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WATER DIFFUSION PERMEABILITY OF ERYTHROCYTES USING AN NMR TECHNIQUE

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SUMMARY

A simple rapid NMR technique requiring little blood is described for measuring the water-exchange time between erythrocytes and plasma doped with Mn^{2+}. The results are reproducible and are within 10 % of published values obtained by isotope tracer techniques.

INTRODUCTION

The water diffusion permeability of the erythrocyte membrane is very high and leads to an exchange time* between cells and plasma of the order of 10 ms. The measurement of such rapid exchange by radio-active tracers is difficult but has been accomplished by Solomon and co-workers[1,2] using a steady-state flow tube apparatus. Probably because of the experimental difficulties involved, the physiology and pathology of erythrocyte water permeability has not been widely investigated. This communication describes a simple NMR method of measuring the water-exchange time. The method is a development of a principle applied to water transfer across nerve cell membranes by Fritz and Swift[3]. The plasma must be doped with Mn^{2+} to a concentration of about 25 mM so that the result, while reproducible, may not be quite the same as *in vivo*.

THEORY

Water protons placed in a static magnetic field can absorb energy from a radio-frequency magnetic field. The decay of the coherent form of this energy can be measured by standard NMR techniques[4] and is normally described in terms of the "spin–spin" relaxation time, T_2. After applying a brief but intense radio-frequency pulse to a blood sample in a suitable magnetic field, the resultant coherence of the proton spins temporarily labels the blood. The spontaneous decay time (T_2) of the label for water inside red blood cells is about 140 ms and is thus much longer than the cell-plasma exchange time (about 10 ms). If T_2 in the plasma could be made very short ($\ll$ 10 ms), the label decay time within the cells would be dominated by the

* Defined as the time for a population to fall to $1/e$ of its initial value.

exchange process: water molecules leaving the cells would lose their label soon after contacting the plasma, and those entering the cells would mostly be unlabelled.

In practice, a short plasma T_2 can be obtained by adding an effectively impermeable paramagnetic material such as Mn^{2+} to the blood. Fig. 1 illustrates the label decay process following an energising radio-frequency pulse at $t = 0$, for a blood sample containing added Mn^{2+}. Initially, the relaxation is dominated by rapid decay of the plasma label. After a few milliseconds the plasma label has effectively disappeared, thereafter only intracellular label remains and this decays more slowly, principally by water diffusion into the plasma.

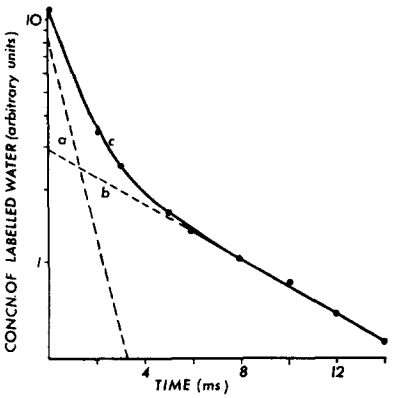

Fig. 1. Decay with time of amount of labelled water in a blood sample whose plasma $MnCl_2$ concentration is 24 mM and packed cell volume is 28%. Label is coherence of proton spins in applied magnetic field following radio-frequency pulse at $t = 0$. Solid curve is sum of component Curves a and b. Component a results from rapid decay of label in plasma. Decay time T_{2a} = 1.15 ms. Component b results from slower decay of label in cells and boundary layers. Decay there is due mainly to water diffusion from cells to plasma. Decay time T_{2b} = 7.7 ms.

Although the slow decay time of Fig. 1 is closely related to the water-exchange time, it can be influenced by (i) spontaneous label decay within the cells and (ii) a back flux of labelled water from the plasma. The effect of (i) can be corrected using Eqn 1:

$$\frac{1}{T_{ae}} = \frac{1}{T_{2b}} - \frac{1}{T_{2i}} \tag{1}$$

where T_{ae} = apparent exchange time; T_{2b} = observed relaxation time of slow decay component (see Fig. 1); T_{2i} = decay time in isolated red blood cells.
The value of T_{2i} inserted in Eqn 1 should incorporate a correction for any diffusion of Mn^{2+} into the cells.

The significance of the back flux of labelled water can be seen qualitatively by solving the one-dimensional diffusion equation for the boundary layer outside the cells.

$$\frac{\partial c}{\partial t} = D\frac{\partial^2 c}{\partial x^2} - \frac{c}{T_{2a}} \tag{2}$$

where x = distance from cell surface; c = label concentration; T_{2a} = label lifetime outside cells (observed rapid decay component, see Fig. 1): D = diffusion coefficient for water in plasma.

Assuming that $T_{2a} \ll T_{ae}$, quasi-steady-state conditions apply and

$$D\frac{\partial^2 c}{\partial x^2} = \frac{c}{T_{2a}} \tag{3}$$

The solution of Eqn 3 if $c = c_0$ at the cell outer surfaces at time t and assuming a distance d between the surfaces of adjacent cells is:

$$c = \frac{c_o}{1 + \exp(d/\delta)} \{\exp[x/\delta] + \exp[(d-x)/\delta]\} \qquad o < x < d/2 \tag{4}$$

where

$$\delta = (DT_{2a})^{\frac{1}{2}} \tag{5}$$

It follows that the net efflux from the cells is J where

$$J = AD\left(\frac{\partial c}{\partial x}\right)_{x=0} = rAc_o(T_{2a}/D)^{\frac{1}{2}} \tag{6}$$

and

$$r = \frac{e^{d/\delta} - 1}{e^{d/\delta} + 1} \tag{7}$$

A is the cell surface area. Also

$$J = -V\frac{\partial c_i}{\partial t} = +V\frac{c_i}{T_{ae}}$$

where V is the cell volume and c_i the internal concentration at time t whence

$$\frac{c_o}{c_i} = \left(\frac{V}{A}\right)\left(\frac{T_{2a}}{D}\right)^{\frac{1}{2}} \Big/ (T_{ae}r) \tag{8}$$

= ratio of back flux to forward flux. Consequently,

$$T_e - T_{ae} = \left(\frac{V}{A}\right)\left(\frac{T_{2a}}{D}\right)^{\frac{1}{2}} \Big/ r \tag{9}$$

(for $T_0 \ll T_{ae}$) where T_e is the actual exchange time.

The right hand side of Eqn 9 is minimised when T_{2a} is small and r is large. The factor r is less than one, approaching unity when $d \gg (DT_{2a})^{\frac{1}{2}}$ (from Eqn 5 and 7). Although Eqn 9 cannot be expected to apply quantitatively to three-dimensional diffusion out of erythrocytes, it can be used to predict qualitatively that if T_e is not affected by the treatment of the sample, the measured exchange time T_{ae} should approach the actual exchange time T_e in the limits of low packed cell volume and high paramagnetic additive concentration in the plasma. The permeability P then follows from Eqn 10:

$$P = V/(AT_e) \tag{10}$$

It will also be of interest to know the total amount of labelled water in the extracellular boundary layers, N_{bdy}. Equating the net outward flux of labelled water to the label annihilation rate in the boundary layer.

$$N_{bdy}/N_{intra} = T_{2a}/T_{ae} \quad (11)$$

where N_{intra} = amount of labelled water remaining inside the cells, and it is assumed that $T_{2a} \ll T_{ae}$.

MATERIALS AND METHODS

Human blood was obtained by venipuncture, stored at 8 °C in sterile, heparinised tubes and used within 8 h of collection. Analytical reagent grade chemicals were used throughout.

Solutions of 20, 50 and 100 mM $MnCl_2$ were prepared and made approximately isotonic by the addition of NaCl. Final adjustment to a tonicity of 300 mosM was achieved by dilution and measurement with a freezing point depression osmometer (Advanced Instruments Inc., Mass. U.S.A.). These unbuffered solutions were found to have a pH of about 6.

Solutions of manganese citrate were prepared by adding a solution of citric acid to solid $MnCO_3$: after a period of boiling and stirring the evolution of CO_2 ceased and subsequently the pH was increased from 4.0 to an average of 6.8 by the addition of NaOH solution. The solutions were diluted until they did not alter the volume of red cells, as determined by packed cell volume measurement. They were then taken to be isotonic. This check was also applied to the $MnCl_2$ solutions.

Cell-washing solutions were isotonic and composed of NaCl, or NaCl and 5 mM EDTA (sodium salt).

The spin–spin relaxation time can be found from line widths in continuous wave NMR, but here was measured by pulsed NMR using 90–180° echo decay[4] at a frequency of 9 MHz. The specimens were made up by adding 1.00 cm^3 of Mn^{2+} solution to 2.00 cm^3 of whole blood and gently mixing. The final plasma Mn^{2+} concentration was determined by calculation from the initial whole blood packed cell volume. The specimen temperature was controlled in the spectrometer to within $\pm$ 1 °C after allowing 10 min for thermal equilibration. Packed cell volume measurements were made at room temperature by the standard microhematocrit technique.

RESULTS

The water proton spin–spin relaxation at 37 °C of blood samples with paramagnetic plasmas showed two-component relaxation behaviour, as illustrated in Fig. 1. A simple indication that the intact cell membrane was responsible for the two-component behaviour was obtained by showing that a short period of homogenisation caused component (b) to disappear. Analysis of Fig. 1 confirms that the expected proportion of protons contributed to each decay component in Fig. 1. The intercept of (b) at $t = 0$ should be proportional to the amount of water in the cells and the adjoining boundary layers. The intercept of (a) should be proportional to the remaining extracellular water. Allowing for the volume occupied by solutes (assumed to be 25 % inside the cells and 5 % in the plasma) and the water in the

TABLE I

RELAXATION TIMES OF LABELLED WATER IN BLOOD AND PLASMA AS FUNCTION OF FREE Mn^{2+} CONCENTRATION IN PLASMA

37 °C, packed cell volume of specimens after dilution 28%.

Manganese additive	*Concn of additive in plasma (mM)*	*Free Mn^{2+} concn in plasma (mM)*	*T_2 in plasma (ms)*	*Blood sample No.*	*Apparent exchange time (ms)*
Nil	—	—	540	—	—
$MnCl_2$	9.5	9.5	2.9	C	9.6**
				D	9.6
$MnCl_2$	24	24	1.2	A	8.5
				B	8.1
				C	7.8
				D	8.5
$MnCl_2$	48	48	0.6	A	7.8
				B	7.9
				C	8.5
				D	8.5
Sodium manganese citrate	100	29*	1.1	B	9.8
				B	8.9
Sodium manganese citrate + 20% excess sodium citrate	95	0.5*	1.9	B	8.6
				B	8.4

* Calculated from stability constant, ref. 5.
** $T_{2a} > 2$ ms, hence overestimate of T_e due to back flux of labelled water (see text).

boundary layer (Eqn 11), the data in Fig. 1 correspond to a packed cell volume of 29 % which is in good agreement with the packed cell volume determined by centrifugation, 28 %.

Values of T_{2b} were calculated from the relaxation data and corrected to apparent exchange times T_{ae} by means of Eqn 1. Estimates of the cell relaxation time T_{2i} employed in these corrections were obtained as follows. After the relaxation measurement on whole blood, plasma and cells were separated by centrifugation and plasma samples were set aside for later T_2 measurements. The cells were washed with isotonic saline and then re-deposited. The value of T_{2i} at the time of T_{2b} measurement was estimated by interpolating between T_2 of the untreated cells, and that of treated cells, which was usually lowered as the result of Mn^{2+} entry. Tests showed that a second wash did not change the extimate of T_{2i}. A saline–EDTA wash was also tried but altered T_{2i} even in the absence of Mn^{2+}.

The calculated values of T_{ae} are listed in column 6 of Table I. The correction to T_{2b} to obtain T_{ae} was approximately 5 % for the manganese citrate samples where there was negligible Mn^{2+} entry, rising to 10 % for 48 mM $MnCl_2$.

As shown in Fig. 2, the apparent exchange times follow the qualitative behaviour predicted from Eqn 9. For plasma relaxation times less than 2 ms and packed cell volumes less than 40 %, T_{ae} approaches a limiting value which we identify with the

actual exchange time T_e. At 28 % packed cell volume, the excess of T_{ae} with the 9.5 mM $MnCl_2$ is of the order expected if it were produced by a back flux. This is evident from Eqn 9, wherein substitution of $T_{2a} = 2.9$ ms, $D = 2.5 \cdot 10^{-5}$ cm$^2 \cdot$s^{-1}, $V/A = 5 \cdot 10^{-5}$ cm and $d = 3 \cdot 10^{-4}$ cm making $r = 0.46$, yields $T_e - T_{ae} = 1.2$ ms.

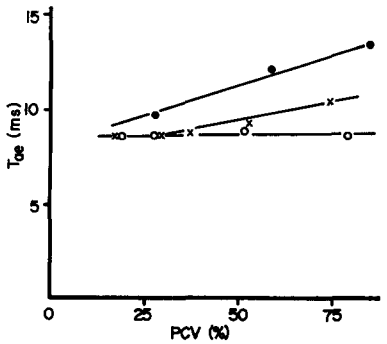

Fig. 2. Apparent exchange time of water between cells and plasma as a function of packed cell volume (PCV) and $MnCl_2$ concentration in plasma. Blood sample No. D. Experimental points refer to plasma $MnCl_2$ concentrations of 9.5 mM (●), 24 mM (×) and 48 mM (○). The lines are intended merely to guide the eye, they have no theoretical significance.

It remains to investigate the extent to which the added Mn^{2+} may have affected T_e. It is likely to be the Mn^{2+} activity rather than concentration which is important. By adding Mn^{2+} in a citrate complex (stability constant = $10^{-3,7}$)[5] the free Mn^{2+} concentration can be varied by a factor of 100 while retaining a short plasma T_2 (see Table I). The lowest free Mn^{2+} concentration in Table I, 0.5 mM, while a factor of 500 above the normal plasma value[5], is one quarter of the normal plasma concentration of Ca^{2+} the only significantly abundant divalent ion under physiological conditions. From Table I, the concomitant change in T_e is at most 20 %.

In Table I the eight readings at 24 and 48 mM yield a mean exchange time of 8.2 ms, S.E. 0.11. This compares reasonably well with a value from tritiated water experiments of 8.9 ms, S.E. 0.45 (calculated from the five points at 37 °C of Fig. 4, ref. 2).

In a search for long term effects of Mn^{2+}, cell volume and T_{2b} were monitored over a period of a few hours in samples containing 48 mM Mn^{2+} in the plasma but very little change was found. T_{2b} decreased by about 10 % probably due to the effect on T_{2i} of Mn^{2+} entering the cells. The cell volume changed by about 3 % which was comparable to the change observed in saline-diluted control samples. The time constant for Mn^{2+} entry was not measured accurately but judging from the relaxation data was of the order of many hours for normal red cells.

It appears therefore that the NMR technique can yield a reproducible measure of the exchange time T_e which is not unduly influenced by the high plasma manganese concentrations employed. The method relies on labelling water protons, although the protons are not permanent components of individual water molecules. Thus, in common with tracer methods using hydrogen isotopes, the exchange time obtained is that of the labelled nuclear species and does not necessarily equal the exchange time for water molecules. In particular a positive error would result if some protons

belonging to intracellular solutes exchanged with the surrounding water protons in times $\lesssim$ 10 ms. However such solute protons would contribute to the height of component (b) in Fig. 1. The close agreement already mentioned between the packed cell volume estimated from Fig. 1 and that determined by centrifugation indicates that the resultant discrepancy between water proton and water molecule exchange times is small. The trans-membrane flux of H^+ could also introduce an error, but this is expected to be negligible.

DISCUSSION

The present NMR technique for measuring the water-exchange time between blood cells and plasma is simpler, faster and more economical of blood than the isotopic tracer techniques hitherto employed. The only preparation the sample requires is the addition of an appropriate manganese salt solution. The interval between sample preparation and measurement could be reduced to 1 min or even less, thermal equilibration being the limiting factor. The measurement itself could be completed in a few seconds if the exchange time was estimated from signal heights at two points during the later part of the decay (Fig. 1, $t \gtrsim 8$ ms). We used blood samples of 2 cm^3 but 0.5 cm^3 would be quite adequate.

By contrast, the tracer technique apparently requires blood samples of about 100 cm^3 which are then subject to cell enrichment and buffering procedures. The technique involves the very rapid mixing of the blood and tritiated water label, pumping through a flow tube and the filtration and counting of several samples.

The NMR technique appears to be more reproducible. The standard deviation of individual readings was found to be 4% (8 readings, Table I) compared with a standard deviation of 11% by the tracer method (5 readings at 37 °C, Fig. 4, ref. 2). For reasons discussed in the previous section it is considered that the NMR–determined water-exchange time is within 20% of the value *in vivo*, despite the necessity to dope the plasma with large concentrations of a paramagnetic salt. The tracer technique avoids that problem, but requires the blood to be in a state of turbulent flow during the period of measurement. It is gratifying that the results obtained by the two techniques agree to within about 10%.

Measurements of the water diffusion permeability of erythrocytes have been used in studies of membrane structure[2,6] and comparison of adult and foetal blood[7]. The NMR method should facilitate the extension of such studies in areas where its particular advantages (speed, simplicity, small sample volume) outweigh the residual uncertainties relating to the use of manganese or other paramagnetic additives. It would appear well suited to situations such as clinical surveys where large numbers of samples must be processed.

The effects of manganese entry would be very small for normal red cells if the exchange-time measurement was completed within a few minutes of mixing the blood with the manganese salt solution. However this would not necessarily be true for pathological specimens. If manganese entry was suspected to have occurred, its extent could be assessed by measuring T_{2i} after washing the cells. The exchange time could then be corrected as described earlier in the text.

CONCLUSIONS

(1) An NMR technique for measuring the water-exchange time of erythrocytes has been described. The results at 37 °C when the plasma was doped with 24 or 48 mM $MnCl_2$ lay in the range 7.8 to 8.5 ms. Their mean, 8.2 ms, was within 10 % of a published figure, 8.9 ms, obtained by tracer techniques.

(2) When $MnCl_2$ was the plasma additive, individual measurements had a standard deviation of about 4 %.

(3) The most important drawback of the method concerns the need to dope the blood plasma with large quantities of a paramagnetic ion Mn^{2+}. However, results varied by at most 20 % when the concentration of free Mn^{2+} in the plasma was varied 100-fold: from 48 to 0.5 mM.

(4) Correction to the exchange time due to Mn^{2+} entry into the cells was at most 5 % under the conditions of the experiment and would be negligible for normal human blood if the measurement was made immediately after sample preparation.

(5) Potentially, measurements could be made on blood samples as small as 0.5 cm^3 and in a time of about 1 min.

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