

Sodium-23 NMR relaxation times in nucleated red blood cells and suspensions of nuclei

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ABSTRACT The relaxation behavior of intracellular ^{23}Na in suspensions of chicken erythrocytes and of their nuclei was investigated. The transverse magnetization was found to decay biexponentially. The average relaxation rates for the nucleated chicken erythrocytes are considerably shorter than the average relaxation rates obtained for dog and human nonnucleated red blood cells. Of particular significance is the twofold decrease in the short component of T_2 . Calculations based on the measured ^{23}Na NMR relaxation rates in suspensions of nuclei indicate that most of the difference between the relaxation rates in the mammalian as compared to the chicken erythrocytes, can be accounted for by the contribution of the nuclei in the latter.

INTRODUCTION

The development of NMR shift reagents for alkali metal ions (1–3) enabled investigation of the properties of intracellular sodium ions in cell suspensions and in intact tissues (4, 5). A study of the intracellular ^{23}Na relaxation in human and dog red blood cells (6) demonstrated that the ^{23}Na transverse magnetization in these cells decays biexponentially. The theory predicts that the short component of the decay, which accounts for 60% of the signal intensity, is sensitive to binding of Na^+ to macromolecules. This may be the reason for the partial invisibility of ^{23}Na NMR in tissues (7).

Human and dog erythrocytes are unnucleated, and therefore not representative of other biological tissues. Thus, we undertook to determine the various factors contributing to intracellular relaxation by studying the relaxation behavior of ^{23}Na in the nucleated chicken erythrocytes and in suspensions of these nuclei.

MATERIALS AND METHODS

NMR spectra

^{23}Na NMR spectra were recorded on a model AM 360-WB, NMR spectrometer, (Bruker Instruments, Karlsruhe, FRG) operating at a frequency of 95.26 MHz. A 10-mm multinuclear probehead was used for all measurements. T_1 and T_2 relaxation times were measured using the inversion recovery and the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequences, respectively. Experiments were performed with repetitive cycling through the delay times to eliminate possible errors due to slow time-dependent phenomena such as cell sedimentation. We found in all our measurements a background signal arising from Na^+ in the glass of the tube and the probe, which was especially pronounced when small concentrations of sodium were measured. This signal was eliminated by conducting one set of measurements on the test sample, and another on a solution of KCl with similar volume and osmolarity. The free induction decay (FID) of the KCl solution

was subtracted from the FID of the sample for each point of the relaxation times measurements.

Treatment of the data

Having a spin of 3/2, the transverse magnetization of free $^{23}\text{Na}^+$ ions exchanging with a slowly rotating bound state is expected to decay biexponentially (8, 9). To determine the slow and fast decay constants T_{2s} and T_{2f} , the curve obtained from the plot of the integrated intensity of the Fourier transform of the echoes in the CPMG sequence as a function of the delay time between the 90° pulse and the acquisition, t , was fitted to Eq. 1:

$$I(t) = I_0 [0.6 \exp(-t/T_{2f}) + 0.4 \exp(-t/T_{2s})]. \quad (1)$$

Thus, the relaxation times T_{2f} and T_{2s} were obtained by a nonlinear least mean square three-parameter computer fitting using the Levenberg and Marquardt method.

In general, for Lorentzian lines, the measurements of peak integrals are more sensitive than peak heights to baseline corrections and to low signal-to-noise ratios. Thus, it is sometimes advantageous to obtain the two components of the T_2 relaxation time from peak heights (h). The lineshape is a superposition of two Lorentzians whose integrated intensities I_1 and I_2 are related to their peak heights h_1 and h_2 and their widths at half heights Δ_1 and Δ_2 by:

$$I_1 = \frac{\pi}{2} h_1 \Delta_1 \quad \text{and} \quad I_2 = \frac{\pi}{2} h_2 \Delta_2. \quad (2)$$

Neglecting shifts between the two Lorentzians, the observed height is given by

$$h = h_1 + h_2 = \frac{2}{\pi} I_0 \left[\frac{0.6}{\Delta_1} \exp(-t/T_{2f}) + \frac{0.4}{\Delta_2} \exp(-t/T_{2s}) \right]. \quad (3a)$$

The widths at half the heights for the two Lorentzians are given by

$$\begin{aligned} \Delta_1 &= \frac{1}{\pi T_{2f}} + LB + \Delta v_{1/2}^{\text{inh}} \\ \Delta_2 &= \frac{1}{\pi T_{2s}} + LB + \Delta v_{1/2}^{\text{inh}}, \end{aligned} \quad (4)$$

where LB is the applied line broadening factor (in hertz) in the exponential multiplication, and $\Delta\nu_{1/2}^{inh}$ is the estimated contribution of field inhomogeneity to the linewidth. LB values were chosen to be $> \Delta\nu_{1/2}^{inh}$ so that inaccuracies in the estimation of $\Delta\nu_{1/2}^{inh}$ will not affect the results of the fitting procedure. Comparison of the two methods for the evaluation of T_{2s} and T_{2f} , i.e., integrals (Eq. 1) and peak heights (Eq. 3), was performed for spectra with good signal-to-noise ratio, and the agreement was good.

Preparation of nuclei from chicken red blood cells

Fresh heparinized blood drawn by vein puncture from large (~5 kg) male Leghorn roosters was centrifuged and the plasma and buffy coat removed. Cells were washed twice in a buffer containing 135 mM NaCl, 2 mM $MgCl_2$, 10 mM TRIS-HCl, pH 7.4, and then once in a buffer containing 125 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 10 mM TRIS-HCl, pH 7.4. The suspension was diluted about fivefold in the latter buffer, and cells were disrupted in the cell disrupter under 400–500 psi argon pressure (10). Here we have followed a procedure developed for oligodendrocytes. The nuclei were washed and centrifuged in the same buffer until the supernatant was clear. In cases where the nuclei were contaminated with red blood cells, the suspension was diluted and passed again through the cell disrupter. Nuclei were suspended in the same buffer in D_2O , to prevent their sedimentation during the NMR experiment.

Determination of DNA Concentration

Nuclei were washed twice with a buffer containing 135 mM NaCl, 2 mM $MgCl_2$, 10 mM TRIS-HCl, pH 7.5. They were then lysed by suspending them in a buffer containing 27 mM EDTA, 10 mM TRIS-HCl, 75 mM NaCl, pH 7.5, and agitated at 37°C for 30 min. 6 ml of 5 M NaCl were added to ~30 ml of this suspension, and further incubated at 37°C for 30 min. At the end of the incubation period, lithium lauryl sulfate was added to a final concentration of 1% (wt/vol), and proteinase K to a final concentration of 200 $\mu g/ml$. The suspension was left at 37°C overnight. In cases where not all the DNA dissolved, the solution was diluted twofold and stirred for a few hours.

DNA was determined from the optical absorption at 260 nm, after purification by phenol extractions and ethanol precipitation (11). The ratio between the absorption at 260 to that at 280 nm was 1.7–1.8 in all cases.

RESULTS

^{23}Na relaxation times in chicken erythrocytes

To separate the intra- and extracellular sodium signals, cells were suspended in a solution containing 4–6 mM $Dy(tripolyphosphate)_2^{7-}$ (3). Typical spectra obtained for a suspension of cells and for the background signal (see Materials and Methods) are shown in Fig. 1. The volume occupied by the cells was measured by ^{59}Co and 2H NMR (12) and used for the calculation of the intracellular Na^+ concentrations from the ^{23}Na signal intensities.

The concentration of Na^+ in chicken erythrocytes increased dramatically during prolonged cold storage.

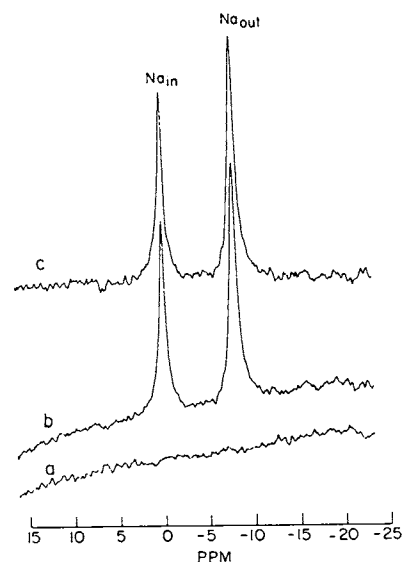


FIGURE 1 (a) ^{23}Na NMR background signal obtained for a solution of 150 mM KCl. (b) ^{23}Na NMR spectrum of a suspension of chicken red blood cells in a medium containing 5 mM $DyCl_3$, 12.5 mM $K_3P_3O_{10}$, 10 mM glucose, 2 mM $K_3Co(CN)_6$, 85 mM KCl, 18 mM NaCl, 2% D_2O , pH 7.4. The fraction of the volume occupied by the cells was 86% and the intracellular sodium concentration was 2.3 mM. (c) Difference spectrum b-a.

When brought back to room temperature, Na^+ is extruded slowly from the cells. We found that this phenomenon follows first-order kinetics with $t_{1/2}$ of ~4 h at 20°C (Fig. 2). The rate is independent of the amount of Na^+ in the suspending medium. The extrusion of the Na^+ ions is

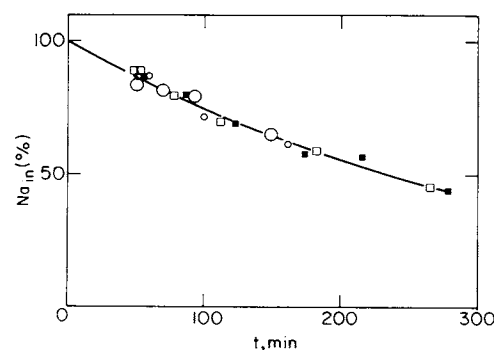


FIGURE 2 Extrusion of sodium ions from chicken red blood cells at 20°C. In order to increase intracellular Na^+ concentration, chicken whole blood was kept at 4°C for a week. Before the NMR measurement the erythrocytes were washed three times in the suspending medium which contained: 5 mM $DyCl_3$, 12.5 mM $K_3P_3O_{10}$, 10 mM glucose, 4 mM $K_3Co(CN)_6$, 2% D_2O , pH 7.4, and 100 mM of various proportions of NaCl and KCl: (■) 3 mM NaCl, 97 mM KCl; (□) 9 mM NaCl, 91 mM KCl; (○) 15 mM NaCl, 85 mM KCl; (○) 21 mM NaCl, 79 mM KCl, pH 7.4.

manifest by an increase in the extracellular ^{23}Na signal and a concomitant decrease in the intracellular one. The sum of the integrals of these two signals was constant throughout the experiment, indicating the same NMR visibility of the transported sodium in the two compartments. Similar results were reported for duck erythrocytes (13).

We examined the dependence of the relaxation times on the intracellular sodium concentrations by measuring the relaxation times of the same batches of cells, after cold storage, on the day of blood delivery and at later times. Results are given in Table 1. T_{2s} and T_{2f} were obtained by resolving the transverse magnetization decay curve to two exponentials, according to Eq. 1. The values of the transverse magnetization were obtained either from the integrated areas of the absorption signals, or from corrected peak heights as described in Materials and Methods. The FID of the background signal was subtracted for each delay time. The longitudinal magnetization was found to decay with a single decay constant.

The following conclusions can be drawn from these experiments: (a) The relaxation rates do not depend on the intracellular sodium concentration. This confirms

our previous findings on dog and human red blood cells (6). The relaxation rates are slightly increased by replacing the H_2O with D_2O as a solvent, which is expected based on the higher viscosity of D_2O . (b) The relaxation times of ^{23}Na in chicken erythrocytes are considerably shorter than those obtained for nonnucleated human and dog erythrocytes. Of particular significance is the twofold decrease in the short component of T_2 , the component which is sensitive to the binding to macromolecules (6).

Nuclei from chicken red blood cells

Because the major difference between mammalian and avian red blood cells is the presence of nuclei in the latter, we tested the possibility that the nuclei are the cause of the increased relaxation.

Experiments were conducted initially in solutions of ficoll-400 to prevent the nuclei from settling to the bottom of the NMR tube, due to their high density. However, control measurements indicated a large effect of the ficoll on the ^{23}Na relaxation times: addition of 18% (wt/vol) of the ficoll to saline caused ^{23}Na T_1 and T_2 to decrease from 54 and 54 ms to 18.5 and 10 ms, respectively, at room temperature. A satisfactory alternative was provided by substituting D_2O for the water in the solution used for suspending the nuclei. Apparently the resultant increase in solution density prevented sedimentation of the nuclei.

As in the case of intracellular ^{23}Na in the intact erythrocytes, the ^{23}Na NMR transverse magnetization decay curve in suspension of nuclei was clearly biexponential. An example of the least mean square fit of the decay curve is given in Fig. 3. We found that the relaxation rates of ^{23}Na in suspensions of nuclei are insensitive to the sodium-potassium ratio of the suspending medium, in agreement with our findings for intracellular sodium in chicken (see above) and mammalian erythrocytes (6). This finding was interpreted (6) as competition of Na^+ and K^+ for the same binding sites. To preclude any contribution from nonexchangeable Na^+ bound in the nuclei, the shift reagent $\text{Dy}(\text{TPP})_2^{7-}$ was added to the suspension of nuclei. The complete intensity of ^{23}Na signal was observed to be shifted by the shift reagent. The relaxation rates are not altered by a 1% Triton treatment of the cells which disrupts the membrane (14) (Table 2). This precludes any role of the nuclear membrane in the relaxation rate of intracellular ^{23}Na . Table 3 summarizes the results of T_1 , T_{2s} , and T_{2f} as a function of the amounts of nuclei, measured by the DNA concentration in the suspension. Fig. 4 presents the correlation between $1/T_{2f}$ and the amount of DNA. The same type of correlation was found for T_1 and T_{2s} .

It is interesting to compare the effect of nuclei on ^{23}Na

TABLE 1 Intracellular ^{23}Na relaxation times

	Na_{in}	T_1	T_{2s}	T_{2f}
	mM	ms	ms	ms
A. Chicken erythrocytes				
Blood A*				
Day 0	2.5	16.0	9.1	2.2
Day 6	8.0	15.9	12.0	4.1
Day 12	18.7	13.6	11.8	2.3
Blood B*				
Day 0	2.3	16.5	13.7	2.7
Day 6	11.5	16.4	12.2	3.0
Day 12	17.0	16.0	10.9	2.4
Average \pm SD* (n = 6)	2-19	15.8 ± 1.0	11.6 ± 1.0	2.8 ± 0.6
Average \pm max. dev.† (n = 2)	18-22	14.9 ± 0.4	12.5 ± 0.4	2.6 ± 0.3
B. Dog and human erythrocytes				
Average \pm SD* (n = 6)	5-100	21.5 ± 4.0	15.0 ± 2.2	6.1 ± 0.6
C. Human erythrocytes				
Average \pm max. dev.† (n = 2)	14-20	21.1 ± 0.1	14.8 ± 0.3	5.1 ± 0.4

*Suspended in an H_2O solution containing 5 mM DyCl_3 , 12.5 mM $\text{K}_2\text{P}_2\text{O}_{10}$, 4 mM $\text{K}_3\text{Co}(\text{CN})_6$, 90 mM KCl, 10 mM NaCl, pH 7.4;

†Suspended in the same solution as in (*) except that the H_2O was replaced by D_2O .

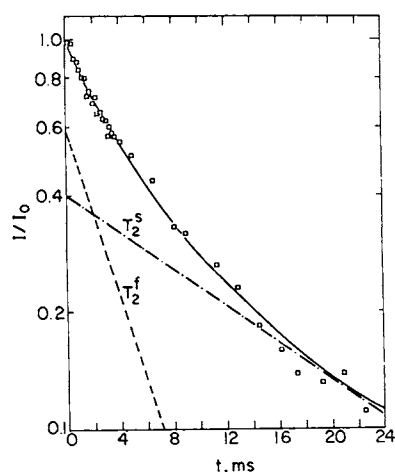


FIGURE 3 Spin echo decay curve of ^{23}Na signal in a suspension of nuclei from chicken red blood cells (32.8 mg/ml DNA). The points are experimental. The calculated curve (—) is resolved into $T_{2f} = 3.9$ ms (---), and $T_{2s} = 18.1$ ms (-.-.-).

relaxation in suspensions of nuclei to that of intracellular ^{23}Na in intact cells. Such a comparison can be made by assuming two contributions to the relaxation of intracellular ^{23}Na : (a) The interactions of ^{23}Na with the various components of the cytoplasm, which can be approximated by the intracellular ^{23}Na relaxation in cells such as human and dog erythrocytes. (b) The contribution to the relaxation originating from the nucleus, which can be calculated from our results for suspensions of nuclei.

Because our results are expressed in terms of DNA concentrations in the suspensions, we estimated the intracellular concentration of DNA in the intact erythrocytes using two measurements: (a) DNA content of the sample of cell suspension, and (b) the intracellular volume in this particular sample measured by ^{59}Co NMR (12). For different suspensions of red blood cells, we found that the red blood cells used in this investigation contained 20 ± 4 mg/ml DNA. This is in good agreement with the value of 16 mg/ml which can be calculated from

TABLE 2 ^{23}Na relaxation rates of suspension of nuclei before and after treatment with 1% triton

		T_1	T_{2s}	T_{2f}
		ms	ms	ms
Sample A	Before	26.5	24.7	3.8
	After	21.0	19.2	4.2
Sample B	Before	26.3	24.2	6.2
	After	25.5	21.3	7.0
Sample C	Before	21.2	24.2	4.1
	After	21.3	19.1	3.1

TABLE 3 ^{23}Na relaxation times in suspensions of nuclei from chicken erythrocytes*

DNA	T_1	T_{2s}	T_{2f}
mg/ml	ms	ms	ms
15.0	26.3	26.8	8.2
17.5	24.5	23.6	6.9
23.5	21.9	20.8	4.8
32.8	18.9	18.1	3.9
40	16.0	15.7	2.3
12.5	29.7	39.8	7.3
19.0	25.2	26.6	6.0
34.7	21.3	19.1	4.5

*Suspended in saline in D_2O .

the average value reported in the literature of 2.6×10^{-12} g DNA/RBC in chicken erythrocytes (15), and the approximate volume occupied by one cell, 1.66×10^{-10} cm^3 (16). By interpolation of the linear curve of $1/T_{2f}$ of ^{23}Na in a suspension of nuclei vs. DNA concentration, given in Fig. 4, we can estimate a value of 163 s^{-1} at DNA concentration of 20 mg/ml. When this contribution is added to the average $1/T_{2f}$, 196 s^{-1} , obtained for human erythrocytes suspended in D_2O , a value of 359 s^{-1} corresponding to $T_{2f} = 2.79$ is obtained. This value is in a good agreement with our average experimental value of $T_{2f} = 2.6$ ms obtained for intracellular sodium in chicken red blood cells in D_2O . One should note that in this calculation we neglected the fact that the higher intracellular viscosity may increase the contribution of the nuclei to $1/T_{2f}$. Thus, the value of 163 s^{-1} based on Fig. 4 may be somewhat low.

Treatment of the intact nuclei with small amounts of Triton X-100 was found to disrupt the nuclear mem-

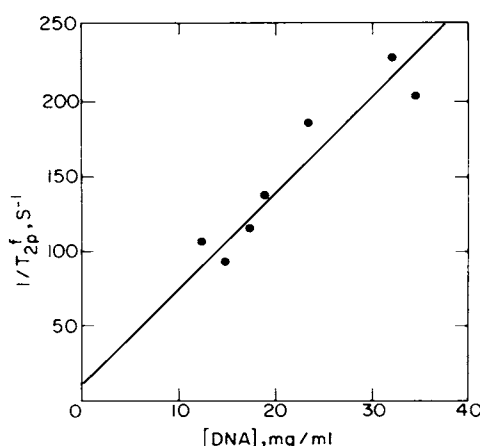


FIGURE 4 The fast component of ^{23}Na transverse relaxation rate, $1/T_{2f}$, in suspensions of nuclei from chicken red blood cells as a function of DNA concentration.

brane leaving the shape of the nuclei unchanged. Similar findings were previously reported for rat liver nuclei (14). In our experiments we observed the shape of the nuclei by microscopic examination and the dissolution of the membrane by NMR. The ^{31}P NMR spectrum of the supernatant of cells treated with 0.5% (a) and later 1% Triton (b) is shown in Fig. 5. Two peaks are detected in the range of the phosphodiester. The peak at the higher field was found to be membranous solubilized lecithin, and that at the lower field, spingomyelin. From the total extracted phospholipids, $\sim 90\%$ was extracted in the first Triton treatment (at a concentration of 0.5%), and $\sim 10\%$ in the second (at a concentration of 1%). No detectable signal of phospholipids was found after further treatment of the same nuclei with 2 or 10% Triton.

DISCUSSION

To the best of our knowledge, this is the first attempt to quantitate the sources of intracellular ^{23}Na relaxation taking into account the contribution of the cell nucleus. Our study focused on the effect of the nuclei on the fast component of T_2 , because this component was found previously to be dominated by the contribution of the slowly rotating bound state. Because it is likely that in the bound state $\omega_0\tau_c \gg 1$, both T_1 and T_2 are expected to be relatively long and their contribution to the overall

relaxation is small. T_{2f} , on the other hand, decreases monotonically when τ_c increases and is expected to be dominated by binding to macromolecules.

Our results indicate that most of the difference between $1/T_{2f}$ in the nucleated chicken erythrocytes and in the unnucleated dog and human erythrocytes can be accounted for by the contribution of the nuclei. The fact that not all the difference is accounted for may be due either to the neglected effect of intracellular viscosity on the relaxation due to the nuclei, or to some contribution of mitochondria.

It should be pointed out that the effect of the nuclei on ^{23}Na relaxation times is considerably smaller than the effect of free DNA solutions when normalized to the same DNA content (17). This is because in the nucleus, the charge of the nucleic acids is largely compensated by the bound nucleoproteins. Our results indicate that the nuclear membrane does not hinder the exchange of the sodium ions enough to affect the averaging of the relaxation times. Thus, removal of the membrane by treatment with a detergent had no significant effect on the measured relaxation times. It is possible that the exchange takes place through the nuclear pores, which are large enough to allow messenger and ribosomal RNA to move from the nucleus to the cytoplasm, and histones to move in the opposite direction.

According to the present findings, the nuclei have a significant effect on the intracellular ^{23}Na relaxation. Such a measurement may give some indication of the state of the DNA in living tissues.

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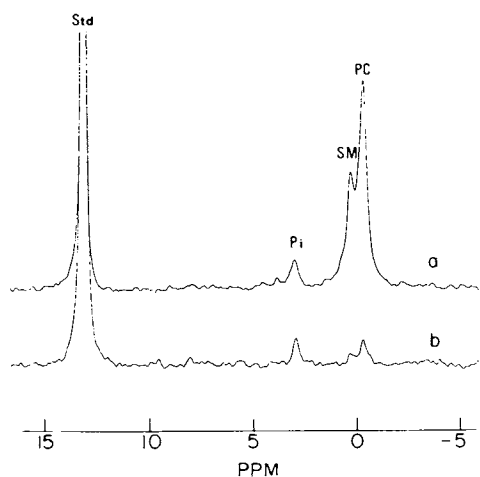


FIGURE 5 (a) ^{31}P NMR spectrum of the supernatant of a suspension of nuclei at the end of 10 min incubation with 0.5% Triton. (b) ^{31}P NMR spectrum of the supernatant in a repeated 10 min incubation of the nuclei with 1.0% Triton. ^{31}P chemical shifts were referred to external ethylenediamine NNN' -tetramethyl phosphonate (EDTMP, $\delta_p = 13.29$ ppm; Kushnir et al., unpublished results). Peak assignments are: Pi, inorganic phosphate; PC, phosphatidylcholine; SM, spingomyelin.

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