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# Effects of Temperature and pH on the Water Exchange through Erythrocyte Membranes: Nuclear Magnetic Resonance Studies

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**Summary.** The temperature and pH dependence of water exchange has been studied on isolated erythrocytes suspended in isotonic buffered solutions. At pH 7.4 a break in the Arrhenius plot of water exchange time at around 26 °C was found. The mean value of the apparent activation energy of the water exchange time at temperatures higher than that of the discontinuity was 5.7 kcal/mole (±0.4); at lower temperatures the values of the apparent activation energy were below 1.4 kcal/mole. The pH dependence of water exchange time of isolated erythrocytes revealed a marked increase of the water exchange time values in the acid range of pH; a much smaller variation of the same parameter occurs between pH 7.0 and 8.0. These finding could be correlated with other processes involving erythrocyte membranes that showed similar pH and temperature dependence and were considered to indicate state transitions in the membranes. It is suggested that the temperature and pH effects on water diffusion indicate that conformational changes and cooperative effects are implicated in the mechanism of this transport process.

The temperature dependence of water exchange through erythrocyte membranes has been investigated by several authors (Vieira, Sha'afi & Solomon, 1970; Shporer & Civan, 1975; Morariu & Benga, 1977). None of these investigators reported significant deflections of the activation energy at a certain temperature. However, other investigators revealed breaks in the temperature dependence of the following erythrocyte membrane parameters: viscosity or microviscosity (Zimmer & Schirmer, 1974; Feinstein, Fernandez & Sha'afi, 1975), osmotic fragility (Aloni, Eitan & Livne, 1977), enzymic incorporation of $^{32}P$ into polyphosphoinositides (Buckley & Hawthorne, 1972), transport of chloride and bromine (Brahm, 1977), exchange transport of glucose (Lacko, Witke & Geck, 1973). These phenomena have been discussed by some authors in terms of phase transitions in erythrocyte membranes, occurring around 19–25 °C. A systematic analysis of the temperature dependence of water transport through erythrocyte membrane should reveal the effect of such transitions on this transport process.

On the other hand, it has been suggested from Raman spectroscopical studies that erythrocyte membranes undergo cooperative, pH-sensitive transitions in the physiological temperature range (Verma & Wallach, 1976). It is important to know if these transitions have any effects on the water transport. However, to our knowledge no studies concerning the effect of pH on the water transport have been reported so far.

The aim of our paper was to present a study of temperature and pH-dependent changes of water transport and to provide a link to the above-mentioned studies of erythrocyte membrane processes. This will give us a better understanding of the molecular mechanisms of erythrocyte water transport. In the present work the water exchange time through isolated erythrocytes, measured by an NMR[1] technique, appeared to be sensitive to changes in both temperature and pH. A break in the Arrhenius plot at 26 °C and a particular shape for the pH dependence of the water exchange time were found. These findings are discussed in relation with other membrane processes that showed a similar behavior, and the signifi-

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[1] *Abbreviations*: NMR = nuclear magnetic resonance; $T_{ae}$ = water exchange time; $t_{disc}$ = the temperature (°C) where a discontinuity in the temperature dependence of the water exchange through erythrocyte membrane occurred.

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cance of pH and temperature effects with regard to the molecular mechanisms proposed for water transport is considered.

## Materials and Methods

Human blood was obtained by venipuncture, in heparinized tubes, and used within 4 hr. The donors were healthy male subjects, 20–40 years old. The determinations have been performed on washed erythrocytes, suspended in solutions of controlled pH. The sample for the NMR measurements was prepared by carefully mixing 1 ml cell suspension with 0.5 ml doping solution (40 nM $MnCl_2$ and 110 mM NaCl).

The water exchange time $T_{ae}$ was measured using the method of Conlon and Outhred (1972), as previously described (Morariu & Benga, 1977). This method will be briefly outlined in this section. The spin-spin relaxation time of the water proton inside the isolated erythrocytes ($T_{2a}$) is about 140 msec and is much longer than the time required for water to exchange through the membrane (the water exchange time, $T_{ae}$). As the relaxation time in plasma ($T_{2b}$) is made much shorter than the exchange time, by adding a paramagnetic ion such as $Mn^{2+}$, the observed relaxation time of the erythrocytes ($T'_{2a}$) will be dominated by the exchange process through the membrane. Thus $T'_{2a}$ will be shortened compared to $T_{2a}$ because of water proton exchange between the erythrocytes and the plasma. The observed relaxation time is related to the exchange time by the equation:

$$T'^{-1}_{2a} = T^{-1}_{2a} + P_b/(P_b\ T_{ab} + T_{2b}) \quad (1)$$

where $P_b$ is the fraction of doped water in the suspending solution or in the plasma (Morariu & Benga, 1977). This has an average value of 0.734 for normal blood. If $P_a$ is the water fraction inside the erythrocytes, then obviously $P_a + P_b = 1$. The value of $T_{2b}$ is about 0.9 msec. In the actual NMR experiment two relaxation times are detected: a short one corresponding to doped water ($T_{2b}$) and a longer component which is $T'_{2a}$. Therefore all parameters in Eq. (1) are known and the exchange time can be calculated.

The spin-spin relaxation time was measured by using a 90° $\tau$ 180° pulse sequence where $\tau$ is the time interval between the radiofrequency pulses. The value of $T'_{2a}$ was measured from the spin-echo attenuation in the region $16 < 2\tau < 26$ msec.

The measurements were performed with a Bruker SXP 4-100/15″ spectrometer at 90 MHz. The temperature was controlled to ±0.2 °C by air flow over an electrical resistance for temperatures higher than room temperature. At lower temperatures a stream of cooled nitrogen was used to control the temperature to ±0.25 °C or better. In both cases the actual temperature in the blood sample was controlled with a thermocouple.

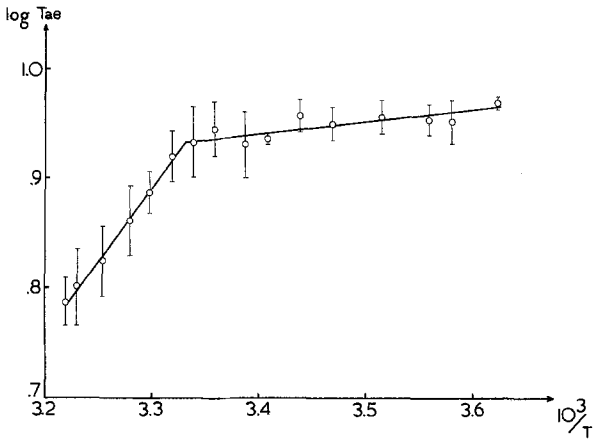


**Fig. 1.** Arrhenius plot of the water exchange time ($T_{ae}$) through erythrocyte membrane. The measurements have been performed on erythrocytes isolated from the blood of five donors. The erythrocytes were concentrated by centrifugation, then washed three times in 150 mM NaCl, 5 mM HEPES (pH 7.4), 1% albumin and 1% glucose to the initial hematocrit of the blood. The results are the mean (o) and the standard deviation (the bars). The plot has been computed by using the least squares method

**Table 1.** Parameters characterizing the temperature dependence of the water exchange time ($T_{ae}$) through erythrocyte membranes at pH 7.4[a]

| Donor | Age (years) | $E_t > t_{disc}$ (kcal/mol) | $E_t < t_{disc}$ (kcal/mol) | $t_{disc}$ (°C) | $T_{ae}$ (msec) |
|---|---|---|---|---|---|
| 1 | 22 | 5.3 | 0 | 26 | 6.8 |
| 2 | 27 | 5.5 | 1.4 | 26 | 6.0 |
| 3 | 30 | 5.8 | 0.6 | 27 | 5.9 |
| 4 | 35 | 6.2 | 0 | 26 | 5.9 |
| 5 | 46 | 5.5 | 0.7 | 26.5 | 6.0 |
| | Mean value | 5.7 ± 0.4[b] | 0.5 ± 0.5[b] | | |

[a] $t_{disc}$ is the temperature of discontinuity in the Arrhenius plot of the water exchange time through erythrocyte membranes. $E_t > t_{disc}$ is the activation energy at temperatures higher than $t_{disc}$, and $E_t < t_{disc}$ is the activation energy at temperatures lower than $t_{disc}$. The measurements were performed on erythrocytes isolated in buffered solutions at pH 7.4, as described in the legend of Fig. 1.

[b] Standard deviation.

## Results

### *The Temperature Dependence of the Water Exchange of Isolated Erythrocytes at pH 7.4*

The temperature dependence of $T_{ae}$ was examined for five samples isolated from different donors. The Arrhenius plot of $T_{ae}$ of these samples is shown in Fig. 1. In the higher temperature range $T_{ae}$ varies markedly with temperature, whereas at lower temperature it is little dependent on temperature. There is an obvious discontinuity in this plot. The break is around 26 °C for erythrocytes isolated and suspended in buffered solutions at pH 7.4. Two values of the apparent activation energy of the water exchange time through the erythrocyte membranes can be calculated on the basis of this plot, one for temperatures lower and the other for temperatures higher than that of the break in the Arrhenius plot. The values of the apparent activation energy, together with the values of temperature at which the discontinuity in the Arrhenius plot is noticed and the value of the water exchange time $T_{ae}$ at 37 °C are given in Table 1.

The effect of temperature on the water exchange time was fully reversible. The same values of $T_{ae}$ were

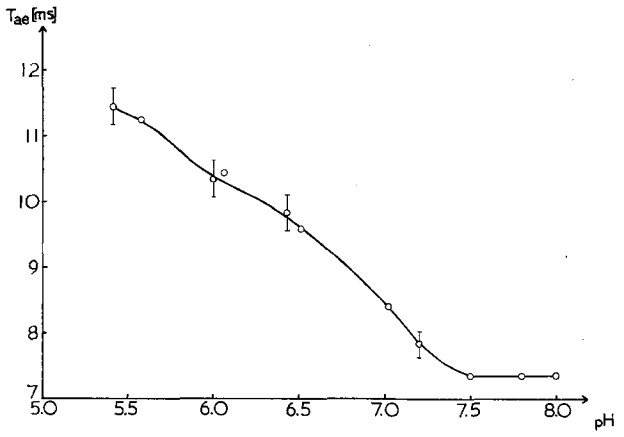


**Fig. 2.** The pH dependence of the water exchange time through erythrocyte membrane at 24 °C. The erythrocytes from the same volume of blood were isolated and washed in 150 mM NaCl buffered with 5 mM HEPES of various pH values and then suspended in the same solution supplemented with albumin (1%) and glucose (1%). The results are the means (○) and standard deviations (the bars) of measurements performed in duplicate or triplicate on blood from two donors

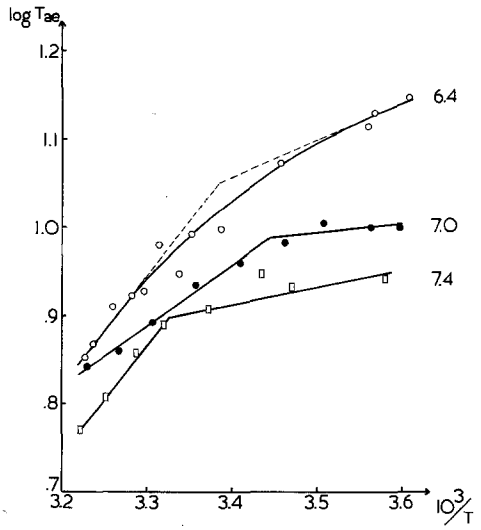


**Fig. 3.** Arrhenius plots of water exchange time through erythrocyte membranes at pH 6.4, 7.0, and 7.4. For each pH value the erythrocytes were isolated and suspended in a buffered solution as described in the legend of Fig. 2

obtained when the samples were run from 37−2 °C and then back to 37 °C.

The mean value of the activation energy of the water exchange time at temperatures higher than that of the discontinuity is 5.7 kcal/mole. This corresponds to the lower limit of the same parameter reported previously by us (Morariu & Benga, 1977) and it is somewhat lower than the corresponding value given by Conlon and Outhred (1978). The values of the apparent activation energy of the $T_{ae}$ at temperatures lower than that of the break vary between zero and 1.4 kcal/mole. It seems that there is no correlation with the age of donor.

### *The pH Dependence of the Water Exchange Time of Isolated Erythrocytes*

The particular shape of the pH dependence at 24 °C of samples of erythrocytes prepared from two donors is shown in Fig. 2. Obviously, in the acid range of pH there is a marked increase of the water exchange time values. A much smaller variation of the same parameter occurs between pH 7.0 and 8.0.

The temperature dependence of $T_{ae}$ was measured on isolated erythrocytes at pH=6.4; 7.0, and 7.4 (Fig. 3). At pH 6.4 the break in the Arrhenius plot is much less evident than at pH 7.4. However, a value of $t_{disc}$ could be estimated by intersecting the tangents at the two extremes of the Arrhenius plot. The activation energies were estimated from the same tangents. The break in the Arrhenius plot is evident at pH 7.0 but the $t_{disc}$ is strongly shifted to lower temperatures. The values of the parameters characterizing the water exchange time at various pH values are listed in Table 2. It is obvious from Fig. 3 and Table 2 that the activation energy and $t_{disc}$ are markedly influenced by the pH of the erythrocyte suspension. It appears also that there is a critical value of pH around 7.0 where both the apparent activation energy and $t_{disc}$ show a minimum.

**Table 2.** Parameters characterizing the water exchange time through erythrocyte membranes at various pH values[a]

| pH | $E_t > t_{disc}$ (kcal/mol) | $E_t < t_{disc}$ (kcal/mol) | $t_{disc}$ (°C) |
|---|---|---|---|
| 6.4 | 4.8 | 2.6 | 22 |
| 7.0 | 3.1 | 0 | 17 |
| 7.4 | 5.9 | 0.5 | 27 |

[a] The notations are the same as in Table 1.

## Discussion

As discussed by Sha'afi (1977), so far two possible mechanisms for water transport in human red cell membrane have been considered. One model is based on the idea that thermal fluctuations in membrane lipid can cause conformational changes in the hydrocarbon chains which lead to the generation of mobile structural defects known as "kinks" (Traüble, 1972), which may act as intrinsic carriers for water and nonelectrolyte transport. The second model assumes the presence of hydrophylic pathways for water transport or "pores" assembled from membrane integral proteins. Sha'afi (1977) presented evidence which sug-

gests that the band 3 protein or glycophorin (nomenclature of Fairbanks, Steck and Wallach (1971) and Marchesi et al. (1972), respectively) or both may be involved in the formation of hydrophilic pathways for water transport.

De Gier (1979) has pointed out that, in studies on membranes, the activation energy can be an indication of whether the permeant is penetrating by the "kink" mechanism or whether a facilitated diffusion mechanism is available. The apparent activation energy of the water diffusion through erythrocyte membranes determined in this paper, in agreement with previously found values (Sha'afi, 1977), is much lower than the activation energy for water permeation in liposomes (12 kcal/mole, De Gier, 1979). This shows that the mechanisms of water permeation which operate in the case of erythrocytes are different from those of liposomes.

Our findings can be explained in terms of a protein channel facilitating the water diffusion. We have noticed an obvious discontinuity around 26 °C in the Arrhenius plot of the water exchange time, the temperature effects being reversible.

Various membrane processes have been reported to show discontinuities as a function of temperature (Raison, Lyons & Thomson, 1971; Lenaz, Curatola & Masotti, 1975; Inesi, Millman & Eletr, 1973; Benga & Strach, 1975). Such discontinuities are often interpreted as indicating conformational changes of the membrane proteins induced by a temperature-dependent phase change in the membrane (*see* Lenaz, 1973; Van Deenen et al., 1975, for reviews). It is not clear whether a phase transition of erythrocyte membrane lipids actually occurs between 0 and 37 °C. While some investigators have found evidence for a phase transition occurring around 20 °C (Johnson, 1975; Zimmer & Schirmer, 1974; Bieri & Wallach, 1976) in recent studies using deuterium nuclear magnetic resonance, it has not been possible to detect any phase transition of the acyl chains of the erythrocyte lipids (Davis, Maraviglia, Weeks & Godin, 1979). However, Bond and Baumann (1979) have recently emphasized that it is not likely that an entire membrane will exhibit a phase transition analogous to lipid phase transitions, but interaction between the lipids surrounding a protein channel and the protein complex *per se* could lead to rather abrupt changes in membrane permeability. It is also possible that the change in the membrane permeability is due to a thermally induced change in the conformation of the protein channel itself. Although the findings described in this paper do not enable us to discriminate between such molecular mechanisms, it is clear that a dependence of the apparent activation energy on the temperature reflects a cooperative process (Volkenstein, 1977).

On the other hand, Verma and Wallach (1976) described pH-sensitive cooperative state transitions of erythrocyte membranes between pH 5.5 and 7. The idea of pH-dependent alterations of erythrocyte membranes is further supported by the observation of Goekoop et al. (1978), by freeze-etch electron microscopy, of a decrease in the number of red cell membrane elevations by changing the pH from 7.5 to 6.5.

It was then interesting to find (Figs. 2 and 3) that significant changes of the water diffusion occur around the pH values which induce state transitions of the erythrocyte membrane. This can be explained in terms of a protein channel mediating the water diffusion. It is well known that facilitated diffusion is strongly dependent on pH. For instance, the rate of dissipation of a pH gradient across the human red cell membrane is a facilitated transport (Jennings, 1978); the plot of the pH dependence of the red cell pH equilibration, mediated by the membrane protein that catalyzes anion exchange (band 3 protein) is very similar to the pH dependence of water diffusion (Fig. 2 in this paper compared to Fig. 3 in Jennings, 1978).

The pathway to water in human red cells is not yet known in detail. While Brown, Feinstein and Sha'afi (1975) suggested the involvement of band 3 protein, other authors (Macey & Farmer, 1970; Brahm & Wieth, 1977) considered that water channels transport water and very little else. However, it is possible that several transport processes are mediated by different polypeptides migrating in the same regions on electrophoregrams or by different segments of the band 3 polypeptide chain. Jennings and Passow (1979) have recently shown that specific regions of the band 3 molecule are involved in the control of anion transport.

The temperature and pH effects on water diffusion reported for the first time in this paper suggest that conformational changes and cooperative effects are implicated in the mechanism of water diffusion through erythrocyte membranes. The NMR measurements combined with other physical and biochemical techniques appear as a useful tool to further investigate this process.

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