^{17}O NUCLEAR MAGNETIC RESONANCE SPECTRUM OF $H_2{}^{17}O$ IN FROG STRIATED MUSCLE

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ABSTRACT Whole striated muscles from the frog *Rana esculenta* were bathed in Ringer's solution enriched with $H_2{}^{17}O$; the muscle water was subsequently collected by vacuum distillation. The integrated intensity of the nuclear magnetic resonance (NMR) signal of ^{17}O in the muscle was measured to be approximately $\frac{3}{4}$ of the signal observed in the distilled water. The phenomenon may arise either from immobilization of a population of the water molecules which may be a very small fraction or as much as $\frac{1}{4}$ of the total, or may reflect tumbling of $\frac{1}{3}$ of the water molecules in a compartment containing an anisotropic medium. Such an effect was demonstrated for $H_2{}^{17}O$ using the model system of sodium linoleate in water.

INTRODUCTION

The intracellular electrolyte composition of biological tissue differs distinctively from that of the bathing medium. For example, the transporting cells of the urinary bladder of the toad accumulate K^+ to a concentration some 40-fold greater than that of the external medium, and extrude Na^+ to a concentration $\frac{1}{2}$–$\frac{1}{3}$ that of the external medium (1), a phenomenon observed for most biological tissue.

This difference in electrolyte composition within and without the cell has been thought to arise from the transport properties of the plasma membrane (2–5). This concept has received strong support from the observations that: (*a*) the plasma membrane of the squid axon continues to constitute a selective barrier to ion movement even after extrusion of the axoplasm (6–9), and (*b*) red blood cells continue to accumulate K^+ (10) and extrude Na^+ (11) even after some 90% of the intracellular hemoglobin has been removed.

Alternatively, the intracellular electrolyte composition may reflect, at least in part, selective binding properties of sites within the cell cytoplasm (12); the two

possible mechanisms are, of course, not necessarily mutually exclusive. The magnitude of the proposed binding, however, and the relationship to maintenance of concentration gradients between cell and extracellular fluid and to transepithelial transport have remained obscure. This position must now be reexamined after the substantial number of recent publications purporting to demonstrate by means of NMR that Na^+ (13–22), K^+ (23), and H_2O (24, 25) are bound within a variety of cells.

The large number of studies of the ^{23}Na NMR spectrum of a great number of tissues have clearly established that only some 40% of the total expected Na^+ signal is visible to NMR. As has been recently observed (26), however, this finding may reflect tumbling of Na^+ within an anisotropic medium or rapid exchange between free Na^+ and a minute fraction of bound Na^+. The literature dealing with the spectrum of ^{39}K is as yet limited because of the technical difficulties in measuring the signal from most biological tissues.

Conclusions concerning the state of water within the cell are particularly tentative at the present time, however, because of lack of agreement concerning the basic observations. Broadening of the line width of ^1H and ^2H in tissue water has been interpreted to reflect either binding of a significant fraction of cell water (24, 25) or local field inhomogeneities (27). Furthermore, reduction of the total anticipated NMR signal of ^1H or ^2H has been reported by some (24, 25), but not by all investigators (18).

In order to help resolve the problem, the NMR spectrum of ^{17}O was studied in water enriched with $H_2{}^{17}O$. Because of the large quadrupolar interaction of the ^{17}O nucleus in $H_2{}^{17}O$ (28), this nucleus is particularly sensitive to changes in the molecular movement. ^{17}O should therefore serve as a far more sensitive NMR probe of structuring or ordering of intracellular water than is provided by protons or deuterium in water. Since the substitution of other oxygen isotopes for ^{16}O produces a negligible change in the chemical properties of water, $H_2{}^{17}O$ is also far less likely to cause the deleterious physiological effects elicited by ^2H_2O (29).

METHODS

The gastrocnemius, sartorius, and semitendinosus muscles were excised intact from two to three doubly pithed native Israeli frogs, *Rana esculenta*, which had been maintained fasting in water at room temperature; in the first experiment, the iliofibularis rather than the semitendinosus was studied, with similar results. The muscles were first rinsed in a slightly modified Ringer's solution (NaCl, 115.5 mM; KCl, 2.5 mM; Na_2HPO_4, 2.5 mM; NaH_2PO_4, 0.5 mM; *d*-tubocurarine chloride, 9 mg/liter; saturated with $Ca_3(PO_4)_2$ at a pH of 7.5). Subsequently, the muscles were transferred to 10 ml of a standard Ringer's solution previously used (30) (NaCl, 115.5 mM; KCl, 2.5 mM; $CaCl_2$, 1.8 mM; Na_2HPO_4, 2.5 mM; NaH_2PO_4, 0.5 mM; and *d*-tubocurarine chloride, 9 mg/liter) containing $H_2{}^{17}O$ at a concentration of 2–7%; the initial incubation solutions contained 7% $H_2{}^{17}O$, but after redistilling the bathing media three to four times in order to conserve ^{17}O, the [$H_2{}^{17}O$] of the incubating media fell to as low as 2%. After incubation for periods of 2.5–5 hr the muscles were blotted dry on

Whatman No. 40 filter paper, and gently packed into 15 mm O.D. Pyrex test tubes. A similar volume of the bathing medium was introduced into a second test tube, serving as a reference solution.

As previously described (26), all NMR measurements were performed with a DP60 Varian Spectrometer (Varian Associates, Palo Alto, Calif.) using a fixed frequency unit (V-4311) operating at 8.13 MHz. In order to detect satellite signals, the derivative of the spectrum was recorded, using the wide line mode of operation and applying a field modulation of 20 Hz.

Intensity measurements were performed by directly recording the absorption signal in the high resolution mode. In order to stabilize the base line, a modulation frequency of 1980 Hz (a value much larger than the line width) was applied, and the receiver output was phase detected by the NMR V-3521 integrator unit; the side bands obtained by this technique served also for chart calibration.

The relative integrated intensity of each absorption spectrum was measured as the product of peak height and width at half-peak height. The ratio R_M was calculated for the relative integrated intensity of the signal from muscle to that from the bathing medium.

After the NMR measurements, the muscles were removed, their wet weights determined, the tissues vacuum-dessicated overnight, and their dry weights measured. The weight of the water distilled and collected from the muscles was always in agreement with the difference in weight between the wet and dry muscles to within ±2%, with the exception of a single sample which differed by 4%. The dry weight ranged from 17.0 to 21.6% of the wet weight (mean ±SEM = 19.4 ±0.6%), in agreement with previous estimates for frog muscle of 18–21% (31), and with the more recent estimate for rat gastrocnemius (32) of 19.5–23.1% of the fat-free weight (the range depending on age).

The volume of the water distilled from muscle was adjusted to equal that of the initial

TABLE I

FRACTION OF SPECTRUM VISIBLE IN COMPARISON WITH REFERENCE BATHING MEDIUM

Experiment	Muscle (R_M)	Water distilled from muscle (R_W)	Ratio (R_V)
I	0.65	1.04	0.62
II	0.61	0.81	0.75
III	0.60	0.87	0.69
IV	0.83	0.83	1.00
V	0.70	0.86	0.81
VI	0.63	0.77	0.82
VII	0.54	0.75	0.73
VIII	0.61	0.73	0.84
IX	0.54	1.00	0.55
Mean	0.63	0.85	0.76
± SEM	±0.03	±0.04	±0.04

Each entry is the mean of 4–13 measurements of ^{17}O spectra. R_M is the ratio of the integrated intensity of the signal from muscle to that from the bathing medium. R_W is the ratio of the integrated intensity of the signal from the water distilled from muscle to that from the same sample of bathing medium for each experiment. $R_V \equiv R_M/R_W$.

muscle sample, by addition of ordinary deionized water. The NMR spectrum of ^{17}O of the distilled water was then determined, using the same sample of bathing medium as the reference solution.

The ratio R_W of the relative integrated intensity of the absorption signal from the distilled water to that from the reference bathing medium was calculated. Because of the volume occupied by the dry residue from the muscle, R_W was necessarily less than 1, within the range of experimental error. From the mean dry muscle weight of 19.4% and the specific gravity of frog muscle of 1.062 (33), it may be calculated that $\bar{R}_W$ should have been approximately 0.815 if $H_2{}^{17}O$ was in equilibrium within and without the muscle cells. The measured value for $\bar{R}_W$ (Table I) of 0.85 $\pm$0.04 agrees favorably with the value predicted, confirming that under the conditions of the current study, the $H_2{}^{17}O$ of the bathing medium was in equilibrium with the total muscle water.

The visible fraction R_V of the total expected ^{17}O signal was calculated as R_M/R_W.

RESULTS

Fig. 1 presents representative absorption spectra of ^{17}O obtained from experiment VI. The spectrum of Fig. 1 *a* was obtained from the packed whole muscles. The width at half-peak height is 222 cps, a markedly broadened value in comparison with the 45 cps characteristic of $H_2{}^{17}O$ in pure water at room temperature (28). Fig. 1 *b* presents the ^{17}O spectrum of an equal volume of solution taken from the bathing medium, examined at the same time with the same experimental settings; although considerably narrower than the muscle spectrum of Fig. 1 *a*, the width at half-peak height is 71 cps, broad in comparison with that in free solution. The integrated intensity of the signal from the muscle is 63% of that from the bathing medium. The mean width of the ^{17}O signal observed from this and several other muscles was 207 cps, while the mean width of the signal from the bathing medium was 73 cps.

Fig. 1 *c* is the absorption spectrum of the water distilled from the same sample of muscle, measured the following day. Fig. 1 *d* is the spectrum observed at the same time as 1 *c*, using the same reference sample taken from the bathing medium as in Fig. 1 *b*. (Note that the calibration for *c* and *d* is slightly different from that for *a* and *b*.) After distillation, the intensity of the experimental ^{17}O spectrum is now 73% of that for the reference sample. The visible fraction of the total expected ^{17}O signal from this muscle sample was therefore 63% $\div$ 73% = 0.86.

Table I presents the results for all nine experiments. Each spectral measurement entered in the table is the mean calculated on the basis of study of 4–13 spectra from the reference and experimental samples. Before distillation, 54–83% of the reference intensity was observed in the muscles. After distillation, 73–104% of the reference intensity was observed in the experimental samples. Taking the ratio of the two fractions for each of the nine experiments, 55–100% of the anticipated ^{17}O signal from muscle was actually observed (mean $\pm$SEM = 76 $\pm$4%). Both experiments IV and V were obtained with water containing the lowest enrichment of $H_2{}^{17}O$ within the present series of experiments. The signal-to-noise ratio was particularly unfavorable in each case, so that these two measurements were the least precise

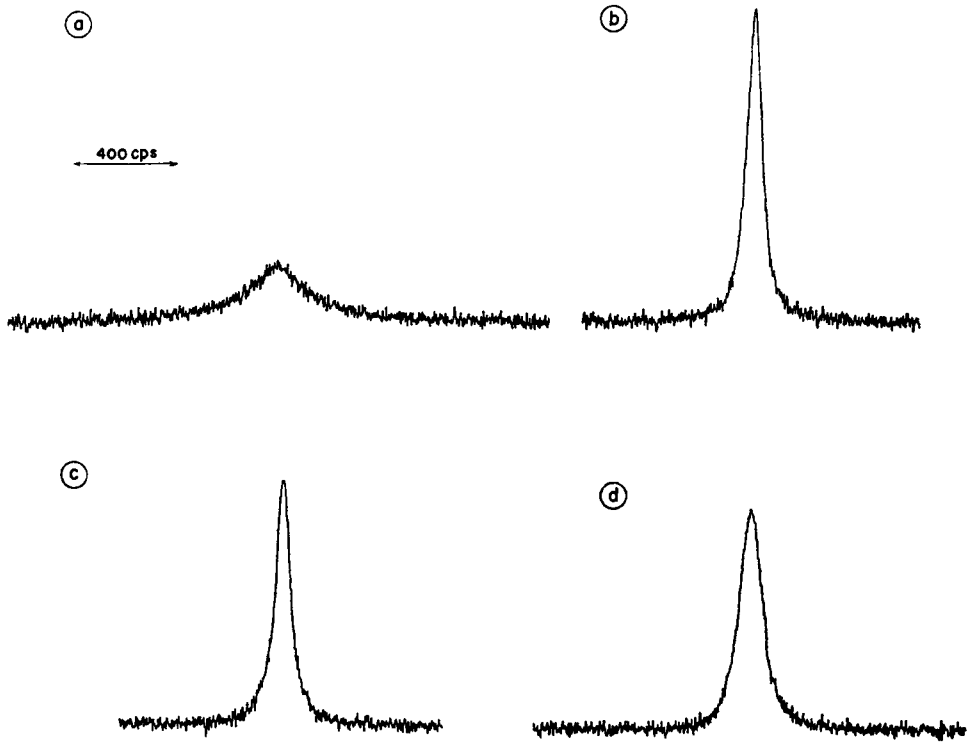

FIGURE 1 Absorption spectra of ^{17}O in $H_2{}^{17}O$. (*a*) Spectrum obtained from the muscle sample of experiment VI. The curve is markedly broadened; the width is 222 cps at half-peak height. (*b*) Spectrum obtained from an equal volume of incubation medium, using the same experimental settings as in *a*. The signal width at half-peak height is 71 cps. The integrated intensity of spectrum *a* is 63% of that for spectrum *b*. (*c*) Spectrum obtained the following day from water distilled from the muscle sample of *a*. (*d*) Spectrum of the same bathing medium studied in *b*, and obtained under the same experimental settings as in *c*. The integrated intensity of spectrum *c* is 73% of that for spectrum *d*. The fraction of the total anticipated signal actually visible in the initial muscle sample was 63%/73% = 0.86. The calibration for spectra *c* and *d* has changed from that of *a* and *b*.

of the entire series. If experiments IV and V are excluded, 55–84% of the anticipated ^{17}O was observed in the muscle samples from the remaining seven experiments (mean $\pm$SEM = 71 $\pm$4%).

The possibility was considered that trace paramagnetic ions such as Mn^{++} might have broadened beyond detection the ^{17}O spectrum of a fraction of the intracellular water. In order to investigate this possibility, experiment III consisted of two parallel operations. The gastrocnemius, semitendinosus, and sartorius muscles were dissected from three frogs; one muscle from each pair was treated according to the standard protocol detailed in the Methods section, while the other muscle from each pair was incubated in a Ringer's solution containing 0.47 mM $MnSO_4$. In comparison with the reference standard taken from the bathing medium, 71%

of the ^{17}O signal was visible from the muscle sample, and 103% of the ^{17}O signal was visible after vacuum distillation. Therefore, the visible fraction of the total anticipated ^{17}O signal from muscle was 71%/103% = 0.68, in agreement with the 0.69 (Table I) obtained for the contralateral muscles incubated in Ringer's solution free of Mn^{++}.

Because of the possibility that the NMR-invisible fraction of the ^{17}O spectrum may have arisen from a first-order quadrupolar effect (26), the ^{17}O signal was also examined in the derivative mode. No satellite signals were observed.

In order to see whether a first-order quadrupolar effect could be demonstrated in a model system, the spectrum of ^{17}O in sodium linoleate in water was also examined. Fig. 2 presents a representative tracing obtained in the derivative mode. Two satellite signals are clearly visible on each side of the central signal. This spectrum is characteristic of the powder pattern for nuclei with a spin number of 5/2, on the basis of a first-order quadrupolar effect (34). The quadrupolar coupling constant e^2qQ/h of the current system may be calculated from the total width of the spectrum. From the measured width of 6900 cps, the estimated quadrupolar coupling constant can be no greater than 23 kHz. The quadrupolar coupling constant for ^{17}O in liquid $H_2{}^{17}O$ may be estimated, however, to be some 8 MHz (28), a value two

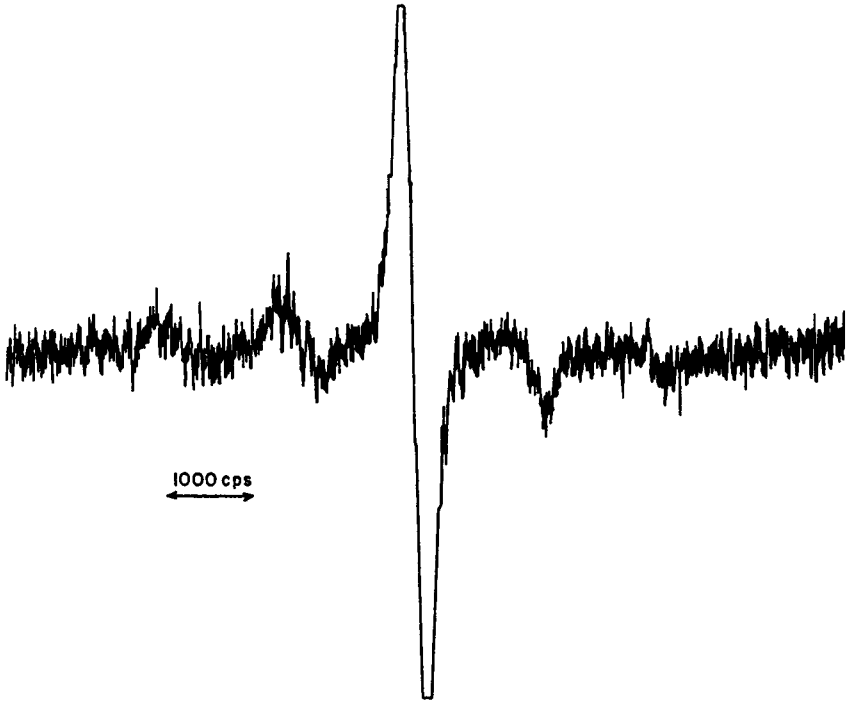

FIGURE 2 Derivative of the ^{17}O signal of $H_2{}^{17}O$ obtained from sodium linoleate in water. Two satellite signals are clearly distinguished on either side of the central signal.

to three orders of magnitude greater. It seems clear, therefore, that the quadrupolar effect for the ^{17}O of $H_2{}^{17}O$ in sodium linoleate in water does not arise from immobilization of all the $H_2{}^{17}O$ molecules.

DISCUSSION

Study of the NMR spectrum of the ^1H and ^2H of water in muscle (24, 25, 27) and brain (25, 27) has indicated a 20-fold and more decrease of the transverse relaxation time T_2 or increase in line width broadening in comparison with the signal in distilled water. These line broadenings have been interpreted to reflect increased organization of tissue water (24, 25), but as Hansen (27) has pointed out, much of the effect probably arises from local magnetic field inhomogeneities induced by the heterogeneity of phase of the sample.

Local field inhomogeneities would not be expected to play a significant role in broadening the signal of ^{17}O in the present studies. The gyromagnetic ratio of ^{17}O is similar to that of ^2H, but the quadrupolar coupling constant of ^{17}O in $H_2{}^{17}O$ is far greater than that for ^2H in water, so that the dominant factor determining the line width in the current experiments is the nuclear quadrupolar interaction.

The relative line broadening of the ^{17}O spectrum of $H_2{}^{17}O$ in muscle of the current study was, in fact, much smaller than that reported in studies of the spectra of protons and deuterium. The average line width of ^{17}O in muscle was 207 cps, some four to five times greater than the 45 cps characteristic of $H_2{}^{17}O$ in pure water at room temperature (28); the average line width of the ^{17}O from the bathing medium was 73 cps. This relatively smaller line broadening of the ^{17}O signal from muscle is in accord with Hansen's proposition that local field inhomogeneities play a major role in the line broadening of protons and deuterium. The ^{17}O broadening may be ascribed to a variety of possible factors such as increased viscosity, exchange with water bound or adsorbed to macromolecules, or to the presence of trace paramagnetic impurities such as Mn^{++}; the increased signal width, per se, need not necessarily arise from structuring of water within the cell.

The intensity of the observable ^{17}O spectrum from $H_2{}^{17}O$ was substantially less in eight of the nine muscle samples examined than in the water subsequently distilled from the same muscles. In experiment IV, the intensities appeared equal; here, however, the precision of the measurements was most in doubt. Since in no case did the muscle emit a stronger signal than the distilled water sample, the difference in signal intensity cannot be ascribed to the experimental uncertainties of the NMR technique. Averaging the results for all nine experiments, the intensity of the signal from muscle was 76 $\pm$4% (mean $\pm$SEM) of the intensity from the distilled sample, a value significantly different from 100%.

In comparing the current results with those from the literature, it must be borne in mind that this reduction of 24% in the NMR signal from muscle water is relatively small, and the range of individual experimental values appreciable (0–45%).

Since Czeisler et al. (18) studied the ^2H signal of ^2H_2O from only two muscle preparations in the course of their study of ^{23}Na, noting an experimental uncertainty of some 15%, they might easily have overlooked the phenomenon. Hazlewood et al. (24) estimated from their studies of ^1H in muscle water that only some 10% of the anticipated signal was not visible to NMR; however, the great disparity in the width of the signals they examined from water and from muscle does not permit precise quantitative analysis. The current results are in quantitative agreement with Cope's (25) pulsed NMR studies of ^2H_2O which indicated that 27 and 13% of the total anticipated signals from rat muscle and brain, respectively, were not visible to NMR.

The current data confirm, therefore, that there must be at least two populations or compartments of water molecules. One population of water molecules is characterized by NMR properties not greatly different from those characterizing aqueous solution. The properties of the second population or compartment of water molecules might reflect: (*a*) the presence of trace quantities of paramagnetic ions within a compartment containing 24% of the $H_2{}^{17}O$ molecules, (*b*) immobilization of 24% of the $H_2{}^{17}O$ by adsorption or binding to a solid phase, (*c*) rapid exchange between free and bound or adsorbed $H_2{}^{17}O$ within a compartment containing 24% of the water, but where the fraction bound may be exceedingly small, or (*d*) a nuclear quadrupolar effect arising from an ordering phenomenon which might reflect tumbling of approximately 33% of the water molecules in an anisotropic medium. Particularly with respect to possibilities *c* and *d*, the $H_2{}^{17}O$ compartments need not necessarily be determined by a rigid barrier, but rather may represent shorter or longer distances for diffusion to a membrane or macromolecular surface.

With respect to the first possibility, trace paramagnetic ions may markedly alter the NMR signal of ^{17}O in $H_2{}^{17}O$. Since Mn^{++} is particularly effective in this regard, it was of interest to study the effect of addition of $MnSO_4$. In the single experiment performed, Mn^{++} was introduced into the bathing medium to a concentration of 0.47 mM, a value some 200 times greater than the reported concentration of endogenous [Mn^{++}] of wet muscle (35); the line width of the ^{17}O signal was broadened, but the relative intensity of the spectrum from muscle was unaffected. This result suggests that the reduction in the intensity of the visible NMR spectrum of ^{17}O does not arise from the effect of paramagnetic ions, but does not entirely preclude this possibility.

The second and third above possible mechanisms would both be entirely consistent with the data presented.

In order to examine the possible significance of the fourth postulated mechanism, molecular tumbling in an anisotropic medium, it was desirable to determine whether a first-order nuclear quadrupolar effect could be demonstrated for ^{17}O under conditions approaching those of biological cells; liquid crystals of soap in water, a system thoroughly characterized by X-ray diffraction techniques (36), was chosen.

Using the derivative mode of analysis (Fig. 2), the signals characteristic of the powder pattern for a nucleus with spin number of 5/2 on the basis of a first-order quadrupolar effect were seen; in this case, the intensity of the central signal is only 27% of the total (34). As noted in the Results section, the basis for the quadrupolar effect in the sodium linoleate–water mixture is not likely to be immobilization of all the $H_2{}^{17}O$ since the quadrupolar coupling constant is two to three orders of magnitude too small. Rather, the quadrupolar effect might arise from molecular tumbling within an anisotropic medium or from rapid exchange between a small fraction of bound $H_2{}^{17}O$ and a larger pool of free $H_2{}^{17}O$ all of which contribute to the visible NMR signal (26).

The fact that satellite signals for ^{17}O were not observed in the muscle samples does not preclude the possibility that a first-order quadrupolar effect is present in muscle as well. The height of the satellite signals is inversely proportional to the width of the total spectrum. For those systems with a very strong quadrupolar interaction, and hence with a very wide total spectrum, the satellite signals would be indistinguishable from the background noise.

The data from the present studies indicate, therefore, that approximately a quarter of the total anticipated intensity of the ^{17}O signal of $H_2{}^{17}O$ in muscle is not visible to NMR. This missing fraction may reflect immobilization of a population of the water molecules, which may be a very small fraction or as much as a quarter of the total, or may reflect tumbling of some third of the water molecules in a compartment containing an anisotropic medium. Further studies will be required to resolve these possibilities.

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