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Interactions of Na^+ with haemoglobin-organic phosphate complexes

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Erythrocyte; intracellular Na^+; ^{23}Na-NMR; Relaxation time

Longitudinal and transverse relaxation times were measured in aqueous solutions containing haemoglobin and 2,3-bisphosphoglycerate and in dilute lysates of human erythrocytes. Analysis of the data in terms of calculated excess relaxation rates shows that Na^+ interacts with the protein-organic phosphate complex. The comparable magnitude of the effect in the model system and in dilute lysate suggests that intracellular Na^+ binds to the haemoglobin-bisphosphate complex. Results obtained with adenosine triphosphate and D-glucose indicate that there is also interaction between Na^+ and haemoglobin complexes of these molecules.

1. Introduction

The interest in understanding the state of Na^+ within the human erythrocyte stems from the role of Na^+ in the normal and pathological metabolism of the cell. A large Na^+ gradient exists across the red cell membrane and the maintenance of this gradient consumes a large proportion of the cell's energy [1].

Intracellular monovalent cation concentrations have a considerable influence on glycolysis in erythrocytes. K^+ stimulates phosphofructokinase [2], a major control point of glycolysis [3], whereas Na^+ has been shown to inhibit glycolysis in reconstituted haemolysates [4]. Also, abnormal Na^+ transport has been correlated with a variety of disorders including manic-depressive psychosis [5], myotonic muscular dystrophy [6], essential hypertension [7] and hyperthyroidism [8].

The Na^+ NMR signal from erythrocytes, unlike that of most other tissues, is not less than that expected from the total Na^+ content [9,10]. The lineshape, however, does indicate the presence of two components centred at approximately the same resonance frequency [11]. The ratio of intensities of the broad to narrow component is about 3 : 2. The Na^+ signal of erythrocytes is therefore in full agreement with the theoretical prediction for a system in which free Na^+, with an average electric field gradient of zero experiencing rotations with very short correlation times (approx. 10^{-11} s), is in fast exchange with a small population of bound ions with long rotational correlation times (approx. 10^{-9} s). The NMR signal differs from that observed in other tissues only in that the broad component is not so broad as to be buried in noise [12,13].

The presence of narrow and broad components of the ^{23}Na-NMR line and the observation of biexponential transverse relaxation indicates that Na^+ binds to membranes, macromolecules, or both within the red cell [9,14]. Indeed, a small but significant amount of binding to erythrocyte membranes has been detected [15,16]. Linewidth and relaxation studies in membrane-free haemolysates [14] reveal that there is also binding to macromolecules within the cell. The obvious contender for this role is haemoglobin which is the most abundant protein of the cell and exists as a polyanion at the pH of the cytoplasm [17]. It has been determined from relaxation studies, however, that pure haemoglobin at physiological pH does not bind Na^+ [14,18]. The specific macromolecules to which Na^+ binds within the red cell are still unknown.

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The knowledge that 2,3-bisphosphoglycerate (DPG) and other organic phosphates which bind Na^+ [19] also bind to haemoglobin [20,21] suggested the hypothesis that the macromolecule to which Na^+ binds within the red cell is the haemoglobin-organic phosphate complex.

The possibility of an interaction between Na^+ and the haemoglobin-organic phosphate compounds was studied first in a model system, under conditions that would favour the interactions of all three species. Measurements of ^{23}Na transverse and longitudinal relaxation times provided information to assist in the understanding of the effect of various experimental parameters on the relaxation of Na^+ in these model systems when organic phosphate or haemoglobin was present alone.

The observed effects when both molecular species are present are synergistic, since the change in relaxation times in the presence of haemoglobin and organic phosphate was different from the effects of each individually.

Comparison of these results with data obtained in experiments with dilute membrane-free red cell lysates indicated that for the same haemoglobin concentration the changes in the relaxation rates of Na^+ were of the same magnitude and had a similar temperature dependence in model systems and in haemolysates.

2. Materials and methods

2.1. Experimental

Deuterium oxide (99.75%) was obtained from the Australian Institute of Nuclear Science and Engineering (Lucas Heights, N.S.W.). 2,3-Bisphosphoglycerate (DPG) was obtained as the pentacyclohexylammonium salt from Boehringer-Mannheim (North Ryde, N.S.W.) and Sigma (St Louis, MO). ATP was purchased from Boehringer-Mannheim. All other reagents were of analytical grade.

Human haemoglobin was purified from red cell concentrates donated by the Sydney Blood Bank; by the method of Scholler and co-workers [22] substituting NaCl by KCl in the buffers, or purchased from Sigma and re-purified by the same method. Haemoglobin concentrations were estimated by the method of Van Kampen and Zijlstra [23] measuring the absorbance of cyanomethaemoglobin at 540 nm [24].

Whatman CM-70 carboxymethylcellulose was obtained from Whatman (Maidstone, U.K.) and Amberlite MB-IA resin was from Sigma.

Lysates were prepared by the freeze-thawing procedure [9] from red blood cells kept in storage for over 3 weeks. Cells were washed six times with 0.5–1 vol. of 0.15 M KCl with centrifugation at $2000 \times g$ for 5 min between washes. Washed cells were lysed by two cycles of freezing in liquid nitrogen. Membrane-free haemolysates were prepared by centrifuging the lysates at $10\,000 \times g$ for 30 min using a Sorvall SS-34 rotor. Low-molecular-weight metabolites were separated from dilute lysates by filtration through a YM-2 membrane in an Amicon 8010 ultrafiltration cell (Amicon, Fawkner, Vic., Australia). P_i (~ 2 mM) was the only detectable ^{31}P-NMR signal of the haemolysates. Na^+ and K^+ contents of lysates were measured with a Corning 430 flame photometer.

Samples containing NaCl and DPG were prepared by adding stock solutions of concentrations 3 and 0.3 M, respectively, to ^2H_2O, and brought to the appropriate pH by adjusting with HCl or KOH. The viscosity of the solutions was changed in the relevant experiments by adding glycerol. Samples containing only low-molecular-weight solutes were filtered using the Amicon ultrafiltration cell with a YM-2 membrane. For samples containing haemoglobin, ultrafiltered NaCl and DPG solutions were added to concentrated haemoglobin solutions.

2.2. NMR methods

Measurements were carried out with 3.5-ml samples dispensed into 10-mm high-precision tubes (703-PP; Wilmad, Buena, NJ).

^{23}Na-NMR spectra were observed at 52.9 and 105.8 MHz with a Varian XL-200 and an XL-400 spectrometer operating in the Fourier transform mode. Spectral width was 4000 Hz over 8192 data points. The 90° pulse was approx. 13.5 μs at 52.9 MHz and 30 μs at 105.8 MHz, and the relaxation delay 2 s. Each spectrum averaged between 16 and

80 transients, depending on the experimental conditions. ^{13}C-NMR spectra were observed at 100.6 MHz. Spectral width was 10000 Hz over 16384 data points. The 90° pulse was approx. 22 μs and the relaxation delay 20 s. Each spectrum averaged 16 transients. Arrays of 18 spectra were measured with interleaved acquisitions for each experiment.

Longitudinal relaxation times (T_1) were measured using the inversion-recovery technique [25], with delay times of 0.001–2 s; T_1 values were calculated by non-linear regression [26]. Transverse relaxation times (T_2) were measured with a Hahn spin-echo pulse sequence [27]; the arrays of echo times chosen depended on the sample to be measured. The shortest time was 0.005 s and the longest varied between 0.02 and 0.11 s. T_2 values were also calculated by non-linear regression analysis. Double-quantum filtered spectra were acquired using the method of Pekar and Leigh [28] with a creation time of 0.002 s and averaging 2000 transients. A concentric tube containing 12 mM bis(tripolyphosphate) dysprosium(III) and 0.45 M NaCl was placed coaxially in standard 10-mm tubes containing lysates or haemoglobin solutions. The reagent in the inner tube complexes with Na^+ shifting its resonance frequency with respect to that of ions in the solutions of the outer tube [29].

2.3. Background theory

For a nucleus with spin quantum number 3/2, such as ^{23}Na, Hubbard [30] has shown that both longitudinal and transverse relaxation times are described by the sum of two exponentially decaying functions. For the case of Lorentzian lines the rate constants are expressed as:

$$\frac{1}{T_1^f} = \frac{(e^2qQ)^2}{10}\left(1+\frac{\eta^2}{3}\right)\tau_c\left(\frac{1}{1+\omega_0^2\tau_c^2}\right) \quad (1a)$$

$$\frac{1}{T_1^s} = \frac{(e^2qQ)^2}{10}\left(1+\frac{\eta^2}{3}\right)\tau_c\left(\frac{1}{1+4\omega_0^2\tau_c^2}\right) \quad (1b)$$

$$\frac{1}{T_2^s} = \frac{(e^2qQ)^2}{20}\left(1+\frac{\eta^2}{3}\right)$$

$$\times\tau_c\left(\frac{1}{1+\omega_0^2\tau_c^2}+\frac{1}{1+4\omega_0^2\tau_c^2}\right) \quad (2a)$$

$$\frac{1}{T_2^f} = \frac{(e^2qQ)^2}{20}\left(1+\frac{\eta^2}{3}\right)\tau_c\left(1+\frac{1}{1+\omega_0^2\tau_c^2}\right) \quad (2b)$$

where T_i^f and T_i^s ($i = 1, 2$) are the fast and slow components of each relaxation time, respectively, η the asymmetry parameter of the electric field gradient, ω_0 the resonance (Larmor) frequency of the nucleus, τ_c the reorientational correlation time, eq the electric field gradient at the nucleus, and eQ the nuclear quadrupole moment.

20% of the signal intensity decays with a longitudinal relaxation time T_1^f while the remaining 80% decays with time T_1^s. 40% of the signal relaxes with the slow component of the transverse relaxation time T_2^s, and the other 60% relaxes with the fast time constant T_2^f.

Experimentally the two components of T_1 are difficult to separate, since they differ at most by a factor of four and the slow component consists of only 20% of the signal intensity. For T_2, however, the two components may be experimentally separable when $\omega_0\tau_c > 1$. In the domain of long correlation times (slow molecular reorientation), the slow component, T_2^s, is proportional to τ_c as are both components of T_1. The fast component, T_2^f, on the other hand, is proportional to the inverse of τ_c.

In the fast-motion domain where $\omega_0\tau_c \ll 1$, all four components are equal to

$$\frac{(e^2qQ)^2}{10}\left(1+\frac{\eta^2}{3}\right)\tau_c$$

Under these conditions $T_1 = T_2$ and each relaxation time decays as a single exponential.

The measured relaxation times, ^MT_i, of Na^+ moving in the extreme narrowing limit in a mixed population of bound and free states in fast exchange (i.e., the average lifetime at the bound site is much less than the relaxation time) is given by [31]

$$\frac{1}{^MT_i} = \frac{X_F}{^FT_i} + \frac{X_B}{^BT_i} \quad (i = 1, 2) \quad (3)$$

where X_F and X_B are the mole fractions and ^FT_i

and ${}^{B}T_i$ the relaxation times of free and bound Na^+, respectively.

Some of the characteristics of the fast exchange limit are: (a) the longitudinal relaxation time is a single exponential, (b) the lineshape is Lorentzian, (c) the measured relaxation time monotonically strictly decreases as ligand is added [31], and (d) the transverse relaxation rate constant is a linear function of reciprocal temperature [12,32,33].

Bull [34] developed equations to describe the experimentally observed relaxation times of spin 3/2 nuclei present in two populations of ions between which there is exchange. Of particular interest in biological systems is the case in which a small fraction of the total population (the bound population) has relaxation times much less than the other (ions free in solution), the chemical shift difference between the two sites is negligible, and there is fast exchange between both populations. If the relaxation process of one or both populations consists of two components, the measured longitudinal and transverse rate constants are:

$$\frac{1}{{}^{M}T_1} = \frac{1}{{}^{F}T_1} + X_B\left(\frac{0.2}{{}^{B}T_1^f + \tau_{ex}} + \frac{0.8}{{}^{B}T_1^s + \tau_{ex}}\right) \quad (4a)$$

$$\frac{1}{{}^{M}T_2} = \frac{1}{{}^{F}T_2} + X_B\left(\frac{0.6}{{}^{B}T_2^f + \tau_{ex}} + \frac{0.4}{{}^{B}T_2^s + \tau_{ex}}\right) \quad (4b)$$

where ${}^{B}T_1^f$, ${}^{B}T_2^f$ and ${}^{B}T_1^s$, ${}^{B}T_2^s$ are, respectively, the fast and slow components of the bound species, and τ_{ex} the residence time of the ion at the bound site.

In the case of canine and human erythrocytes, Shinar and Navon [14] found that: (a) the fast motion condition held for intracellular Na^+ which is either free or bound to small molecules; (b) the measured T_2 of intracellular Na^+ was significantly shorter than T_1 indicating binding of ions to macromolecules; and (c) the temperature dependence of the relaxation rates suggested that the exchange between free and bound Na^+ is in the fast limit. They were able to analyze the transverse relaxation curves of the intracellular ^{23}Na signal from erythrocytes in terms of the two components using a multiparameter fit, and concluded that T_2 values obtained from a fit to one exponential only may be considered as weighted average values of the two components. In dilute haemolysates binding of Na^+ to macromolecules was established by employing the 'excess' relaxation rate constant relative to that in free salt solutions defined as

$$R_i = \frac{1}{T_i} = \frac{1}{{}^{M}T_i} - \frac{1}{{}^{F}T_i} \quad (i = 1, 2) \quad (5)$$

and observing that there is a difference between the excess transverse and excess longitudinal rate constants. The measured relaxation times for free sodium salt solutions was 55 ms, corresponding to a rate constant of 18.2 s^{-1}.

In the model systems we studied, the two time constants of the transverse relaxation differed by less than a factor of two. Consequently, the errors incurred in a two-exponential analysis may be quite large. Since the data yielded a very good one-exponential fit, we used the difference in excess rate constants, $R_2 - R_1$, as a measure of the interaction of Na^+ with macromolecules. The difference between the excess rate constants may arise from several mechanisms. A first mechanism is the relative immobilization of Na^+ by binding to macromolecules or membranes [14,35]. Slow exchange between compartments in which the ions would be located in different environments is another factor which may contribute to decrease the transverse relaxation time relative to the longitudinal relaxation time. A third mechanism proposed by Berendsen and Edzes [36] involves diffusion of Na^+ through regions with a molecular order in which the electrostatic field gradients are different. The non-zero average field gradient would vary slowly with time affecting only the transverse relaxation time, and resulting in a non-zero excess rate constant difference.

The systems we studied were isotropic samples in one compartment, in which it is reasonable to assume that the average field gradient is zero. Thus, the enhancement of the transverse relaxation rate constant relative to the longitudinal relaxation rate constant is ascribed to the interaction of Na^+ with a macromolecule. Without attempting a rigorous definition of 'binding', the term has been used to denote specifically restricted molecular motion.

3. Results

3.1. Effect of viscosity

Under experimental conditions in which the quadrupolar coupling constant (e^2qQ) does not change, the relaxation rates of Na^+ given by eqs. 1 and 2 depend on the correlation time. At 105.8 MHz and room temperature the motion of these ions in aqueous solutions is in the extreme narrowing limit, and the relaxation times are inversely proportional to τ_c. Changes in viscosity induce changes in the correlation time which result in variation of the observed rates. Fig. 1 shows the variation of relaxation times with viscosity in aqueous NaCl solutions titrated with glycerol. The measurements span a 5-fold decrease in the relaxation times with increasing glycerol concentration in which both time constants change equally. It should be noted that the difference between the excess transverse and longitudinal rate constants is zero even in the very viscous solutions.

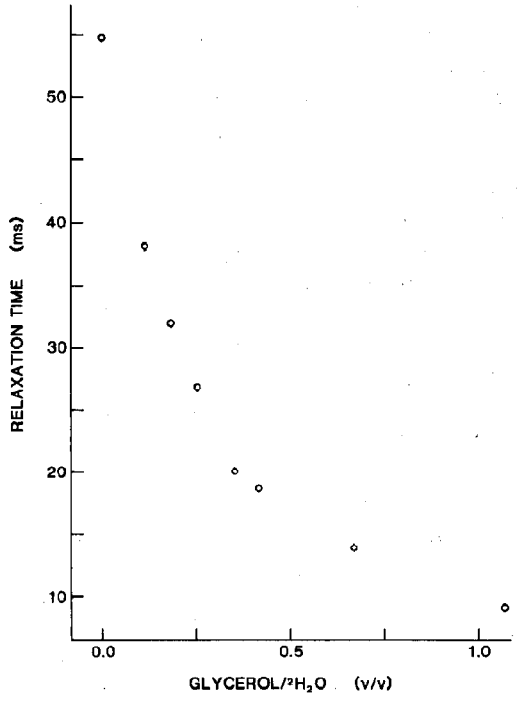

Fig. 1. Dependence of ^{23}Na longitudinal (○) and transverse (◇) relaxation times on glycerol concentration. Measurements were carried out at pH 7.4 and 297 K in 5 mM NaCl solutions.

3.2 Binding of Na^+ to DPG

When DPG is added to NaCl solutions the ^{23}Na NMR signal remains Lorentzian and the data from transverse and longitudinal relaxation measurements yield excellent fits to single-exponential curves. Fig. 2 shows that the dependence of measured relaxation times on ligand concentration is a monotonically strictly decreasing function.

The dependence of measured relaxation times on pH and temperature is shown in table 1 for aqueous solutions of NaCl (5 mM) and DPG (20 mM). Binding between Na^+ and DPG is indicated by the pH dependence of the observed relaxation times. DPG contains five ionisable groups. Three of these, the carboxyl and the first ionisation of the two phosphate groups, have an average pK_a of less than 3 [37]. The average pK_a of the remaining two ionisable groups appears to vary inversely with ionic strength [37,38] and has been reported

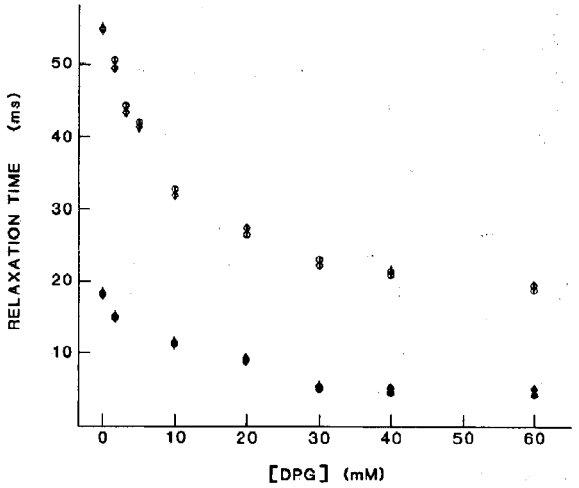

Fig. 2. Effect on ^{23}Na longitudinal (○) and transverse (◇) relaxation times of binding to DPG in aqueous solutions and in water/glycerol mixtures. Experimental conditions: pH 7.4, 297 K and 7 mM NaCl. Open symbols represent data obtained in aqueous solutions; full symbols are data from 2.5:1 (v/v) water/glycerol mixtures. Error bars denote 1 S.D.

Table 1

Temperature and pH dependence of relaxation times and excess relaxation rate constants of Na^+ binding to DPG
Solutions contained 5 mM NaCl and 20 mM DPG.

pH	T (K)	$^{M}T_2$ (±0.7) (ms)	$^{M}T_1$ (±0.7) (ms)	R_2 (±1.5) (s^{-1})	R_1 (±1.5) (s^{-1})
6.60	286	30.1	29.6	15.0	15.6
	298	39.2	38.8	7.3	7.6
	310	47.0	46.6	3.1	3.3
	322	55.0	55.5	0.0	0.0
7.27	286	19.0	19.2	34.4	33.9
	298	27.1	27.5	18.7	18.2
	310	34.5	34.0	10.8	11.2
	322	38.2	37.8	8.0	8.3
8.12	286	14.9	15.2	48.9	47.6
	298	22.8	22.3	25.7	26.6
	310	28.1	27.7	17.4	17.9
	322	33.3	33.8	11.8	11.4

to be 7.1 at $I = 0.1$ M [37]. At the low ionic strength of the solutions employed in the experiments shown in table 1, most DPG molecules would have only three negative charges at pH 6.6, and would have undergone the second ionisation of the phosphate groups at pH 8.12. The decrease in relaxation times as the pH was raised indicated increasing binding of Na^+ to DPG; the apparently sigmoidal binding curve has a pK_a of 6.7 ± 0.1. Both relaxation times increased with temperature and the transverse relaxation rates did not decrease at low temperatures; and, within experimental error, the transverse relaxation rate constant is a linear function of reciprocal temperature.

Thus, in the range of temperatures measured, the data are consistent with the system being in fast exchange, notwithstanding a 3-fold change in the relaxation time values. The association constant, K_a, in the formation of the Na-DPG complex is defined in terms of the concentrations of Na^+, DPG and the complex as

$$K_a = [\mathrm{Na-DPG}]_B/([Na^+]_F[\mathrm{DPG}]_F) \qquad (6)$$

From this relationship it is possible to solve for X_B and obtain a quadratic expression in terms of K_a and the concentrations of free and liganded Na^+ and DPG. Assuming the equilibrium to be in fast exchange, eq. 3 can be employed; solving for X_B yields a relationship in terms of $^{M}T_i$, $^{F}T_i$ and $^{B}T_i$. The system of equations thus obtained contains two undetermined parameters, K_a and $^{B}T_i$. From the binding data of Na^+ to DPG in aqueous solutions in fig. 2, it is possible to calculate a value of 16.5 ± 0.1 M^{-1} for K_a and of 9.9 ± 0.7 ms for $^{B}T_i$. Evidence for the existence of fast exchange was obtained from measurements of ^{13}C longitudinal relaxation times in solutions containing 7 mM NaCl and 60 mM DPG. Application of the NT_1 equation for a rigid rotor [39] to the measured longitudinal relaxation time of 2.51 ± 0.09 s corresponding to the C-2 carbon of DPG gave a correlation time for the overall isotropic motion of the DPG molecule of $1.9 \pm 0.1 \times 10^{-11}$ s based on a C–H bond length of 109 pm. Taking a realistic value of 1.2 MHz [40,41] for the quadrupolar coupling constant of bound Na^+, the relaxation time of ions in the complex is 9.3 ± 0.6 ms. The good agreement of the calculated values for $^{B}T_i$ suggests that the equilibrium is in fast exchange.

The variation of the transverse and longitudinal relaxation times of Na^+ with DPG concentration is shown in fig. 2 for aqueous solutions and for water/glycerol mixtures. In the conditions used for these experiments the measured relaxation times decreased monotonically as ligand was added, until they became independent of DPG con-

centration at about 40 mM for both types of solutions. Taking the limit for the extreme narrowing region as $\omega_o\tau_c = 0.1$, the calculated correlation time at the Larmor frequency of 105.8 MHz is 0.15 ns. The corresponding value of the relaxation times for a quadrupolar coupling constant of 1.2 MHz is 1.1 ms. Consequently, even in the case where the measured relaxation times are as low as 5 ms, the motion of Na^+ remains within the fast motional domain. Assuming that the presence of glycerol does not affect substantially the association constant, from the binding data for water/glycerol mixtures in fig. 2, one obtains a value of 2.0 ± 0.3 ms for ${}^{B}T_i$. In this instance of Na^+ binding to a small molecule the difference between the excess relaxation rate constants is also zero for viscous solutions.

3.3. Binding of Na^+ to the haemoglobin-DPG complex

The ^{23}Na NMR signals of free NaCl in aqueous solution with DPG, haemoglobin, or the protein-organic phosphate complex are shown in fig. 3. At a given pH the increased broadening of the peak, in solutions containing haemoglobin and DPG, could be interpreted as the cumulative effect of the fast exchange between free and DPG-bound Na^+ and the increased viscosity owing to the presence of the protein. The decrease in transverse relaxation time of the Na^+ resonance with increasing pH in solutions with haemoglobin and DPG cannot be ascribed to a hydrodynamic effect, but can be explained by a change in the binding constant of the exchange between free and DPG-bound Na^+. An increase in linewidth as the pH was raised was also observed in solutions containing only the bisphosphate.

To elucidate how the protein-phosphate complex affects the relaxation of Na^+, transverse and longitudinal relaxation times were measured at constant pH in NaCl solutions containing 5 mM DPG, with different amounts of haemoglobin. The results are listed in table 2. The observed decrease in longitudinal relaxation times with increasing haemoglobin concentration can be explained by

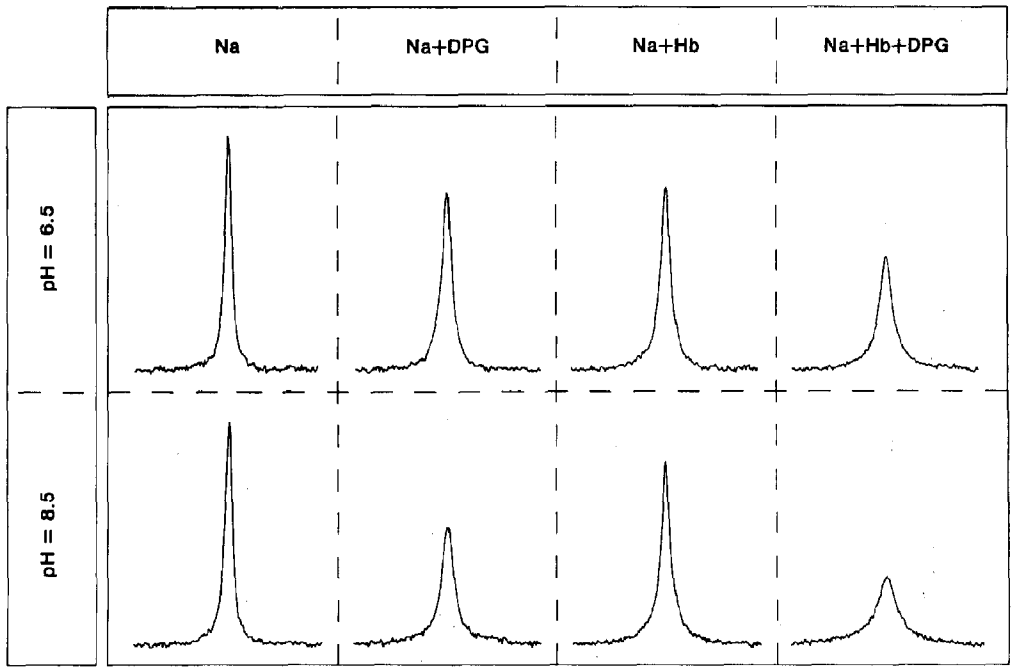

Fig. 3. ^{23}Na-NMR spectra of 7 mM NaCl solutions in ^2H_2O at 297 K. Spectral width, 211.6 Hz; DPG concentration, 5 mM; haemoglobin concentration, 11% (w/v).

Table 2

Relaxation times and excess relaxation rate constants of Na^+ in solutions with different haemoglobin concentrations

Solutions at pH 7.2 contained 7 mM NaCl, 5 mM DPG, and 140 mM KCl.

[Hb] (% w/v)	$^{M}T_2$ (±0.7) (ms)	$^{M}T_1$ (±0.7) (ms)	R_2 (±1.5) (s^{-1})	R_1 (±1.5) (s^{-1})	$R_2 - R_1$ (s^{-1})
0	43.0	43.2	5.1	5.0	0.1
4	28.7	37.8	16.6	8.3	8.3
8	25.1	36.6	21.6	9.2	12.4
10	22.7	35.3	25.7	10.1	15.6
12	21.9	34.9	27.9	10.5	17.4
16	20.9	33.7	29.7	11.5	18.2

the increase in viscosity of the solutions, but the change effected on both relaxation times as measured by the difference in excess relaxation rate constants reveals that Na^+ is bound to a macromolecule, presumably the haemoglobin-DPG complex. The measured longitudinal and transverse relaxation times in a dilute lysate containing 13% haemoglobin, 40 mM KCl, 17 mM NaCl and 4.7 mM DPG were 38.2 and 17.2 ms, respectively.

The role of the haemoglobin-DPG complex in binding intracellular Na^+ was investigated by comparing the observed relaxation rates at different temperatures in dilute lysates with and without DPG and in solutions prepared with the same haemoglobin and DPG concentrations. The results are given in table 3 for haemoglobin prepared with buffers thoroughly gassed with carbon monoxide. It should be noted that the value of the difference excess relaxation rate constant measured for similar concentrations of Na^+, DPG and haemoglobin is about half of that presented in table 2. The transverse relaxation rate constant

Table 3

Measured relaxation times and calculated excess rate constants

Solutions at pH 7.4 contained 12% (w/v) haemoglobin, 6 mM NaCl, 120 mM KCl and 5 mM of either ATP, DPG or D-glucose. The haemoglobin employed in these measurements was prepared in buffers saturated with carbon monoxide.

	T (K)	$^{M}T_2$ (±1) (ms)	$^{M}T_1$ (±1) (ms)	R_2 (±1.7) (s^{-1})	R_1 (±1.7) (s^{-1})	$R_2 - R_1$ (s^{-1})
	285	28.3	30.2	35.3	33.2	2.1
	297	39.0	39.2	25.6	25.5	0.1
	311	41.3	48.5	24.2	20.6	3.6
DPG	285	20.2	23.2	49.4	43.1	6.3
	291.5	20.9	25.9	47.7	32.6	9.1
	297	30.2	31.3	33.1	32.0	1.1
	304.5	27.1	34.7	36.9	26.2	8.1
	311	34.0	39.6	29.4	25.3	4.1
ATP	285	17.7	21.1	56.5	47.3	9.2
	297	24.6	30.6	40.7	32.6	8.1
	311	28.3	38.1	35.3	26.2	9.1
D-Glucose	285	27.1	29.4	36.9	34.0	2.9
	297	38.8	39.3	25.8	25.4	0.4
	311	42.7	50.0	23.4	20.0	3.4

shows a linear dependence on reciprocal temperature suggesting an equilibrium in fast exchange.

The relaxation times of a sample at pH 7.4 containing 6% haemoglobin, 42 mM KCl, 3 mM NaCl and 4 mM DPG were examined at two Larmor frequencies at 297 K. The longitudinal relaxation times measured at 52.9 and 105.8 MHz were 35 ± 1 and 37 ± 1 ms, respectively; and the transverse relaxation times were 19 ± 1 and 27 ± 1 ms, respectively. Shporer and Civan [33] examined the effect of Larmor frequency on the relaxation constants of Na^+ in frog striated muscle. They interpreted a very small change in the longitudinal relaxation rate constant and a small change in the slow component of the transverse relaxation rate constant in terms of a model consisting of fast exchange between ions immobilised to macromolecules and free species of Na^+ in solution within the cell. We interpret our qualitatively similar results as suggesting fast exchange between free ions and ions immobilised at the haemoglobin-DPG complex.

Selective detection of the biexponential transverse relaxation of the ^{23}Na nuclei was achieved by the application of double-quantum filtering. Fig. 4 shows the spectra of samples with two compartments; one small, containing the tripolyphosphate-dysprosium complex and the other compartment containing either dilute lysates (A) or haemoglobin solutions (B). The double-quantum filtered spectra of the same samples are shown in fig. 4C and D, respectively. The ions in the compartment with the shift reagent relax monoexponentially and their NMR signal cannot pass through the filter. The double-quantum signals observed for lysates and for the haemoglobin-DPG solutions reveal that Na^+ relaxes biexponentially in this compartment.

3.4. Binding of Na^+ to ATP and to glucose

The interaction of Na^+ with other haemoglobin-organic phosphate complexes was tested by measuring relaxation times of ^{23}Na in haemoglobin solutions in the presence of ATP, which binds Na^+ [20] and is found in normal human red cells at concentrations of about 1.2 mM [20,21]. Relaxation times, rate constants and difference excess rate constants at several temperatures are given in table 3. The difference excess rate con-

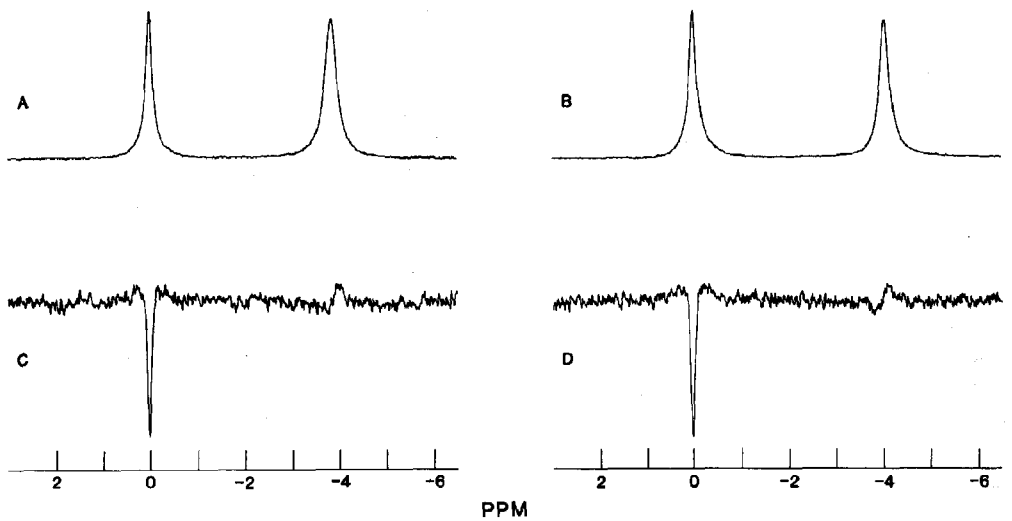

Fig. 4. Single (A,B) and double-quantum filtered (C,D) ^{23}Na-NMR spectra of haemolysates (A,C) and haemoglobin (B,D) solutions containing DPG. Experimental conditions are the same as for fig. 3. The spectra are referenced at 0 ppm to the ^{23}Na resonances in the above solutions. The other resonance appearing in each spectrum at 4 ppm corresponds to Na^+ complexed with $[Dy(P_3O_{10})_2]^{7-}$ contained in a concentric tube.

rate constants are smaller than those obtained for solutions containing DPG under the same experimental conditions (table 3).

Glucose is another abundant metabolite in red cells capable of binding Na^+ [32] and haemoglobin. Rheological investigation of membrane-free haemoglobin showed that D-glucose promotes the formation of a visco-plastic gel structure [42,43]. Gel strength is a function of glucose concentration, haemoglobin concentration and temperature. Relaxation rate constants measured in haemoglobin solutions containing D-glucose, and the difference excess rate constants are listed in table 3. The experimental conditions employed were the same as for the measurements in solutions with ATP. The rates are much smaller than those obtained for DPG and ATP, thus indicating that the contribution of glucose to intracellular Na^+ binding is negligible.

4. Discussion

Measurement of relaxation times of Na^+ in viscous solutions and in the presence of small ligand molecules demonstrate that, under the experimental conditions employed, the motion of Na^+ remains within the fast limit domain. That is, under these conditions, hydrodynamic effects, or binding to molecules moving with short correlation times, do not result in different excess transverse and excess longitudinal relaxation rates.

The presence of macromolecules in sodium salt solutions can increase the viscosity to the extent of inducing observable effects in the relaxation constants of the ions. In solutions containing haemoglobin, the measured decrease in both relaxation times can be accounted for by the increased viscosity. However, in this case too there is no significant increase in the difference between excess relaxation rate constants.

Establishing the existence of Na^+ binding to complexes of haemoglobin and organic-phosphate ligands like DPG, capable of binding Na^+ and the protein, presents practical difficulties. Experimentally, the analysis of a measured signal decay in terms of multiexponential functions with real exponents has severe constraints owing to the sensitivity of exponents and amplitudes to small changes in the data, and it is realistic only in a limited number of cases [34,44]. In the systems studied here, the values of the two different components in the transverse and longitudinal relaxation times could not be obtained reliably from the data. Hence, eqs. 1 and 2 cannot be employed to derive approximate values of the correlation time τ_c. Eq. 4 provides another avenue to gain insight into possible interactions between Na^+ and the complex. The interpretation of relaxation data obtained at different relative concentrations of Na^+, ligand and protein may suggest or preclude the existence of binding to macromolecules. An obstacle to this line of analysis is the lack of information about reasonable approximations in a given set of experimental conditions for the value of the mole fraction of bound ions and for the residence time. A second difficulty with the application of eq. 4 arises from the variation of the relaxation times with temperature. The observed linear decrease of the transverse rate constant with reciprocal temperature (table 3) suggests that the Na^+ is in fast exchange between sites. However, the change of the transverse rate is smaller at low than at high temperatures, thus raising the possibility that the exchange may not be in the fast limit, in which case eq. 4 would not apply. As it is suggested by the results of measurements at different Larmor frequencies, it is possible that Na^+ is in fast exchange between three sites, free, DPG-bound and complex-bound, and the change in the equilibrium populations with temperature results in the observed changes in relaxation rates. But until experimental evidence is obtained to support this hypothesis, expressions of the type given by eq. 4 cannot be used to establish the existence of binding of Na^+ to the complex.

Shinar and Navon [14] employed the difference between transverse and longitudinal relaxation rate constants as a criterion for detecting binding of Na^+ to macromolecules, and concluded that the excess transverse relaxation rate of intracellular Na^+ was caused by binding to molecules other than haemoglobin. Employing the same criterion and from the data given in tables 2 and 3, we conclude that in model systems consisting of NaCl, DPG and haemoglobin there is a synergistic bind-

ing of the ions to the protein-organic phosphate complex. Considering that the difference excess rate constants measured in dilute lysates and in the model system are of the same order of magnitude and that the differences in excess rate constants in both systems show similar temperature dependence, we suggest that intracellular Na^+ also interacts synergistically with haemoglobin. The existence of biexponential relaxation, characteristic of binding to macromolecules, was made apparent in lysate/ and haemoglobin/DPG solutions with double-quantum filtered spectra (fig. 4), in which the observed signal is the result of subtracting both components of the relaxing magnetisation. The binding to the haemoglobin-DPG complex does not exclude the possibility of interactions with other macromolecules as well. The data given in table 2 suggest that the 20-fold excess of intracellular K^+ relative to Na^+ would not result in the complete displacement of Na^+ from the complex.

The data in table 3 indicate that in haemoglobin solutions there is binding of Na^+ to macromolecules in the presence of ATP and very little binding in the presence of D-glucose. Taking the calculated difference in excess rate constants as a measure of the strength of the interaction, binding in the presence of DPG is stronger than in the presence of ATP, and weakest in the presence of D-glucose. Since in normal human erythrocytes the concentration of free ATP is about one-quarter of that of DPG, the contribution of the triphosphate to intracellular Na^+ binding is likely to be small. The considerably weaker effect observed in the presence of D-glucose suggests that its contribution is also probably very small.

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