DIRECT EVIDENCE FROM NUCLEAR MAGNETIC RESONANCE STUDIES FOR BOUND SODIUM IN FROG SKELETAL MUSCLE

J. L. CZEISLER, O. G. FRITZ, JR., *and* T. J. SWIFT

From the Department of Chemistry, Case Western Reserve University, Cleveland, Ohio 44106

ABSTRACT The recent work of Cope on ^{23}Na magnetic resonance studies of frog muscle has been repeated with the view of investigating certain objections which can be raised concerning the original studies. The present work leads to the conclusion that Cope's results concerning bound sodium are essentially correct in that a large fraction of the ^{23}Na present does not contribute normally to a detectable nuclear magnetic resonance (NMR) signal. This "missing" signal can be detected at high radio-frequency intensity however, and a signal-saturation study distinctly reveals its presence.

INTRODUCTION

The transcellular potential of any excitable cell is a function of two distinct sets of properties of the charged species which it contains. The first set defines the specific chemical environments and associated chemical potentials for each of the cellular ionic species. The second set defines the transport characteristics of each of these species and, in particular, as they pertain to transport in and out of the cell, reflects the nature of the cell membrane.

Neither of these sets has yielded to highly definitive experimental measurement up to the present, but the first set has proved to be particularly elusive because of the difficulty of measurements in living cells. Measurement of these sets of properties is particularly important in view of the rather specific proposals of Ling (1) concerning the roles of water organization and ion association in the existence and alteration of the cellular potential.

The technique to be employed for these measurements must be such that the cellular potential is not disturbed; it must be sensitive, selective, and capable of quite rapid measurement if it is to be used, for instance, to study chemical changes during the propagation of an action potential. In spite of numerous pitfalls inherent in the application to very complicated systems, nuclear magnetic resonance seems to be a technique of great potential applicability. Proton magnetic resonance techniques have been developed recently which enable one to observe changes in cellular water as a function of the transcellular potential (2–4).

Cope (5, 6) has recently employed ^{23}Na NMR to investigate the state of Na^+ in frog muscle. (Reference 6 contains a number of pertinent references to work on ^{23}Na NMR.) He compared the ^{23}Na signal intensity with the proven sodium content of his samples and concluded that the major fraction of the sample ^{23}Na was not contributing to his measured NMR signal. His explanation for this result was that this "NMR-invisible sodium" was bound to cellular macromolecules yielding a broad signal which would not be detected under his conditions for signal recording.

Such a conclusion is an extremely important one; however, a number of objections can be raised concerning the experimental procedures which led to it. These objections center around the fact that the muscle ^{23}Na signal had to be compared to a ^{23}Na signal from a homogeneous solution of known concentration for calibration purposes. In Cope's work this standard was a dilute sodium chloride solution. Unfortunately, the muscle ^{23}Na signal is considerably broader (on a high resolution instrument) than this standard signal and hence, questions arise concerning possible differential saturation and modulation effects in the original work. These concerns are compounded by the fact that limited signal:noise ratio is a distinct problem with the muscle resonance and the investigator is strongly tempted to use radio-frequency power levels and modulation amplitudes which yield the optimum signal:noise ratio for the muscle sample. Our experience has shown that this invalidates the use of the dilute sodium chloride solution as a calibration standard for quantitative work. The fact tnat the Cope paper did not specifically deal with this problem has been a source of uncertainty concerning the conclusions reached.

A second serious problem centers around the fact that the muscle sample is heterogeneous and the standard is homogeneous. The muscle could alter the quality factor, Q, of the NMR receiver coil sufficiently, compared to the aqueous solution, that the signal:noise ratio from the two samples are not comparable. What is needed is proof that in the absence of "binding" effects the sodium signal from the muscle would correspond to the total concentration of ^{23}Na.

The third point to be made is perhaps the most important one: if bound sodium exists in muscle, its NMR signal should be detectable even if it is quite broad, although not under the conditions employed by Cope. The principal requirement for the observation of very broad signals is a high radio-frequency field level.

The first problem outlined above was handled in the present work by employing a dilute solution of Na_2SO_4 in 95% H_2SO_4 (by weight) as the standard. The high viscosity of this solution produces a signal of approximately the same line width as that of the muscle and with quite similar saturation and modulation characteristics. A crystal control was installed on the radio-frequency unit of the Varian VF-16 Wide Line Spectrometer (Varian, Palo Alto, Calif.) which was employed in this work and the field spread was reduced by shimming to *circa* 20 milligauss over the sample employed. The signal:noise ratio was then sufficient to allow the use of a level of radio-frequency intensity significantly below saturation and of a modulation amplitude significantly below the line width. Differential modulation and saturation effects are not significant under these conditions.

The second problem is somewhat more difficult to handle since it basically involves the introduction of a suitable standard into the muscle tissue itself. Properly, this should be done in a nondestructive manner. Deuterium substitution proved to be eminently useful here since deuterium could be easily added through the use of deuterated Ringer's solution as the bathing solution. The deuterium resonance could then be observed under nearly the same instrument conditions employed for the ^{23}Na study. Subsequently the muscle water (containing over 90% of the deuterium) was recovered by distillation and the actual deuterium content of the muscle was compared with the NMR result. This study proves to be an excellent check on the effect of the muscle sample on the receiver circuit and hence precludes the possibility that the observations are, in any part, due to sample artifact.

The third point discussed above concerned the direct detection of more than one sodium signal in muscle. A saturation study proved to be sufficient for these purposes. The intensity, v, of a single lorentzian absorption signal varies with radio-frequency power according to equation 1 (for derivation of equation 1, see reference 7).

$$v = \frac{AH_1}{1 + B\Delta\omega^2 + CH_1^2}, \tag{1}$$

where A, B, and C are constants, H_1 is the radio-frequency intensity, and $\Delta\omega$ is the difference between the applied radiofrequency and the nuclear resonance frequency. This signal maximizes when $\Delta\omega = 0$; the variation of $v_M = v\ (\Delta\omega = 0)$ with H_1 can be expressed in the form of equation 2.

$$v_{Mr} = 2H_{1r}/(1 + H_{1r}^2), \tag{2}$$

where v_{Mr} is the signal intensity v_M relative to the maximum value (which occurs when $CH_1^2 = 1$) and H_{1r} is the power level relative to H_1 of the maximum value. The two parameter equation 2 can be plotted as a universal curve of $\log v_{Mr}$ vs. $\log H_{1r}$ as is shown in Fig. 1. For any single experimental lorentzian absorption signal, a plot of $\log v_M$ vs. $\log H_1$ must be superimposable on the universal curve with, at most, a horizontal and vertical shift of axes required. A similar treatment can be made for signal widths. However, in the experiments reported here, signal amplitudes are somewhat more precisely measurable than signal width and hence the former were employed in the saturation study.

If two absorption signals are superimposed in some experimental situation and one signal is sufficiently broader than the other, a plot of $\log v_M$ vs. $\log H_1$ will yield two maxima, one at lower power for the narrower signal and one at higher power for the broader signal. If the conclusion of Cope is correct and if the bound sodium signal is not too broad, this two maxima plot is what one would expect to see for the magnetic resonance saturation behavior of sodium in muscle.

In addition, excellent checks for artifacts are provided by the Na_2SO_4 standard

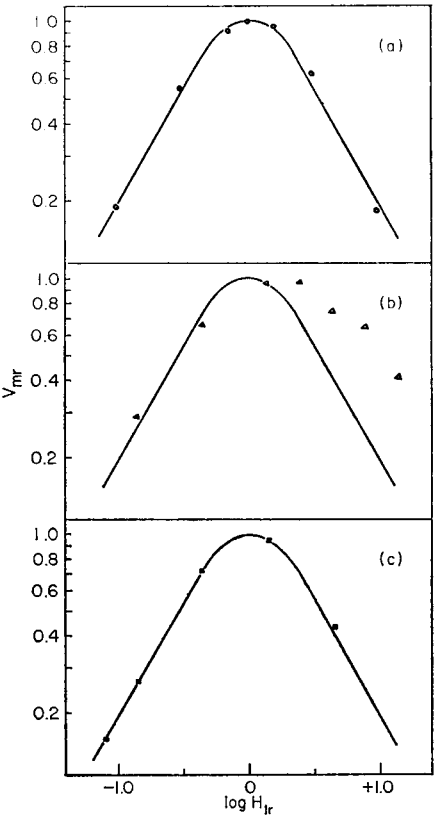

FIGURE 1 Saturation behavior of NMR signals: (*a*) sodium ion (31 mM) in 60% H_2SO_4; (*b*) sodium ion in frog skeletal muscle; (*c*) deuterium in frog skeletal muscle.

solution, which should saturate as a single lorentzian line, and by the signal of deuterium in muscle, which tests the behavior of the muscle in a saturation study.

Procedural details for each of these studies are given in the experimental section.

EXPERIMENTAL

Materials

All chemicals used in these studies were reagent grade. The studies in which deuterium was employed made use of 99.7% D_2O (Columbia Organic Chemicals, Inc., Columbia, S. C.). Skeletal muscle was obtained from *Rana catesbeiana* (Lemberger Company, Oshkosh, Wis.). The intact semimembranosus muscle (dorsal thigh) was used in all experiments.

Tissue Preparation

Bullfrogs were killed by pithing and one, or both, semimembranosus muscles were excised according to the quantity of tissue required. Care was taken to avoid unnecessary mechanical strain on the muscle during its preparation. After excision the muscles were placed in a Ringer's solution appropriate to the purpose of the experiment (see below).

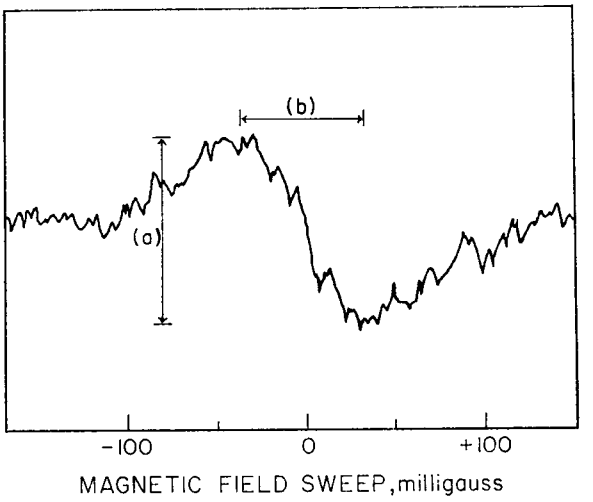

FIGURE 2 Signal of sodium ion in frog skeletal muscle as recorded by Varian VF-16 NMR Spectrometer (derivative output): (*a*) peak-to-peak height; (*b*) peak-to-peak width.

Measurements by Concentration Comparison

All measurements in this section were carried out, at ambient temperature, using a Varian VF-16 Wideline NMR Spectrometer. The modulation frequency employed in these studies was 20 cycles/sec. Under these conditions the output plot of this instrument has the mathematical form of the *derivative* with respect to $\Delta\omega$ of equation 1. A sample signal of muscle sodium is presented in Fig. 2. The concentration of the nuclei under observation is proportional to the peak-to-peak height multiplied by the square of the peak-to-peak width, a relationship which may be shown to be exact for a signal of derivative form.

Sodium ion in muscle bathed in Ringer's solution. Two muscles were bathed in Ringer's solution for 10 min and then introduced into a thin - walled glass tube (15 mm I.D.) which was then placed in the spectrometer. The NMR absorption signal of ^{23}Na was observed at 7.88 MHz. The signal was scanned and recorded several times. The ^{23}Na signal from a standard solution (Na_2SO_4 in 95% H_2SO_4) of known sodium concentration was then recorded using the same tube and identical instrument control settings.

After recording the sodium signal of both sample and standard, the muscle sample was desiccated under vacuum and then incinerated in a crucible until essentially all organic matter had been burned away. The residue, a small amount of white powder, was dissolved in a weighed quantity of H_2SO_4 (95%). The sodium NMR signal of this solution was recorded. The standard was also recorded for comparison.

Sodium ion in muscle bathed in K^+-rich Ringer's solution. Two muscles were bathed in K^+-rich Ringer's solution (Ringer's made 0.113 M in KCl) for 10 min. The remainder of the procedure for this muscle sample was identical to that described above for muscle bathed in regular Ringer's solution.

Deuterium in muscle bathed in D_2O-rich Ringer's solution. Two experiments were carried out using muscles bathed in a Ringer's solution which was prepared to be 1% in D_2O. The relatively low deuterium content was chosen to produce signals which had a signal:

noise ratio comparable to that observed in the NMR studies of sodium in muscle. This provided a further check on saturation and modulation effects.

One pair of muscles was bathed for 20 min, while a second pair was bathed for 3 hr. The exchange of the H_2O in the muscle with D_2O in the bathing solution provided the muscle with a deuterium content which was observable using NMR. The NMR signal (at 6.91 MHz) from deuterium in each muscle sample was recorded and the deuterium signal from each bathing solution was also recorded. The total water content of each muscle sample was then distilled under vacuum and the quantity of distillate was measured. The deuterium signal of each distillate was recorded and the deuterium signal of each bathing solution was again recorded for comparison purposes.

Saturation Behavior of NMR Signals

All experiments described in this section were carried out with a Varian HA-60 High Resolution NMR Spectrometer which employed fixed frequency rf oscillators, one of 15.1 MHz for purposes of sodium NMR, and another of 8.1 MHz for purposes of deuterium NMR. The output of this instrument is in the form of the NMR *absorption mode*, having a shape given by equation 1. In the experiments described below exact line shape was a key factor. Thus, in order to minimize distortion of the line shape due to field inhomogeneity, it was found to be necessary to employ a high resolution instrument.

Saturation behavior of the signal of sodium ion in solution. The NMR signal of ^{23}Na ($\sim$31 mM) in a solution of Na_2SO_4 in 60% H_2SO_4 was recorded at each of several rf field intensities over a relative range of two orders of magnitude of rf field intensity.

Saturation behavior of the signal of sodium ion in muscle. A pair of muscles was prepared by bathing in Ringer's solution for 10 min. The saturation behavior of the signal was recorded as described above.

Saturation behavior of the signal of deuterium in muscle. A single muscle was bathed for 4 hr in Ringer's solution made 5% in D_2O. The muscle was then introduced into a glass tube (12 mm I.D.) and placed in the spectrometer. The saturation behavior of the signal was recorded as described above.

RESULTS

The results of the studies of ^{23}Na NMR using the Varian VF-16 Spectrometer are presented in Table I.

The results of the determinations of the deuterium content of muscles bathed in Ringer's made 1% in D_2O are presented in Table II.

TABLE I

SODIUM CONCENTRATION OF INTACT AND ASHED FROG SKELETAL MUSCLE AS DETERMINED BY SODIUM NMR

Bath	Intact	Ashed
	mM	*mM**
Ringer's	15	40
K^+-rich Ringer's	14	31

* Corrected to volume of intact muscle.

TABLE II

DEUTERIUM CONCENTRATIONS IN MUSCLE RELATIVE TO DEUTERIUM CONCENTRATIONS OF BATHING SOLUTION AS DETERMINED BY DEUTERIUM NMR

Bathing time	Intact	Distilled
	%	%*
20 *min*	40	39
3 *hr*	73	79

* Corrected to volume of intact muscle.

The signal:noise ratio in the studies of sodium and deuterium (20 min bathing time) in muscle was approximately 5. Due to this limited signal:noise ratio, the results presented above for intact muscle are the average values of several measurements and are reproducible to $\pm 15\%$. The precision of the total muscle Na^+ was significantly greater, the values presented being reproducible to $\pm 5\%$.

The results of the studies of the saturation behavior of NMR signals of nuclei in muscle and in solution are presented in Fig. 1. No attempt was made in any of these plots to obtain a best fit by mathematical curve-fitting procedures. The optimum signal:noise ratio in measurements on ^{23}Na NMR of intact muscle using the Varian HA-60 Spectrometer was found to be 15. The signal:noise ratio for the deuterated muscle and for sodium in solution was 30–50.

DISCUSSION

We commence this section by summarizing the findings presented above. A study, by ^{23}Na NMR, of the concentration of sodium ion in frog skeletal muscle after brief bathing in Ringer's solution reveals that, under the condition of low rf field intensity, only a part of the sodium content is detectable. The NMR-visible sodium ion amounts to only approximately 37% of the total sodium ion when the muscle has been bathed in Ringer's solution. Bathing of the muscle in K^+-rich Ringer's solution, however, reduces the total sodium ion concentration without markedly affecting the sodium ion concentration detectable by the NMR method. When the muscle has been bathed in K^+-rich Ringer's solution, the NMR-visible sodium constitutes approximately 45% of the total sodium in the tissue. These results confirm and extend those of the earlier work of Cope (5, 6) and the recent work of Ling and Cope (8). Measurements by NMR of the deuterium concentration of muscle containing small amounts of deuterium verify our belief that these results cannot be discounted on the grounds of artifacts due to instrumentation or to the bulk nature of the tissue studied.

As was pointed out earlier, the behavior of the amplitude of an NMR signal as a function of rf field intensity is theoretically predictable. Our studies have shown that the saturation behavior of NMR signals of sodium ion in solution and deuterium in muscle follow the theoretical prediction well. While no significance may be attached to the position of the universal curve in Fig. 2 *b*, it is clear that the experimental points cannot be fit by a curve of this type. These observations strongly indicate that the medium in which the sodium ion exists in muscle tissue is quite different than than in a simple aqueous electrolyte solution.

On the basis of the studies of the sodium ion concentration in intact frog skeletal muscle, it is reasonable to propose that there are two or more distinguishable states of sodium ion in muscle. In one, or several, of these states the sodium ion is detectable by NMR at low rf field intensity while in the other state, or states, the sodium ion is NMR-invisible under these conditions.

The observation of an anomalous saturation behavior of the NMR signal of sodium ion in muscle indicates that at least two signals do exist, each representing a state, or range of states, of the ion. The study further indicates that sodium ions in all of these possible states have the same, or very similar, resonant frequencies, i.e., the NMR signals appear at very closely the same place in the spectrum. The signals have, however, quite disparate line widths when observed using low rf field intensities. This accounts for only one of the two signals being observable under these conditions. While the presence of the broad signal may be accounted for in a number of ways, it is consistent with the view of Cope that some sodium ions in the muscle are bound to other molecules, presumably macromolecules, in the system. Preliminary studies in our laboratory have shown that the NMR signal of sodium ion bound to a wet cation exchange resin (Dowex 50W-X8 (Dow Chemical Co., Midland, Mich.), sodium form) has a peak-to-peak width of approximately 150 milligauss when observed on the Varian VF-16 Wideline NMR Spectrometer (signal output in derivative form). The peak-to-peak width of aqueous NaCl ($\sim$2 M) was found to be 45 milligauss under similar instrumental conditions. This comparison reinforces the proposal that the presence of a quite broad ^{23}Na signal in the NMR study of skeletal muscle represents sodium ion which is strongly associated with other molecules in the system. Although no characterization of this association can be made at the present time with any degree of certainty, such characterization is possible and remains the next major step in this investigation.

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