WATER AND IONS IN

MUSCLES AND MODEL SYSTEMS

R. K. OUTHRED *and* E. P. GEORGE

From the School of Physics, University of New South Wales, Kensington, Australia 2033

ABSTRACT   The nuclear magnetic reasonance (NMR) relaxation times of protons in toad muscle water have been measured at three frequencies: 2.3, 8.9, and 30 MHz. The results are analyzed in terms of a distribution of correlation times, and it is found that only a few percent of the observed protons have mobilities more than two orders of magnitude smaller than normal. Sodium and chloride ion chemical potentials in some hydrated materials with similar proton NMR characteristics to toad muscle have been found to be heightened, but not sufficiently to account for the distribution of sodium ions in muscle.

INTRODUCTION

Cope (1) has claimed that low values of ^1H and ^2H NMR relaxation times in tissue water indicate "crystallinity" although he attempted no quantitative analysis of the degree of modification to the water that the data might imply. In fact, considered in isolation, the data at a particular resonant frequency are consistent with a simple model in which more than 90% of the water is in a normal state (2).

When proton data at more than one resonant frequency are compared, the simple model is no longer adequate. NMR relaxation times measured at three frequencies for water in toad muscle are presented here. Using techniques described in a previous paper (3), referred to hereafter as paper I, the implications for the state of the water are examined.

Also, the states of sodium and chloride ions in some nonliving systems with similar NMR characteristics have been investigated using ion-specific electrodes in an effort to detect the ion exclusion effects postulated for muscle water (4).

MATERIALS

Whole gastrocnemius muscles dissected from decapitated toads of the species *Bufo marinus* were ligated, gently blotted, and placed in stoppered sample tubes. Agarose and gelatin samples were prepared as described in paper I except that sodium chloride solutions were substituted for distilled water.

Actomyosin precipitates were prepared from toad thigh muscles by the method of Szent-Gyorgyi (5) as described by Cope (6). The precipitation from dilute potassium chloride was repeated. Samples containing additional sodium chloride were obtained by adding small amounts of concentrated sodium chloride.

METHODS

The NMR techniques have been described in paper I. Chloride ion chemical potential was measured using a silver/silver chloride electrode; for the sodium ion potential an Orion model 96-11 combination sodium electrode (Orion Research, Inc., Cambridge, Mass.) was used. This also provided a calomel reference potential. Potential differences were displayed on a chart recorder. Response time for the sodium electrode was on occasions as long as 15 min. The temperature was 21.5 ±0.5°C for the measurements on agarose and gelatin samples and 18 ±0.5°C for the actomyosin measurements. Standard solutions used in the electrochemical measurements on agarose and gelatin contained only analytical reagent grade sodium chloride. For the actomyosin experiments sufficient potassium chloride was included to match the ion concentration in the samples.

Sodium and potassium analyses were by flame photometry after digestion in 1 N nitric acid. Chloride was determined by potentiometric titration. Water content was determined by drying to constant mass *in vacuo* at 100°C.

RESULTS AND DISCUSSION

Proton NMR Measurements on Toad Muscle

Pulsed NMR measurements of T_1 for protons in toad muscle water are shown in Fig. 1. Whole gastrocnemius muscles were dissected from three batches of toads A, B, and C. In the first experiment, on a toad from batch A, a single muscle was examined at three frequencies 2.3, 8.9, and 30 MHz, the temperature being 2°C. The measurements were completed within 6 h of decapitating the toad.
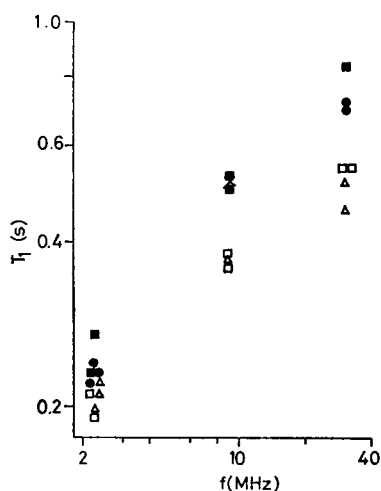

FIGURE 1

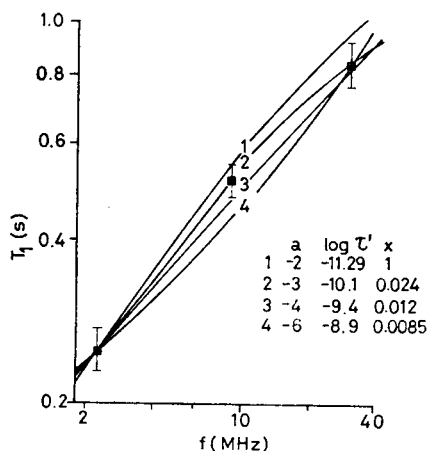FIGURE 2

FIGURE 1 Toad muscle water proton spin-lattice relaxation times as a function of resonant frequency. Filled symbols, 25°C; open symbols, 2°C. $\triangle$, batch A; $\bullet$, $\bigcirc$, batch B; $\blacksquare$, $\square$, batch C. $\times$, a muscle from batch C, not ligated. For batches B and C, points represent individual specimens. For batch A a single specimen was observed at all three frequencies.

FIGURE 2 The average batch C toad muscle T_1 data at 25°C fitted by BLP distribution of proton correlation times.

In later experiments, on toads from batches B and C, fresh specimens were used at each frequency and temperature (2 and 25°C). Observations commenced 15–30 min after the death of the toad. Although T_1 sometimes changed slightly over a period of several hours the fluctuations followed no definite pattern. The points in Fig. 1 for toads from batches B and C represent extrapolations to zero time. When not in the spectrometer, the muscles were stored (a) in Ringer's solution at 25°C, (b) at 25°C in Ringer's solution containing 6 mM glucose, (c) at 2°C in Ringer's solution, or (d) in the sample tube at 25 or 2°C. The results did not reflect the method of storage. The muscles remained contractile 8 h after dissection.

The specimens were handled with great care during preparation, as injury can lead to the generation of free radicals (7) which might interfere with the NMR relaxation processes. Normally the muscles were ligated after dissection but a single specimen from batch C was observed at 8.9 MHz without ligating to test whether the injury caused thereby was sufficient to upset the NMR measurements. The result (marked $\times$ in Fig. 1) was not significantly different from others obtained at the same frequency and temperature.

Spin-spin relaxation determined from echo decay after a 90°–180° pulse sequence was exponential. Values for the relaxation time lay in the range 40–55 ms, but the sample-to-sample variations within this range made it impossible to draw any conclusions concerning the temperature or frequency dependence of T_2, except the general one that within the limits of our experiment, 2–25°C and 2.3–30 MHz, the variation of T_2 must be quite small. For reasons discussed in paper I it is thought unlikely that the T_2 data were significantly in error due to diffusion in magnetic field gradients.

Water content of the batch C muscles was 82 ± 1 %. Sodium and potassium concentrations in two specimens from batch C were 42 ± 5 mmol/kg H_2O and 91 ± 10 mmol/kg H_2O.

The T_1 data were analyzed in terms of a distribution $p(\tau)$ of proton correlation times using the methods described in paper I. As in paper I, for curve-fitting purposes $p(\tau)$ was assumed to be a bimodal log power (BLP) distribution:

$$p(\tau) = x \frac{A}{\tau} (\log_{10} [\tau/\tau_2])^a \quad \text{if} \quad \tau'' > \tau > \tau',$$

$$= (1 - x)\delta(\tau - \tau_2) \quad \text{otherwise,}$$

where $A \int_{\tau'}^{\tau''} (\log_{10} [\tau/\tau_2])^a (d\tau/\tau) = 1$, τ_2 is the proton correlation time in bulk water, x is the fraction of protons in modified states, and τ'', τ' are the upper and lower limits of τ for modified states. It is assumed that $\tau'' > \tau' > \tau_2$. This definition imposes a minimum of form on $p(\tau)$ while allowing the curve-fitting procedure to be systematized.

The BLP distributions which are consistent with the batch C T_1 data at 25°C (see Figs. 2 and 3) have several common features.
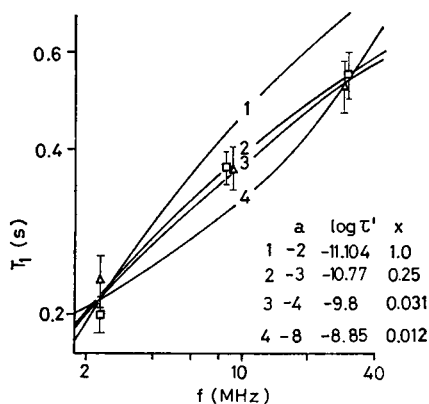
FIGURE 3

FIGURE 4

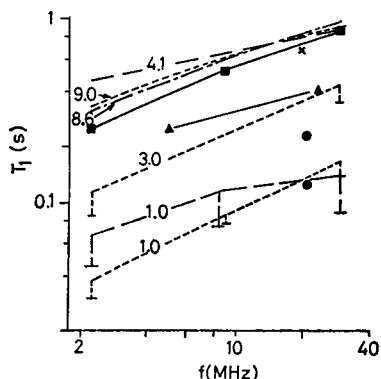FIGURE 3 Toad muscle T_1 data at 2°C fitted by BLP distributions of proton correlation times.

FIGURE 4 Comparison of water proton spin-lattice relaxation in muscles and nonliving hydrated systems. ■, batch C toad muscle data, 25°C; ▲, frog muscle data of Bratton et al. (11); ●, toad muscle data of Walter and Hope (2), 21°C; ✕, frog muscle datum of Abetsedarskaya et al. (12). Broken lines indicate behavior of actomyosin precipitates, — · — · —; hydrated agarose, ········; and hydrated gelatin, — — — —. Figures are water concentration in grams per gram dry matter. Vertical bars indicate reduction in T_1 due to replacement of water by 3 M NaCl. T_1 in actomyosin precipitates was not affected (±10%) by the introduction of 0.05 M NaCl.

(a) $\tau' \lesssim 10^{-9}$ s and $-a > 2$. We conclude that for states with $\tau \gtrsim 10^{-9}$ s, the lowest correlation times are the most prevalent and that there are an unknown number of proton states with correlation times less than 10^{-9} s but different from that of normal water. The lack of information about states with $\tau < 10^{-9}$ s is a consequence of the fact that T_1 was not measured at frequencies greater than 30 MHz as discussed more fully in paper I.

(b) Comparing for the various $p(\tau)$ the fraction with $\tau > 10^{-9}$ s, we find that not more than 2% of the observed protons are in such states.

(c) The best fitting BLP distribution satisfactorily accommodate the T_2 data if $\tau'' \simeq 10^{-5.5}$ s in accordance with the hypothesis that fast exchange for T_2 ceases when the relaxation time becomes comparable with the correlation time.

The above analysis is based on the assumption that the spin-lattice relaxation was not dominated by interactions with paramagnetic centers, the effects of confinement to small pores (8, 9) or coupling by spin diffusion between water and macromolecular protons (10). All of these possibilities can to some extent be tested by comparing proton and deuteron relaxation data. Unlike the proton, deuteron relaxation in water is insensitive to spin diffusion, translational motion, and interaction with paramagnetic centers (8). At a resonant frequency of 4 MHz, the deuteron spin-lattice relaxation time in rat muscle water at 25°C was found by Cope (1) to be 0.092 s, a factor of 4.9 times less than in normal water. This is smaller than the

equivalent ratio of 10 for protons in toad muscle water at 4 MHz obtained by inter-polation from the present data but is still much greater than unity. It is concluded that contributions to spin-lattice relaxation from causes other than spin diffusion, translational restrictions, or paramagnetic materials have a major role in rat muscle and presumably also in toad muscle. Nevertheless any or all of the above three mechanisms may still have contributed significantly to the present data. If so, the remaining relaxation rate, to be accounted for by modified water or other sources of long τ protons would be diminished. So also would the number of long τ protons required. Since relaxation rates are additive, for any particular value of τ, the requi-site proton population might be diminished or remain unaltered. It could not be increased.

The present study does not reveal whether the proton states with $\tau > 10^{-9}$ s rep-resent modified water or macromolecular protons coupled to the water.

As not more than 2% of the protons have relaxation times greater than 10^{-9} s, compared with a free water value of 10^{-11} s, it is difficult to reconcile our results with the hypothesis (4) that intracellular water is semicrystalline. It is not impossible that a significant fraction of the water exists in states with $\tau \leq 10^{-10}$ s as they would have escaped detection in our observations. Such correlation times are 10^{5} times smaller than in ice and not more than 10 times the value for normal water. Proton T_1 measurements at >200 MHz might detect such a fraction, but were its existence demonstrated, it is not clear that it could be regarded as semicrystalline.

Proton NMR and Electrochemical Experiments with Nonliving Materials

In Fig. 4, proton T_1 data for three types of hydrated nonliving materials are com-pared with the data for muscles. The T_1 characteristics of these materials are quali-tatively similar to those of the muscle specimens and are not seriously affected by the introduction of 0.05 M NaCl (actomyosin) or 3 M NaCl (agarose and gelatin). Their sodium ion and chloride ion potentials were investigated with the results shown in Figs. 5 and 6. Except when the water content is very low there is no evi-dence for the ion exclusion effect postulated by Ling and Cope (4). For a particu-lar set of conditions, ion exclusion could have been masked by a compensating amount of ion binding. It is very improbable that such compensation persisted over the range of conditions studied. Even in the samples of lowest water content where the T_1 values are much lower than those reported here in muscle water (Fig. 4) the enhancement of the ion chemical potentials, which is equivalent to exclusion of salt from about 30% of the water, is far too small to account for the sodium ion distri-bution in tissue. Moreover, the observations could probably be explained in terms of steric effects without recourse to "crystalline" water.

The single ion chemical potentials can be criticized because of the uncertainty which possible variations in the liquid junction potential at the tip of the calomel electrode always introduce into such measurements. The same conclusions, how-
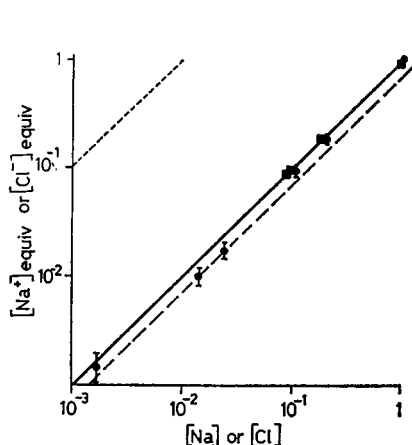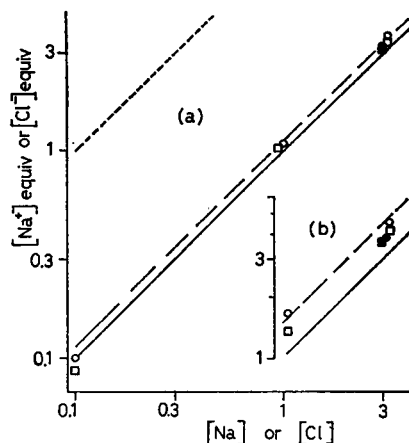
FIGURE 5   FIGURE 6

FIGURE 5   Sodium ion (●) and chloride ion (■) chemical potentials in actomyosin pre-
cipitates at 18°C. Water content = 9 g/g actomyosin. [Na], [Cl]: ion concentration (mol/kg
H₂O) in actomyosin samples $[Na^+]_{equiv}$, $[Cl^-]_{equiv}$: concentration (mol/kg H₂O) in
standard salt solution with equivalent ion chemical potential. Theoretical lines: — — — ,
30% of ions inactive; ——— , ions excluded from 90% of sample water (only approximate;
change of activity coefficient with ionic strength not considered). To account for sodium
ion distribution in muscle at least this degree of effective exclusion would be required. ——— ,
no exclusion, no association.

FIGURE 6   Sodium ion (●, ○) and chloride ion (■, □) chemical potentials in water asso-
ciated with agarose (open symbols) and gelatin (filled symbols) at 21°C. Quantities plotted
and theoretical lines ——— and ——— as in Fig. 5; — — — , ions excluded from 0.33 g
water/g agarose or gelatin. (a) 3 g, and (b) 1 g solution/g agarose or gelatin.

ever, may be deduced from the sum of the sodium ion and chloride ion chemical
potentials where the only uncertainty concerns the specificity of the sodium and
chloride electrodes.

In view of the results obtained for actomyosin, a major component of muscle, it
seems likely that the muscle proton NMR characteristics result from whatever
macromolecule-water interactions (possibly including proton exchange) are opera-
tive in actomyosin precipitates. If so they are probably not associated with appre-
ciable ion exclusion.

To our knowledge, the only other published measurements of muscle proton T_1
at more than one frequency are those of Bratton et al. (11). As shown in Fig. 4, they
observed shorter relaxation times but the same qualitative argument can still be
applied. Working at 20 MHz, Abetsedarskaya et al. (12) found T_1 for frog muscle
water to be comparable with the values reported here but Walter and Hope (2)
found much shorter relaxation times (Fig. 4). Even Walter and Hope's data, how-
ever, need not imply a high degree of crystallinity or ion exclusion.

The work of Derbyshire and coworkers (13) on the muscle protein tropomyosin
and Hazlewood et al. (14) on whole muscle suggests that minor fractions of struc-
tured water may exist. The present results do not preclude that possibility.

CONCLUSIONS

(a) Not more than 2% of the NMR-visible protons in toad muscle water have correlation times more than 100 times larger than in normal water (i.e., $\gtrsim 10^{-9}$ s).

(b) The results of NMR measurements on water protons in toad muscle do not require that the bulk of the intracellular water be semicrystalline.

(c) Hydrated actomyosin, agarose, and gelatin have qualitatively similar proton and deuteron NMR characteristics to muscle tissue water. Ionic chemical potentials can be significantly heightened in these systems but not sufficiently to account for sodium ion distribution in muscle tissue.

(d) Nonliving materials, including actomyosin, a principal component of muscle, exhibit similar NMR characteristics to muscle but do not significantly exclude ions from their constituent water. Therefore the known NMR properties of muscle cannot justifiably be said to favor the hypothesis that sodium ions are excluded from the bulk of the water in muscle cells. Nevertheless, they do not totally exclude this possibility.

REFERENCES

1. COPE, F. W. 1969. *Biophys. J.* 9:303.
2. WALTER, J. A., and A. B. HOPE. 1971. *Aust. J. Biol. Sci.* 24:497.
3. OUTHRED, R. K., and E. P. GEORGE. 1973. *Biophys. J.* 13:83.
4. COPE, F. W. 1970. *Biophys. J.* 10:843.
5. SZENT-GYORGYI, A. 1951. Chemistry of Muscular Contraction. Academic Press, Inc., New York, 2nd edition.
6. COPE, F. W. 1967. *J. Gen. Physiol.* 50:1353.
7. COMMONER, B., J. C. WOOLUM, and E. LARSSON. 1969. *Science (Wash. D. C.).* 165:703.
8. GLASEL, J. A. 1970. *Nature (Lond.).* 227:704.
9. MICHEL, D., and H. PFEIFER. 1965. *Z. Naturforsch. Teil A.* 20:220.
10. ANDERSON, J. E. and W. P. SLICHTER. 1965. *J. Phys. Chem.* 69:3099.
11. BRATTON, C. B., A. L. HOPKINS, and J. W. WEINBERG. 1965. *Science (Wash. D. C.).* 147:738.
12. ABETSEDARSKAYA, L. A., F. G. MIFTAKHUTDINOVA, and V. D. FEDOTOV. 1968. *Biophysics.* 13:750.
13. CYR, T. J., W. DERBYSHIRE, J. L. PARSONS, J. M. V. BLANSHARD, and R. A. LAWRIE. 1971. *Faraday Soc. Trans.* 67:1887.
14. HAZLEWOOD, C. F., B. L. NICHOLS, and N. F. CHAMBERLAIN. 1969. *Nature (Lond.).* 222:747.