WATER EXCHANGE BETWEEN RED CELLS AND PLASMA

MEASUREMENT BY NUCLEAR MAGNETIC RELAXATION

M. E. FABRY *and* M. EISENSTADT

From the Division of Genetic Medicine, Department of Medicine, Albert Einstein College of Medicine, Bronx, New York 10461

ABSTRACT Water exchange between human red blood cells and the plasma phase was measured by water proton nuclear magnetic resonance relaxation in the presence of low concentrations of Mn(II) and by ^{17}O relaxation of $H_2{}^{17}O$ in the absence of added Mn(II). The results were analyzed as a classic case of two-compartment exchange. The half-life for cell water at 25°C was found to be 15 ms ± 2 ms, longer than the time determined by other techniques. The T_1 of the hemoglobin protons in the red cell and the volume of exchangeable water were also measured. The method appears to be a sensitive tool for the study of membrane permeability to water and other small molecules undergoing rapid exchange.

INTRODUCTION

In 1957 Paganelli and Solomon (1) used tritiated water (THO) and a rapid flow device to measure the rate of water diffusion into red cells. They found a half-life of 5.8 ms for the equilibration of THO with free erythrocytic water. Using an improved flow apparatus, Barton and Brown (2) obtained a half-life of 7.6 ms at 25°C in 1964. In 1972, Conlon and Outhred (3) measured water diffusion through red cell membranes by means of nuclear magnetic resonance (NMR) relaxation (T_2) and found a half-life of cell water of 5.7 ms at 37°C. Recently, Shporer and Civan (4) used $H_2{}^{17}O$ to measure the water half-life and found 11.5 ms at 25°C and 6.4 ms at 37°C. In this paper we report new results using a method similar to that of Conlon and Outhred.

Hydrogen nuclei are weakly paramagnetic and, if placed in a strong magnetic field, will show a small net magnetization along the direction of the field. A short pulse of electromagnetic radiation, at an appropriate frequency, can invert this net magnetization. The rate at which the net magnetization returns to its normal orientation is called the spin-lattice relaxation time, T_1, and is the fundamental quantity measured in our study.

Our present experiments may be thought of as measurements of the rate of escape of labeled molecules from the cell. The water is "labeled" by inverting the direction of the equilibrium nuclear magnetization of its protons. Because of the high intracellular protein concentration, water protons in the cell reorient with a T_1 of 570 ms, which is

short relative to the T_1 of 1,700 ms for the water protons in the plasma phase. Plasma protons, however, can be made to reorient with a T_1 of only a few milliseconds by adding Mn(II) to the plasma, forming a paramagnetic Mn-albumin complex, which is one of the most effective agents for decreasing T_1's (5). Since the complex cannot enter the red cell, cell water can regain its normal orientation in a time faster than 570 ms only by passing through the membrane into the plasma. If the half-life for escape of the cell water is much longer than the T_1 in the plasma, but much shorter than the intrinsic T_1 of the cell water, the measured T_1 of the cells and plasma consists of a fast component, due to the relaxation of plasma water protons, and a slow component whose T_1 is determined solely by the rate of water escape. At lower Mn(II) concentrations, the plasma T_1 is closer to the half-life of cell water, and a more complex analysis, described in this paper, is necessary.

The NMR method has several advantages over other procedures: First, it is done at equilibrium, so that the question of mixing time of stagnant layers does not arise. Second, the measurement may be repeated as often as desired, thus allowing for signal averaging. Third, only small sample volumes, as little as 0.5 ml, are required. Fourth, processes which have half-lives of milliseconds or shorter may be studied. Finally, the technique is applicable both to other biological two-compartment systems and to certain other nuclei, provided only that the system can be arranged such that the half-life of exchange is intermediate between the relaxation times in the two compartments.

EXPERIMENTAL METHODS

Samples were prepared from freshly drawn heparinized human blood by addition of various amounts of 0.5 M $MnCl_2$ to a suspension of plasma and cells. They were then separated by centrifugation and the appropriate amounts of Mn(II)-plasma and settled cells were withdrawn and mixed to reach the desired hematocrit. In this way a series of suspensions with constant plasma T_1 and different hematocrits could be prepared. Albumin present in serum both reduces the concentration of free Mn(II) and enhances the relaxation effect of the Mn(II) (5). During measurements the samples were frequently mixed to prevent settling. After the T_1 measurements, aliquots were withdrawn in microhematocrit tubes for determination of the fractional cell volume, H_t. The remaining cell suspension was centrifuged and the T_1 of the plasma in the absence of the cells, T_{1b}, was determined. The relaxation time of cell water alone, T_{1a}, was measured as 570 ms in a tightly packed button of cells.

Measurements were generally done within a half hour or less after addition of Mn(II). To determine whether some Mn(II) might enter the cells, some samples were allowed to stand for up to 24 h at 5°C, and then were washed with human serum albumin (HSA) in normal saline. The T_{1a} of packed cells, and the half-life of water exchange following a second addition of Mn(II) were determined.

Mn(II) solutions were prepared at least 2 days before use, since we found, as had Weed and Rothstein (6) that freshly prepared solutions penetrate the cell more rapidly, an undesirable property in our experiments. Some experiments were also performed with 0.5 M Mn(II) stock solutions prepared either less than 4 h or less than 24 h prior to their addition to cell suspensions. All measurements were made at 25°C. T_1 was measured at 25 MHz using standard pulse techniques (7). A 180° pulse inverted the magnetization, and its recovery was monitored after a known delay time by the free induction decay following a 90° pulse. The delay time

was varied and results such as those shown in Fig. 1 were obtained. We have verified that essentially all protons in the sample were being observed by comparing the absolute magnitude of the blood signal with that of an equal volume of distilled water. All measurements were corrected for the signal due to hemoglobin protons which was determined by relaxation measurements on several high H_t samples with very short T_{1b}'s. The residual signal out to several hundred milliseconds was measured and extrapolated back to zero time. This correction, made proportional to H_t, was subtracted from all measurements.

The measurements in D_2O were carried out by first equilibrating 1 ml of packed cells with 1 ml of D_2O saline for 2 h and then centrifuging the suspension and measuring the T_1 of loosely packed cells with no added Mn(II). The cells were then recovered and reequilibrated with 2 ml of D_2O saline for 1½ h, centrifuged, and T_1 was again measured with no added Mn(II).

For measurements with $H_2{}^{17}O$, paramagnetic impurities were removed from 5% $H_2{}^{17}O$ with a small Chelex column adjusted to pH 7.4. 1 ml aliquots of heparinized plasma were lyophilized and the plasma reconstituted with $H_2{}^{17}O$. Packed cells were added to the $H_2{}^{17}O$ enriched plasma and allowed to equilibrate. Measurements were made at 10 MHz. With signal averaging data could be collected over 1.5–2 decades of magnetization recovery.

THEORY

The decay of magnetization in a system of two well-mixed compartments with exchange may be described by the coupled rate equations

$$\begin{aligned} d(\Delta M_a)/dt &= -(k_a + k_x)\Delta M_a + k_y \Delta M_b, \\ d(\Delta M_b)/dt &= -(k_b + k_y)\Delta M_b + k_x \Delta M_a, \end{aligned} \tag{1}$$

where ΔM_a and ΔM_b are the deviations from equilibrium magnetization of cell and plasma water, respectively, $k_a(\equiv T_{1a}^{-1})$ and $k_b(\equiv T_{1b}^{-1})$ are the NMR relaxation rates of water in isolated cells and plasma, and k_x and k_y are the probabilities per unit time for the transfer of water from cells to plasma and vice versa. However at equilibrium the number of molecules passing into the cell compartment from the plasma compartment must equal the number leaving, therefore

$$\begin{aligned} k_x \text{ (cell volume) (exchangeable } H_2O \text{ conc. in cell)} \\ = k_y \text{ (plasma volume) (exchangeable } H_2O \text{ conc. in plasma).} \end{aligned} \tag{2}$$

"Well-mixed" means the average lifetime of a water molecule in either compartment does not depend on its location, i.e. the mean time to diffuse between cell boundaries in either compartment is short compared with the lifetime in that compartment. The time to diffuse 0.7 μm, the average half-thickness of the erythrocyte, is about 0.2 ms, using $D = 1 \times 10^{-5}$ cm^2/s (8). Thus mixing of cell water is complete in a time much less than the measured k_x^{-1}. For equal volumes of cell and plasma water, the lifetime of a water molecule in both compartments is about the same, as shown by Eq. 2, so the above argument applies to either compartment. For greater dilutions of cells, the lifetime in the plasma phase increases as the cube of the intercellular distance, Eq. 2, but the diffusion time, which is proportional to r^2/D, increases only as the square of the

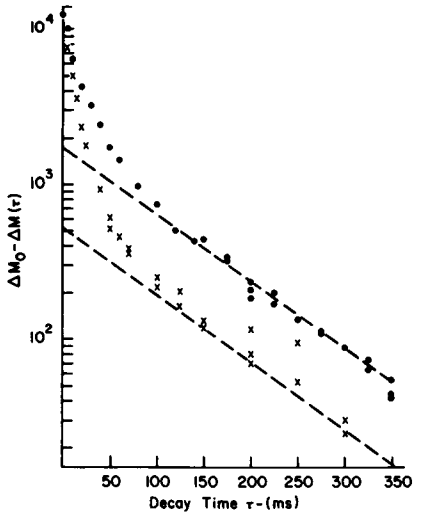

FIGURE 1

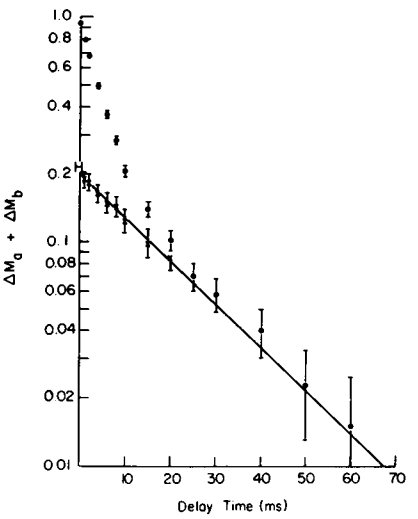

FIGURE 2

FIGURE 1 T_1 decay data for two samples: (•) hematocrit 81.5, 10 mM $MnCl_2$; (×) hematocrit 18.5, 2mM $MnCl_2$. Magnetization in arbitrary units.

FIGURE 2 Typical decay for a sample of cells in plasma at 25°C, H_t = 19, with 5 × 10^{-3} M total $MnCl_2$, and T_{1b} = 5.3 ms. The contribution of hemoglobin protons has been subtracted as described in the text. The fast component, ϕ_+^{-1}, has been subtracted from points with delay times of 1–20 ms (these points are represented by ×'s) leaving only a slow component ϕ_-^{-1} of 22.2 ms. The H value is calculated from Eq. 6. A standard error of ± 10% of the full scale signal is shown. Total magnetization normalized to unity.

distance. Hence the assumption of complete mixing remains valid for all further dilution.

Woessner (9) has considered such systems in detail, and the general solutions of Eq. 1 are

$$\Delta M_a = A_+ \exp(-\phi_+ t) + A_- \exp(-\phi_- t)$$
$$\Delta M_b = B_+ \exp(-\phi_+ t) + B_- \exp(-\phi_- t), \tag{3}$$

where

$$2\phi_\pm = (k_a + k_b + k_x + k_y) \pm [(k_a - k_b + k_x - k_y)^2 + 4k_x k_y]^{1/2}, \tag{4}$$

and the coefficients A and B are related to the fractional volume of cells and plasma as well as to $\phi_\pm$. The quantity determined experimentally is $\Delta M_a + \Delta M_b$ as a function of time. In the limit of $k_a \ll k_x, k_y \ll k_b$, the fast and slow decay constants become

$$\phi_+ = k_b + k_y \approx k_b$$
$$\phi_- = k_a + k_x \approx k_x. \tag{5}$$

In addition to the fast and slow relaxation times, a third independent quantity may be obtained from the extrapolation of the slow component to zero time, denoted as H. It is related to the fraction of the total volume occupied by cell water, P_a, by

$$H = [P_a(k_b - k_a) + \phi_+ - k_b]/(\phi_+ - \phi_-), \tag{6}$$

from which P_a may be determined. When ϕ_+ is equal to ϕ_-, another equation applies (9), but this case does not arise here. We began with an initial assumption that the volume of exchangeable water is 0.72 times the total cell volume, based on an average water content of the red blood cell of 72% (10). For low hematocrits, the calculated value of k_x is quite insensitive to the precise value of this fraction, and $k_y \ll k_x$, so that it is adequate to use 0.72 in Eq. 2 as well. Once the value of k_x has been accurately determined, experiments at high hematocrit can yield information on the exchangeable water content of the cell.

RESULTS

Fig. 1 shows two typical decays of $\Delta M_o - \Delta M(\tau)$ vs. time after the 180° inverting pulse. The dotted line fits a slow decay (T_1 = 100 ms) which we attribute to the protons incorporated in hemoglobin. Before the data were analyzed further, this slowly decaying signal was subtracted, as described in the experimental section. The validity of the correction was confirmed by the observation that all measurements at long delay times approached the correction asymptotically, and the corrected results gave consistently good agreement with theory for a single value of the cell water life-time over a wide range of H_t and T_{1b}. This signal was also present in tightly packed samples containing no Mn(II), where it appeared as an initial fast component with a slope of 100 ms. Furthermore, when red cells are equilibrated with several changes of D_2O saline, a slow decay of approximately the predicted magnitude is observed. The magnitude is only approximate because some of the hemoglobin protons do exchange during the equilibration period. The measured T_1 from loosely packed cells in D_2O with no added Mn(II) can be very accurately fit with two exponentials; one representing the slow decay of the water protons, now primarily HDO, and the other representing the faster decay of the unexchanged hemoglobin protons. That the T_1 of the hemoglobin protons can be fit with a single exponential is somewhat surprising in view of the presumed diversity of environments and relaxation mechanisms. We realize, of course, that by using only two exponentials for the D_2O case, subtle effects will be overlooked; however, we estimate that if more than 25% of the hemoglobin protons have a $T_1 >$ 150 ms or a $T_1 <$ 50 ms they would be observable. For the purpose of correcting the raw data, analysis in terms of a single exponential is quite adequate.

Fig. 2 shows a typical decay of $\Delta M_a + \Delta M_b$ vs. time after the raw data have been corrected for the signal due to hemoglobin, as described in the experimental section. Additional points at short delay times for fitting the slow component are obtained by calculating the fast component from the independently measured, T_{1b}, H_t and an

approximate value of k_x, and subtracting it from the results. These points, and the H value calculated from H_t as described above are than used to fit a single exponential of slope ϕ_-. Using the new ϕ_- the process is repeated; at most two iterations are needed for convergence.

Fig. 3 shows ϕ_-^{-1}, which we shall refer to as the slow component, as a function of T_{1b} for various hematocrits. The solid lines were calculated for an exchange time, k_x^{-1}, of 22 ms; the dashed lines were calculated for a k_x^{-1} of 10 ms. Experimental points are shown on this graph only for $H_t \approx 20$, since ϕ_-^{-1} becomes increasingly sensitive to the precise value as H_t increases. For $T_{1b} \rightarrow 0$, the theoretical curve asymptotically approaches k_x^{-1}. ϕ_-^{-1} is most sensitive to k_x^{-1} at relatively short T_{1b}, although surprisingly little sensitivity is lost until T_{1b} becomes longer than 7–8 ms for the cases considered here. Results for T_{1b} less than 5 ms (i.e. Mn(II) greater than 10 mM) depart from this asymptote; consequently, we have only used results for T_{1b} greater than 5 ms to determine k_x. We find a value of 0.046 ± 0.006 ms^{-1} corresponding to a cell water half-life, $t_{1/2} = 15 \pm 2$ ms, at 25°C in normal plasma.

Fig. 4 shows ϕ_-^{-1} as a function of hematocrit for various T_{1b}. The solid lines were calculated on the basis of a fractional water content of the red cell of 0.73 for the total cell volume and a value of k_x^{-1} of 22 ms derived from the results at low hematocrit. The dashed lines above and below the $T_{1b} = 20.1$ ms curve were calculated for fractional cell water content of 0.78 and 0.68, respectively. We conclude that a fractional cell water content of 0.70 ± 0.05 gives a best fit for the results.

The cell water lifetime may be measured with ^{17}O enriched H_2O in the absence of added Mn(II), since for this case both T_{1a} and T_{1b} are shorter than k_x^{-1}. We found that the T_1 of $H_2{}^{17}O$ in plasma at 25°C is 3.9 ms and that T_1 in the red cell interior is 1.7 ms. With our measurements, we were only able to set a lower limit of 20 ms on the cell water lifetime, k_x^{-1}. Calculated curves of magnetization recovery ($\Delta M_a + \Delta M_b$) using Eqs. 3, 4, and 6 for $k_x^{-1} > 20$ ms do not differ from the zero exchange limit ($k_x^{-1} \rightarrow \infty$) by more than 2 or 3% in the first $1\frac{1}{2}$ decades of decay. However, the sensitivity of the calculated curves to the lifetime for $k_x^{-1} < 20$ ms is sufficient to establish a lower limit.

Human red cells are known to take up Mn(II) slowly under some conditions (6); however, we observed no change in ϕ_- or T_{1b} over a 4 h period for cells in their own plasma, containing Mn(II) as high as 10^{-2} M. At lower Mn(II) no change was observed for even longer periods. However, if cells are exposed to 5×10^{-2} M Mn(II) in saline, ϕ^{-1} drops from an initial value of 13 ms to 9 ms after 4 h. Measurements were also made on cells which were allowed to stand in Mn(II)-plasma solutions overnight. When such cells were washed three times in isotonic saline-HSA solutions, it was found that T_{1a} had been shortened from 570 ms to values as low as 80 ms, depending on the time of exposure and the Mn(II) concentration. When Mn(II)-HSA was reintroduced to the washed cells, the decrease in ϕ_- was greater than that predicted from the measured change in T_{1a}.

Experiments with Mn(II) stock solutions prepared either less than 4 or less than 24 h prior to use were also done. The value of k_x^{-1} calculated from these experiments

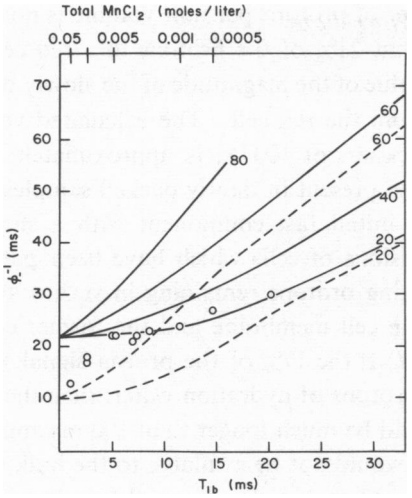

FIGURE 3

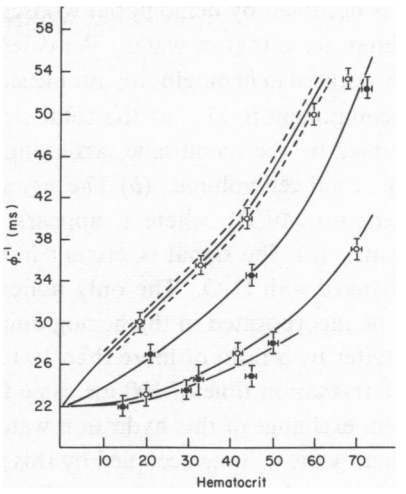

FIGURE 4

FIGURE 3 The experimental points are the slow component, ϕ_-^{-1}, for various T_{1b} at 25°C, in normal plasma, at H_t = 19–24. The theoretical curves were calculated for the hematocrits indicated on the right-hand side. The solid curves were calculated for k_x^{-1} = 22 ms; the dashed curves were calculated for k_x^{-1} = 10 ms. T_{1b} is a nonlinear function of total $MnCl_2$ for the reasons discussed in ref. 4.

FIGURE 4 The experimental points are ϕ_-^{-1} for various T_{1b} as a function of hematocrit in normal human plasma at 25°C. The experimental points and theoretical curves, reading from bottom to top represent T_{1b} of (●) 7.4 ms, (○) 8.5 ms, (●) 14.7 ms, and (○) 20.1 ms. A fractional cell water content of 0.73 was used in all calculations except for the dashed curves above and below the T_{1b} = 20.1ms curve where fractional cell water contents of 0.78 and 0.68, respectively, were assumed.

was identical to the value calculated for experiments using older stock solutions. However, if these cell suspensions were allowed to stand, T_{1b}^- was observed to decrease and T_{1a}^{-1} increase; this effect was greater for the 4 h stock solutions than for the 24 h stock solutions.

Some preliminary studies showed that k_x^{-1} decreased with temperature, but changed little with pH over the range of 7.4 ± 0.5. No variation was observed among samples from five healthy donors. Additions of 2 mM *p*-chloromercuribenzene sulfonic acid (PCMBS) caused an increase in k_x^{-1} by about a factor of two, similar to the findings of Macey et al. (11). When the volume of the cells was decreased by 10% by the addition of a small amount of 1 M NaCl to the plasma, ϕ_-^{-1} decreased from 22 ms to about 20 ms.

DISCUSSION

Signal Due to Hemoglobin Protons

We believe that the signal with a T_1 of 100 ms is due to the hemoglobin protons for the following reasons: (*a*) The magnitude is correct. Some 28% of the volume of the red

cell is occupied by hemoglobin whose number of protons per unit volume is not very different from that of water. We calculate that 21% of the protons in a red cell are incorporated in hemoglobin; our measured value of the magnitude of the slowly decaying component is 17% of the total signal from the red cell. The calculated volume occupied by the membrane, assuming a thickness of 100 Å, is approximately 1.7% of the total cell volume. (*b*) The signal is also present in tightly packed samples containing no Mn(II), where it appears as an initial fast component with a slope of 100 ms. (*c*) The signal is present in suspensions of cells which have been partially exchanged with D_2O. The only nonexchanging protons remaining in such a system will be incorporated in the hemoglobin or the cell membrane and the former exceed the latter by a ratio of more than 10 to 1. (*d*) If the 17% of the proton signal which has a relaxation time of 100 ms came from protons of hydration water, then the half-life for exchange of this hydration water would be much longer than 100 ms and consequently the volume occupied by this water would not be available to the bulk water which has a half-life of 15 ms. This additional excluded volume above the volume excluded by hemoglobin itself would be reflected in P_a and would result in a value of P_a of 0.53 instead of the 0.70 which was experimentally determined.

Comparison of Theory and Results

As can be seen from Fig. 4, the agreement between the theoretical curves and the experimental results is extremely good. The results at high hematocrit indicate that 0.70 ± 0.05 of the volume of the red cell is occupied by exchangeable water. Since this fraction does not differ appreciably from the total water content of the cell, we conclude that all of the cell water exchanges with a half-life of 15 ms. This does not preclude the existence of hydration water associated with the hemoglobin; it does imply that such water exchanges with the rest of the water in the cell with a half-life much less than 15 ms.

Comparison with the Results of Others

Our value of the cell water half-life is about twice as long as that obtained from rapid flow measurements (1, 2, 11). Barton and Brown (2) obtained 7.6 ms ($t_{1/2} = 0.693\ k_x^{-1}$ and $k_x \equiv k/v_q$ in the notation of Barton and Brown and Paganelli and Solomon; and $k_x \equiv T_e^{-1}$ in the notation of Conlon and Outhred). The discrepancy with our results is in an unexpected direction since most errors in T_1 measurements tend to make measured values too short because of extraneous relaxation mechanisms. Conlon and Outhred (3) obtained a half-life of 5.7 ms, working at a higher temperature, 37°C, and with Mn(II) concentrations of 0.01 M and higher, necessitated by considering only the limiting case (low hematocrit and short T_{1b}).

We believe that the primary source of the discrepancy between our value of k_x and that of Conlon and Outhred is the high concentration of free Mn(II) present in their cell suspensions. Several other experimental conditions, such as increased temperature, increased osmotic strength, and Mn(II) entering the cells will cause small changes in ϕ_-^{-1} but none of these will cause a decrease as large as that which we observe when

the free Mn(II) concentration is increased from 2 mM to 48 mM (see Fig. 3) when ϕ_-^{-1} decreases from 22 ms to 12 ms.

In addition, Conlon and Outhred measured the transverse relaxation time, T_2, while we measured T_1. The difference adds considerable complication to an already complex two-component decay, since the Mn(II) introduces a chemical shift (12) in the plasma phase as well as shortening T_{2b}. For Mn(II) concentrations of about 10^{-2}M, the shift would be of the same order of magnitude as the exchange rate, leading to beats in the transverse decay (9).

Our $H_2{}^{17}O$ results are in agreement with those of Shporer and Civan (4). The ^{17}O method is inherently less sensitive because both T_{1a} and T_{1b} are shorter than k_x^{-1}; however, it is capable of setting a clear lower limit on k_x^{-1} which is particularly valuable since these measurements are made without the addition of Mn(II).

The source of the discrepancy between our results and the flow experiments is more difficult to understand. The most likely source of systematic error in our experiments is the possibility that Mn(II) may affect the membrane; however, two observations seem to rule out this as a source of error: (*a*) Measurements made with $H_2{}^{17}O$ without added Mn(II) clearly show that $k_x^{-1} > 20$ ms at 25°C. (*b*) High Mn(II) concentrations give too short a value for k_x^{-1}, thus it seems unlikely that lower Mn(II) concentrations could result in k_x^{-1} too long.

To summarize, we have demonstrated good agreement between the predictions of the NMR theory for two-compartment exchange and our experimental results, which allows us to determine both the water exchange time and the fractional content of exchanging water accurately. Although our exchange time, determined at low Mn(II), differs from that determined by Conlon and Outhred, our results under similar conditions are similar to theirs; and our results with both ^1H and ^{17}O agree with the $H_2{}^{17}O$ results of Shporer and Civan. Our value for the exchange time k_x^{-1} of cell water is longer than that determined in flow experiments, but it seems unlikely that the use of Mn(II) at low concentration is the origin of this discrepancy.

We are indebted to Professor John T. Edsall for reviewing an earlier version of this paper. We also wish to thank Professor I. H. Scheinberg for his advice and encouragement of this work.

This work was partially supported by the following grants: NIH AM 01059, NIH GM 18298, Office of Naval Research N 00014-69-C 0131, American Cancer Society Institutional Grant IN-28-0.
Dr. Fabry held a New York Heart Association Senior Investigatorship during this work.

Received for publication 24 April 1975.

REFERENCES

1. PAGANELLI, C. V., and A. K. SOLOMON. 1957. The rate of exchange of tritiated water across the human red cell membrane. *J. Gen. Physiol.* **41**:259.
2. BARTON, T. C., and D. A. J. BROWN. 1964. Water permeability of the fetal erythrocyte. *J. Gen. Physiol.* **47**:839.
3. CONLON, T., and R. OUTHRED. 1972. Water diffusion permeability of erythrocytes using an NMR technique. *Biochem. Biophys. Acta.* **288**:354.

4. SHPORER, M., and M. M. CIVAN. 1975. NMR Study of ^{17}O from $H_2{}^{17}O$ in Human Erythrocytes. *Biochem. Biophys. Acta.* **385**:81.
5. MILDVAN, A. S., and M. COHN. 1963. Magnetic resonance studies of the interaction of manganous ion with bovine serum albumin. *Biochemistry.* **2**:910.
6. WEED, R. I., and A. ROTHSTEIN. 1961. The uptake of divalent manganese ion by mature normal human blood cells. *J. Gen. Physiol.* **44**:301.
7. FARRAR, T. C., and E. D. BECKER. 1971. *In* Pulse and Fourier Transform NMR. Academic Press, Inc., New York.
8. WANG, J. H., C. B. ANFINSEN, and F. M. POLESTRA. 1954. The self-diffusion coefficients of water and ovalbumin in aqueous ovalbumin solution. *J. Am. Chem. Soc.* **76**:4763.
9. WOESSNER, D. E. 1960. Nuclear transfer effects in nmr pulse experiments. *J. Chem. Phys.* **35**:41.
10. PONDER, E. 1948. *In* Hemolysis and Related Phenomena. Grune & Stratton, Inc., New York. 120.
11. MACEY, R. I., D. M. KARAN, and R. E. L. FARMER. 1972. Properties of water channels in red cell membranes. *Biomembranes.* **3**:331.
12. FRAIT, Z., D. FRAITORA, and D. DOSKOCILOVA. 1973. Measurement of the proton nmr field shift in aqueous solutions containing Cu^{2+}, Cr^{3+}, Fe^{3+} and Mn^{2+} ions. *Czech. J. Phys.* **B23**:908.