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NMR RELAXATION STUDIES OF INTRACELLULAR Na^+ IN RED BLOOD CELLS

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The state of intracellular Na^+ in human and dog erythrocytes was characterized by ^{23}Na-NMR using dysprosium complexes as shift reagents. Intracellular Na^+ concentrations were determined using integration of the inner Na^+ NMR signals and measurements of the intracellular volume using ^{59}Co-NMR of extracellular $Co(CN)_6^{3-}$. T_2 was found to be significantly shorter than T_1, indicating some binding to macromolecules. While the longitudinal magnetization decay follows a single exponential, the transverse magnetization could be fitted with a double-exponential function. It was shown that neither the binding to the inner side of the membrane nor binding to hemoglobin contributes to the relaxation enhancement.

Key words: ^{23}Na-NMR; ^{59}Co-NMR; Relaxation time; Shift reagent; Intracellular Na^+; Red blood cell

1. Introduction

The state of the alkali metal ions inside living cells has been a subject of intense research for many years [1,2]. Unfortunately, the resonances of intra- and extracellular $^{23}Na^+$ in cell suspensions occur at the same frequency in the NMR spectrum due to the relative insensitivity of the ^{23}Na chemical shift to the environment. Recently, it has been found [3–5] that a few anionic complexes of Dy^{3+} cause considerable shifts of the NMR signals of alkali metal ions. Pike et al. [6] have demonstrated the use of these complexes in a system of vesicles where they obtained a good separation between the Na_{in} and Na_{out} signals. Gupta and Gupta [7] have introduced dysprosium(III) (tripolyphosphate)$_2^{7-}$ ($Dy(TPP)_2^{7-}$) as a shift reagent. This compound is the most efficient shift reagent known so far. The Na^+ content of human red blood cells was monitored by using this shift reagent [7] and was found to be in good agreement with the known values.

The existence of an Na^+/K^+ concentration gradient across the cell membrane is a well known fact. While human erythrocytes contain relatively low concentrations of Na^+, cat and dog erythrocytes have a high Na^+ content [8]. NMR studies of Na^+ in red blood cells by Yeh et al. [9] revealed that there is no tightly bound NMR-invisible intracellular Na^+.

In the present work we have used dysprosium complexes with the EDTA or with TPP to monitor the intracellular Na^+ concentration and relaxation times in dog (high-Na^+) and human (high-K^+) erythrocytes.

2. Experimental

2.1. Preparation of samples

Blood was drawn from dogs and humans into tubes containing EDTA in a final concentration of 1 mg/1 ml blood. Shortly before the NMR measurement, the plasma and buffy coat were aspirated and the cells washed three times with an isotonic solution of shift reagent containing 10 mM glucose

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and 25% ^2H_2O (pH 7.4). In most experiments, the solution also contained 2 mM $K_3Co(CN)_6$, and the volume of the cells in each sample was determined by ^{59}Co-NMR. In order to avoid sedimentation of the cells during measurements, each sample was mixed before placing it in the NMR probe and measurements were performed within a few minutes.

2.2. Preparation of hemolyzates

Red blood cell hemolyzates were prepared by two procedures. (i) Freeze-thawing [9]: cells were washed three times in an isotonic saline solution containing 10 mM NaCl, 136 mM KCl, 10 mM glucose, 10 mM Hepes, 1 mM Na_2HPO_4. After two cycles of freezing in liquid N_2 and thawing (38°C), the cells were centrifuged at 20000 rpm in a Sorvall centrifuge head no. SS34 for 1 h. (ii) Osmotic shock: cells were washed as in the procedure above and then diluted in 4 vols. distilled water. After 30 min incubation at room temperature the sample was centrifuged as above.

2.3. Preparation of hemoglobin

Hemoglobin was purified from human red blood cells using CM-cellulose chromatography by the method of Scholler et al. [10]. The concentration of hemoglobin was determined using $\epsilon_{415} = 1.25 \times 10^4$ M^{-1} cm^{-1} [11].

2.4. NMR spectra

Spectra were obtained on Bruker AM 360-WB, WH-300 and WH-90 NMR spectrometers. The field was locked using the deuterium signal. The standard high-range multinuclear probe head was used for both ^{23}Na and ^{59}Co measurements with 10-mm sample tubes. T_1 and T_2 relaxation times were measured using the standard inversion recovery and the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequences, respectively.

3. Results

Typical ^{23}Na-NMR spectra obtained from a suspension of dog and human erythrocytes in solutions of Dy-EDTA and Dy-TPP shift reagents are shown in figs. 1 and 2, respectively. The intracellular Na^+ peak (assigned an arbitrary zero frequency) has about the same position as in aqueous NaCl solution. The extracellular Na^+ resonance which is shifted to low field in the presence of Dy-EDTA is shifted to high field in the presence of Dy-TPP. The high Na^+ content of dog erythrocytes [9] and the low Na^+ content of human erythrocytes [12] are clearly visible from the figures.

A summary of the NMR parameters for the inner and outer ions in suspensions of dog and human erythrocytes is given in tables 1 and 2. It is seen that the chemical shift difference between the inner and outer signals $\Delta\delta$ as well as the linewidth of the inner signal $\Delta\nu_{1/2}^{in}$ and outer signal $\Delta\nu_{1/2}^{out}$ increase with the concentration of the shift reagent. The increase in linewidths is most probably caused by the increase in magnetic susceptibility difference between the cells and bulk solution. A discussion of this effect was recently given by Fabry and San George [13]. The susceptibility

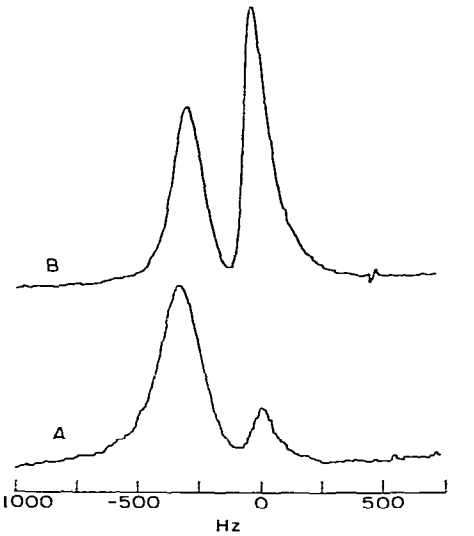

Fig. 1. ^{23}Na-NMR spectra of suspension of human (A) and dog (B) erythrocytes in a solution of 0.02 M $DyCl_3$, 0.056 M Na_2H_2EDTA, 0.028 M NaCl, 0.01 M glucose, 0.002 M $K_3Co(CN)_6$ (pH 7.5).

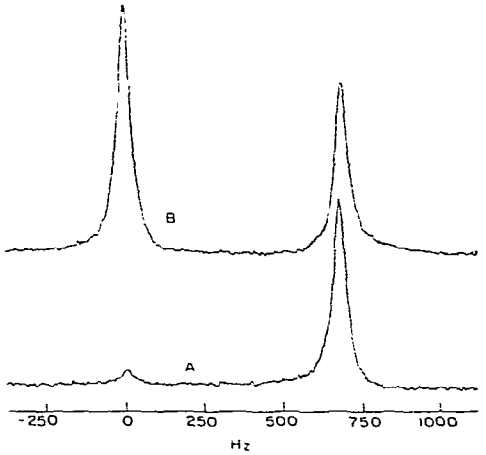

Fig. 2. ^{23}Na-NMR spectra of suspension of human (A) and dog (B) erythrocytes in a solution of 0.004 M $DyCl_3$, 0.01 M Na_5TPP, 0.162 M NaCl, 0.01 M glucose, 0.002 M $K_3Co(CN)_6$ (pH 7.5).

difference should not affect the linewidth of nuclei inside the spherical cells [14], thus the fact that the signal of the inner Na^+ is also broadened is explained by the concave shape of the erythrocytes. As the broadening of the intracellular Na^+ was found to increase with the concentration of cells in the suspension, we conclude that a significant contribution to the linewidth originates from field inhomogeneities caused by the neighboring cells.

In order to determine the molarity of the internal Na^+ from the integral of the Na_{in} peak, one needs to know the volume of the intracellular fluid. The volume occupied by the cells is traditionally determined by the use of macromolecules such as inulin or serum albumin labeled with radioactive isotopes. In the present work we calibrated the volume of the cells by ^{59}Co-NMR of $Co(CN)_6^{3-}$ which was performed on the same sample tube in the same probe as for the ^{23}Na-NMR experiment (Shinar, H. and Navon, G., unpublished data). The method consists of adding to the solution used for suspending and washing the cells a certain nonpenetrating inert cobalt(III)-containing complex. The NMR integral (I^{Co}) of the ^{59}Co signal in the cell suspension is compared with that of the original washing solution (I_o^{Co}). In this way the free volume not occupied by the cells is determined. Denoting the integrals of intracellular ^{23}Na signal as I^{Na} and that of a standard solution containing a known sodium concentration, $[Na_o]$, as I_o^{Na} the intracellular concentration of Na^+ is given by:

$$[Na]_{in} = \frac{I^{Na}[Na]_o}{I_o^{Na}(1 - I^{Co}/I_o^{Co})} \quad (1)$$

Table 1

^{23}Na shifts and T_1 relaxation times in suspensions of canine and human red blood cells in the presence of Dy-EDTA shift reagent

Conditions: $\nu_0 = 79.38$ MHz; temperature $21 \pm 3°$C. Ratio of EDTA to Dy(III) was 2.6:1. Solutions also contained 10 mM glucose, 2 mM $K_3Co(CN)_6$ and NaCl which was added to maintain the osmolarity at 0.3 (pH 7.5).

Blood sample	$[Dy^{(III)}]$ (mM)	$\Delta\delta$ (Hz)	$\Delta\nu_{1/2}^{out}$ (Hz)	$\Delta\nu_{1/2}^{in}$ (Hz)	$[Na^{in}]$ (mM)	T_1^{out} (ms)	T_1^{in} (ms)
Dog A	0			39			21 6
Dog B	9	165	109	61			
Dob B	15	221	147	68		28	21.5
Dog C	21	263	157	117	86	26.5	21.5
Dog B	23	302	223	105		29	21.8
$DyCl_3$	15					29	
$DyCl_3$	21					26	
Human A	15	207	138	74	7.1	31.5	25.5
Human A	21	302	197	110	7.6		
Human B	10 [a,c]	156	122	62	24	32.5	26.5
Human C	10 [b,c]	153	106	82	33	31.5	25.5

[a] Kept refrigerated for 48 h.

[b] Kept refrigerated for 1 week.

[c] Cells were washed only once with the shift reagent solution and therefore the Dy(III) concentration is not accurately known.

Table 2

^{23}Na shifts and relaxation times in suspensions of canine and human red blood cells in the presence of Dy-TPP shift reagent

Conditions: $\nu_0 = 79.38$ MHz; temperature: $21 \pm 3°C$. Ratio of TPP to Dy(III), 2.5: 1. Solution also contained 10 mM glucose, 2 mM $K_3Co(CN)_6$ and NaCl which was added to maintain the osmolarity at 0.3 (pH 7.5).

	$[Dy^{(III)}]$ (mM)	$T_{1,in}$ (ms)	$T_{1,out}$ (ms)	$T_{2,in}$ (ms)	$T_{2,out}$ (ms)	$\Delta\nu_{1/2}^{in}$ (Hz)	$\Delta\nu_{1/2}^{out}$ (Hz)	$\Delta\delta$ (Hz)
Human	5	24.5	19.5	12.5	11	45	60	785
Human	7.5	24	14.5	12.6	6	45	80	1250
Dog	5	22.7	18.4	12.0	9.7	57.5	70	850
Dog	7.5	23	14	11.7	7.5	55	77.2	1295
$DyCl_3$	5		20.5		17			
$DyCl_3$	7.5		14		12			
NaCl			55		55		22	

A suitable complex found by us is $Co(CN)_6^{3-}$ which is relatively inert and gives a single sharp signal. The use of ^{59}Co-NMR has several advantages, among which are its very high sensitivity (28% of that of protons), the fact that its resonance frequency is close to that of ^{23}Na and therefore liable for tuning in the same probe, and finally, that unlike ^{13}C or ^{31}P, there are no intracellular ^{59}Co signals which may interfere with the measurements.

In estimating the free volume in the cell suspension from the ^{23}Na signal intensity of the outer solution [7] one assumes that the outer Na^+ concentration does not change during the experiment. This assumption is not always valid, since the Na^+ concentration gradient across the membrane is maintained by an active process which is sensitive to the experimental conditions.

Some examples of the calculated intracellular Na^+ concentrations in dog and human erythrocytes, based on the volume calibration of the ^{59}Co signal of 2 mM $Co(CN)_6^{3-}$ added to the washing solutions, are given in table 1. It is seen that the concentration of Na^+ in human erythrocytes increases dramatically upon prolonged cold storage. In various samples where fresh blood was kept at room temperature for 3–4 h and then washed with the shift reagent and immediately measured, the intracellular Na^+ concentration was in the range 5–8 mM. These values fall within the normal range given in the literature [12]. The range of intracellular Na^+ obtained for canine blood was 86–144 mM, again within known values [8].

Values of the longitudinal and transverse relaxation times of intracellular and extracellular Na^+ are listed in tables 1 and 2. It is observed that, unlike T_1 and T_2 of extracellular Na^+, the relaxation times of intracellular Na^+ do not vary considerably for different samples of the same species and are independent of the concentration and nature of the shift reagent. It is interesting to note that the intracellular relaxation times are practically independent of the intracellular Na^+ concentration. Since the increase of intracellular Na^+ is accompanied by a parallel decrease of K^+, the lack of change of relaxation time indicates binding of Na^+ and K^+ with the same affinity so that the fraction of bound Na^+ is independent of the Na^+/K^+ ratio. The values for T_1 ($\pm$S.D.) were 20.7 ± 2.4 ms ($n = 16$) and 23.0 ± 2.6 ms ($n = 15$) for canine and human erythrocytes, respectively, at $21 \pm 3°C$. Similarly, the T_2 values were found to be 11.3 ± 1.3 ms ($n = 5$) and 11.6 ± 1.0 ms ($n = 4$) for these two species, respectively. These values should be compared with the value of $T_1 = T_2 = 55$ ms obtained for 0.15 M NaCl under the same conditions. Thus, both T_1 and T_2 values of Na^+ inside erythrocytes are significantly shorter than in comparable aqueous salt solutions.

The longitudinal and transverse relaxation of ^{23}Na nuclei are described by sums of two exponentials. The general expression for the decay of the longitudinal magnetization is given by [15]:

$$M_z(t) - M_{z0} = (M_z(0) - M_{z0})\{0.2 \exp(-t/T_1^f) + 0.8 \exp(-t/T_1^s)\} \quad (2)$$

and that for the transverse magnetization by:

$$M_x(t) = M_x(0)\{0.6\exp(-t/T_2^f) + 0.4\exp(-t/T_2^s)\} \quad (3)$$

In the case of an exchange between bulk Na^+ and small fractions (P_i) of bound Na^+ with relaxation times T_{Bi} and exchange lifetimes τ_{exi}, the decay times are given by [16]:

$$\frac{1}{T_1^f} = \frac{1}{T_1(0)} + \sum_i \left(\frac{P_i}{T_{Bi} + \tau_{exi}} \right) \quad (4)$$

(and similar expressions for T_1^s, T_2^f and T_2^s) where $T_1(0)$ is the relaxation time of Na^+ in the bulk solution. The relaxation rates of the bound Na^+ are:

$$\frac{1}{T_{B1}^f} = 0.4\pi^2\chi^2 J(\omega_0) \quad (5)$$

$$\frac{1}{T_{B1}^s} = 0.4\pi^2\chi^2 J(2\omega_0) \quad (6)$$

$$\frac{1}{T_{B2}^f} = 0.2\pi^2\chi^2 [J(0) + J(\omega_0)] \quad (7)$$

$$\frac{1}{T_{B2}^s} = 0.2\pi^2\chi^2 [J(\omega_0) + J(2\omega_0)] \quad (8)$$

$$J(\omega) = \frac{\tau_c}{1 + \omega^2\tau_c^2} \quad (9)$$

where $\chi = e^2qQ/h$ is the quadrupolar coupling constant (in Hz).

For the interpretation of the relaxation rates of intracellular Na^+ it is convenient to divide the various contributing factors into three groups: (a) effect of viscosity on the rotational correlation time; (b) binding to small molecules; (c) binding to macromolecules and membranes.

Within the frequency range used in the present work the condition $\omega_0\tau_c \ll 1$ is expected to hold for the first two groups but not for the third. Since the rotational correlation times of hydrated ions and their complexes with small molecules in aqueous solutions are in the range of $(2-6)\times 10^{-11}$ s, it is only when τ_c changes by a factor of 30–100 that $\omega_0\tau_c$ becomes equal to 1. The diffusion constants and mobility of small ions in muscle cells [17] as well as in erythrocytes [18] were found to be smaller by a factor of 2 compared to those in free solutions. Therefore, the condition $\omega_0\tau_c \ll 1$ is expected to hold for intracellular Na^+ which is either free or bound to small molecules.

In the case of the third group of possible contributions to the relaxation rates, i.e., binding of Na^+ to macromolecules or membranes, the longitudinal relaxation time is expected to be dependent on the frequency. Measurements of T_1 of intracellular Na^+ in the case of canine and human erythrocytes showed no such dependence at $\omega_0/2\pi = 95.26$, 79.38 and 23.81 MHz within experimental error. These results indicate that T_1 of intracellular Na^+ is not affected by binding to macromolecules but is determined by the viscosity of the intracellular medium or by binding to small molecules. However, if this were the only effect, one would expect T_1 to be equal to T_2. The fact that the measured T_2 of intracellular Na^+ is significantly shorter than T_1 clearly indicates an effect of Na^+ binding to macromolecules or membranes on T_2. The temperature dependencies of the longitudinal and transverse relaxation rates of intracellular Na^+ are given in fig. 3. The rates decrease at higher temperatures, a trend characteristic of the fast exchange limit. A quantitative treatment of the temperature dependence is not possible, since the degree of binding may also be temperature dependent. However, since T_2^{-1} does not show any tendency to decrease at low temperatures, a slow exchange limit may be safely excluded.

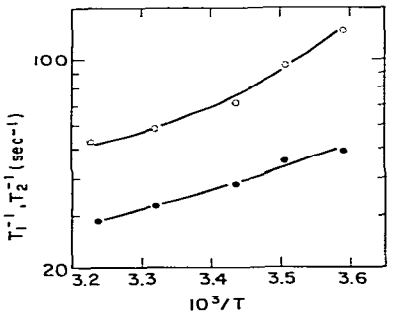

Fig. 3. Temperature dependence of T_1 and T_2 relaxation rates of intracellular Na^+ in dog erythrocytes. Cells were washed with a solution of 0.005 M $DyCl_3$ 0.0125 M Na_5TPP, 0.098 M Na^+, 0.01 M glucose (pH 7.5).

Examining eqs. 2–9 one can see that in cases where T_2 is significantly shorter than T_1 there are contributions from terms with $\omega\tau_c > 1$ and therefore the two components of T_2 should be significantly different and easily separable. It was noted previously [2] that T_1 is not easily separable because its two components may differ by less than a factor of 4 and the fast relaxing component accounts for only 20% of the total intensity. However, in our system, an alternative explanation is probably applicable. Since for the bound Na^+, $\omega\tau_c \gg 1$, both $J(\omega_0)$ and $J(2\omega_0)$ are very small and the effect of bound Na^+ on T_1 (eq. 4) is negligible, i.e., $T_1^f \simeq T_1^s \simeq T_1(0)$.

Experiments to analyze the transverse relaxation curve in terms of the two components (eq. 3) were performed with an AM360-WB spectrometer equipped with a pulse programmer which enabled us to measure T_2 in a CPMG experiment with short delay times. The results are given in table 3. In spite of the considerable error caused by the multiparameter best fitting (an example is given in fig. 4) of the decay curve, it is seen that T_2^s is rather similar to T_1 while T_2^f is significantly shorter. The results for the dog and human erythrocytes are similar within experimental error. The measured T_2 values given in tables 1 and 2 may be considered as weighted average values of the two components.

In order to determine whether the relaxation of intracellular Na^+ is caused by binding to the inner side of the membrane or to soluble macromolecules, we measured relaxation times of lysed red blood cells where the membrane fragments were removed. The results are given in table 4. Since the hemolyzates were diluted as compared to the cell contents in intact cells, we corrected for the dilution by multiplying the excess relaxation relative to free salt solutions (T_{1p}^{-1} and T_{2p}^{-1}) by the dilution factor. The dilution factor was determined using the optical absorbance of hemoglobin with the basic assumption that all the constituents of the red blood cell contents are diluted by the same factor. Thus the hemoglobin optical absorbance of the hemolyzate was compared with that of lysed red blood cells (taken from the same blood sample) whose internal volumes were determined by the NMR method described in the present work. The results shown in the last two columns of table 4 show that the corrected T_{1p}^{-1} and T_{2p}^{-1}, of two hemolyzate solutions prepared in two different ways, agree, within experimental error, with that of intact cells. This result clearly indicates that binding to the inner side of the membrane does not contribute to the relaxation enhancement of intracellular Na^+.

One of the first candidates for Na^+ binding inside red blood cells is hemoglobin, which is the

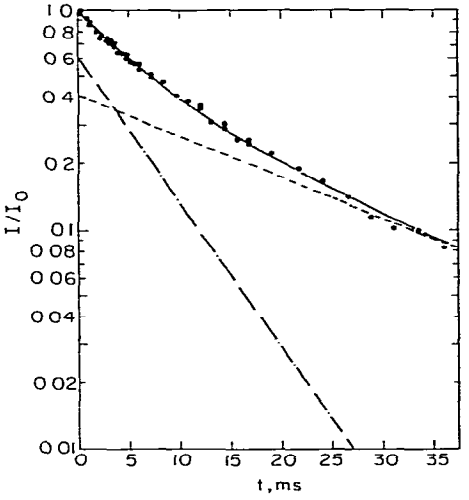

Fig. 4. Spin echo decay curve of intracellular ^{23}Na signal in human erythrocytes measured by CPMG sequence. The points are experimental. (———) Calculated curve, fast component $T_2^f = 6.6$ ms. (– · – · –), slow component $T_2^s = 23.6$ ms (- - - -).

Table 3

The fast and the slow components of the T_2 relaxation curve of ^{23}Na in red blood cells

Measurements were performed at 95.26 MHz, $t = 21 \pm 1°C$.

	T_2^f (ms)	T_2^s (ms)	T_1 (ms)
Human A	7.3	18.7	28.0
Human B	6.0	15.2	23.0
Human C	6.0	16.0	23.0
Dog A	5.9	14.3	20.0
Dog B	5.9	13.6	18.0

Table 4

Relaxation times in suspension of human red blood cells, hemolyzates and solution of hemoglobin

Measurements were made at 79.38 MHz.

Sample	Dilution	T_1 (ms)	T_2 (ms)	T_{1p}^{-1} (s^{-1})	T_{2p}^{-1} (s^{-1})	Dilution $\times T_{1p}^{-1}$	Dilution $\times T_{2p}^{-1}$
Inside cells	–	24.3	12.5	23.2	62		
Hemolyzate [a]	2.04	34.5	22.5	11.0	26.4	22.4	53.9
Hemolyzate [b]	1.52	30.5	16.0	14.8	44.5	22.5	67.6
Hemoglobin [c]	1.5	40	35	7.0	10.6	10.5	15.8

[a] Prepared by osmotic shock.

[b] Prepared by freeze-thawing.

[c] Concentration of hemoglobin was 3 mM (about 2/3 of that of intact cells). The ^{23}Na relaxation times were essentially independent of Na^+/K^+ ratio upon varying [NaCl] from 10 to 140 mM and [KCl] from 140 to 10 mM keeping their sum as 150 mM.

predominant intracellular protein. Previous experiments using ^{23}Na T_1 relaxation [19] did not give any indication of Na^+ binding to hemoglobin. As discussed above, T_2 is expected to be more sensitive to such binding. Thus, both T_1 and T_2 ^{23}Na relaxation times in solutions of purified human hemoglobin were measured by us and are listed in table 4. The small effect on T_1 and T_2 may be ascribed to the increased viscosity of the hemoglobin solution [19]. The corrected T_1 relaxation rate accounts for about half of the observed effect in either intact cells or in hemolyzates. The difference between T_{2p}^{-1} and T_{1p}^{-1} was ascribed by us to binding to macromolecules. In the case of hemoglobin this difference is about 5 s^{-1} and about 40 s^{-1} for cells and hemolyzates. Thus, the excess T_2 relaxation rate of intracellular Na^+ is caused by binding to macromolecules other than hemoglobin.

4. Discussion

The new possibility of observing separate signals from internal and external Na^+ enabled us to measure longitudinal and transverse relaxation times of intracellular Na^+ without any complications and unnecessary assumptions concerning Na^+ outside the cells. Furthermore, the new technique of calibration of the intracellular volume using ^{59}Co-NMR is a convenient and rapid way of determining the concentrations of intracellular Na^+.

We have found that both T_1 and T_2 of intracellular Na^+ are significantly shorter than those in aqueous salt solutions. However, T_1 was found to be an insensitive measure of Na^+ binding to macromolecules, as might be expected for systems where $\omega_0\tau_c \gg 1$. The frequency independence of T_1 of intracellular Na^+ indicates that the contributions to T_1 relaxation result from viscosity effects and from binding of Na^+ to small molecules. About half of the effect is brought about by the increased viscosity due to intracellular hemoglobin. The fact that T_2 is significantly shorter than T_1 reflects the binding of Na^+ either to macromolecules or membranes where $\omega_0\tau_c > 1$. This was confirmed by the separation of the transverse magnetization decay curve into two components. We could eliminate the possible contribution of binding to membranes by preparing membrane-free hemolyzates and making proper corrections for the dilution to the observed relaxation times. The specific macromolecules that cause the T_2 relaxation enhancement of intracellular Na^+ are still unknown. We have confirmed the statement made by Bull et al. [19] that Na^+ does not bind to hemoglobin by using the more sensitive T_2 measurements. Yeh et al. [9] report increased Na^+-NMR linewidth upon addition of hemoglobin. However, an examination of their procedure indicates that their measurements were performed on membrane-free hemolyzates and not on purified hemoglobin, as was done in the present work. No binding of Na^+ to hemoglobin was observed by electrochemical measurements of free

Na^+ using collodion membrane electrodes [20]. However, as discussed later, this method is relatively insensitive as compared with NMR. Rose and Bryant [21] also report that there is no binding of Na^+ to bovine carbonic anhydrase. Methods such as membrane electrode [20] and equilibrium dialysis [22] are not expected to be very sensitive to the direct binding of Na to proteins. For instance, 1% binding of Na^+ to the macromolecules is not likely to be detected by these methods. On the other hand, electrostatic attraction of Na^+ to highly charged macromolecules may not be detected by NMR if the attracted Na^+ retains its hydration shell and freedom of rotation.

Since we have no data on the nature of the macromolecules that bind Na^+ in red blood cells and their rotational correlation times, it is impossible to estimate the fraction of bound Na^+. Still, we would like to emphasize some facts that are relevant to such an estimation. Following our previous discussion in the cases where $\omega_0\tau_c \gg 1$ and thus $J(2\omega_0) \ll J(0)$, we obtain:

$$\frac{1}{T_2^f} - \frac{1}{T_2^s} = 0.2\pi^2 \sum_i P_i \chi_i^2 \tau_{ci} \qquad (10)$$

For the estimation of P_i the values of χ_i and the rotational correlation times τ_{ci} must be known. The fact that T_2 is significantly shorter than T_1 indicates that $\omega_0\tau_c$ is greater than unity for a resonance frequency of 23.81 MHz, thus τ_c must be greater than 7×10^{-9} s. As for the quadrupolar coupling constants for bound Na^+ in biological systems no experimental values are available. However, for complexes of Na^+ with ionophores and other small molecules few quadrupolar coupling constants were estimated [23]. The values of χ for ionophores are in the range 1.3–1.8 MHz [24], and for the channel ionophore gramicidin χ 1.7 MHz [25]. Although James and Noggle [26] report a value of $\chi = 4$ MHz for Na-HEDTA (hydroxyethylethylenediaminetriacetic acid), a recalculation using their experimental relaxation time and rotational correlation time obtained for Mn-HEDTA (Gradsztajn, S. and Navon, G., unpublished results), $\tau_c = 5.2 \times 10^{-11}$ s, yields a value of χ 1.7 MHz. Much smaller values of χ were estimated for systems containing polyelectrolytes such as polymethacrylic acid [27], mucopolysaccharides [28] and nucleic acids [26,28]. However, these values may be weighted averages of small fractions of truly immobilized ions and large fractions of condensed but freely rotating ions (see ref. 1 for a discussion). Since binding of Na^+ to both gramicidin and HEDTA involves the same functional groups as in proteins, we shall adopt the value of 1.7 MHz for our calculation, bearing in mind that this value may be considerably different. Inserting the lower limit of $\tau_c = 7 \times 10^{-9}$ s, $\chi = 1.7$ MHz and our experimental value $T_2^{f-1} - T_2^{s-1} \simeq 100\ s^{-1}$ in eq. 10 gives us a value of $P_B < 2.5 \times 10^{-3}$. Since our results indicate that the fraction of bound Na^+ does not depend on the intracellular Na^+/K^+ ratio, inserting the value of 120 mM for the total concentration of intracellular alkali metals gives us an upper limit of 0.3 mM for the concentration of metal ions bound to macromolecules. The small fraction of exchangeable bound Na^+ found in the present work, as well as the absence of invisible Na^+ reported by Yeh et al. [9], indicates that binding of Na^+ cannot control the concentration of intracellular Na^+. Apparently, the important factors are the relative efficiencies of various active and passive transport mechanisms. Urry et al. [29] report some variations of ^{23}Na T_1 values caused by unsealed erythrocyte ghosts obtained from normotensive and hypertensive patients. The differences of T_1 we obtain between intracellular Na^+ and hemolyzate (see table 4) are too small to indicate any effect of the cytoplasmic surface of the erythrocyte membrane on the T_1 of intracellular Na^+. Moreover, no significant effect was found for T_2 which is expected to be much more sensitive to binding to membranes.

In many tissues the intensity of the observed Na^+ signal amounts to about 40% of the total intracellular Na^+ (see ref. 1). This is probably due to the large fraction of Na^+ bound to macromolecules causing T_2^f to be too short and thus the broad component of the absorption is too broad to be observed. Red blood cells are unique amongst other cells in having no organelles such as nuclei and mitochondria. The low degree of Na^+ bound in red blood cells may be attributed to the absence of such organelles. This hypothesis is supported by

our preliminary findings that the ^{23}Na relaxation times in nucleated chicken red blood cells are considerably shorter than those in human or dog red blood cells which are reported in the present work.

Note added in proof

A measurement of ^{23}Na relaxation times in human erythrocytes was recently reported by Pettegrew et al. [30]. They did not resolve the T_2 decay into two exponentials. Their relaxation times were slightly longer than those reported in the present work.

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