Biochimica et Biophysica Acta, 511 (1978) 408—418

BBA 78111

THE TEMPERATURE DEPENDENCE OF ERYTHROCYTE WATER DIFFUSION PERMEABILITY

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(Received September 21st, 1977)

Summary

1. The activation energy of the diffusion water exchange in red blood cells increases with temperature.

2. Fetal blood has a higher activation energy for diffusion water exchange than adult blood.

3. Treatment of red cells with *p*-chloromercuribenzoate alters the activation energy and apparently allows a lipid and a protein pore pathway to be resolved. The permeability and activation energy of the treated cells is in the range found for lipid membranes; and the difference between treated and untreated cells, the "protein" pathway, has a diffusion activation energy comparable with that of free water.

4. A resolution of the discrepancies between the NMR methods of measuring diffusion water exchange is suggested.

Introduction

The diffusion of water across the erythrocyte membrane has been studied for twenty years. Interest has centred on whether the mechanism is similar to that of the free diffusion of water, on whether the pathway is located in membrane lipid or protein or both, and on whether there exist water filled pores in the membrane. The temperature dependence of water diffusion in the membrane, usually expressed as an activation energy, has been measured in the course of these studies.

At the molecular level, the mechanism of diffusion in liquids is not settled. The validity of the activated complex theory [1], once a mainstay in the area has been called into question [2]. Although activation energies may remain

Abbreviations: PCMB, *p*-chloromercuribenzoate; PCMBS *p*-chloromercuribenzene sulfonate.

useful for purposes of comparison, caution is necessary in interpreting them in terms of molecular events. If two processes have the same mechanism then their activation energies should be the same. The converse is, of course, not necessarily true, but in the the case of the diffusion of the one species, water, similar activation energies increase the likelihood that the mechanisms are similar.

We report here that a single activation energy does not give the best fit to the observed temperature dependence of the water diffusion: two activation energies, one for low and one for high temperatures, give a better fit. In the light of this and other results, we examine the case for a dual lipid-protein pathway for the water flow.

Methods

The NMR technique used here has already been described [3,4]. Transverse relaxation of water proton was monitored in whole blood diluted with isotonic manganese saline solution. The observed relaxation is resolvable into two exponential components. The more rapid, with a time constant T_{2a} of approx. 0.5 ms, derives from relaxation of extracellular water protons. The second component, with a time constant T_{2b} of the order of 10 ms, is due to intracellular water. The water diffusion exchange time T_e is extracted from the latter component after correcting for intracellular decay, which takes place with a time constant T_{2i} of the order of 100 ms, by using the relationship

$$1/T_e = 1/T_{2b} - 1/T_{2i} \tag{1}$$

The membrane permeability for water diffusion, P, is related to $1/T_e$ and the cell water volume, V, and the cell surface area, A, by

$$P = (V/A)(1/T_e) \tag{2}$$

Transverse relaxation was measured by the standard 90°—180° echo decay technique at a frequency of 8 MHz.

Blood was collected by venipuncture and stored in heparinised tubes. The effect of storage conditions was tested. After 24 h at 20°C, blood then doped with Mn solution was found to have a T_{2b} the same as at the time of collection; after 48 h, T_{2b} had risen by 5%. After 48 h storage at approximately 4°C, T_{2b} had not changed, but after 90 h it had increased by 10%. T_{2i} did not change significantly. In subsequent measurements then, refrigerated blood generally 24 h old, and not more than 48 h old, was used.

Transverse relaxation in Mn doped whole blood was the same as in cells washed twice in saline, resuspended in saline and then doped.

The volumes used were approximately 0.4 ml of whole blood and 0.9 ml of Mn solution (50 or 100 mM $MnCl_2$ made isotonic with NaCl), giving a packed cell volume of about 18%. It was found necessary to keep the packed cell volume below 20% in order to eliminate any dependence of relaxation time on the packed cell volume.

The transverse relaxation time of the cell interior, T_{2i}, was also measured by

TABLE I

RED CELL T_{2i} AS A FUNCTION OF TEMPERATURE

Values at 3, 25 and 45°C were measured; the other values are interpolated.

Temperature (°C)	3	5	10	15	20	25	30	35	40	45
T_{2i} (ms)	90	90	105	115	125	135	145	155	160	165
S.D. (n)	5 (3)					12 (8)				20 (8)

the 90°—180° method, using packed cells from which the supernatant, with no added Mn, had been removed by centrifuging twice. The results for blood from 3 persons at 3°C and 8 persons at 25 and 45°C are shown in Table I. It can be seen that the variability is not large. The addition of PCMB to the cells in the absence of Mn did not affect T_{2i}.

The contribution of errors in T_{2i} to the measurement of T_e is reduced by a factor of T_{2b}/T_{2i}, compared to the contribution of errors in T_{2b}, as can be seen from Eqn. 1. In view of this, values for T_{2i} at temperatures other than those mentioned above were obtained by interpolation; the values are shown in Table I.

In the upper temperature range, the T_{2b} of Mn doped blood fell slowly with time, the more so if the blood had been incubated in PCMB. As previously discussed [3], this is probably due to Mn slowly entering the cells and shortening T_{2i}. The effect was dealt with either by making the measurement quickly enough to obviate it, or where this was not possible, by following the time course of the decline in T_{2b} and correcting by extrapolation back to time zero. It was thus possible to use values of T_{2i} obtained from cells which had not been in contact with Mn.

It was found, with Macey and Farmer [5], that the inhibiting effect on water exchange of incubating cells for 1 h in PCMB, or the sulphonate derivative PCMBS, increased with concentration up to about 1 mM; above this, the effect was independent of concentration. Concentrations of 2 mM in isotonic NaCl were therefore used. The effect of PCMBS was indistinguishable from PCMB. Washing the cells in saline before or after incubation did not alter the effect.

The specimen temperature in the spectrometer was controlled to within

TABLE II

EFFECT OF TEMPERATURE ON RED CELL VOLUME

(a) Isotope dilution method. Hematocrits relative to that at 22°C. (Results of Vieira et al. [6]).

Temperature (°C)	0	22	37
Mean	0.97	1.00	1.02
S.D. (n = 4)	0.024	0.000	0.022

(b) Centrifugation method. Hemotocrits relative to that at 25°C.

Temperature	25	40	44
Mean	1.00	1.02	1.01
S.D. (n = 4)	0.000	0.032	0.017

±1°C below and 20°C and ±0.5°C above. Sequential and random temperature orders were used: the results were the same.

Vieira et al. [6] have measured the dependence of red cell volume on temperature by an isotope dilution method. This was redetermined by measuring the packed cell volumes with a microhematocrit centrifuge placed in an air oven. The results are similar (Table II).

Results

(a) Temperature dependence

Fig. 1 shows the Arrhenius plot of the water diffusion exchange rate from the blood of several donors. The activation energies are shown in Table IV. It is clear by inspection of Fig. 1 and from the correlation coefficients that a two line fit is superior to a one line fit. The two lines were obtained by varying the junction between them so as to maximise the correlation coefficient of each. The junctions are in the range 20 to 30°C. The mean overall activation energy is 22.0 ± 0.6 kJ · mol^{-1}; and two line means are 17.6 ± 1.0 kJ · mol^{-1} at lower temperatures and 28.1 ± 1.3 kJ · mol^{-1} at higher temperatures.

The activation energy found by the triated water method [6] was 25.1 ± 0.8 kJ · mol^{-1}, higher than our overall value. The large volume of blood needed with this method meant that it was not possible to measure the same blood at the three different temperatures used, 4, 22, and 37°C. The NMR (B) method [10] (see Table III) yielded a value of 36.4 ± kJ · mol^{-1} over four temperatures between 23 and 37°C, above both our overall and higher temperature values.

One of the samples in Fig. 1 was from a patient with Duchenne muscular dystrophy and two were from mothers of patients. The water exchange rate is the same as in normal cells. The implications will be discussed elsewhere.

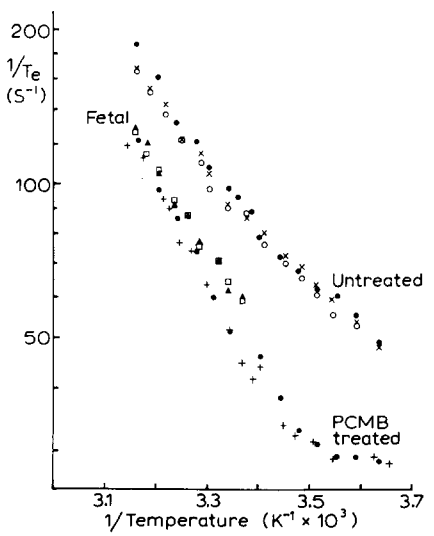

Fig. 1. Red cell water diffusion exchange rate ($1/T_e$) as a function of temperature, including the effect of PCMB. (● ♂ 34 yrs., normal; ○ ♀ 34 yrs., mother of a DMD patient but probably not a carrier; X ♀ 27 yrs., DMD carrier; + ♂ 6 yrs. DMD patient; ▲ □, normal fetal blood).

TABLE III

HUMAN RED CELL WATER DIFFUSION EXCHANGE TIMES

Units ms. Standard deviations are quoted where available.

Room temperature	37°C	Method	Reference
Adult cells			
8.4 ± 2.0 (23°C, n = 7)	—	^{3}HHO [a]	Paganelli and Solomon [9]
11.0 ± 2.7 (23°C, n = 10) [b]	—	^{3}HHO	Barton and Brown [7]
13.0 ± 4.0 (22°C, n = 10) [c]	8.9 ± 1.6 (n = 5) [c]	^{3}HHO	Vieira et al. [6]
—	8.2 ± 0.31 (n = 8)	NMR (A) [d]	Conlon and Outhred [3]
11.0 ± 0.4 (25°C, n = 9)	7.15 ± 0.15 (n = 10)	NMR (A)	This paper
12.2 ± 1.0 (20°C, 12 subjects)	—	NMR (A)	This paper
16.7 (25°C)	9.4	NMR (B)	Shporer and Civan [10]
21.7 ± 2.9 (25°C)	—	NMR (C)	Fabry and Eisenstadt [11]
Fetal cells			
17.9 ± 4.1 (23°C, n = 17) [b]	—	^{3}HHO	Barton and Brown [7]
16.4 (25°C)	10.4	NMR (A)	This paper

[a] This value is low for reasons discussed by Barton and Brown.

[b] The temperature is assumed to be 23°C from the discussion of the results of Paganelli and Solomon and from the discussion by Vieira et al. of their results.

[c] Values calculated by us from data points in their Fig. 4.

[d] NMR method (A): transverse relaxation (T_2) of water protons with added extracellular Mn. (B): longitudinal relaxation (T_1) of added $H_2{}^{17}O$. (C): longitudinal relaxation (T_1) of water protons with added extracellular Mn.

It can be seen from Fig. 1 that at say 40°C, the value of $1/T_e$ expected by extrapolation from the low temperature range would be about 125 s^{-1}, compared with the observed value of about 150 s^{-1}, an increment of 20%. This increment could be caused by temperature dependence of any of the parameters V, A or P in Eqn. 2. The change in cell water volume, V, with temperature is negligible (Table II). No evidence is to hand on the behaviour of the cell area, A, with temperature. To explain the result, however, A would have to decrease as the temperature was raised, and in such a way that V did not change. Neither seems likely. We conclude then, that the most likely cause is an increase in P.

Fetal blood has a higher activation energy than adult blood in the upper temperature range. (Not enough blood was available to examine the lower temperature range.) The exchange rate for fetal blood is lower than that of adult blood by a factor of about 1.5 at 25°C, in agreement with the ^{3}HHO result [7] (Table III). The permeability is lower by the same factor since the value of the ratio V/A is the same for adult and fetal blood [7], namely 0.37 μm.

Effect of PCMB

At 22°C, treatment with 2 mM PCMB lowered $1/T_e$ by a factor of 0.54 (S.D. = 0.025, n = 7 persons). Macey et al. [8], using the ^{3}HHO method report a single measurement of this factor of about 0.4. Their temperature and concentration were not specified, so the significance of the difference between these results cannot be assessed.

The Arrhenius plot of the water diffusion exchange of blood treated with

TABLE IV

RED CELL WATER TRANSPORT ACTIVATION ENERGIES

Sample	Overall E^a (kJ · mol^{-1})	r^2 (n)	Low temperature E^a (kJ · mol^{-1})	r^2 (n)	High temperature E^a (kJ · mol^{-1})	r^2 (n)
● TC	22.7	0.973 (15)	16.4	0.991 (7)	28.5	0.983 (8)
X RP	21.6	0.988 (15)	18.2	0.996 (9)	26.6	0.981 (6)
○ MMcD	21.6	0.983 (15)	18.2	0.981 (8)	29.1	0.994 (7)
Mean ± S.D.	22.0 ± 0.6		17.6 ± 1.0		18.1 ± 1.3	
With PCMB						
● TC	—		—		40.5	0.993 (7)
+ APa	—		—		37.3	0.990 (9)
Fetal						
□ PC	—		—		31.5	0.997 (9)
▲ LP	—		—		31.7	0.986 (9)
Other methods (for comparison)						
	25.1 ± 0.8 (^{3}HHO [6])		—		—	
	36.4 ± 4 (NMR (B) [10])		—		—	
Osmotic transport (for comparison)						
	14 [6]		—		—	
	20 [8]		—		—	
With PCMB	48 [8]		—		—	

PCMB (Fig. 1) is linear in the high temperature range, the activation energy being about 40 kJ · mol^{-1}, significantly higher than that of untreated cells. The slope decreases below 30°C and approaches zero near 0°C.

Discussion

(a) Comparison with other results

The water diffusion permeability of the erythrocyte membrane has now been measured by four different methods, one (^{3}HHO) using triated water as tracer [6,7,9], and three NMR techniques, which we shall refer to as NMR (A) [3,4], (B) [10,11] and (C) [11] (see Table III for details), using magnetically labelled water as tracer. The available results at room temperature and 37°C are collected in Table III. (One published ^{3}HHO result [8] is excluded because of uncertainty about the temperature.)

The earliest measurements were by the ^{3}HHO method. There is a minor inconsistency in the ^{3}HHO results at room temperature. Vieira et al. [6] state that they find a value of ω $\{= (V/A)/(T_eRT)\}$ in agreement with that of Barton and Brown [7], yet the exchange time found from the data points in their Fig. 4 is some 18% higher than that quoted by Barton and Brown. The reason for this difference is not known. Our present results agree with those of Barton and Brown, both for adult and fetal cells.

The later NMR methods have in some instances given results substantially higher. To understand the discrepancies between the NMR results, it is necessary to consider the techniques in some detail.

There are at least three substantive differences between the NMR (A) method and the other two. In the NMR (A) method, transverse relaxation (T_2) is observed, conditions are such that there is a significant flux of labelled water in one direction only and there is a substantial extracellular concentration of added Mn. In the NMR (B) and (C) methods, the longitudinal relaxation (T_1) is measured, appreciable fluxes of labelled water occur both into and out of the cell and there is little or no added Mn.

As has been pointed out [11], transverse relaxation can, under certain circumstances, be complicated by the effect of chemical shifts. To eliminate this in the NMR (A) method, we measured the relaxation only after the plasma signal had become negligible, and when using phase sensitive detection were careful to optimise the reference frequency. Diode detection yielded substantially similar relaxation times, and the second phase of the relaxation always appeared to be strictly exponential. In view of these precautions, we feel it unlikely that chemical shifts were a source of error.

The longitudinal relaxation used in methods (B) and (C) avoids the above problems, but is open to the possible difficulty that transport of the magnetisation can occur through spin diffusion without material transport [12].

In order to determine a permeability, it is necessary to know the flux and the concentration gradient. In NMR (A), the conditions were such that the external concentration of labelled water was effectively zero at the time of measurement. This simplified the determination of the concentration gradient. On the other hand in methods (B) and (C), there was a significant concentration of labelled water on both sides of the membrane. The label concentration

will then, in general, be non-uniform over the extra cellular space, and as noted previously [3], a rigorous evaluation of the label concentration at the membrane boundary requires the solution of a diffusion equation. To avoid this, the simplifying assumption of uniform concentration has been used in methods (B) and (C).

With methods (B) and (C) Fabry and Eisenstadt [11] maintain that this assumption is valid. Our own calculations based on a 1 dimensional diffusion equation suggest that the consequent errors would be not more than about 10% if the cells were evenly spaced. It is well known, however, that in whole blood not subject to shear forces, the red cells rapidly aggregate into rouleaux [13]. Our own observations indicate that rouleaux formation was likely under the conditions of NMR (B) and (C) as used by Fabry and Eisenstadt. In the presence of rouleaux, the assumption of uniform extracellular concentration of labelled water is unlikely to be valid. Qualitatively, we would expect that the concentration of labelled water in the dead spaces between adjacent cells in the rouleaux would be higher than in the bulk extracellular phase. For long extracellular relaxation times, a rouleau of aggregated cells would tend to behave as a single exchanging unit. Only when the extracellular relaxation time was short would sufficient decay occur in the intercellular dead spaces for the cells to be resolved into discrete exchanging units. The surface/volume ratio of a rouleau composed of more than 4 or 5 cells would be approximately half that of a single cell, giving a corresponding doubling of the exchange time. Thus we would expect that at long extracellular relaxation times, the observations should fit a simple two compartment model but with an exchange time approximately double that of a single cell. As the relaxation time is shortened, a transition should occur to the single cell result. The results of Fabry and Eisenstadt (their Fig. 3) appear to show such a transition from an exchange time of about 22 ms to a limiting value at short relaxation times of about 10 ms. Results using NMR (A) would not suffer from this effect for two reasons: the extracellular relaxation time is very short and rouleaux do not form, apparently due to the saline dilution of the plasma. In addition, it is not clear whether Fabry and Eisenstadt allowed for the effect of the varying tonicity of the external solution on the cell volume and hence on the exchange time. Such effects could explain the discrepancy between Fabry and Eisenstadt's NMR (B) and (C) results on the one hand, and the NMR (A) and the ^{3}HHO results on the other. We feel this is at least as likely an explanation as invoking a possible effect of Mn on the membrane [11]. As noted previously, the exchange time does not depend on the Mn activity over a 100 fold range [3], and the results obtained by NMR methods (A) are comparable with those obtained by the ^{3}HHP method in the absence of Mn.

Shporer and Civan [10] used method (B) with cells resuspended in saline, thus possibly avoiding rouleaux formation and their exchange times are lower than those of Fabry and Eistenstadt using the same method, although still higher than the ^{3}HHO and NMR (A) results. Their results appear to show an unexplained dependence of water turnover rate on packed cell volume (their Fig. 3). In addition, "outdated blood" was used; as noted above we found that the exchange time increased with the age of the blood.

(b) Activation energies and diffusion pathways

The activation energy for the self diffusion of free water has recently been remeasured with care [14]. The values are 19.7 kJ · mol^{-1} in the range 1° to 15°C and 17.6 kJ · mol^{-1} in the range 15° to 45°C. Changes in the properties of water with temperature can therefore be ruled out as causing an increase in red cell water diffusion activation energy. In fact, in the low temperature range, we find an activation energy, 17.6 kJ · mol^{-1}, apparently a little lower than that for free diffusion. Changes in the membrane, may be the cause of this (see below).

Macey et al. [8] have proposed that PCMB blocks the protein pathway for water flow, leaving a lipid pathway only. In support of this proposal, they have shown that with PCMBS-treated cells, the ratio of osmotic to diffusion flow approaches unity, and that the activation energy for osmotic flow approaches that for a lipid bilayer, namely 50 to 60 kJ · mol^{-1}. We now examine our results in the light of this hypothesis.

If water diffusion proceeds by parallel lipid and protein pathways characterised by exchange rates $1/T_l$ and $1/T_p$ respectively, then the observed exchange rate $1/T_e$ would be given by the relation

$$1/T_e = 1/T_l + 1/T_p \tag{3}$$

From Fig. 1, at 20°, $1/T_e$ is 77 s^{-1} and $1/T_l$, estimated from the PCMB curve would be 40 s^{-1}, giving a value for $1/T_p$ of 37 s^{-1}. Thus, on this hypothesis, the water flow is roughly evenly divided between the lipid and protein pathways. The permeability of the membrane lipid to water would be about 15 μm · s^{-1} {from Eqn. 1 with V/A = 0.37 μm.} and the activation energy at higher temperatures about 40 kJ · mol^{-1}.

How do these values compare with the water permeabilities of lipid membranes? Relevant data are collected in Table V. The range of values is large and no clear conclusion is possible except perhaps that Macey's hypothesis is not refuted. Close agreement would not be expected unless the membranes had the

TABLE V

WATER DIFFUSION PERMEABILITIES OF LIPID MEMBRANES

Membrane	Permeability		Activation energy	Method	Reference
	μm · s^{-1}	°C	(kJ · mol^{-1})		
Red cell lipid (?)	15	20	40	NMR (A)	This paper
Bilayer of lecithin, cholesterol, *n*-decane	18—21	20	—	^{3}HHO	Everitt et al. [17]
Vesicle of lecithin, cholesterol	8	22	50 ± 8	NMR (B)	Haran and Shporer [16]
Bilayer of lecithin	4—5	20	—	^{3}HHO	Vreeman [18]
Vesicle of dipalmitoyl lecithin	1.7	20	63 ± 4	NMR (A) and NMR (C) modified	Andrasko and Forsen [19]

same lipid composition as red blood cell membranes and it was demonstrated that PCMB completely blocked the protein pathway.

Other properties of red cell lipid alter near 20°C. The activation energy for spin label motion in the membrane, as indicated by the esr detection of a probe molecule, shows changes at 18 and 24°C (Raison, J., unpublished results); and the viscosity of membrane fragments shows a discontinuous increase at 18 to 19°C [15]. Near 0°C, a horizontal Arrhenius plot has been found for the water permeability of phospholipid vesicle membranes [16].

It is easy to show algebraically that the Arrhenius plot of a process which is the sum of two parallel processes is, in general, non-linear. If the two pathway model for water diffusion is correct, it would not be surprising if the overall Arrhenius plot were non-linear. The plot of the separate pathways might be expected to be linear, however, unless special factors intervene, as is probably the case with the membrane lipid. To examine the activation energy of the putative protein pathway, we subtract measurements of $1/T_1$ (PCMB treated cells) from $1/T_e$ (untreated cells) to obtain $1/T_p$ as a function of temperature. The result is shown in Fig. 2. The slope corresponds to an activation energy of 16.9 ± 1.1 kJ · mol^{-1} ($r^2 = 0.915$) comparable with that for water self diffusion. This is consistent with water diffusing through protein channels wide enough as not to cause hindrance. If the channels did hinder the diffusion, it would be expected that at least the activation free energy would rise, as has been shown in a model system [20].

As for the osmotic water exchange in the red cell, Vieira et al. [6] report a value of 14 ± 1.7 kJ · mol^{-1} for the activation energy based on measurements at close temperature intervals in the range 5 to 40°C. They reported no dependence of the activation energy on temperature, although the scatter of their data is high and could conceivably mask a small effect on temperature (their Fig. 6). Macey et al. [8] have quoted a higher value of 20 kJ · mol^{-1} for the same parameter, rising to 48 kJ · mol^{-1} on treatment with PCMB (their Fig. 3) based

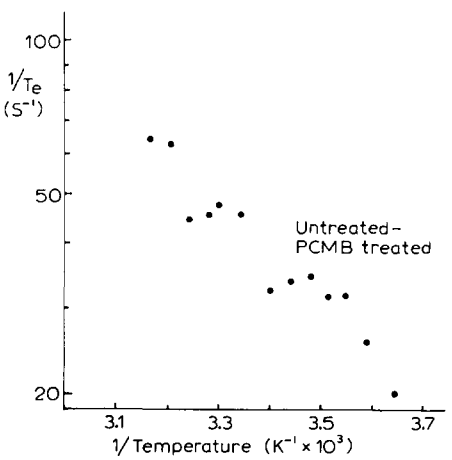

Fig. 2. Residual red cell water diffusion exchange rate (1/*Te*) as a function of temperature. The residual is the difference between the rates for untreated cells and cells treated with PCMB, for the data points • in Fig. 1.

on measurements at four temperatures in the range 10 to 37°C. It would be useful to have this data for PCMB treated cells at small temperature intervals to see if there was any similarity to the temperature dependence of diffusion exchange.

Acknowledgements

This work was supported by the National Health and Medical Research Council of Australia and the Muscular Dystrophy Association of America. We thank Drs. A. Kidman, G. Morgan and J. Raison for their generous advice and assistance, and the School of Physics and Material Science, N.S.W. Institute of Technology for the use of NMR equipment.

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