VARIED MAGNETIC FIELD, MULTIPLE-PULSE, AND MAGIC-ANGLE SPINNING PROTON NUCLEAR MAGNETIC RESONANCE STUDY OF MUSCLE WATER

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

L. M. RYAN and B. C. GERSTEIN, *Ames Laboratory, U. S. Department of Energy, and Department of Chemistry, Iowa State University, Ames, Iowa 50011 U.S.A.*

ABSTRACT The nuclear magnetic resonance linewidth of ^1H in water of frog muscle was studied as a function of magnetic field strength and angle of orientation. The results suggest that the observed spectra are dominated by demagnetization field anisotropy and dispersion, but a small static dipolar interaction of the order of a few hertz may be present. Data from line-narrowing, multiple-pulse experiments also indicate the presence of a small dipolar broadening.

INTRODUCTION

In many biological systems, water is present as a separate heterogeneous phase. In some systems in which the macromolecules are oriented, such as collagen (1, 2), oriented DNA (3, 4), silk fibroin (3), and keratin (3, 5), dipolar splitting of H_2O ($\Delta\nu_H$) and quadrupolar splitting of D_2O ($\Delta\nu_D$) have been observed in the nuclear magnetic resonance (NMR) spectra. On the other hand, the existence of dipolar or quadrupolar splitting for water in biological cells has not been unequivocally demonstrated. An observation of orientation-dependent NMR patterns for water protons in nerve fiber (6) was later attributed to the effect of demagnetization fields (7). Recently, Fung (8) reported changes in the linewidths of both proton and deuteron in muscle water as a function of the angle between the muscle fiber and the magnetic field. However, the interpretation of the results as an indication of water orientation has been questioned (9), and it was suggested that the change in the proton line shape of muscle water with orientation may also be the result of anisotropy in magnetic susceptibility (9).

In collagen (1, 2, 10), Li-DNA (4), and other nonbiological systems such as clay (11) and rayon (12), $\Delta\nu_H/\Delta\nu_D \sim 0.3$. However, the observed linewidths for H_2O in nerve (6, 7) and muscle (8) are larger than those for D_2O. $\Delta\nu_H$ and $\Delta\nu_D$ in hydrated collagen decrease as the water content increases (2, 13–16). Native collagen contains ~1 g H_2O/g dry protein. At this water content, the proton doublets overlap each other and are not resolvable (1). Skeletal muscle contains ~75% water, or 3 g H_2O/g dry weight. Since the water contents in muscle and nerve are much larger than that in native collagen, it is surprising that their proton peaks show apparent splittings (6, 8). These comparisons seem to favor the interpretation that the

B. M. Fung is the recipient of a Research Career Development Award from the National Institutes of Health.

multiple proton peaks in these cell systems are due to dispersion of the demagnetization field (7, 9) rather than true dipolar splitting. It was suggested that the arguments can be settled by examining the proton linewidth as a function of the strength of the external magnetic field, H_0 (9).

Homonuclear dipolar splittings in solid or partially ordered systems can be reduced or eliminated by spinning the sample at the "magic angle" (54° 44′) with respect to H_0 (17–19), at a frequency large compared to the dipolar interaction, $\mathbf{H}_D$ (s^{-1}), by using multiple pulse sequences (20–22), or by a combination of both (23, 24). The use of these techniques and a measurement of the variation of proton linewidth with H_0 might enable us to better understand certain characteristics in the NMR study of muscle water.

EXPERIMENTAL

Muscle of frogs (*Xenopus laevis*) was used in this study. Immediately after a frog was killed, the tibialis muscle was excised from the leg, and a striated piece was cut and aligned on a Teflon (DuPont de Nemours & Co., Inc., Wilmington, Del.) plug, which was then inserted into a NMR sample tube, placed upside down. Then, the sample was placed in the NMR probe in a magnetic field, and was rotated so that the axes of the muscle fibers could form specific angles with respect to H_0. A proton NMR study at H_0 = 23,487 G was made by using a Varian XL-100 spectrometer (Varian Associates, Palo Alto, Calif.) in the continuous wave mode with external ^{19}F lock. Measurements at H_0 = 11,744, 5,872, and 2,936 G, respectively, were made on a home-built pulse spectrometer with a Bruker high-resolution (unshimmed) magnet (Bruker Instruments, Inc., Billerca, Mass.) at the University of Oklahoma. The total time for studying each sample at four frequencies was 2–3 h. Except at 23,487 G, the linewidth was defined as $\Delta\nu = 1/\pi T_2^*$, where T_2^* is the decay constant of the free induction decay (FID). Since at exact resonance the FID signal is sensitive to small drifts of H_0 for a system without field-frequency lock, T_2^* was determined from the envelope of the FID when the frequency was set at 1 kHz off resonance. $\Delta\nu$ determined this way was probably not exactly the same as the halfwidth of the Fourier transform of the FID. However, the error introduced was much smaller than uncertainties due to the variation of sample shape and size and the lack of perfect alignment. The values of $\Delta\nu$ reported are the average values of two measurements for the same relative θ (0° and 180°, 30° and 150°, etc.). Multiple-pulse and magic-angle spinning experiments were performed on a home-built spectrometer at Iowa State University. The spectrometer operated at 56 MHz for protons (H_0 = 13,153 G), with a deuterium lock. 5-mm (OD) oriented samples were used in the multiple-pulse experiments, and unoriented samples were used for the magic-angle spinning experiments. The spin-spin relaxation time, T_2, was obtained by using the Carr-Purcell-Meiboom-Gill sequence (25, 26). The constructions of the spectrometer and the special probe are described in detail elsewhere (24, 27).

RESULTS

In a previous study of oriented frog muscle at H_0 = 23,487 G (8), the sample size was quite large (fitting a 12-mm OD tube). Considerable variations of proton linewidth and small changes in the peak center with the angle between the fiber axes and H_0 (θ) were observed. In the present work, samples of smaller sizes (fitting a 5-mm OD tube) were studied. The spectra differed somewhat from sample to sample, but the maximum linewidth $\Delta\nu$ (for θ = 0°) was generally smaller than that for the larger samples (8), and there was less variation in $\Delta\nu$ with respect to θ. The spectra of one sample are shown in Fig. 1. The variation in the position of the peak is 1.2 ppm. The maximum variation in the linewidth is 30 Hz. Changes in the center of spectrum and linewidth with orientation also observed in the proton spectra for a sample of liquid water which was placed in a container ~5 × 2 × 7 mm, constructed by shaping a 5-mm

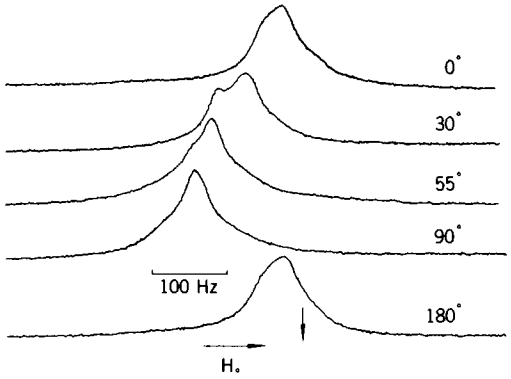

FIGURE 1 Proton NMR spectra of frog muscle oriented at different angles with respect to H_0. The sample was placed in a 5-mm (OD) tube and the spectra were taken at 100 MHz and 25°C. The arrow indicates the peak position for liquid water.

NMR tube. The results of this experiment are given in Fig. 2, which shows the spectrum with the long side of the rectangular parallelopiped at angles 0°, 55°, and 90° to the external field. The variation in the position of the peak is 1.2 ppm. The maximum variation in the linewidth is 25 Hz.

To determine whether the line broadening of protons in muscle water is only due to dispersion of demagnetization, a study of the proton linewidth of muscle water was studied as a function of magnetic field. The results for two samples at four different fields are given in Table I.

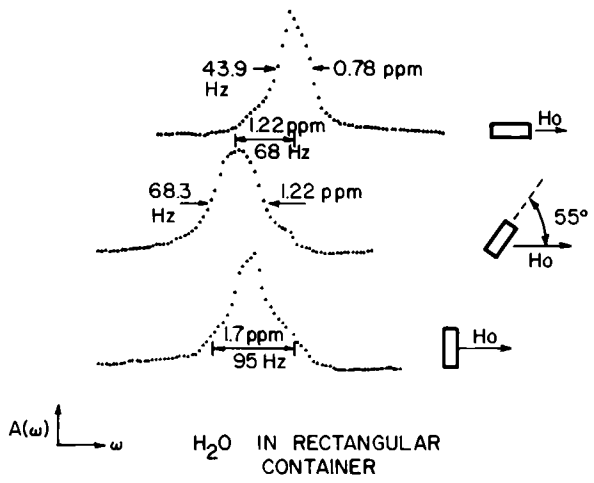

FIGURE 2

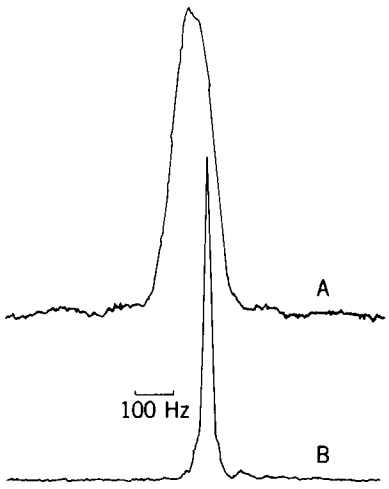

FIGURE 3

FIGURE 2 Line shape of a rectangular water sample as a function of orientation to static field at 56 MHz. Note change in linewidth due to dispersion of demagnetization field, and change in center of spectrum due to change in average demagnetization field.

FIGURE 3 Proton NMR spectra of frog muscle at 56 MHz and 25°C. (A) Without spinning. (B) With magic-angle spinning at a rate of 2 kHz. The linewidth was reduced only by a factor of 2 when the sample was spun at the same rate about an axis perpendicular to H_0.

TABLE I
PROTON LINEWIDTH OF WATER AT DIFFERENT VALUES OF H_0 AT 25°C

H_0	Muscle sample A				Muscle sample B				Liquid water, spherical
	0°	30°	55°	90°	0°	30°	55°	90°	
G	*Hz*								
23,487	76	82	70	62	130	160	108	132	4
11,744	53	47	41	56	132	83	74	55	30
5,872	46	42	28	33	53	39	34	39	20
2,936	25	27	21	28	27	24	20	25	16

The orientations of the muscle axes with respect to H_0 are shown.

In another set of experiments, the possible existence of dipolar broadening in the proton spectrum width of muscle water was studied by using the Rhim-Ellman-Vaughn eight (REV-8) pulse cycle (20). In this method, a series of phase-shifted $P = \pi/2$ pulses, P_x-τ-(τ-P_x-τ-P_y-2τ-P_{-y}-τ-P_{-x}-2τ-P_{-x}-τ-P_y-2τ-P_{-y}-τ-P_x-τ)$_n$, is applied to the sample, and the echo amplitudes at the end of each 8-pulse cycle are collected and Fourier-transformed. Under optimum conditions, the static homonuclear dipolar interaction is removed to first order, and interactions involving H_0 (chemical shift, magnetic susceptibility and H_0 inhomogeneity) are scaled by roughly $3/\sqrt{2}$ in the resulting spectrum (20). This scaling factor was indeed observed when the Fourier-transformed spectra of a sample of liquid water for a single pulse and for the REV-8 sequence were compared. The results for a muscle sample are given in Table II.

It is well known (17–19) that dipolar interactions can be removed by spinning the sample at the magic angle, 54.77° with respect to H_0, at a speed greater than the dipolar splitting (both expressed in hertz). Anisotropy in chemical shift and in magnetic susceptibility would also be averaged to their respective isotropic values provided that the anisotropy is smaller than the spinning rate (28). The spectra in Fig. 3 show the effect of magic angle spinning on a sample of randomly oriented muscle. The linewidth was reduced from 128 to 23 Hz under magic-angle spinning. When the sample was spun at the same rate (2 kHz) about an axis perpendicular to H_0, the linewidth was reduced only by a factor of 2. A study of the spin-spin relaxation time, T_2, with magic-angle spinning was also made. For a randomly oriented muscle sample, T_2 for the water protons at 56 MHz and 25°C was 35 ± 3 ms without spinning, and 43 ± 4 ms with magic-angle spinning. The corresponding results for another sample was 36 ± 3 and 35 ± 3 ms, respectively.

TABLE II
PROTON LINEWIDTH OF A SAMPLE OF MUSCLE WATER AT 56 MHz AND 25°C

Orientation vs H_0		0°	30°	55°	90°
		Hz			
Single-pulse linewidth	$\Delta\nu_1$	100	97	90	83
REV-8 linewidth	$\Delta\nu_2$	41	39	37	31
$\Delta\nu_1/\Delta\nu_2$		2.4	2.5	2.4	2.7

DISCUSSION

Spectra at High Field

To discuss the results of the proton linewidth study, it is necessary to realize that the effective magnetic field at a given proton in sample within an external field consists of a number of contributions:

$$\mathbf{H}_{\text{eff}} = \mathbf{H}_0 + \mathbf{H}_{\text{demag}} + \mathbf{H}_{\text{shield}} + \mathbf{H}_{\text{dipole}}. \tag{1}$$

$\mathbf{H}_0$ is the external field of the magnet. $\mathbf{H}_{\text{demag}}$ is the demagnetization field associated with the surface geometry of the sample. $\mathbf{H}_{\text{shield}}$ is the field associated with the currents induced in the local electronic environment by the external field $\mathbf{H}_0$. $\mathbf{H}_{\text{dipole}}$ is the field due to the through space interaction with all other nuclear magnetic moments in the sample.

In terms of the demagnetization factor, n, the volume susceptibility tensor $\bar{\bar{\chi}}_v$, the shielding tensor $\bar{\bar{\sigma}}/\text{s}^{-1}$, the nuclear gyromagnetic ratio γ, and the internuclear vector $\mathbf{r}_{ik}$ between the ith moment and the kth moment, the field at the ith nucleus is given by:

$$\mathbf{H}^i_{\text{eff}} = \mathbf{H}_0(\bar{\bar{I}} - n\rho\bar{\bar{\chi}}_v - \bar{\bar{\sigma}}_i) - \sum_k [3r_{ik}(\mu_k \cdot \mathbf{r}_{ik})/r^5_{ik} - \mu_k/r^3_{ik}]. \tag{2}$$

For a sample with appropriate geometry, e.g., an ellipsoid of revolution, n is exactly calculable. For a needle with H_0 parellel to the needle axis, $n = 0$; for a needle with H_0 perpendicular to the needle axis, $n = 2\pi$ (24). A sample with nontractable boundary conditions, e.g., a cube, will have a demagnetization field dispersion in the sample. This dispersion will have the effect of an external field inhomogeneity, and will result in a corresponding broadening of the NMR spectrum associated with dephasing of the transverse components of spin throughout the sample. For a particular sample geometry and orientation with respect to the external field, the dispersion of the demagnetization field within the sample may be calculated by, e.g., relaxation techniques applied to solutions of the appropriate Maxwell equations. Alternatively, in the absence of exact knowledge of the boundary conditions for the sample in question, one may choose to estimate this dispersion via an experiment on an appropriately chosen model system.

The example of the rectangular cell (Fig. 2) shows the effect of sample geometry on the spectrum. In general, the average value of the demagnetization field determines the center of the spectrum, and dispersion of the field determines the line shape and width. Muscle cells are elongated and highly anisotropic. By comparing the spectra in Figs. 1 and 2, we conclude that the main contribution to the linewidth and the change in the peak position for muscle water is the demagnetization field. The larger linewidth and apparent splitting previously observed (8) are most likely due to the larger size of samples used.

Variation of Linewidth with Magnetic Field

To determine whether there are other contributions to the proton linewidth of muscle water, we studied the linewidth at different magnetic field strengths (Table I). If the line broadening is due to the dispersion of demagnetization only, $\Delta\nu$ would be directly proportional to H_0 for each sample. The data in Table I show that $\Delta\nu$ does decrease with the decrease of H_0, but the change in $\Delta\nu$ is somewhat slower. It is interesting to notice that, at low values of H_0, $\Delta\nu$ was

smallest for $\theta = 55°$, at which $(3\cos^2\theta - 1) \simeq 0$. This is difficult to explain by the effect of dispersion in the demagnetization field, because the factor $(3\cos^2\theta - 1)$ appears in the dipolar term but not in the demagnetization term of the spin Hamiltonian (Eq. 2). At this angle, the proton linewidth of muscle water approached that of a spherical sample of liquid water of comparable size when H_0 was lowered. The variation in $\Delta\nu$ from one sample to another was also much less at low values of H_0. These facts suggest that, whereas the dispersion of demagnetization is the main factor of the increased proton linewidth and its variation with θ for muscle water, there may also be a small contribution to line broadening from nonzero static dipolar interaction. The data in Table I indicate that the broadening for $\theta = 0°$ would be of the order of only a few hertz. This is much smaller than that suggested by the apparent splitting pattern shown by Fung (8). Packer has suggested (30) that there is a small residual quadrupole splitting of several hertz for a fraction of D_2O (~20%) in muscle. Since neither of these estimations is accurate, it is not clear whether the ratio $\Delta\nu_H/\Delta\nu_D$ for muscle water is comparable to that in other oriented heterogeneous systems (1, 2, 4, 10–12) or not.

Multiple Pulse Experiments

The possible existence of a small static dipolar interaction in muscle water is further demonstrated in the results of the eight-pulse experiment (Table II). If the line broadening of the proton signal of muscle water is due to dispersion in the demagnetization field only, the ratio $\Delta\nu$ for the signal obtained under REV-8 sequence and that for a single-pulse experiment would be $3/\sqrt{2} = 2.12$ or less. If the proton signal under a single pulse is further broadened by nonzero static dipolar interaction, which vanishes in the REV-8 sequence, this ratio would be larger than 2.12. The data in Table II show that this was indeed the case, indicating the existence of dipolar broadening in the single-pulse linewidth. It is to be noted that, for a perfectly aligned sample, there would be no diplor broadening at $\theta = 54.7°$ for both the single-pulse and the REV-8 results. The fact that the linewidth ratio is larger than 2.12 at this angle (Table II) is probably due to the lack of perfect alignment for the muscle sample. Considering that the uncertainties in the linewidths shown in Table II are ~5%, the dipolar broadening for the single pulse data would be of the order of a few hertz, consistent with the result of the varied H_0 experiments.

Magic-Angle Spinning

The magic-angle spinning spectrum (Fig. 3) also demonstrated the large contribution of anisotropic magnetic susceptibility to the proton linewidth of muscle water at high fields. On the other hand, proton T_2 did not change substantially with magic-angle spinning at a rate of 2 kHz. This implies that the longest correlation time of the motion of part (or all) of water molecules is $<8 \times 10^{-5}$ s, but tells nothing about other details of the molecular motion. In a previous study by one of us, it was suggested that nonzero static dipolar interaction in muscle water may have a large contribution to $1/T_2$ of protons (8). However, since we have demonstrated that the nonzeroth dipolar interaction for muscle water is likely to be very small, this contribution would not be substantial. Other explanations of the large ratio of T_1/T_2 for proton in muscle water have been offered recently (31, 32).

In summary, we conclude that the proton spectra of muscle water in high magnetic fields are mainly determined by demagnetization field anisotropy and dispersion. The varied magnetic field and multiple-pulse experiments demonstrated that a small static dipolar

interaction of the order of a few hertz may be present and would have a small contribution to the proton linewidth.

The work of B. M. Fung is partly supported by National Institutes of Health grant R01 AG00199. The work of B. C. Gerstein and L. M. Ryan is supported by the Division of Chemical Sciences of the Office of Basic Energy Science of the U.S. Department of Energy.

Received for publication 14 August 1979.

REFERENCES

1. BERENDSEN, H. J. C. 1962. Nuclear magnetic resonance study of collagen hydration. *J. Chem. Phys.* **66**:3297.
2. MIGCHELSEN, C., and H. J. C. BERENDSEN. 1967. Deuteron magnetic resonance on hydrated collagen. *In* Magnetic Resonance and Relaxation. R. Blinc, editor. North-Holland Publishing Co., Amsterdam, The Netherlands. 761–766.
3. BERENDSEN, H. J. C., and C. MIGCHELSEN. 1965. Hydration structure of fibrous macromolecules. *Ann. N.Y. Acad. Sci.* **125**:365.
4. MIGCHELSEN, C., H. J. C. BERENDSEN, and A. RUPPRECHT. 1968. Hydration of DNA. Comparison of nuclear magnetic resonance results of oriented DNA in A, B, and C form. *J. Mol. Biol.* **37**:235.
5. LYNCH, L. J., and A. R. HALY. 1970. NMR study of the anisotropy of water absorbed by keratin. *Kolloid Z. Z. Polym.* **239**:581.
6. CHAPMAN, G. E., and K. A. MCLAUCHLAN. 1967. Oriented water in the sciatic nerve of rabbit: *Nature (Lond.).* **215**:391.
7. KLEIN, M. P., and D. E. PHELPS. 1969. Evidence against orientation of water in rat phrenic nerve. *Nature (Lond.).* **224**:70.
8. FUNG, B. M. 1975. Orientation of water in striated frog muscle. *Science (Wash. D. C.).* **190**:800.
9. SHPORER, M., and M. M. CIVAN. 1977. The state of water and alkali cations within the intracellular fluids: the contribution of NMR spectroscopy. *In* Current Topics in Membranes and Transport F. Bronner and A. Kleinzeller, editors. Academic Press, New York. **9**:1.
10. CHAPMAN, G. E., and K. A. MCLAUCHLAN. 1969. The hydration structure of collagen. *Proc. R. Soc. Lond. Biol. B Sci.* **173**:223.
11. WOESSNER, D. E., and B. S. SNOWDEN. 1969. NMR doublet splitting in aqueous montmorillonite gels. *J. Chem. Phys.* **50**:1516.
12. DEHL, R. E. 1968. Broad line NMR study of H_2O and D_2O in oriented rayon fibers. *J. Chem. Phys.* **48**:831.
13. CHAPMAN, G. E., S. S. DANYLUK, and K. A. MCLAUCHLAN. 1969. A model for collagen hydration. *Proc. R. Soc. Lond. B Biol. Sci.* **178**:465.
14. DEHL, R. E. 1970. Collagen: mobile water content of frozen fibers. *Science (Wash. D. C.).* **170**:738.
15. FUNG, B. M., and M. M. SIEGEL. 1972. The ordering and relaxation times of water adsorbed on collagen fibers. *Biochim. Biophys. Acta.* **278**:185.
16. MIGCHELSEN, C., and H. J. C. BERENDSEN. 1973. Proton exchange and molecular orientation of water in hydrated collagen fibers. An NMR study of H_2O and D_2O. *J. Chem. Phys.* **59**:296.
17. ANDREW, E. R., A. BRADBURY, and R. G. EADES. 1958. Nuclear magnetic resonance spectra from a crystal rotated at high speed. *Nature (Lond.).* **182**:1659.
18. ANDREW, E. R., A. BRADBURY, and R. G. EADES. 1959. Removal of dipolar broadening of nuclear magnetic resonance spectra of solids of specimen rotation. *Nature (Lond.).* **183**:1802.
19. LOWE, I. J. 1959. Free induction decays of rotating solids. *Phys. Rev. Letters.* **2**:285.
20. WAUGH, J. S., L. M. HUBER, and U. HAEBERLEN. 1968. Approach to high resolution nmr in solids. *Phys. Rev. Letters.* **20**:180.
21. MANSFIELD, P. 1971. Symmetrized pulse sequences in high resolution nmr in solids. *J. Phys. C.* **4**:1444.
22. RHIM, W. K., D. D. ELLEMAN, and R. W. VAUGHAN. 1973. Enhanced resolution for solid state NMR. *J. Chem. Phys.* **58**:1772.
23. GERSTEIN, B. C., R. G. PEMBELTON, R. C. WILSON, and L. M. RYAN. 1977. High resolution NMR in randomly oriented solids with homonuclear dipolar broadening: combined multiple pulse NMR and magic angle spinning. *J. Chem. Phys.* **66**:361.
24. PEMBLETON, R. G., L. M. RYAN, and B. C. GERSTEIN. 1977. NMR probe for combined homonuclear multiple pulse decoupling and magic angle spinning. *Rev. Sci. Instrum.* **48**:1286.

25. CARR, H. Y., and E. M. PURCELL. 1954. Effects of diffusion on free precession in nuclear magnetic resonance experiments. *Physiol. Rev.* **94**:630.
26. MEIBOOM, S., and D. GILL. 1958. Modified spin-echo method for measuring nuclear relaxation times. *Rev. Sci. Instrum.* **29**:688.
27. GERSTEIN, B. C., C. CHOW, R. G. PEMBLETON, and R. C. WILSON. 1977. Utility of pulse nuclear magnetic resonance in studying protons in coals. *J. Phys. Chem.* ***81:565.***
28. HAEBERLEN, U., and J. S. WAUGH. 1968. Coherent averaging effects in magnetic resonance. *Physiol. Rev.* **175**:453.
29. FEHER, G., and W. D. KNIGHT. 1955. Measurement of electronic susceptibilities by means of nuclear resonance absorption. *Rev. Sci. Instrum.* **26**:293.
30. PACKER, K. J. 1977. The dynamics of water in heterogeneous systems. *Philos. Trans. R. Soc. London B Biol. Sci.* **278**:59.
31. CIVAN, M. M., A. M. ACHLAMA, and M. SHPORER. 1978. The relationship between the transverse and longitudinal nuclear magnetic resonance relaxation rates of muscle water. *Biophys. J.* **21**:127.
32. FUNG, B. M., and T. W. MCGAUGHY. 1979. Study of spin-lattice and spin-spin relaxation times of ^{1}H, ^{2}H, and ^{17}O in muscle water. *Biophys. J.* **28**:293.