Biochimica et Biophysica Acta 815 (1985) 189–195
Elsevier

BBA 72548

Nuclear magnetic resonance investigation of human erythrocytes in the presence of manganese ions. Evidence for a thermal transition

Vasile V. Morariu [a,*], Mihai S. Ionescu [b], Maria Frangopol [b], Radu Grosescu [b], Mihaela Lupu [b] and Petre T. Frangopol [b]

[a] *Institute of Isotopic and Molecular Technology, P.O. Box 700, R-3400 Cluj-Napoca and* [b] *Institute of Physics and Nuclear Engineering, P.O. Box MG-6, R-76900 Măgurele-Bucharest (Romania)*

(Received August 7th, 1984)
(Revised manuscript received January 3rd, 1985)

Key words: Erythrocyte membrane; Water exchange; Mn^{2+}; Phase transition; NMR

Water proton transverse relaxation was investigated in whole blood and washed erythrocytes samples, respectively, at various temperatures and manganese concentrations. Water diffusional exchange controls proton relaxation in whole blood samples at higher Mn^{2+} concentrations (20–30 mM) or in washed erythrocyte samples at low Mn^{2+} content (1–5 mM). Mn^{2+} uptake is significant in washed normal erythrocyte samples when its concentration is about 18 mM or higher in the medium, at temperatures below about 26°C. The thermal transition as revealed by the NMR doping method represents a switch from a water exchange process, mainly seen in the higher temperature range, to a paramagnetic ion controlled water proton relaxation in the lower temperature range.

Introduction

Various experimental methods of investigation have been developed for the study of the diffusional transport of water across both biological and model membranes [1,2]. Since this passive process occurs rapidly, in a time interval of the order of milliseconds, it requires methods of investigation which are typical for fast reactions. The nuclear magnetic resonance (NMR) technique has gained an increasingly wide use for this purpose during the last decade.

The original NMR method for measurement of water exchange through human erythrocyte membranes is based on the doping of blood samples with such paramagnetic ions as manganese divalent cations and was first reported by Conlon and Outhred [3]. Improvements in the calculation of the exchange time were subsequently proposed [4,5].

Our previous work has shown that the apparent water diffusional exchange time through human erythrocyte membranes undergoes a rapid change around 26°C [6,7]. This result was interpreted in terms of a structural transition occurring in the biomembrane and a mathematical description of the phenomenon was attempted [8]. The same idea is also supported by various other properties of the erythrocyte membrane such as: microviscosity, osmotic fragility, anion and glucose transport which show particular changes around the same temperature (see literature cited in Ref. 6). More evidence have gathered in the meanwhile from lithium efflux [9], spin label [10] and fluorescent probes [11] investigations of the erythrocyte membranes.

The apparent exchange time of human

* To whom correspondence should be addressed.

erythrocyte membranes was also investigated in cases of various diseases and significant alterations were found including also the temperature dependence. These data were obtained in the experimental conditions mentioned in Ref. 4 and refer to: liver diseases, Gaucher disease, hyperlipemia, nephrotic syndrome [4], malignant tumors [12], leukemias [13,14], hemolytic anemia [15] and epilepsy [16,17].

Other investigators using an apparently similar NMR method reported no breaks in the temperature dependence of the diffusional exchange. Non-NMR investigations of water transport have also failed to support any structural transition within the same temperature region [1]. A closer look at the literature revealed that methods for NMR sample preparation varied significantly among different laboratories. Therefore the aim of this paper is to perform a systematic investigation of the NMR manganese doping method under various experimental conditions mainly to elucidate the existence of a temperature induced transition of the membrane. This work reports for the first time results of NMR measurements of whole blood and washed samples at various manganese ion concentrations in the extracellular environment, performed in the same laboratory. In addition the particular experiment which reveals the thermal transition was checked by the authors in two different laboratories by different teams.

This work mainly suggests that the transition is only evident in washed samples of erythrocytes at higher manganese concentrations and it represents the onset of Mn^{2+} penetration into the cells when lowering the temperature. At higher temperatures than about 26°C water exchange is however the main process which is observed by the NMR doping method. The penetration of erythrocytes by Mn^{2+} is little or not at all evident in samples of whole blood regardless of Mn^{2+} concentration (as shown by the NMR method).

Materials and Methods

N-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) was from Sigma Chemical Co., bovine serum albumin from Miles Laboratories, sodium chloride from Merck, manganese(II) chloride from BDH, and glucose from Fluka. All solutions were made in doubly distilled water. All other chemicals were of analytical grade.

Human blood obtained by venipuncture from male healthy donors and normal infants less than 48 h of age was collected in heparinised tubes and used within 4 h. NMR measurements were carried out on both whole blood and washed erythrocytes. In order to obtain the washed erythrocytes, whole blood was centrifuged for 5 min at $2000 \times g$ and plasma and buffy coat were carefully removed by aspiration. Subsequently the red blood cell pellet was washed three times with an isotonic buffered solution 5 mM (Hepes/150 mM sodium chloride/0.1% glucose (pH 7.4)).

The final washed erythrocytes sediment was resuspended to the initial hematocrit in the same buffer solution supplemented with 0.5% bovine serum albumin.

The samples for NMR were prepared prior to the measurement by thoroughly mixing an aliquot of either whole blood or washed erythrocytes suspension with an appropriate volume of an isotonic doping solution (40 mM manganese(II) chloride/110 mM sodium chloride) so as to obtain the desired final Mn^{2+} concentration in the aqueous extracellular environment. It is essential for this paramagnetic doping method for evaluation of water permeability that manganese ions are confined into the extracellular aqueous milieu only [3]. Their presence shortens the transverse relaxation time of water protons outside the cells and makes it possible for the NMR method to distinguish between intra- and extracellular water molecules. Therefore, the doping solutions were always prepared at least 1 week in advance in order to reduce Mn^{2+} penetration inside the red blood cells [5]. Manganese binding to serum albumin present in the suspending medium also hampers its penetration into the intracellular compartment [18,19].

All measurements were performed within 30 min of paramagnetic ions addition to the erythrocyte aliquots, except for the investigation of the temperature dependence of transmembrane water exchange rate, which took about 3 h to perform.

Hematocrit measurements were performed by standard techniques.

Pulsed NMR measurements were performed by two different teams working in different laboratories (laboratory A and B, respectively). Some NMR

data obtained in laboratory A have been already published [6,7]. Both groups used a 90 MHz Bruker pulsed NMR spectrometer. The time evolution of the transverse magnetization due to the water protons in the sample was followed either with a spin-echo method [20] (lab. A), or with a standard Carr-Purcell-Meiboom (CPMG) radiofrequency pulse sequence [20] (lab. B), with a $\pi/2$ pulse of 8 μs and a spacing of 200 μs between the π pulses. In the case of the spin-echo method, the echo amplitude was recorded with a Box-car integrator without signal accumulations. The long relaxation time was estimated at $8 < 2\tau < 16$ ms where τ is the pulse spacing. In the case of the CPMG sequence, the NMR signal was sampled with an analog-digital converter and transferred into the memory unit of a Nicolet B-NC 12 computer. This procedure enabled us to perform computer-aided signal averaging: three to five scans were averaged for each measurement.

The sample temperature was controlled to within ± 0.2 degree C by a flow gas system using nitrogen gas evaporated from a Dewar flask containing liquid nitrogen. The actual temperature was measured with a glass-coated thermocouple placed inside the blood sample.

The relaxation of the water proton transverse magnetization of the sample follows a two compartment exponential decay pattern, which is due to the paramagnetic doping of the extracellular aqueous environment. A two-exponential nonlinear regression program was used to fit experi-mental data with the theoretical curve corresponding to the exponential decay and extract the long apparent relaxation time, T'_{2a}. The water exchange time through the membrane τ_a is related to T'_{2a} by the equation describing the two site exchange theory [21,4,5]. The exact calculation of τ_a needs also T_{2a} and T_{2b} to be known. Here T_{2a} is the spin-spin relaxation time of the water protons inside the isolated red blood cells and T_{2b} is the relaxation time of the water protons in the paramagnetically doped aqueous suspending environment of the erythrocytes (the extracellular compartment). T_{2a} and T_{2b} were measured separately in a red blood cell packed sample and in plasma or suspending buffer containing appropriate concentrations of Mn^{2+}, respectively. The results of these measurements were corrected by 1.6% for trapped plasma volume [22]. Intracellular water molecule populations were corrected by 5% [19].

Results

Results of measurements performed on samples of red blood cells were shown to be closely similar to those corresponding to samples of undoped whole blood [5]. Therefore, we have investigated the temperature dependence of T_{2a} for unwashed sedimented erythrocytes. Our results show that T_{2a} has a strong temperature dependence, which remains linear over the entire temperature range between 10°C and 40°C, in good agreement with previous results [5].

The relaxation time of water protons in the extracellular paramagnetically doped aqueous environment, T_{2b}, also depends strongly on both temperature and manganese ion concentration in the case of the isotonic BSA buffered solution (Table I, entries 17–19) as well as for human plasma (not shown here). The temperature dependence is linear over the entire range of temperatures for all Mn^{2+} concentrations we have investigated. Measurements of T_{2b} versus temperature have not been reported so far.

The temperature dependence of transmembrane water permeability and the possible occurrence of conformational transitions at the level of the molecular structures involved in the diffusional exchange of water are still subject of controversy. The Arrhenius plots $\ln \tau_a = f(10^3/T)$ were shown to change slope and activation energy at about 26°C in the case of both whole blood and washed erythrocytes suspensions doped with a rather high concentration of paramagnetic ions [23,6]. However, similar measurements performed for whole blood samples containing low manganese concentrations showed that the Arrhenius plots remained linear and no such 'breaks' occured between 5°C and 40°C [5].

In order to further clarify this point, we have investigated comparatively the temperature dependence of water permeability for both whole blood and washed cell samples containing different Mn^{2+} concentrations. Every τ_a value was computed using Eqn. 12 [5]. Data shown in the following plots were also corrected for the temperature dependence of both T_{2a} and T_{2b}. Previous data reported

TABLE I

ACTIVATION ENERGIES FOR THE APPARENT WATER EXCHANGE TIME THROUGH HUMAN RED BLOOD CELL MEMBRANES IN WHOLE BLOOD AND IN SAMPLES OF WASHED ERYTHROCYTES

No.	Method	Sample	Mn^{2+} in the extracellular environment (mM)	Temperature range (°C)	Discontinuity temperature (°C)	$\Delta G^{\neq}$ activation energy ($kJ \cdot mol^{-1}$)			Ref.
						Overall activation energy	Low-temperature activation energy	High-temperature activation energy	
1	NMR, T_2 (^1H)	Whole blood	1.7	40	–	20 ±0.2	–	–	5
2	NMR, T_2 (^1H)	Whole blood	1.7	40	–	22.4	–	–	24
3	NMR, T_2 (^1H)	Whole blood	1.7	40	–	19.8	–	–	25
4	NMR, T_2 (^1H)	Whole blood	5.0	30	–	12.1	–	–	This work
5	NMR, T_2 (^1H)	Whole blood	30.0	30	–	24.3	–	–	This work
6	NMR, T_2 (^1H)	Whole blood	39.0–79.0	40	20–30	22 ±0.6	17.7 ± 1.0	28.1 ± 1.3	23
7	NMR, T_2 (^1H)	Whole blood	Dextran-magnetite 1.3	40	–	22.2	–	–	24
8	NMR, T_2 (^1H)	Washed erythrocytes	5.0	30	–	21.0	–	–	This work
9	NMR, T_2 (^1H)	Washed erythrocytes	8.0	35	–	25.0	–	–	This work
10	NMR, T_2 (^1H)	Washed erythrocytes	18.0	35	26.5	–	2.1 ± 2.1	23.8 ± 1.7	6
11	NMR, T_2 (^1H)	Washed erythrocytes	30.0	35	28	–	16.8	37.5	This work
12	NMR, T_1 (^{17}O)	Washed erythrocytes (from outdated blood)	–	14	–	36.4 ± 4.2	–	–	26
13	Radiotracer ^3HHO	Washed erythrocytes	–	35	–	25.0 ± 0.7	–	–	27
14	Radiotracer	Washed erythrocytes	–	35	–	21.1 ± 1.7	–	–	1
15	Radiotracer ^3HHO	Bulk water (self-diffusion)	–	44	15	18.3	19.7	17.6	28

TABLE I (continued)

No.	Method	Sample	Mn^{2+} in the extracellular environment (mM)	Temperature range (°C)	Discontinuity temperature (°C)	$\Delta G^{\neq}$ activation energy ($kJ \cdot mol^{-1}$)			Ref.
						Overall activation energy	Low-temperature activation energy	High-temperature activation energy	
16	NMR, T_2 (^1H)	Washed erythrocytes (infants)	18	35	14	–	-2.1 ± 0.6	3.9 ± 0.4	This work
17	NMR, T_2 (^1H)	$MnCl_2$ aq. solution	5 mM	40	–	-2.5	–	–	This work
18	NMR, T_2 (^1H)	$MnCl_2$ aq. solution	8 mM	40	–	-2.7	–	–	This work
19	NMR, T_2 (^1H)	$MnCl_2$ aq. solution	18 mM	40	–	-2.0	–	–	This work

in the literature have been only corrected for T_{2a} temperature dependence. Both T'_{2a} and the corrected values of the water exchange time, τ_a, corresponding to a given manganese concentration were plotted together throughout in order to facilitate comparison of the data. Fig. 1 shows the Arrhenius plot of these parameters for a sample of washed erythrocytes doped with 5 mM Mn^{2+}, and 30 mM, respectively. The plots are linear and the exchange process is characterized by a single value of the activation energy over the entire temperature range at 5 mM Mn^{2+}. However, when measurements were performed in the presence of a high concentration of paramagnetic ions, a sharp break in the Arrhenius plot became apparent for both T'_{2a} and τ_a, in good agreement with previous results [6].

In the case of whole blood samples, no breaks in the Arrhenius plots were noticed, either at high (30 mM) or at low (5 mM) manganese concentrations. The $\Delta G^{\neq}$ values are included in Table I (entries 4, 5).

The investigation of washed red blood cells collected from infants has shown that their membranes behave quite differently with respect to the diffusional transport of water, as compared to adult erythrocytes. The Arrhenius plot in Fig. 2 consists of two different linear regions with slopes of opposite signs. These straight lines intersect at about 13.5°C. The average $\Delta G^{\neq}$ value for four cases are included in Table I.

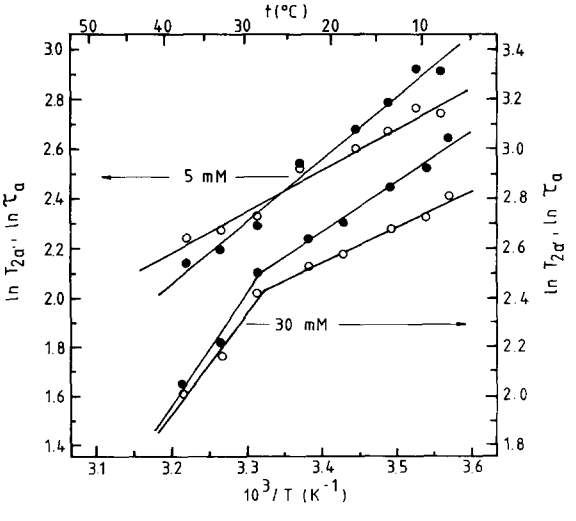

Fig. 1. Arrhenius plot of the apparent transverse relaxation time ln T'_{2a} (○——○), and of the transmembrane water exchange time, ln τ_a (●——●), versus reciprocal temperature for samples of washed erythrocytes containing: 5 mM and 30 mM Mn^{2+}, respectively, in the extracellular environment.

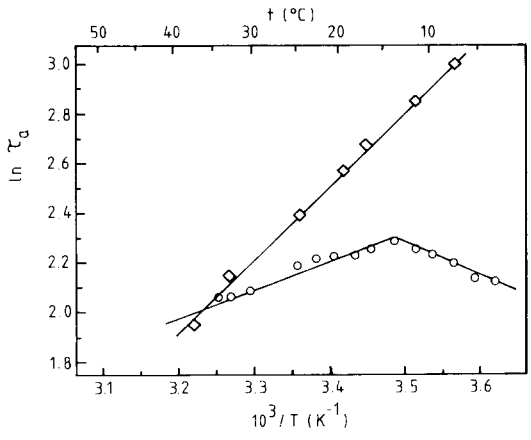

Fig. 2. Arrhenius plot of the water exchange time, ln τ_a, versus reciprocal temperature for a sample of infant blood paramagnetically doped with 18 mM Mn^{2+} (○——○) compared to a sample of adult blood (◇——◇).

Discussion

Our present data show that a break in the Arrhenius plot of the apparent water exchange time is evident only in the case of samples consisting of washed erythrocytes which are doped with relatively high manganese concentrations. No such discontinuity could be noticed for washed erythrocytes either at lower manganese concentrations or in cases when alternative experimental methods (other than NMR) were used [1]. The Arrhenius plots also remained linear in the case of adult whole blood, even at a relatively high manganese concentration (30 mM). The NMR doping method applied to erythrocytes from infants (performed only in Lab. A) has shown that these membranes have even a more peculiar behavior in that the Arrhenius temperature dependence has two linear regions and a maximum centered at about 13°C.

If all parameters are taken into consideration and used to calculate the apparent water exchange time, its value should be independent for example of the manganese concentration or cell fraction. We have for the first time considered every parameter and its temperature dependence for various experimental conditions yet the Arrhenius plots depend on both sample preparation and manganese concentration. We however conclude that certain experimental conditions are not appropriate to investigate the water diffusional process by the NMR doping method. The examination of Table I shows that in certain cases there is a good agreement between the NMR and non-NMR methods. The non-NMR methods give an activation energy $\Delta G^{\neq}$ ranging between 21–25 kJ/mol while similar values by the NMR doping methods are obtained with whole blood samples at low Mn^{2+} concentration (Table I, entries No. 1, 2, 3, 7); medium and high Mn^{2+} concentration (entries No. 5, 6); washed samples at low Mn^{2+} concentration (entry No. 8); and medium concentrations only in the higher temperature range (entry No. 10). Higher or lower values are obtained in two cases (entries No. 4 and 11). As the great majority of the activation energies are in good agreement with non-NMR values we may conclude that the NMR doping method works well from the point of view of the activation energy, regardless of manganese concentrations or sample preparation excepting washed erythrocyte samples at higher Mn^{2+} concentration in the lower temperature region. In the latter case, the activation energies for adult samples of blood are lower than for bulk selfdiffusion (Table I). Infant samples show even negative activation. These low positive or negative $\Delta G^{\neq}$ values have clearly no meaning for water diffusional exchange. The particular negative values for infant blood strongly suggest that manganese ions enter the cells. A comparison of $\Delta G^{\neq}$ for Mn^{2+} solutions of various concentrations show a good agreement (Table I) with the negative energies for infant blood in the lower temperature region. Entry of manganese is characterized by an activation energy of 54.4 kJ · mol^{-1} on the other hand [29].

We therefore suggest that the transition observed by the NMR method is caused by the presence of paramagnetic ions into the cells. As a result, water proton relaxation is caused by electron spin-nuclear spin dipolar coupling of Mn^{2+} with water protons and the apparent exchange time becomes shorter. In other words, the thermal transition revealed by the NMR method is artefactual from the point of view of the water exchange. It is essentially caused by the fact that two different processes are operative in the upper and lower range of temperature, respectively: in

the former case water exchange is the main process controlling the observed relaxation time while in the latter electron-nuclear spin coupling due to Mn^{2+} is operative. The average temperature of transition of about 26°C represents the onset of a significant Mn^{2+} entry into the cells when lowering the temperature. This is a different opinion to that expressed previously when the transition was attributed entirely to the water 'channels' of the membrane [6]. If the entry of Mn^{2+} is relatively fast, and a quasiequilibrium is achieved during the NMR experiment, then a further temperature decrease should reveal a typical $\Delta G^{\neq}$ value as for a manganese solution. This particular case appears to be valid for infant blood. A slower uptake should correspond to various small apparent activation energies as found in our experiments below the transition.

The fact that at lower Mn^{2+} concentration in washed samples no transition is observed may reflect the sensitivity limit of the NMR method to detect low Mn^{2+} concentrations. At the same time we know that Mn^{2+} uptake increases strongly with its concentration in the medium [29].

The lack of transition in whole blood samples regardless of Mn^{2+} concentration suggests that plasma prevents the uptake.

In conclusion the NMR doping method is potentially useful to investigate two distinct phenomena related to human erythrocytes: (a) water diffusional exchange and (b) Mn^{2+} uptake in washed erythrocyte samples.

References

1 Brahm, J. (1982) J. Gen. Physiol. 79, 791–819
2 Kuchel, P.W. (1981) Critical Rev. Anal. Chem., 12, 155–231
3 Conlon, T. and Outhred, R. (1972) Biochim. Biophys. Acta 288, 354–361
4 Morariu, V.V. and Benga, G. (1977) Biochim. Biophys. Acta 469, 301–310
5 Pirkle, J.L., Ashley, D.L. and Goldstein, J.H. (1979) Biophys. J. 25, 389–406
6 Morariu, V.V., Pop, V.I., Popescu, O. and Benga, G. (1981) J. Membrane Biol. 62, 1–5
7 Morariu, V.V. (1981) Rev. Roum. Phys. (Bucharest) 26, 617–625
8 Jalobeanu, C., Jalobeanu, M. and Morariu, V.V. (1983) Rev. Roum. Phys. (Bucharest) 28, 831–835
9 Levy, R., Zimlichman, R., Keynan, A. and Livne, A. (1982) Biochim. Biophys. Acta 685, 214–218
10 Cassoly, R., Daveloose, D. and Leterrier, F. (1980) Biochim. Biophys. Acta 601, 478–489
11 Fomenko, B.S. (1983) Stud. Biophys. 98, 169–174
12 Morariu, V.V. and Bucur, D.R. (1979) Oncologia (Bucharest) 18, 207–211
13 Morariu, V.V., Petrov, L. and Ordean, R. (1979) Oncologia (Bucharest) 18, 19–20
14 Morariu, V.V. and Petrov, L. (1982) Rev. Roum. Phys. (Bucharest) 27, 857–863
15 Lupea, I., Morariu, C.L., Morariu, V.V. and Ordean, R. (1978) Pediatria (Bucharest) 27, 261–263
16 Benga, G. and Morariu, V.V. (1977) Nature (Lond.) 265, 636–638
17 Morariu, V.V. and Benga, G. (1984) in Membrane Processes: Molecular Biology and Medical Applications (Benga, G., Baum, H., and Kummerow, F.A., eds.), pp. 121–139, Springer Verlag, New York
18 Mildvan, A.S. and Cohn, M. (1963) Biochemistry 2, 910–918
19 Fabry, M.E. and Eisenstandt, M. (1975) Biophys. J. 15, 1101–1110
20 Farrar, T.C. and Becker, E.D. (1971) Pulse and Fourier Transform NMR, Academic Press, New York
21 Woessner, D.E. (1963) J. Chem. Phys. 39, 2783–2787
22 Giberman, E. (1973) Experientia (Basel) 29, 1083–1085
23 Conlon, T. and Outhred, R. (1978) Biochim. Biophys. Acta 511, 408–418
24 Ashley, D.L. and Goldstein, J.H. (1980) Biochem. Biophys. Res. Commun. 97, 114–120
25 Small, W.C. and Goldstein, J.H. (1982) Biochim. Biophys. Acta 720, 81–86
26 Shporer, M. and Civan, M.M. (1975) Biochim. Biophys. Acta 385, 81–87
27 Vieira, F.L., Sha'afi, R.I. and Soloman, A.K. (1970) J. Gen. Physiol. 55, 451–466
28 Mills, R. (1973) J. Phys. Chem. 77, 685–688
29 Weed, R.I. and Rothstein, A. (1960) J. Gen. Physiol. 44, 301–313