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CATION NUCLEAR MAGNETIC RESONANCE (NMR). ^{7}Li- and ^{23}Na-NMR RESULTS OBTAINED WITH HUMAN ERYTHROCYTES

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Abstract

A short discussion of practical results and theoretical aspects of nuclear magnetic resonance (NMR) on intracellular monovalent cations is presented. ^{7}Li- and ^{23}Na-NMR relaxation studies on human erythrocytes are described which indicate that lithium and sodium ions are essentially free inside the cell. However, there is a contribution to transverse relaxation originating from cations diffusing through electric field gradients imposed by the cytoskeleton, which is reflected in the NMR line shape. ^{39}K-NMR results obtained by other authors for intracellular potassium are compared with the ^{7}Li- and ^{23}Na-NMR results presented here. The few ^{39}K-NMR results obtained so far do not suggest that significant differences in dynamic behavior exist between intracellular potassium and sodium. ^{39}K-NMR studies will, in the future, certainly enhance our understanding of the intracellular state of potassium ions.

Key Words: Nuclear magnetic resonance (NMR), red blood cells, ^{7}Li-NMR, ^{23}Na-NMR, ^{39}K-NMR, relaxation times, dynamic ion behavior, erythrocytes, intracellular order.

Introduction

The physical behavior of the intracellular monovalent cations Na^+ and K^+ continues to be subject of intense debate. It has been proposed by several workers that an appreciable fraction of Na^+ and K^+ is bound to specific cites in the cell [14, 16, 26]. This idea forms part of the "Association - Induction Hypothesis" proposed by Ling [26]. By the majority of scientists, however, the cell cytoplasm is seen as an aqueous solution in which the ions and water molecules can diffuse freely although the properties of cell water are somewhat different from those of free water because of solvation interaction with proteins [36]. The intention of this paper is to discuss how the technique of nuclear magnetic resonance (NMR) can provide insight into the intracellular behavior of Na^+ and K^+ and whether any binding of those ions takes place.

Mammalian cells contain a membrane associated cytoskeleton which has been shown by electron microscopy to be composed of a highly disordered, interconnected network of filamentous proteins [43]. As has been shown experimentally, large protein molecules may be severely restricted in their intracellular mobility because of this network [42]. Smaller molecules, such as size-fractionated dextrans have more motional freedom but still experience the presence of the intracellular network, which imposes a certain degree of local order [27]. A very useful property of sodium and potassium ions is that these nuclei have a nuclear magnetic moment, which makes it possible to perform NMR studies on these ions in vivo. NMR studies of intracellular monovalent cations are valuable because NMR experiments provide insight into the intracellular order experienced by these cations [2]. For example, it has been found that in cancerous tissues ^{23}Na-NMR T_1 relaxation times are longer than in normal tissue [21], indicative of a loss of intracellular structure because of higher water-content.

A subject that comes up regularly in cation NMR is the "invisibility - problem" [39]. This problem consists of the observation that in many cells and tissues only 40% of sodium or potassium ions is detected by NMR, as compared to other analytical techniques such as flame photometry. In the early days of cation NMR this phenomenon was explained by binding of 60% of the cations present, which by the particular NMR properties of sodium and potassium would make the NMR signal of these bound nuclei so broad as to make it undetectable above the

instrumental noise [15]. Later it was shown by Berendsen and Edzes [2], that such results could also be explained by the existence of only one population of nuclei but present in an environment which is structurally and electrostatically heterogeneous on the NMR time scale. We will discuss this important point further in the theoretical section.

In several cell systems the partial NMR invisibility of sodium or potassium has been observed. Examples are yeast cells [30] and E. coli [35]. In erythrocytes generally all of the sodium [33] or potassium [4, 29] is visible by NMR. Intracellular viscosity is related to water content. Thus, changes in water content can lead to changes in relaxation times and NMR visibility. Experimentally it is found that in cells with high intracellular viscosity such as yeast cells (13.6 cP) [5] only 40% of intracellular ions are visible, while in erythrocytes with a lower intracellular viscosity of 5.5 cP [28] all of the sodium and potassium can be detected.

The study of intracellular monovalent cations has been greatly facilitated by the development of cell impermeable shift reagents [12, 23]. Without shift reagents the resonances for intra- and extracellular ions overlap. Shift reagents are paramagnetic inorganic ion chelators which shift the extracellular resonance of the complex ions up- or downfield making it possible to study the intracellular ions separately. Two popular shift reagents are $Dy(PPP)_2^{7-}$ (dysprosium-tripolyphosphate) and $Dy(TTHA)^{3-}$ (dysprosium-triethylenetetraminehexaacetate). $Dy(PPP)_2^{7-}$ produces the largest shift but decomposes in certain physiological media, thus possibly releasing toxic products [39]. $Dy(TTHA)^{3-}$ produces a smaller shift but is physiologically more benign and is therefore used in animal studies. The use of shift reagents for the study of intracellular cations has been reviewed by several authors [22, 24, 39].

Most NMR results seem to indicate that intracellular sodium and potassium ions are only weakly restricted in their motion and diffuse freely to and from various macromolecules inside the cell. Local order is created by the fact that the majority of intracellular proteins are negatively charged at physiological pH, thus creating electric field gradients affecting the NMR line shapes of diffusing nuclei. In the next sections theoretical aspects of NMR of intracellular quadrupolar nuclei will be discussed and results obtained for Li^+ and Na^+ in erythrocytes under various experimental conditions will be given.

Theoretical Aspects

Three monovalent cations of physiological interest are $^{23}Na^+$, $^{39}K^+$, and $^{7}Li^+$. These ions have spin 3/2 nuclei, which give rise to four equidistant energy levels in an external magnetic field. In an NMR spectrum one absorption line would be observed in that case. However, spin 3/2 nuclei possess an electric quadrupole moment which in the case of non-averaged quadrupolar interaction perturbs the equidistance between the 4 energy levels and thereby splits the NMR absorption line into three lines with intensities 3:4:3. These general NMR properties of spin 3/2 quadrupolar nuclei have been reviewed extensively [2, 13, 17].

Intracellular mobility of Na^+, K^+, or Li^+ can be studied by NMR relaxation experiments. Relaxation of intracellular spin 3/2 occurs by interaction between the nuclear electric quadrupole moments and electrostatic field gradients caused by localized negative charges on intracellular macromolecules. A model that explains the obtained relaxation results is that the ions diffuse through the cell interior, thus experiencing fluctuating electric field gradients which affect their relaxation properties. The relaxation times T_1 and T_2 give, therefore, experimental access to intracellular order.

Quadrupolar relaxation of a spin-3/2 nucleus has been shown to consist of the sum of two components [25]. However, in practice, deviations from single-exponential relaxation are often hard to detect and it can then be justified to define effective relaxation times as:

$$\left(\frac{1}{T_1}\right)_{eff} = \frac{2\pi^2}{25}\left(\frac{e^2qQ}{h}\right)^2 [J(\omega) + 4J(2\omega)] \qquad (1a),$$

and

$$\left(\frac{1}{T_2}\right)_{eff} = \frac{\pi^2}{25}\left(\frac{e^2qQ}{h}\right)^2 [3J(0) + 5J(\omega) + 2J(\omega)] \qquad (1b),$$

where $J(\omega) = \tau_c / (1 + \omega^2\tau_c^2)$ is the spectral density function and e^2qQ/h is the quadrupolar coupling constant (qcc) in frequency units [32]. In the fast motional region where $\omega^2\tau_c^2 << 1$ equations (1a) and (1b) reduce to:

$$\frac{1}{T_1} = \frac{1}{T_2} = \frac{4\pi^2}{10}\left(\frac{e^2qQ}{h}\right)^2 \tau_c \qquad (2)$$

If two different motional frequencies affect the relaxation, one can write, in the case of uncorrelated motions:

$$J(\omega) = \frac{\tau_{c1}}{1 + (\omega\,\tau_{c1})^2} + \frac{\tau_{c2}}{1 + (\omega\,\tau_{c2})^2} \qquad (3)$$

When these two motions are of a different nature, two different quadrupole coupling constants qcc_1 and qcc_2 have to be assigned to τ_{c1} and τ_{c2}, respectively [11].

The general observation of T_2 values shorter than T_1 values for quadrupolar spin 3/2 nuclei inside cells can be explained by the existence of two different motional frequencies affecting the relaxation. One motion has a short correlation time τ_{c1} and probably reflects the rotational motion of water molecules in the vicinity of the cations. As shown for human erythrocytes, $\tau_{c1} << \omega^{-1}$ for both intracellular Na^+ and Li^+, where ω is the resonance frequency in angular frequency units [32]. The other motion is slow with the conditions $T_2 >> \tau_{c2} >> \omega^{-1}$ and possibly reflects intracellular diffusion of Na^+ and Li^+ in a structurally and electrostatically heterogeneous

environment [32].

The explanation of the relaxation behavior of quadrupolar nuclei in terms of two different frequencies of motion was originally given by Berendsen and Edzes [2] and later applied by Chang and Woessner [11] to muscle tissue. Under the condition $\tau_{c1} << \omega^{-1} << \tau_{c2}$ it was shown by these workers that the T_1 longitudinal relaxation should follow a single exponential giving a single T_1, while the T_2 transverse relaxation should be a biexponential with intercepts of 40% for the slow component and 60% for the fast component. From this analysis, these workers demonstrated that ^{23}Na $T_1 = T_2$ (long). The 40% and 60% intercepts correspond to the central and two outer transitions, respectively, in the NMR frequency spectrum [2, 11].

Experimental Procedures

Erythrocyte Preparation

Twenty millimeter of venous blood drawn into 20 ml plastic syringes containing 3.0 ml of anticoagulant citrate dextrose as described previously [32]. Lithium chloride was added to the blood to give an overall concentration of 50.0 mM. Dysprosium tripolyphosphate shift reagent (Dy:2TPP) was added to a 5.0 mM final concentration for the samples with osmotic values of 200, 300, and 400 mOsm, and a 9.0 mM final concentration for the samples with 500 and 600 mOsm. The blood osmolality was then adjusted to the desired value by either diluting the blood with a 5.0 mM Dy:2TPP solution (for the 200, 300, and 400 mOsm samples), or by adding a saturated NaCl solution (for the 500 and 600 mOsm samples) [44]. Osmotic values were measured with a Wescor 5100 B vapor pressure osmometer, after calibration with Wescor standard solutions. The mean of four separate measurements was taken as the osmotic value. After 22-24 hours of Li^+ incubation at 23° C at a given osmolality, the osmolality was measured again and adjusted if necessary. A small aliquot of the blood was then examined by light microscopy after which the blood was centrifuged at 600 g for 20 minutes to gently pack the erythrocytes. The plasma was then removed and the packed erythrocytes were transferred to a 12 mm NMR sample tube for ^{31}P-NMR followed by ^{23}Na- and ^{7}Li-NMR analysis. The hematocrit did not decrease significantly any further after packing the cells in the NMR tube. ^{31}P-NMR spectra indicate that the erythrocytes are viable after 22-24 hours of Li^+ incubation.

^{23}Na-^{7}Li NMR

The NMR experiments were conducted at resonance frequencies of 77.4 MHz for ^{7}Li, 52.7 MHz for ^{23}Na, and 80.9 MHz for ^{31}P. The T_1 values were measured with the $180°-\tau-90°$ inversion recovery sequence and T_2 values with the $90°_x-(\tau-180°_y-\tau)_n$ Carr-Purcell-Meiboom-Gill sequence with n = 10 and τ varying from 0.05 to 1.0 msec. Prior to the ^{23}Na-NMR and ^{7}Li-NMR relaxation experiments, ^{31}P-NMR spectra were obtained for each sample to determine 2,3-diphosphoglycerate (2,3-DPG), inorganic orthophosphate (P_i) and ATP levels in order to assess the physiological condition of the erythrocytes. ^{23}Na- and ^{7}Li-NMR relaxation experiments were carried out overnight at room temperature. The next morning another ^{31}P-NMR spectrum was obtained. In general 2,3-DPG levels decreased considerably overnight while P_i levels increased. ATP levels, however, remained constant. By these NMR criteria the erythrocytes were physiologically intact during the relaxation studies. All erythrocyte samples were also inspected by light microscopy. It was observed that at lower osmotic values (200-300 mOsm) the majority of erythrocytes exhibited a biconcave discocyte shape and tended to aggregate in rouleaux formation. At higher osmotic values (> 400 mOsm) the erythrocytes tended to assume an echinocyte shape, characterized by rounding of the cells and spicule formation [3].

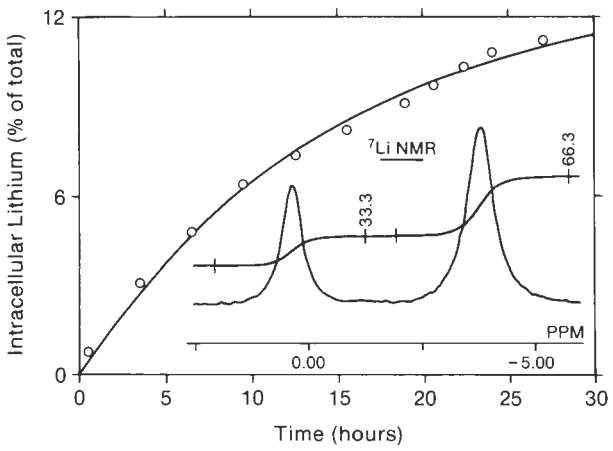

Figure 1. Intracellular Li^+ concentration as a percentage of the total Li^+ concentration, plotted as a function of time t (in hours). The data were fitted with the function A [1-exp(-t/T)], which yields a time constant T = 14.7 ± 1.2 hours.
Insert: ^{7}Li-NMR spectrum of erythrocytes incubated for 24 hours in plasma containing 50 mM LiCl. Resonance frequency was 77.4 MHz. 5 mM dysprosium: tripolyphosphate shift reagent was added just before NMR experiments. Number of scans is 100.

^{7}Li- and ^{23}Na-NMR results obtained with human erythrocytes

Lithium (Li^+) is widely used as a psychotherapeutic drug. Little is known about the molecular basis for the biological action of Li^+ at present. It has been postulated that Li^+ competes for the binding sites of biological cations such as Na^+, K^+, Mg^{2+}, and/or Ca^{2+} [20]. Another postulated action of Li^+ is that it interacts with membrane phospholipids [18]. Recently it has been reported that Li^+ effects the intracellular inositol and cyclic AMP signalling systems, because it can alter the function of GTP binding proteins [1]. To obtain more insight into the behavior of intracellular Li^+ at the molecular level, Li^+ inside red blood cells was studied by NMR. For comparison, several other Li^+ solutions were studied.

Human erythrocytes were incubated with lithium chloride as described above. The uptake of lithium ions was followed by ^{7}Li-NMR, using dysprosium shift reagent, and followed single exponential kinetics with a time constant of 14.7 hours (Fig. 1). While for concentrated hemoglobin solutions the relaxation times T_1 and T_2 were of the same order of magnitude, it was found that $T_1 \cong 5$ sec and $T_2 \cong 0.15$ sec for intracellular lithium. T_1 is three times shorter than for free aqueous Li^+, which can be explained by intracellular viscosity η as is to be expected for the fast motional region ($\tau_c << 2 \times 10^{-9}$ sec). From these experiments it is estimated that

TABLE 1. Summary of ^{7}Li-NMR Results

Sample	T_1 (sec)	T_2(sec)	T_1/T_2
LiCl (1 M)	16.65 ± 0.06	15.50 ± 0.06	1.07
LiCl (10 M)	6.80 ± 0.06	5.40 ± 0.05	1.26
LiCl (50 mM) in 26 wt% sucrose	6.89 ± 0.08	4.46 ± 0.05	1.54
LiCl (50 mM) in 38 wt% sucrose	4.03 ± 0.04	3.29 ± 0.08	1.22
LiCl (50 mM) in 46 wt% sucrose	2.29 ± 0.02	2.08 ± 0.03	1.10
1 M LiCl in 10% agar	6.63 ± 0.05	0.655 ± 0.02	10.12
1 M LiCl in 20% agar	4.09 ± 0.03	0.655 ± 0.01	19.95
5 mM LiCl in 32 wt% hemoglobin A	5.15 ± 0.06	2.29 ± 0.09	2.25
25 mM LiCl in 32 wt% hemoglobin A	4.86 ± 0.08	1.84 ± 0.04	2.64
25 mM LiCl in 32 wt% hemoglobin A dilute 2x with Tris buffer	7.07 ± 0.11	3.69 ± 0.05	1.92
LiCl 50 mM in blood plasma (N = 2)[a]	4.89 ± 0.49	2.93 ± 0.09	1.67
Erythrocytes + 50 mM LiCl (N = 5)	5.10 ± 0.56	0.145 ± 0.02	35.17
Erythrocytes in isotonic sucrose / PIPES buffer	4.26 ± 0.07	0.089 ± 0.01	47.86

[a]Values given are the ± standard deviation. All values obtained at 25°C. N = number of human subjects.

the intracellular viscosity is 4 cP. The very short T_2 has to be caused by an additional low frequency interaction affecting the lithium ions. Lithium does not seem to interact significantly with hemoglobin or phospholipid membranes. Using agar gels as a model for the cytoskeleton always $T_2 << T_1$, and for the 20% gel similar values are found as for intracellular Li^+. The results of these experiments are summarized in Table 1 [32, 34].

The results in Table 1 lead to the following five conclusions for intact normal human erythrocytes:

(1) Intact erythrocyte membranes are permeable to Li^+ with an uptake following single exponential kinetics and a time constant of 14.7 hours.

(2) Li^+ does not significantly bind to hemoglobin as demonstrated by experiments on concentrated hemoglobin solutions.

(3) Preliminary ^{7}Li-NMR and differential scanning calorimetry results on dispersions of dipalmitoylphosphatidylcholine or dipalmitoylphosphatidyl glycerol do not indicate that the interactions of Li^+ with those phospholipids are significantly stronger than the interactions of Na^+ or K^+. Therefore, it is not expected that such interactions play a role in erythrocytes.

(4) The presence of Li^+ does not affect the Na^+ NMR relaxation behavior in erythrocytes. (T_1 = 25.0 ± 1.8 msec and 24.5 ± 1.2 msec, T_2 = 11.2 ± 0.9 msec and 12.3 ± 0.9 msec, before and after incubation with LiCl, respectively). Therefore Li^+ behaves as Na^+ intracellularly and competition for interaction sites between these ions occur on a basis of equality.

(5) Intracellular viscosity, about 4 cP, can explain the reduction in the intracellular ^{7}Li T_1, but not in T_2. Because the T_1 and T_2 values of Li^+ in erythrocytes and agar gels are similar, it was proposed that heterogeneous diffusion through electrostatic field gradients imposed by the membrane-associated cytoskeleton may explain the relaxation behavior [32].

The experiments outlined above were extended later to ^{23}Na- and ^{7}Li-NMR relaxation experiments on human erythrocytes under various osmotic conditions (J.F.M. Post, unpublished results). An increase in osmolality of the external medium results in a decrease in the intracellular water content and an increase in the density of the cytoplasmic matrix. This has been demonstrated by fluorescence polarization studies on human lymphocytes [10]. Alterations of intracellular structure by osmotic manipulations should be readily detectable by ^{7}Li- and ^{23}Na-NMR relaxation experiments.

20 cc blood samples were obtained from five volunteers on five different occasions, giving a total of twenty five samples. The osmotic values were varied in steps of 100 mOsm from 200 mOsm to 600 mOsm as described in the experimental section.

The results of these experiments are summarized in Figures 2 and 3. The main observation is that the intracellular T_1 values for both ^{23}Na and ^{7}Li vary almost linearly with osmotic pressure, but the T_2 values, especially for ^{23}Na, are more or less constant. The ^{23}Na T_1 values range from 24.0 ± 0.8 msec at 200 mOsm to 15.6 ± 1.5 msec at 600 mOsm; the ^{23}Na T_2 values remain relatively unchanged with a value of 12.9 ± 1.1 msec at 200 mOsm and a value of 11.2 ± 1.0 msec at 600 mOsm. The ^{7}Li T_1 value at 200 mOsm is 5.4 ± 0.6 sec compared to a value of 3.6 ± 0.2 sec at 600 mOsm. The ^{7}Li T_2 goes from a value of 158.9 ± 106.6 msec at 200 mOsm to a value of 61.1 ± 33.8 msec at 600 mOsm.

In dilute aqueous solution the T_1 for ^{23}Na is shorter (60 msec) than for ^{7}Li (16 sec) [32]. This observation reflects the larger quadrupolar coupling constant for ^{23}Na (500-700 kHz) [40] compared to a value of 35 kHz for ^{7}Li in D_2O [41]. The intracellular T_1 value for ^{23}Na averaged over all samples (20 msec) is reduced by a factor of 3 compared to dilute aqueous solution (60 msec). The intracellular T_1 value for ^{7}Li averaged over all samples (4.5 sec) is

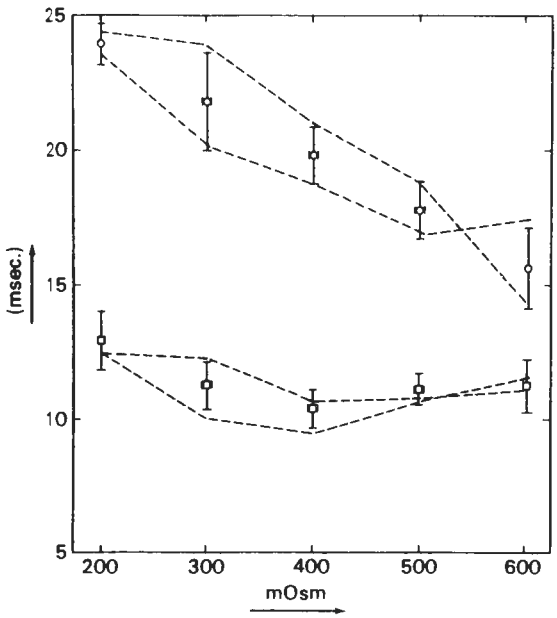

Figure 2. Effective ^{23}Na T_1 (circles) and T_2 (squares) relaxation times as a function of osmolality. Means and Standard deviations of values from five donors are given. Dashed lines in Figs. 2, 3a, and 3b represent donors with the highest and lowest average values, respectively, and are indicative of different erythrocyte properties between different donors.

reduced by a factor of 3.7 compared to dilute aqueous solution (16.5 sec). Both the ^{23}Na and ^{7}Li intracellular T_1 values yield τ_{c1} values in the fast motional region ($< 2 \times 10^{-9}$ sec) [32] using equation (2). The comparable reductions in ^{7}Li T_1 and ^{23}Na T_1 upon going from dilute aqueous solution to the intracellular environment suggest no significant differences between intracellular Na^+ and Li^+ mobilities.

From the ^{7}Li T_1 results presented herein and using previously published $1/T_1$ versus viscosity calibration curve [32], the intracellular viscosity appears to increase from 3.5 cP to 6.0 cP when the osmolality increases from 200 mOsm to 600 mOsm.

In Figs. 2 and 3 the effective relaxation times as defined in the theoretical section, have been plotted. However, many of ^{23}Na T_2 relaxation curves can best be described by a sum of two exponentials, especially at the osmotic values of 400, 500, and 600 mOsm. The results of fitting the ^{23}Na T_2 data where possible to a biexponential function are given in Table 2. Similar ^{23}Na T_2 results for erythrocytes have been published elsewhere [38]. The fact that many of the ^{23}Na-NMR T_2 curves can be described by a sum of two exponentials, while T_1 always follows a single exponential behavior, indicates that the model of two motional frequencies with $\tau_{c1} << \omega^{-1} << \tau_{c2}$ provides an adequate description for the dynamic behavior of intracellular Na^+ [2, 11].

The intracellular ^{7}Li relaxation is different in that none of the ^{7}Li T_2 relaxation curves appear to be a biexponential, while in principle, the same theoretical considerations given for ^{23}Na should apply to ^{7}Li. A single exponential T_2 relaxation curve for intracellular Li^+ has been reported for frog heart tissue [6]. The T_2 was 31 msec in that study. In

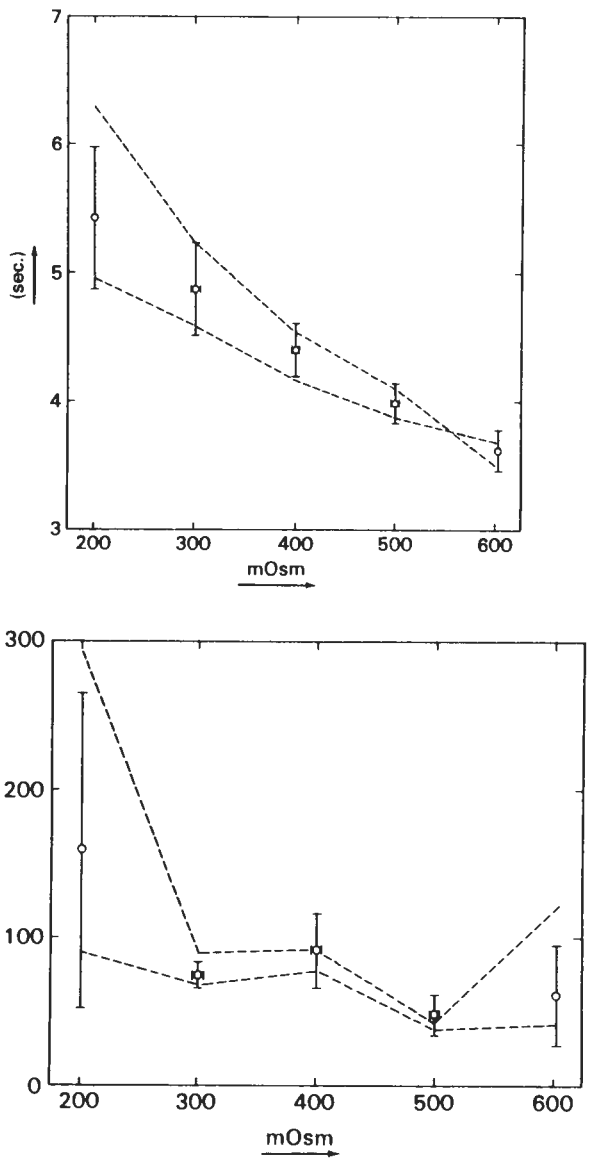

Figure 3. Effective ^{7}Li T_1 (Fig. **3a**) and T_2 (Fig. **3b**) relaxation times. Means and standard deviations from five donors.

general, the ^{7}Li T_2 values are much shorter than the T_1 values, i.e., on the order of 100 msec as compared to seconds.

Discussion

The ^{23}Na and ^{7}Li intracellular T_1's are directly influenced by extracellular osmotic changes. The most likely explanation for this is that τ_{c1}, which determines T_1, is affected by the intracellular viscosity. The more or less constant ^{23}Na and ^{7}Li T_2 values over the osmolality range studied indicate that the intracellular erythrocyte structure responsible for determining τ_{c2} for Na^+ and Li^+ is not altered by extracellular osmotic change.

If the erythrocyte membrane cytoskeleton is responsible for the long τ_{c2} of intracellular Na^+ and Li^+, the electron microscopy findings on the spread

TABLE 2. Analysis of the ^{23}Na-NMR transverse relaxation as a sum of two exponentials. The numbers represent means and standard deviations.

	n*	A	T_2^A(msec)	B	T_2^B(msec)	T_1(msec)
400 mOsm	3	53.1 ± 15.5	3.7 ± 1.2	46.9 ± 15.5	25.6 ± 15.5	20.6 ± 0.5
500 mOsm	4	59.4 ± 12.4	4.1 ± 0.4	40.6 ± 12.4	28.2 ± 9.9	17.6 ± 1.1
600 mOsm	5	63.8 ± 13.0	4.3 ± 1.1	36.2 ± 13.0	34.9 ± 18.5	15.2 ± 1.3

*n = number of samples. Signal intensity $I = A.\exp(-t/T_2^A) + B.\exp(-t/T_2^B)$. Theoretically A=60 and B=40.

membrane cytoskeleton of reticulocytes and discocytes [19] could explain why the ^{23}Na and ^{7}Li T_2 values are not significantly altered by osmotic pressure-induced changes in erythrocyte shape and intracellular viscosity. If one assumes the erythrocyte membrane cytoskeleton to consist of hexagons with 180×10^{-7} cm long sides [19] and assumes an intracellular diffusion constant (D) for Na^+ and Li^+ to be approximately half the value in water [31], i.e., 5×10^{-6} cm^2sec^{-1}, then since $\sqrt{< x^2 >} = \sqrt{6Dt}$, $t \cong 1 \times 10^{-5}$ sec, which is $>> \omega^{-1}$. Therefore, diffusional constraints provided by the membrane cytoskeleton could give a τ_{c2} value in the long correlation time region.

The electric field gradients associated with slow diffusional motions act over a much larger distance than the radius of the hydration sphere and are much smaller; thus, $qcc_1 >> qcc_2$. The maximum qcc for the first hydration sphere around Li^+ in 7M LiCl has been determined to be 155 kHz; the maximum qcc for the remainder of the solution has been determined to be 15 kHz [41]. Therefore, as a first approximation: $qcc_2(Li^+) \cong 0.1\ qcc_1(Li^+)$. By comparison, the quadrupole coupling constant qcc_2 for Na^+ in muscle tissue has been estimated to be between 5.3 and 169 kHz [11]. If τ_{c2} is too long, the line shape will not be Lorentzian and equations (1b) and (3) will not be valid. An upper limit for $\tau_{c2}(Li^+)$ can be estimated from the requirement that [11]:

$$(qcc_2)\tau_{c2} \leq 0.1 \qquad (4)$$

Using equation (4) and a value of 3.5 kHz for $qcc_2(Li^+)$ gives a maximum value of 3×10^{-5} sec for $\tau_{c2}(Li^+)$.

An estimate for the value of $\tau_{c2}(Li^+)$ can be derived from the experimental effective T_1 and T_2 values. If $qcc_1 >> qcc_2$ and $\tau_{c1} << \omega^{-1} << \tau_{c2}$, it can be shown, using equations (1) and (3), that:

$$T_1/T_2 \cong 1 + 0.3\ \frac{(qcc_2)^2\tau_{c2}}{(qcc_1)^2\tau_{c1}} \qquad (5)$$

From equations (2) it is found, for a T_1 value of 5 sec, that $\tau_{c1} \cong 4 \times 10^{-11}$ sec. Taking T_2 = 100 msec and using an approximation that $qcc_2(Li^+) \cong 0.1\ qcc_1(Li^+)$, it is found that $\tau_{c2}(Li^+) \cong 6.5 \times 10^{-7}$ sec, much shorter than the upper limit of 3×10^{-5} sec but longer than 2×10^{-9} sec (ω^{-1}). This τ_{c2} value for intracellular Li^+ falls within the range of τ_{c2} values for intracellular Na^+ in muscle tissue given in reference [11] in which $10^{-8} < \tau_{c2} < 10^{-5}$ sec. It appears that $\tau_{c2}(Li^+)$ is of the same order of magnitude as $\tau_{c2}(Na^+)$, suggesting that significant differences in dynamic behavior do not exist between Li^+ and Na^+ inside normal human erythrocytes.

The results and analysis given above for Li^+ and Na^+ in erythrocytes strongly suggest that these ions are essentially free inside the cell. The addition of an electrostatic influence by fixed charges on intracellular macromolecules may affect the quadrupolar NMR line shape, but this does not correlate with a restriction of mobility. The T_1 relaxation times indicate that the dynamic behavior of the ions is mainly determined by intracellular viscosity, thus confirming that they are essentially free.

With regard to the intracellular state of potassium, NMR studies of intracellular potassium are not routinely done yet. The main reason for this is the low sensitivity of the ^{39}K nucleus. The BIONMR receptivity (product of NMR receptivity and ^{39}K concentration in the human body) has been reported to be 0.23×10^{-4} as compared to $4.08 \times 10^{-}$for ^{23}Na [39]. With improvements in equipment and instrumental techniques, however, NMR studies of intracellular potassium will become more common.

The NMR results obtained so far for intracellular K^+ indicate that the dynamic behavior of K^+ is similar to that of Na^+. In Saccharomyces cerevisiae and E. coli, Na^+ and K^+ were both 40-45% visible by NMR [9, 30, 35], consistent with the existence of a low frequency motion for both ions. In erythrocytes both Na^+ and K^+ are 100% NMR visible [4, 29, 33]. A ^{39}K-NMR T_1 of 18.3 ± 5 msec has been reported for human erythrocytes at 25°C. The ^{39}K-NMR T_2 could be decomposed into two components with intercepts of 0.61 ± 0.13 and 0.39 ± 0.13, with relaxation times of 5.0 ± 4.3 msec and 22.5 ± 5.5 msec, respectively [7].

^{23}Na and ^{39}K in free aqueous solution have identical T_1 and T_2 values of 59 msec at 25°C [7]. The intracellular ^{39}K-NMR T_1 and T_2 values for erythrocytes are almost identical to those reported in Table 2 for intracellular Na^+, suggesting that in human erythrocytes Na^+ and K^+ have identical dynamic behavior. Effective ^{39}K-NMR T_1 and T_2 values of 3 msec have been reported for intracellular potassium of perfused rat salivary gland [37]. No ^{23}Na-NMR T_1 and T_2 values are available for comparison. The authors concluded from the short relaxation times that some interaction between the potassium ions and intracellular macromolecules might be present. For perfused rat hearts the T_1 for intracellular potassium was found to be 11.8 ± 0.6 msec, while the T_2 could be decomposed in two components with intercepts 33 ± 8% and 67 ± 4% with relaxation times T_2 of 1.3 ± 0.6 msec and 10.1 ± 1.9 msec, respectively [8]. Because the intercept ratio

of 33:67 deviated strongly from the 60:40 ratio expected for a single pool of potassium, it was concluded by these authors that within the cardiac cell at least two distinct pools of potassium are present.

The results obtained so far for intracellular K^+ do not suggest that important differences in intracellular behavior between Na^+ and K^+ exist. Undoubtedly more ^{39}K-NMR studies will be undertaken in the future which will increase our understanding of the state of intracellular potassium.

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Discussion with Reviewers

H.J. Vogel: The Dysprosium tripolyphosphate complex carries a high negative charge and hence a large number of cations are added along, which function as counterions. What was the counterion used and was this amount taken into consideration by the calculation of the ionic strength?

Author: The counterion used was Na^+. The osmolality of the erythrocyte suspension was adjusted as described under methods after addition of the shift reagent, so that the final value was not affected. Shift reagent and counterions form an integral part of the medium determining the ionic strengths.

H.J. Vogel: With the relativity long T_1 values for ^{7}Li, it would appear that paramagnetic relaxation or other relaxation mechanisms could contribute to the overall relaxation, which would normally be dominated by the quadrupole moment in the case of, for example, $^{23}Na^+$. Could these explain, for example, the differences between the biexponential and monoexponential T_2 curves for Na^+ and Li^+? How were alternative relaxation mechanisms excluded for Li^+?

Author: It has been well established that the only two contributions to the spin-lattice relaxation of $^{7}Li^+$ in aqueous solution are nuclear quadrupolar relaxation (67%) and dipolar relaxation between $^{7}Li^+$ and surrounding water molecules (33%) (see reference [41] and references therein). Both contributions peak at the same temperature and are caused by the reorientational motions of the water molecules with a τ_c in the fast motional region. The spin-spin relaxation of $^{7}Li^+$ in erythrocytes is dominated by quadrupolar coupling with a τ_c in the slow motional region, which I ascribe to transient interactions with macromolecular charges (see discussion with reviewer W. Negendank). During these transient interactions the lithium ions presumably retain their hydration water molecules, which remain in fast exchange with bulk water. Therefore the dipolar $^{7}Li^+$-H_2O contribution to the spin-spin relaxation is negligible. Formally I should include the dipolar contribution to the T_1 in estimating the slow τ_c, but this is not essential to obtain an order of magnitude, which is the best we can hope for anyway.

H.J. Vogel: A major problem in determining intracellular concentrations of cations by NMR is the exact measurement of the intra- and extracellular volumes. Although concentrations are not discussed in detail in this review, it would be useful for the sake of completeness to mention the best methods to calculate these from the NMR data.

Author: Intracellular ion concentrations cannot be calculated from NMR data without an independent measurement of intra- and extracellular volume in the cell suspension. Furthermore, it is necessary to establish what fraction of the intracellular volume is accessible to the ion. The problem has been dealt with for the hypophosphite anion ($H_2PO_2^-$) in an erythrocyte suspension (Kirk K, Kuchel PW, Labotka RJ (1988), Biophys. J. 54: 241-247). These authors showed that the ratio of concentrations $r = [H_2PO_2^-]_i / [H_2PO_2^-]_o$ is given by

$$r = (I_i / I_o) ((1 - H_t) / \alpha H_t),$$

where I_i and I_o are the NMR intensities corresponding with intra and extracellular ions respectively, H_t is the hematocrit, and α is the cell fraction accessible to the ion. It was assumed that all of the intracellular water acts as solvent for the intracellular hypophosphite population. α was given the gravimetrically determined value of the fractional water volume of the cell. So even for "simple" cells like erythrocytes assumptions have to be made about accessibility, in order to calculate intracellular ionic concentrations.

J.A. den Hollander: Your results seem to suggest that the intracellular behavior of Li^+ is very similar to that of Na^+. Does Li^+ also enter the erythrocyte by the same mechanism as Na^+ does? How do ouabain and nystatin influence Li^+ movement across the membrane?

Author: A major conclusion of these experiments is indeed that Li^+ and Na^+ behave similarly. No evidence for strong binding of Li^+ was found and the T_1 values for $^{23}Na^+$ and $^{7}Li^+$ indicate that both ions are essentially free and mainly influenced by the intracellular viscosity. Li^+ transport in erythrocytes takes place by the same four pathways used for Na^+ transport: 1. (Na^+, K^+)-ATPase; 2. chloride-dependent Na^+-K^+ cotransport system; 3. Na^+-Na^+ exchange system; and 4. Passive leakage. In addition, Li^+ can also be transported in the form of $LiCO_3^-$ by the HCO_3^--Cl^- anion exchange system of erythrocytes (Wiley JS, et al. (1984). Hypertension 6: 360-368). I have no information available concerning the effects of ouabain and nystatin on Li^+ transport, but as Li^+ and Na^+ share the same transport pathways, these effects may be very similar to the effects of Na^+ transport.

J.A. den Hollander: ^{39}K-NMR is hard to do. Have you considered using thallium as a substitute for potassium?

Authors: ^{39}K-NMR is indeed hard to do as you probably know better than I do. However, it is not impossible. An acceptable spectrum for intracellular K^+ in erythrocytes was obtained in 20 minutes [4], using standard NMR equipment. A factor in favor of ^{39}K-NMR is the relatively high intracellular K^+ concentration as compared to Na^+ (150 mM versus 20 mM in erythrocytes). The NMR receptivity for ^{39}K is given as 0.48×10^{-1} [39], while for ^{23}Na the receptivity is 9.27. ^{1}H has the value 100.0. However, the seven-fold higher intracellular K^+ concentration produces a roughly fifty-fold reduction in scanning time (using equal T_1 relaxation times) to obtain the same signal to noise ratio possible at equal concentrations of Na^+ and K^+. Therefore, a "modified" NMR-receptivity for intracellular $^{39}K^+$ of $50 \times 0.48 \times 10^{-1} \cong 2.5$ can be postulated as compared to 9.27 for $^{23}Na^+$. In whole tissue the story is different, of course, as $^{23}Na^+$ and $^{39}K^+$ concentrations are roughly equal [39].

Thallium has been used as a substitute for potassium in several NMR studies of enzyme activation by K^+. Pyruvate kinase, for example, is 60% activated by Tl^+ as compared to 100% by K^+ (Villafranca JJ, Raushel FM (1980). Federation Proc. 41: 2961-2965). However, other authors have found that the "potassium-thallium-analogy" may be misleading, as Tl^+ and K^+ follow different transport routes for entering Ehrlich mouse ascites tumor cells. Also, Tl^+ was found to be present at a 30% higher concentration than K^+ (Sessler MJ, Geck P, Maul FD, Hor G, Munz DL (1986). Nucl. Med. 25: 24-27). Because of these differences, due caution should be exercised in comparing intracellular Tl^+ with K^+. I would prefer to improve the technique and study intracellular K^+ directly by ^{39}K-NMR.

W. Negendank: There is, in my opinion, a rather strong model dependence of the interpretation of the results of quadrupolar NMR relaxation observations in systems as complex as cells and tissues. This problem is manifested by the fact that in spite of the very high quality of Dr. Post's experiments, the value of τ_{c2} obtained by fitting the data has an uncertainty that spans 3 orders of magnitude (10^{-7}-10^{-5} sec). Thus, although the data are compatible with interpretation within the context of a single fraction of freely dissolved ion able to diffuse between regions of different electrical field gradients and quadrupolar coupling constants, I believe that they are also compatible with any number of two- (or multi) site models in one site of which the ion is spending a sufficient amount of time to be considered bound by physical adsorption (e.g., with a lifetime in the bound site on the order of 10^{-5} - 10^{-3} sec, which is the range of values derived by a jump diffusion model from isotope kinetic studies in cells (Negendank W, this issue)). In even a simple two-site model, the observed T_2 decay is a complex function of not only the correlation times and the quadrupolar coupling constants, but of the relative lifetimes in the two sites (i.e., the rate of exchange between them) and the relative fractions in the two sites. Moreover, in such systems, variations in the quadrupolar coupling constant or the correlation time over a short range of one order of magnitude can have marked effects on the rates and fractions of exponential decays. This may be realized by reference to the equations derived by T. Bull ((1972) J. Mag. Reson. 8: 344). The correlation time within the bound site may be shorter than the lifetime of the ion in the site because of motions within the site (Marshall AJ (1970). J. Chem. Phys. 52: 2527; Bull T (1978) J. Mag. Reson. 31: 453), and detailed NMR studies of ^{23}Na in polyelectrolyte solutions, which are much less complex than cells, require the assumption of physical absorption, with correlation times in the bound site in the same range as τ_{c2} in cells (10^{-9} - 10^{-6} sec) (see e.g., Gustavsson H, et al. (1978). J. Am. Chem. Soc. 100: 4655; Levij M, et al. (1982). Chem. Phys. Lett. 87: 34). In my opinion, the only way to overcome the model dependence of the interpretation of quadrupolar NMR relaxation in cells and tissues is to determine whether or not there is site-specific physical absorption of ions, and what are the lifetimes and fractions in the sites, by means other than NMR.

Author: The debate on the interpretation of NMR results on intracellular monovalent cations has been going on for about 20 years now, and it will probably continue for many years to come. I agree with the reviewer that most interpretations of quadrupolar relaxation have been strongly model dependent and that techniques other than NMR should be employed to gather independent information about intracellular cation behavior. The strong point of NMR, however, is that it gives you dynamic information rather than a time averaged picture and that it can be used to directly measure the nuclei of interest in vivo without having to resort to the use of probe molecules or destructive analysis of the sample. It is hard to envisage what other technique shares these advantages.

As an experimental scientist I prefer to look at the data first and see what they are trying to tell me, rather than starting with a model to interpret them. The observation of a single T_1 and a double exponential T_2 curve leads, by using the relaxation theory for spin 3/2 quadrupolar nuclei, to the necessity of two correlation times, one in the fast motional region ($\tau << \omega^{-1}$) and the other in the slow motional region ($T_2 > \tau > \omega^{-1}$). This approach was employed for tissue [2, 11], but was used recently also for interpretation of quadrupolar relaxation of sodium ions in DNA solutions [40]. The result of correlation times in two very different frequency

domains is beautiful in its simplicity and offers the advantage that no a priori assumptions about the character of the motions have to be made. The model of fast exchange, elaborated by Bull, involves too many parameters to be of practical value. This is the case even for a relatively simple system such as a charged polymer in solution.

Having accepted two correlation times the character of the fast and slow motions is open for interpretation. The fast correlation time is most likely reflecting the rotational motions of the hydration water molecules surrounding the ions. The correlation time for this process is ~ 10^{-11} sec and therefore falls in the fast motional region for quadrupolar relaxation. This is confirmed by the viscosity dependent T_1 results obtained with erythrocytes. Changing the intracellular viscosity, and therefore the τ_c of the water molecules, affects only T_1 and not T_2. T_2 is mainly determined by the $J(0)$ spectral density contribution, involving a slow motion. The slow motion can be interpreted in two ways: diffusion of the ions through anisotropic regions, or binding of a small fraction of the ions at specific sites for a time τ_c at least comparable to ω_o^{-1} [17]. The distinction between these two models may be rather artificial as it is known that ions retain their hydration water and rotational mobility during site specific adsorption, so that the binding in this case can only be considered to be of a very transient nature. Diffusion through anisotropic regions may be, then, just another way of describing the same process. Because the NMR results indicate the existence of only one pool of ions, I prefer to use the model of diffusion through electrostatically heterogenous regions inside the cell, but I admit that this point is open for further discussion.

The fact that we can only give an order of magnitude for τ_{c2}, between 10^{-5} and 10^{-8} sec, does not limit the value of this result in my opinion. What could be gained from being able to pin down τ_{c2} accurately? We would still be saddled with the problem of having to interpret the slow motion. Knowledge of τ_{c2} would not increase our understanding of the cell interior. Putting more and more effort in determining τ_{c2} would be like applying the law of diminishing returns, applied in economic theory.