of the spin-echo train in muscle (3, 4). The prominent sharp peak at −33 ppm in the ^{13}C spectra is most likely due to fast-rotating methyl groups, the proton T_2's of which would be somewhat longer. However, the intensity of the peak is quite small and the contribution of the methyl groups would not be important in the proton spin-echoes.

To ascertain that the fast-decaying part of the proton signal is indeed due to protons in organic molecules rather than protons in "bound water," we now consider the problem of the signal intensity. It is well known that the accurate determination of carbon-13 intensities is subject to problems such as different nuclear Overhauser effects and relaxation rates. However, these problems are less severe for fast-relaxing carbon-

13 nuclei, such as those observed here. We compared the total intensity of the carbon signals in Fig. 1A to that of a sample of 63% dipalmitoyl lecithin in water, and found that for each gram of muscle roughly 4 mmol of carbon atoms are relatively mobile, of which about 12% is carbonyl, 12% is aromatic, and 76% is aliphatic. (The sample of dipalmitoyl lecithin in D_2O was prepared by centrifuging the mixture back and forth at 50°C through a constriction in a tube. The carbon-13 spectrum was taken under the same conditions as for the dehydrated muscle.) If each of the aromatic and aliphatic carbon atoms has on average 1.5–2 protons attached, the quantity of nonrigid organic protons would be 5–7 mmol/g muscle. Since normal mouse muscle contains 75% water (6), the number of water protons is 83 mmol/g muscle. Therefore, the percentage of nonrigid organic protons in the proton signal of muscle (excluding the contribution from the immobile organic protons in the microsecond range) is about 6–8%. This again falls in the range of the values (3–8%) quoted for the fast-decaying part of the proton spin-echo (3, 4).

Thus, by studying the line-width and the intensity of the ^{13}C spectra of natural muscle, we have made plausible that nonrigid organic protons are indeed responsible for the fast-decaying or "millisecond" part of the proton spin echoes, as suggested by Foster et al. (4). The proton signal attributed to tightly bound water molecules (2, 3) that do not exchange with D_2O or cannot be evacuated (1) is actually due to nonrigid organic protons. Even for dehydrated muscle, some fast rotating methyl groups have relatively narrow ^{13}C (Fig. 1B) and proton (1) peaks. This organic fraction would be particularly important when nonfreezable water is studied at low temperature (7–10) and when dehydrated muscle is investigated (11, 12). It has been shown that the deuteron signal does not have a millisecond fraction in the free induction decay (14) or spin echoes (4). An experiment which claimed that part of the deuteron signal for D_2O in deuterated muscle was "NMR-invisible" (13) could not be reproduced in other laboratories (14, 15).

We now proceed to give further evidence that the nonexponential behavior of the intermediate and slowly decaying portions of the proton spin-echoes may not be caused by the superposition of signals of two slowly exchanging factions (16).

Fig. 2 shows the amplitudes of the spin-echo train of the water protons (organic or millisecond fraction excluded) of mouse muscle at 37°C as a function of time. The proton NMR spectra were taken with a home-built pulse spectrometer with a Bruker high-resolution magnet and probe (Bruker Instruments, Inc., Billerica, Mass.). Eight scans were accumulated with a Nicolet 1072 signal averager for each spectrum. The muscle was dissected and quickly put inside a sample tube preheated to 37°C in the spectrometer probe. The sample was allowed to equilibrate for 5 min before the first reading was recorded. Shortly after the muscle was dissected, the spin-echo decay was exponential. It became slower and turned nonexponential with time in the first 40 min. Over the next 24 h, the signal changed very slowly as the muscle physically deteriorated (a slower initial change was reported for porcine muscle at 17°C [17]). To use the two-component analysis (2–4), one would have to interpret the result in terms of a rapid change in the amount of extracellular water from zero to an equilibrium value

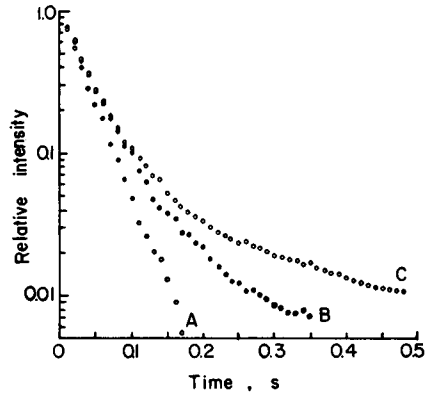

FIGURE 2 ^{1}H spin-echo data for mouse muscle water at 30 MHz and 37°C. The initial intensities are not included because the contribution from organic protons is not negligible. (A) 5 min, (B) 10 min, (C) 30 min.

shortly after death. However, the extracellular space in muscle is estimated to be 10–20% (18, 19), and is not known to change drastically after the animal is dead. On the other hand, it is well known that the amount of phosphocreatine in mammalian muscle decreases rapidly after death, and the lack of ATP regeneration causes post-mortem shortening during rigor (20). The effect is more obvious at 37°C for animals killed without prior immobilization (20), as in our experiments. We have suggested that the average motional anistropy and proton relaxation of tissue water are determined by the water molecules in the hydration layer of macromolecules (15, 16). Therefore, the change in the proton spin-echoes after death (Fig. 2) may simply reflect the effect of the conformational change of the muscle proteins on the relaxation behavior of the water molecules in the hydration layer, rather than a rapid redistribution of water in different parts of the tissue. Further study is being made to test this point. As a comparison, we observed very little change in the proton spin-echo train of brain tissues at 37°C during the 1st h after death.

The author is a recipient of a Research Career Development Award from the National Institutes of Health. This work was partly supported by NIH grant RO1 AG00199.

Received for publication 2 May 1977 and in revised form 21 June 1977.

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