Biochimica et Biophysica Acta, 464 (1977) 45—52

BBA 77552

DIFFUSIONAL WATER PERMEABILITY OF RED CELLS

INDEPENDENCE ON OSMOLALITY

DAVID Y. CHIEN and ROBERT I. MACEY *

Department of Physiology and Anatomy, University of California, Berkeley, Calif. (U.S.A.)

(Received June 15th, 1976)

Summary

The osmotic permeability coefficient (L_p) for human red cells has been reported to depend on the osmolality of the suspending solution. These results are also consistent with the view that the value of L_p depends on flow rectification. In this report an NMR method is used to measure the dependence of water exchange times at constant cell volume on osmolality. Our results indicate that the diffusion water permeability is constant over a large range of osmolality (300—1000 mosM) produced by the permeable solutes urea, methanol, ethanol, and glycerol. The results support the view that the apparent dependence of L_p on osmolality is due to flow rectification.

Introduction

Two different laboratories [1,2] have reported that the osmotic permeability coefficient for human red cells depends on the osmolality of the suspending solution. This finding, if substantiated, is important in several different contexts. In the first place it has been used to infer the existence of an outer skin on the membrane which can swell and shrink osmotically [1,3]. Secondly, a dependence on osmolality is an implicit but essential assumption in the demonstration that the Onsager cross coefficient L_{pd} is independent of concentration [3]. Further, the constancy of L_{pd} is an implicit assumption in the demonstration that the solute permeability coefficient P_s is a function of the volume flow J_v [3]. Finally, the assumed dependence of L_p on osmolality has been used to provide correction factors to demonstrate effects of ethanol on permeability which would vanish if the correction was not applied [4].

The primary basis for the assertion that osmotic permeability coefficient L_p

* To whom correspondences should be addressed.

is dependent on osmolality consists of a scattergram where many measurements of log L_p are plotted against the reciprocal of osmolality. The L_{ps} taken from swelling experiments (from isotonic to hypotonic medium) tend to cluster around a value of L_p that is higher than the cluster of L_{ps} from shrinkage experiments (isotonic to hypertonic medium). If a regression line is drawn between these clusters, there is an apparent correlation between log L_p and the reciprocal of osmolality. However, these results are also consistent with the view that the value of L_p depends on the direction of flow, i.e. whether the cells are swelling or shrinking [5]. A dependence of L_p on direction of flow is referred to as flow rectification; it has been predicted theoretically for asymetric membranes [6] and it has been demonstrated experimentally in a number of systems [7].

An alternative approach to this problem involves measurements of exchange times (diffusional permeability, P_w) for water across the membrane as a function of osmolality. Since this measurement is made under conditions of osmotic equilibrium, factors involved in rectification cannot play a role. If water permeability does depend on osmolality, then the exchange time should also show this dependence. On the other hand, if the results are due to rectification then the exchange time should be independent of the medium osmolality. Using the impermeable solutes NaCl or sucrose to alter osmolality, Outhred and Conlon [8] have shown that exchange times vary directly with cell volume over volumes ranging from 0.8 to 1.4 isotonic volume. This is precisely what is predicted if P_w is independent of osmolality (see Eqn. 2). However, the range of osmolality is narrow and the conclusion requires independent proof that P_w is independent of volume.

In this paper we remove these equivocations by taking advantage of the fact that the apparent dependence of L_p on osmolality is reported to be controlled solely by the osmotic pressure (as determined by freezing point) and is independent of the reflection coefficients or permeability of the solutes composing the external solutions [3]. Using permeable solutes it is then possible to measure water exchange times at a constant volume and over a much larger range of osmolalities. Our results indicate that P_w is constant over a large range of osmolality produced by the permeable solutes urea, methanol, ethanol, and glycerol.

Methods

Sample preparation

Human blood obtained from four healthy subjects was collected in (acid-citrate-dextrose) and stored at 4° C until used (no longer than two days). Blood was then prepared by washing three times in isotonic buffered saline (150 mM NaCl, 5 mM Hepes, pH 7.4) and resuspended in isotonic saline to a final hematocrit of 65—70%. Sufficient quantities of a concentrated solution of test solutes were slowly added to the washed blood in order to reach the desired osmolality. Each sample was allowed at least 15—30 min for full equilibration which was verified by comparing the hematocrit of each sample with a corresponding control suspended in isotonic saline. Osmolality of the supernatant

from each sample was determined to within 2% by freezing point depression (Fiske Mark III Osmometer).

Spin-lattice (T_1) and spin-spin (T_2) NMR relaxation times were measured at room temperature on blood samples containing 20 mM (T_1) and 53 mM (T_2) $MnCl_2$. The $MnCl_2$ was added from concentrated solutions just prior to measurement. Following the relaxation measurement, cells were separated by centrifugation, washed three times with isotonic saline, and then redeposited. The relaxation times of water left in tightly packed cells, T_{1p} or T_{2p}, were 550 ms and 100 ms.

Spectral measurements

Water exchange times were measured by nuclear magnetic resonance techniques which are based on the following [9]. When water protons are placed in a magnetic field, their spins become oriented. Application of a radiofrequency pulse reorients the spin and this reorientation serves as a label which can be detected by NMR techniques. Following application of the pulse, this label slowly decays. However, if paramagnetic impermeable Mn^{2+} are placed in the plasma, the label within the plasma is very quickly quenched (within a few ms). Thus application of a radiofrequency pulse labels both plasma and cellular water. Soon after, the label in plasma disappears because of the quenching action of Mn^{2+}; now only cellular water is labelled. From here on the decay of label from the total sample (cells and plasma) is almost entirely due to the exchange process; water leaving the cells lose label soon after contacting plasma, water entering the cells is mostly unlabelled.

Water exchance times were estimated from T_2 measurements by the method of Conlon and Outhred [9]. Exchange times were estimated from T_1 measurements with a method described by Fabry, M.E. and Eisenstadt, M. (submitted for publication) with the exception that slightly higher concentrations of Mn^{2+} were employed and serum albumin was removed.

Water proton relaxation time measurements were carried out by using a Spin-Lock CP-2 pulse spectrometer (44.4 MHz) together with a Jeol JNM-4H-100 magnet and power supply. Spin-lattice relaxation times (T_1) were measured with a 180—90° pulse sequence; the light of the free induction decay signal following the 90° pulse was monitored with a Princeton Applied Research Model CW-1 Boxcar Integrator combined with a Hewlett-Packard Model 3430 A Digital voltmeter. The Carr-Purcell pulse sequence incorporating the Meiboom-Gill modification was used for water proton spin-spin relaxation time (T_2) measurements.

At room temperature raw T_1 and T_2 data both showed a biphasic character. The slow decay data were fitted into a single exponential decay. The water exchange time, T_{ex}, is calculated from T_1 or T_2 by the expression

$$\frac{1}{T_{ex}} = \frac{1}{T_{1,2}} - \frac{1}{T^{p}_{1,2}} \tag{1}$$

where $T^{p}_{1,2}$ represents the value of T_1 or T_2 obtained with isolated packed cells [9]. Inclusion of the term $1/T^{p}_{1,2}$ corrects for spontaneous decay of label in the cells, and as measured, it incorporates a correction for leakage of Mn^{2+} into the cells.

Results and Discussion

Both spin-lattice relaxation times T_1 and spin-spin relaxation times T_2 were measured at different hematocrits and at different medium osmolalities. Tables I, II, and III show that the water exchange time T_{ex} estimated from either T_1 or T_2 is independent of hematocrit and medium osmolality.

The values of T_{ex} as calculated from T_1 and T_2 agree reasonably well with other workers. Thus our T_{ex} from T_1 measurements is estimated to be 18 ms in good agreement with the value of 16.7 ms of Shporer and Civan's [10] T_1 study of the $H_2{}^{17}O$ in human erythrocytes and also in reasonable agreement with the value of 22 ms obtained by Fabry and Eisenstadt who measured T_1 of water protons in the presence of serum albumin and with lower extracellular concentrations of $MnCl_2$. Our value of T_{ex} obtained from T_2 is about 11 ms at 25° C. The corresponding value obtained by Conlon and Outhred [9] at 37° C was 8.2 ms. Assuming an activation energy for P_w of 6 kcal/mol [11], Conlon and Outhred's figure at 25° C would be expected to be about 12.1 ms which is close to our value. The basis for the discrepancy between values of T_{ex} obtained from T_1 and T_2 measurements is complex. (Possibly it is related to differences in the free Mn^{2+} concentration which could induce a proton chemical shift in comparison with the half-line width [10]). However, it is not our intent to enter into any controversy regarding which estimate of T_{ex} is best. Rather, we are content to establish the fact that both estimates show T_{ex} is independent of medium osmolality.

The water diffusion permeability P_w is related to T_{ex} by

$$P_w = V_w/AT_{ex} \tag{2}$$

TABLE I

EXCHANGE TIME OF WATER BETWEEN CELLS AND PLASMA AS A FUNCTION OF HEMATOCRIT and $MnCl_2$ CONCENTRATION IN PLASMA

Manganese additive, $MnCl_2$. The osmotic environment of the cells, isotonic saline. Age of blood, 1 day. Temperature, 25° C.

Blood sample	Hematocrit (%)	Free Mn^{2+} conc. in plasma (mM)	T_1 * (ms)	T_{1ex} * (ms)	T_2 ** (ms)	T_{2ex} ** (ms)
A	38	20	17.4	17.9		
A	30	20	17.3	17.8		
A	25	20	17.1	17.7		
A	20	20	17.1	17.7		
B	20	20	17.1	17.5		
D	50	35			8.7	10.5
D	35	35			9.3	10.4
D	10	35			9.1	10.2
B	50	53			8.8	10.6
B	44	53			9.3	10.8
C	33	53			10.1	11.8
C	22	53			10.2	11.6
C	10	53			10.1	11.2
B	22	53			10.6	11.7

* Free Mn^{2+} conc. 20 mM.

** Free Mn^{2+} conc. 53 mM or 35 mM.

TABLE II

RELAXATION TIMES OF LABELLED WATER FROM T_1 MEASUREMENTS IN BLOOD AND PLASMA AS FUNCTION OF EXTRACELLULAR OSMOLALITY

Age of blood, 1 day. Hematocrit, 20%. Free Mn^{2+} conc. in plasma, 20 mM. Temperature, 25°C. No bovine serum albumin or human serum albumin.

Sample	Osmolality (mosM) ± 2%	T_1 (ms)	Blood Sample	T_{1ex} (ms)
(1) Erythrocytes + isotonic saline	310	17.1	A	17.7
	305	17.1	B	17.5
(2) Erythrocytes + saline * + urea	482	16.5	A	17.1
	606	16.8	A	17.5
	928	16.1	A	16.7
(3) Erythrocytes + saline * + glycerol	481	16.5	A	17.1
	653	16.9	A	17.8
	996	16.6	A	17.4
(4) Erythrocytes + saline * + ethanol	480	17.4	B	18.2
	655	18.1	B	18.9
	1000	17.5	B	18.5

* Isotonic saline (150 mM NaCl, 5 mM Hepes, pH 7.4).

where V_w equals the volume of cell water and A equals the cell membrane area. All measurements were made at equilibrium with a constant concentration of impermeable solutes in the medium so that V_w and A are the same in each experiment (hematocrit measurements showed that the equilibrium cell volume did not deviate from the isotonic volume). Since T_{ex} is independent of osmolality, it follows that P_w is also independent of osmolality. This is illustrated by the data in Fig. 1 which represents a composite of both T_1 and T_2 experiments.

TABLE III

RELAXATION TIMES OF LABELLED WATER FROM T_2 MEASUREMENTS IN BLOOD AND PLASMA AS FUNCTION OF EXTRACELLULAR OSMOLALITY

Age of blood, 1 to 2 days. Hematocrit, 22%. Free Mn^{2+} conc. in plasma, 53 mM. Temperature, 25°C. No bovine serum albumin or human serum albumin.

Sample	Osmolality (mosM) ± 2%	T_2 (ms)	Blood Sample	T_{2ex} (ms)
(1) Erythrocytes + isotonic saline	298	10.6	B	11.7
	302	10.2	C	11.6
(2) Erythrocytes + saline * + urea	577	10.5	B	11.8
	750	10.6	B	11.8
	895	10.5	B	11.6
(3) Erythrocytes + saline * + methanol	618	10.7	C	11.9
	850	9.9	C	11.8
	1030	9.8	C	11.4
(4) Erythrocytes + saline * + glycerol	622	9.9	B	11.4
	855	10.6	B	11.9
(5) Erythrocytes + saline * + ethanol	650	8.6	C	11.8
	849	8.3	C	11.6
	1050	8.4	C	12.1

* Isotonic saline (150 mM NaCl, 5 mM Hepes, pH 7.4).

The relative sensitivity of P_w and L_p to changes in water transport requires comment, especially if the membrane contains pores. On the one hand, if the change in L_p with osmolality reported by Rich et al. and Blum and Forster [1,2] was due to changes in the number of pores, or their length, or the local viscosity, we would expect these changes to be reflected in both L_p and P_w measurements with equal sensitivity. On the other hand, if L_p changes are due to a change in aqueous pore radius r then it could be argued that these changes would not be as apparent with P_w measurements, because P_w is much less sensitive than L_p to changes in pore radii. This follows because P_w will vary as the square of the pore radius, r^2, but the variation in L_p will lie somewhere between r^2 (very narrow channels) and r^4 (wide channels where Poiseuille flow is applicable) [12].

To illustrate, let

$$P_w = ar^2 \tag{3}$$

$$L_p = br^n$$

where a and b are constants of proportionality and $2 \leqslant n \leqslant 4$. Eliminating r from Eqn. 3 leaves

$$P_w = [a/b^{2/n}]L_p^{2/n} \tag{4}$$

When $n = 2$, P_w varies directly as L_p. However, the greatest discrepancy between P_w and L_p occurs when n assumes its maximal value which is 4. In that case, P_w will vary as the square root of L_p; a doubling of L_p, for example, corresponds to only a 1.4-fold increase in P_w. The sensitivity of P_w on r will be compromised even further if we take into account the fact that both measurements of

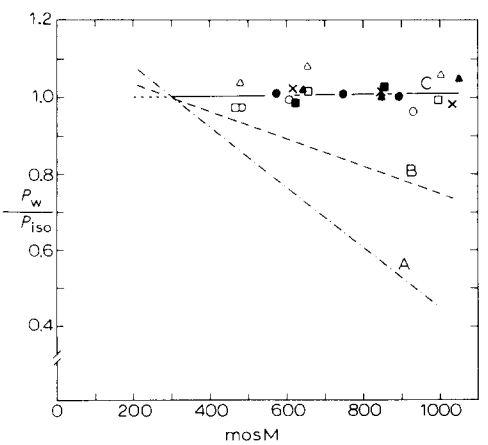

Fig. 1. Composite of water permeabilities P_w obtained from spin-lattice, and spin-spin relaxation data listed in Tables II and III, curve C. All data points are normalized by P_{iso}, the control value of P_w obtained in isotonic saline. ●○, represent data with variable extracellular urea; ■□, data with variable extracellular glycerol; ▲△, data with variable extracellular ethanol; ×, data with variable extracellular methanol. Open figures represent spin-lattice data, solid figures spin-spin data. Curve A, constructed (and extrapolated, dashed line) from data of Blum and Forster [2], represents anticipated results if both P_w and L_p have similar dependencies on osmolality. Curve B, constructed (and extrapolated) from same data, represents anticipated results if the "pore" radius changes with osmolality. If P_w depends on osmolality the experimental points should fall within the area limited by A and B.

P_w and L_p represent water transport through two parallel paths, the aqueous pores and the lipid bilayer. These two extreme cases are illustrated in Fig. 1 by the dotted curves A and B. A was constructed from the reported dependence of L_p on osmolality by Blum and Forster [2], i.e. as though P_w varied directly as L_p. B represents anticipated results for P_w if the pore radius was changing with osmolality. It was constructed from the data of Blum and Forster [2] by assuming that L_p varies as r^4, P_w varies as r^2, and that the ratio of osmotic to diffusional permeability (at isotonic conditions) equals 3.3 (Sha'afi and Gary-Bobo ref. 15). In addition, a small correction was applied assuming 10% of the osmotic water flow takes place through the lipid portions of the membrane [14]. If in fact there is a dependence of water permeability on osmolality, then the experimental points should lie somewhere between the two extremes represented by curves A and B. They clearly do not. (If curves A and B were based on the data of Rich et al. [1], instead of Blum and Forster [2], then the deviation of C from the area defined by A and B would be even more prominent.) We can conclude that the methods used in this study are sufficiently sensitive to detect changes in P_w that would be obtained if L_p varied with osmolality to the extent reported, and that these changes are not observed.

These findings support the argument that the apparent dependence of L_p on osmolality is due to rectification. This interpretation has been considered by Rich et al. [1], but their arguments that there is no rectification are not compelling because they are based on very few experiments which show considerable scatter and depend on the a priori assumption that L_p depends on osmolality. As stated by Forster [13], neither Rich et al. nor Blum and Forster [1,2] provided sufficient overlap of swelling and shrinking at the same osmolality to test for rectification. However, the problem has been examined in detail by Farmer and Macey [5] using a small perturbation method to span a large range of osmolalities with considerable overlap of swelling and shrinking at the same osmolality. Treating swelling and shrinking experiments as independent data it was shown that L_p was independent of osmolality in both human and beef red cells. The value of L_p for shrinkage experiments (inward flow) was consistently 40—50% greater than corresponding values for swelling experiments (outward flow). Further, an analysis of the data of Rich et al. [1] showed that their results were fully consistent with this interpretation. Outhred and Conlon's [8] measurements of the dependence of T_{ex} on cell volume also support this conclusion. Cell volume was changed by altering the concentrations of impermeable solutes in the medium. Under these conditions T_{ex} was found to be directly proportional to V_w which implies that the product $P_w \times A$ is constant despite the changes in medium osmolality.

The water exchange experiments demonstrate that, under equilibrium conditions, there is no dependence of P_w on medium osmolality. There remains a remote possibility that sudden changes in medium osmolality exert a short transient effect on water permeability which escaped detection by these experiments. We consider this an unlikely explanation because these effects did not appear in the osmotic transient experiments described above [5].

The present studies together with earlier reports of Farmer and Macey [5] and Outhred and Conlon [8] present a formidable block of evidence that there is no significant dependence of water permeability (either L_p or P_w) on the

osmolality of the suspending medium. It follows that there is no evidence for the existence of many of the anomolous features of red cell transport (e.g. dependence of solute permeability on flow, independence of L_{PD} on concentration, dependence of σ on concentration) which are demonstrated with the assumption that L_p does depend on osmolality [3].

Acknowledgements

We are grateful to Dr. I.D. Kuntz and Dr. Adam Zipp for the kind permission to use their NMR equipment. We would like to give our special thanks to Dr. Zipp for his enthusiastic assistance. This research was supported by N.I.H. Grant # GM 18819-04.

References

1 Rich, G.T., Sha'afi, R.I., Romualdez, A. and Solomon, A.K. (1968) J. Gen. Physiol. 52, 941—954
2 Blum, R.M. and Forster, R.E. (1970) Biochim. Biophys. Acta 203, 410—423
3 Sha'afi, R.I., Rich, G.T., Mikulecky, D.C. and Solomon, A.K. (1970) J. Gen. Physiol. 55, 427—450
4 Seeman, P., Sha'afi, R.I., Galey, W.R. and Solomon, A.K. (1970) Biochim. Biophys. Acta 211, 365—368
5 Farmer, R.E.L. and Macey, R.I. (1970) Biochim. Biophys. Acta 196, 53—65
6 Patlak, C.S., Goldstein, D.A. and Hoffman, J.F. (1963) J. Theor. Biol. 5, 426—442
7 Dainty, J. (1963) Adv. Botan. Res. 1, 279—326
8 Outhred, R. and Conlon, T. (1973) Biochim. Biophys. Acta 318, 446—450
9 Conlon, T. and Outhred, R. (1972) Biochim. Biophys. Acta 288, 354—361
10 Shporer, M. and Civan, M.M. (1975) Biochim. Biophys. Acta 385, 81—87
11 Vieira, F.L., Sha'afi, R.I. and Solomon, A.K. (1970) J. Gen. Physiol. 55, 451—466
12 Solomon, A.K. (1968) J. Gen. Physiol. 51, 335S—364S
13 Forster, R.E. (1971) Current Topics in Membranes and Transport 2, 41—98
14 Macey, R.I., Karan, D. and Farmer, R.E.L. (1972) Biomembranes 3, 331—340
15 Sha'afi, R.I. and Gary-Bobo, C.M. (1973) Prog. Biophys. Mol. Biol. 26, 105—146