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THE WATER PERMEABILITY OF ERYTHROCYTES*

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SUMMARY

1. We have used a stopped-flow rapid-reaction apparatus to record rapid changes in light transmission of cell suspensions following abrupt alterations in osmolality. These changes in osmolality can be related to average cell volume to give the kinetics of water transfer.

2. The sudden cessation of forward movement of a cell suspension in the apparatus, in the absence of any change in osmolality, produced a consistent pattern of variation in light transmission which we reduced in magnitude but could not eliminate completely by the introduction of glass capillaries into the observation tube. Therefore in each experiment a control record following the mixing of the cell suspension under study with isosmotic fluid was obtained and subtracted from the record following a change in osmolality.

3. Water permeability, P_w, of human red cells at room temperature averaged 0.30 $cm^4/osm \cdot sec$ during exosmosis and 0.36 $cm^4/osm \cdot sec$ during endosmosis. P_w decreased with increasing extracellular osmolality according to the regression, $P_w = 0.41 - 0.00029$ mosm/l. P_w did not vary with changes in intracellular osmolality although a slight dependency could not be ruled out.

4. P_w for horse red cells: $-0.28 - 0.00026 \times$ extracellular osmolality (mosm/l). P_w for nucleated eel and chicken cells averaged 0.054 $cm^4/osm \cdot sec$, one-fifth of that for human cells, and in the case of the eel cells only, did not vary with extracellular osmolality.

5. From arguments based in part on measurements of the rates of gas exchange of red cells, we conclude that any stagnant layer of fluid must be less than 1 μ thick.

6. If the cell membranes are heterogeneous in the direction of water flux and water permeability varies with osmolality to a different degree in the different layers, our experimental results are explicable by classical diffusion theory.

Abbreviations: osm, osmoles; mosm, milliosmoles.

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INTRODUCTION

At the present time the model of the biological membrane which describes its passive permeability properties most comprehensively is that in which aqueous pathways ('pores') are dispersed throughout a matrix of hydrophobic lipids. This model has been used to interpret the permeability characteristics of the erythrocyte membrane by HÖBER[2], PARPART AND BALLENTINE[3], and GOLDSTEIN AND SOLOMON[4]. There are two sets of experimental evidence which unambiguously support this scheme. The first is that a volume of water crosses the membrane of red cells faster in the presence of an osmotic pressure gradient than labeled water exchanges between intracellular and extracellular compartments during osmotic equilibrium[5,6]. The second is that the experimentally determined osmotic driving force exerted by small hydrophilic molecules is less than one would expect from their cell permeability[4,7].

Because of the importance of the estimates of red cell water permeability, which had been obtained from measurements of changes in light scattering in a continuous-flow rapid-reaction apparatus by SIDEL AND SOLOMON[8], we thought it advisable to repeat and extend them using a stopped-flow rapid-reaction apparatus[9]. This technique permits the measurement of changes in light transmission as early as 0.003 sec after mixing, an important point since the exchange parameters can be determined with greater accuracy and convenience at the beginning as contrasted with the later portions of the transient process. The stopped-flow instrument has the additional advantage of great economy of reacting fluid; a complete reaction curve can be obtained with less than 1 ml of reactants. At the same time we were aware from earlier work in this laboratory that large changes in the light scattering of red cell suspensions were produced in rapid-reaction apparatus by fluid movements[10,11] which we expected could be largely neutralized by subtracting suitable control records. However, this technical problem turned out to be a major obstacle which required extensive effort to overcome. SHA'AFI *et al.*[12] have reported similar application of a stopped-flow apparatus to the problem, but there are significant differences between these apparatuses.

METHODS

General

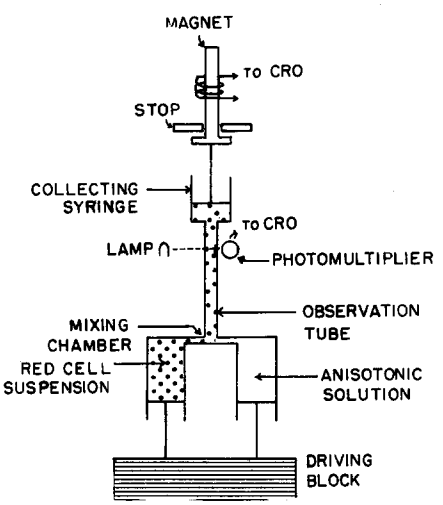
The human red cell suspensions were prepared by drawing fresh venous blood into a syringe whose dead space was filled with heparin, washing the cells twice in a buffer composed of 2.2 mM KH_2PO_4, 7.8 mM K_2HPO_4 and sufficient NaCl to adjust the osmolarity to the desired value, ending up with a 12.5 % suspension. In some experiments sucrose was substituted for part of the NaCl. The pH was titrated to 7.5 with HCl or NaOH. Fresh horse blood was obtained from animals kept at the School of Veterinary Medicine several blocks away and was suspended in the same medium as the human cells. Eel blood was collected from live Anguilla anguilla by the technique of STEEN[13] and with his cooperation. The suspending medium used was the same as for human cells but the pH was adjusted to 5.9 and 7.8 with HCl or NaOH. Chicken blood was drawn from normal animals and suspended in the same medium as for human cells.

Osmolality was measured by freezing point depression to a precision of ± 2 mosm/ml.

The ultimate measure of cell volume and changes therein was the hematocrit which was estimated using a variation of the method of VAN ALLEN[14]. The suspension was centrifuged for 45 min at 900 × *g*; by experience the hematocrit after 1.5 h was only 1 %, or less, smaller. No correction was made for trapped fluid volume. The technique was tested by measuring the hematocrit of known dilutions of a single cell suspension, checked against hemoglobin concentration measured with the cyanmethemoglobin method[15], and was reproducible to ± 2 % of the expected value. We obtained the effective water volume, *RW*, of the red cells of each individual donor by plotting the hematocrit against the reciprocal of the osmolality and in all cases obtained a straight line. In addition the wet weight : dry weight was estimated by drying an aliquot of packed cells at about 90°.

Stopped-flow rapid-reaction apparatus

The basic instrument was that of SIRS AND ROUGHTON[16] with modifications described before by HOLLAND AND FORSTER[11] but its essential features will be reviewed here because of their importance in interpreting the changes in light scattering. The cell suspension and an isotonic diluent are introduced (Fig. 1) into the mixing chamber through four tangentially directed jets, by pushing the driving block. In addition to providing the turbulence necessary for efficient mixing, this chamber creates a helical pattern of flow which continues along the entire length of the obser-



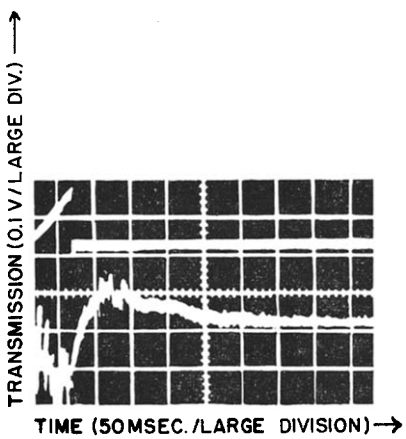


Fig. 1. A schematic diagram of the rapid-reaction apparatus. See text for detailed explanation.

Fig. 2. Oscilloscope traces of the voltage developed in the solenoid by the magnet moving within it (upper record) and photomultiplier output (lower record) when red cells suspended in normal saline were mixed with isotonic buffer in the stopped-flow rapid-reaction apparatus. Linear flow rate down the observation tube was 180 cm/sec per large division above zero, represented by the horizontal part of the record. Flow rate increases rapidly until the syringe hits the stop after about 0.07 sec. As the flow rate of the mixture increased, the transmission decreased, reaching a minimum 0.15 V below the value when the fluid was stationary (the right hand end of the trace, about 0.8 V) at the instant of fluid stoppage. Transmission then rose to 0.9 V in 0.05 sec, falling to the stationary transmission value in 0.3–0.4 sec.

vation tube. As solution passes through the observation tube, the collecting syringe fills, pushing the magnet above it through a solenoid coil, thereby developing a voltage which provides an instantaneous measure of flow. When the magnet flange hits the mechanical stop net linear flow ceases within a millisecond. The 'dead time' of the instrument at the flow rates used was 0.003 sec. Monochromatic light was obtained by inserting a monochromator or interference filter between the observation tube and the photomultiplier. Both the output of the phototube and the potential induced across the solenoid were recorded on a dual-trace storage oscilloscope (Fig. 2). Although not included in the diagram the light passing through the observation tube could also be split and one half passed through a second monochromator and onto a second photomultiplier when it was desired to use the apparatus as a double-beam instrument.

The syringes, mixing chamber and observation tube were in contact with metal blocks and covered with a metal plate through which water could be circulated to maintain the temperature at any desired value. Experiments were run at a room temperature of 26–27° unless otherwise indicated.

Changes in light transmission of red cell suspensions

Whenever a cell suspension was mixed in the rapid-reaction apparatus with buffer of the same osmolality (hereinafter designated 'control'), so that there was no change in volume at equilibrium, there was still a series of striking and consistent changes in light transmission before and after suddenly stopping net forward movement (Fig. 2). At the start of the record, as flow through the apparatus is increased, light transmission decreases. At the instant flow stops, transmission suddenly increases, rising to a maximum then falling slowly to a fixed equilibrium value by about 0.3 sec, which is maintained for about 30 sec.

Unfortunately the artefact is greatest at the start of a volume change, the most important part of the record. Therefore we devoted considerable effort to its study and possible means of eliminating it. It was not present when hemoglobin solution was substituted for the cell suspension and therefore did not result from mechanical or electrical disturbance of the instrument *per se*. It was substantially less when human red cells were suspended in saline of 0.57 tonicity, in which the cells were largely sphered, than when they were suspended in isotonic or hypertonic (1.45 tonicity) saline. It was almost completely eliminated when a suspension of polyvinylbenzene spheres with a radius of from 6 to 14 μ and equivalent absorbance was substituted for the cell suspension. On the other hand a suspension of *Escherichia coli* of comparable absorbance gave a control artefact similar to that of human red cells. We assumed from these results that the phenomenon was probably related to flow-dependent orientation of large domains of non-spherical cells, although it was also possible that the scattering properties of individual cells were altering[17].

A high-frequency noise, 500 cycles/sec or greater, appeared where there was net flow, was markedly reduced after stoppage (see first 0.05 sec of Fig. 2), and could be eliminated by filling both syringes with the same cell suspension. We concluded that this noise is produced by small, about 1 %, oscillatory variations in the relative delivery rates of cell suspension and buffer to the mixing chamber, a suggestion of Dr. J. B. Steen.

The amount of transmitted light varied with net linear flow rate through

the observation tube as demonstrated in Fig. 3. The data were obtained from records similar to that in Fig. 2 during the period before stoppage. In general light transmission decreases as linear flow rate increases, similar to the reports of others on continuously flowing red cell suspensions[18–21]. It was technically difficult to obtain reliable

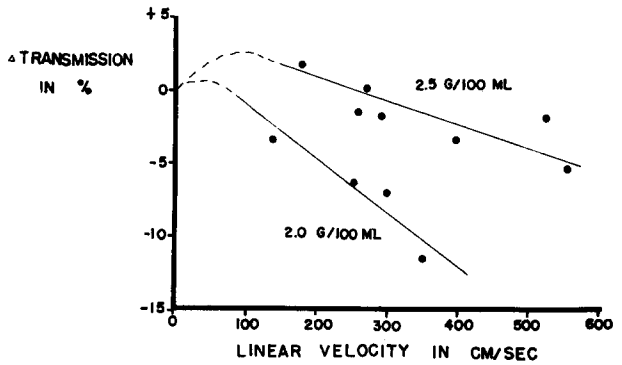


Fig. 3. Change in transmission of light of wavelength 600 nm through a cell suspension as a function of the instantaneous linear velocity of the fluid through the observation tube of the stopped-flow rapid-reaction apparatus at the instant of stopping. Figures refer to the total hemoglobin concentration of the mixture. The experiments were carried out by placing cell suspensions of twice the indicated concentration in one syringe and isotonic buffer in the other.

values at flow rates much less than 150 cm/sec, but the graphs do not extrapolate to zero change in transmission. We found similar results with steady-state flow through the instrument. A reasonable explanation for the artefact is that at the instant of stopping the net linear flow rate is 300 cm/sec or greater, and the light transmission must move up a curve analogous to those in Fig. 3 to reach a state of zero flow, producing first a rise, then a fall in transmission. The time required for the light transmission of the control artefact to rise to its maximum increased with increasing linear velocity at the instant of stopping, compatible with the notion that it took longer to slow down to the same effective velocity. The time required for the output to decrease from its maximum to its final value was independent of initial flow velocity, as would be expected from Fig. 3. This description is not precise, because the data in this figure pertain to net linear flow rate, which ceases on stoppage. However, rotational movements of cells are visible in the observation tube with a microscope after stopping and KURODA AND FUJINO[22] found that light transmission perpendicular to the axis of rotation of a stirred cell suspension increased and then decreased following cessation of stirring, very similar to the artefact in Fig. 2.

Since the magnitude of the artefact was not affected by varying wavelength from 476 to 650 nm, 650 nm was chosen for the practical reason that the light source and photomultiplier gave maximum output at this color.

The relative magnitude of the artefact, that is the change in *O.D.* of the artefact: change in *O.D.* for change in volume, decreased with increasing cell concentration. We chose 1:16 as optimal.

We investigated the effect of several modifications of the instrument on the relative magnitude of the artefact. A light diffusor interposed between the light source and the observation tube reduced the *O.D.* of the cell suspension as expected[18,23], but did not affect the relative size of the artefact. Substitution of a ball-in-seat

mixing chamber, which should impart less rotational velocity to the fluid in the observation tube, for the four-jet chamber reduced the relative size of the artefact, although not as much as other modifications.

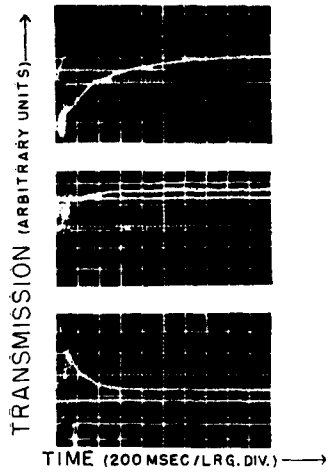


Fig. 4. Records of a swelling experiment, a control artefact, and a shrinking experiment. *Top.* The increase in transmission when the external osmolality was changed from 323 to 241 mosm/l. *Middle.* A control record in which the same cell suspension in 323 mosM was mixed with isotonic saline. *Bottom.* The decrease in transmission when the external osmolality increased from 323 to 430 mosm/l. In all records the third horizontal line extending across the record is the resweep at 30 sec, considered the completion of process.

The most effective alteration was the insertion of a bundle (seven) of fine glass capillaries in the observation tube in order to limit rotational flow, a suggestion of Dr. Gibson. This reduced the relative magnitude of the artefact about 40 % and therefore was used in all our experiments unless noted otherwise. The glass capillaries may have altered the flow characteristics of the observation tube, increasing its washout time, so we tested the apparatus by measuring the rate of de-oxygenation of an oxygenated red cell suspension by dithionite at 28°, following the difference in light transmission at 475 and 587 nm, and obtained a value of 8.0 sec^{-1}, not significantly different from the values of 8.0–9.1 sec^{-1} reported by LAWSON, HOLLAND AND FORSTER[24], after correction to the same temperature. The reliability of these figures is only of the order of $\pm$ 10 %, but the chemical process is as fast or faster than any changes in cell volume we measured, so that any adverse effect of the presence of the capillaries in the observation tube should have been exaggerated. Extrapolations of the reaction curve indicated that any time delay produced by the addition of the capillaries was less than several milliseconds and could be neglected.

The procedure used was to obtain a control record in each experiment and subtract it graphically from the records of light transmission during changes in volume. The changes in light transmission with swelling and shrinking were of a monotonic nature and reached a final end point in 0.5–1.5 sec (Fig. 4). However, in all records, including the controls, there was a slow secondary fall in light transmission of unknown cause from approx. 4 to 20 sec (not shown in Fig. 4), after which the output was stable until about 5 min, when it started to rise steadily, coincident

with settling of the cells. It often was not possible to reach a reliable end point at a sweep speed that permitted the necessary definition of the early portions of the curve (see for example the uppermost curve in Fig. 4). We therefore reswept the record between 30 sec and 4 min and used this value as the end point. Although this resweep was always less than the apparent end point for the change in volume, this was also true for the control record and since this last one is subtracted from the shrinking and swelling records, the secondary decrease in light output is effectively eliminated. Light transmission changes were a linear function of hematocrit after subtraction of the control record. These experimental results indicate that changes in chemical composition or in refractive index of cells or suspending fluid during our experiments do not influence significantly the relation between cell volume and light transmission.

SHA'AFI *et al.*[12] also found a repeatable waveform in light scattering upon stopping net forward flow of the cell suspension in the absence of a change in average cell volume in their apparatus. They do not give enough information to compare their records with our control artefact, but it is reasonable to conclude that we are both dealing with the same phenomenon, in spite of the fact that their stop is much slower and our fluid system presumably more rigid.

Calculation of water permeability

The osmotic water permeability, P_w (in cm⁴/osm·sec), was calculated from a variation of the standard equation of JACOBS[25].

$$tP_w = \frac{V^\circ\left(\frac{V^\infty}{V^\circ} + \frac{RW-1}{V^\circ}\right)}{C_1{}^\circ A \frac{RW}{V^\circ}}\left[\left(1-\frac{V^\infty}{V^\circ}\right)n + \left(\frac{V^\infty}{V^\circ} + \frac{RW}{V^\circ} - 1\right)\ln(1-n)\right] \qquad (1)$$

t is the time in seconds; V° is the initial volume of the cells, V^∞ that at final equilibrium, and V that at time t, all in cm³; R is the fraction of cell water that appears to be taking part in the osmotic changes[26]; W is the initial volume of cell water; $C_1{}^\circ$ is its osmolality; A is the surface area of the red cells in cm²; n is the fraction of the volume change completed at time t, equal to $(V-V^\circ)/(V^\infty-V^\circ)$.

For each experiment, 2–4 records and an equal number of controls were made. The curves were extremely reproducible. A graph relating cell volume and light transmission was constructed from (a) half the hematocrit of the unmixed cell suspension and the corrected light transmission immediately after mixing, and (b) the hematocrit of the mixture and corrected final light transmission (30 sec to 4 min). RW was derived from a plot of cell volume *versus* reciprocal osmolality experimentally determined for each given sample of blood. In order to calculated P_w the right hand side of Eqn. 1, referred to henceforth as the 'volume function', was plotted against time.

In deriving Eqn. 1 it was assumed (a) that the surface area of the cell remains constant as the volume changes, which has not been seriously challenged[26]; (b) that extracellular osmolality is constant after mixing, which is reasonable because of the small volume of cells, and (c) that the water flux produced by the osmotic gradient is established instantaneously. It is also assumed that the factors which determine RW act instantaneously. The recent report of GARY-BOBO AND SOLOMON[27] leaves some doubt about the validity of this last assumption. If P_w, or any of the other

values assumed constant in Eqn. 1, vary during a reaction, it should be immediately apparent because the plot of the volume function against time will not be a straight line. Failure to obtain linearity could also result from errors in the estimation of the initial or final values of light transmission. The P_w at the half-time would then give the most accurate measure of permeability.

RESULTS

Osmotic permeability of human red cells

Table I presents results of 18 swelling and 18 shrinking experiments on human red cells in which the cells had been equilibrated initially with hypotonic (242–270 mosm/l), isotonic (about 300 mosm/l) and hypertonic (321 and 341 mosm/l) media. The final column gives the maximum and minimum values of P_w in 13 experiments (36 %) in which the volume function *versus* time was alinear. All the measurements of P_w are plotted against external osmolality in Fig. 5. Although there is considerable scatter the data as a whole show a significant decrease with increasing extracellular osmolality (correlation coefficient -0.558; $P < 0.01$). The regression equation is

$$P_w = 0.414 - 0.00029 \text{ mosm/l} \quad (2)$$

While one obvious conclusion is that P_w depends primarily on extracellular osmolality, an equally reasonable possibility is that the difference in concentration across the membrane is of primary importance. In an attempt to distinguish between these alternatives we varied the initial equilibrated intracellular osmolality between 240 mosm/l and 340 mosm/l, to provide permeabilities at the same extracellular osmolality but at differing intracellular osmolality. The results are included in Fig. 5 and distinguished by different symbols. There was no detectable effect of changing intracellular osmolality upon P_w. Since the electrolyte concentration of the suspending fluid could not be varied more than from -60 to $+40$ mosm/l without producing prohibitive hemolysis, P_w's dependence on internal osmolality would have to have

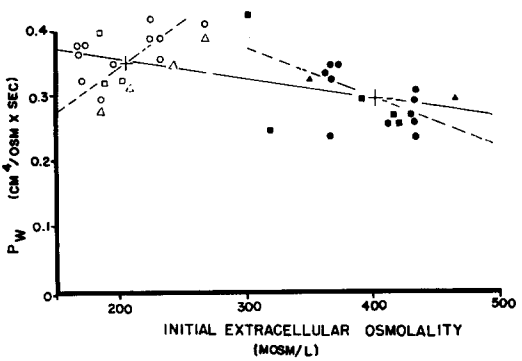


Fig. 5. Water permeability of human red cells (P_w) as a function of initial extracellular osmolality. The circles are the reactions in which the initial osmolality was normal, approx. 300 mosm/l, the squares are those in which the initial osmolality was hypotonic (242–270 mosm/l) and the triangles are those in which the initial osmolality was hypertonic (321 and 341 mosm/l). The open symbols indicate swelling reactions (reduced extracellular osmolality) and the filled symbols shrinking reactions (increased extracellular osmolality). The two crosses are the average values for shrinking and for swelling and the two dotted lines are the corresponding regression curves, whose slopes are significantly different from zero and from each other ($P < 0.25$). The solid line is the regression for all points.

TABLE I

OSMOTIC WATER PERMEABILITY (P_w) OF HUMAN ERYTHROCYTES

The suspension contained 2.2 mM KH_2PO_4 and 7.8 mM K_2HPO_4 *plus* sufficient NaCl to produce the required osmolality. Temperature was 26–27°. 'Range' refers to variations in the slope of the volume function (Eqn. 1) during one experiment. Where no range of P_w is given, it was constant throughout the experiment.

Blood sample	*Initial osmolality*		*Initial osmolal gradient* (*mosm/l*)	P_w (*cm*⁴/*osm·sec*)	*Range* (*cm*⁴/*osm·sec*)
	Extracellular (*mosm/l*)	*Intracellular* (*mosm/l*)			
H–1	226	298	− 72	0.42	0.35–0.42
	435		+ 137	0.31	0.26–0.34
	366		+ 68	0.33	
H–3	167	300	− 133	0.36	
	228		− 72	0.39	0.35–0.39
	433		+ 133	0.27	0.22–0.27
	365		+ 65	0.35	
H–4	167	300	− 133	0.38	
	228		− 72	0.38	
	433		+ 133	0.24	
	365		+ 65	0.35	
H-6	168	303	− 135	0.32	
	197		− 106	0.35	
	231		− 72	0.36	
	267		− 36	0.41	
	364		+ 61	0.24	
H–7	433	304	+ 129	0.29	
	365		+ 61	0.34	
H–9	167	301	− 134	0.38	
	434		+ 133	0.26	
H–10	185	285	− 100	0.29	
	412		+ 127	0.26	
H–11	189	270	− 81	0.32	0.32–0.50
	416		+ 146	0.27	0.27–0.32
H–12	185	261	− 76	0.40	
	317		+ 156	0.25	0.25–0.32
H–13	201	242	− 41	0.32	0.25–0.36
	442		+ 200	0.26	0.20–0.26
	394		+ 152	0.30	
	300		+ 58	0.43	
H–14	185	341	− 156	0.28	0.27–0.30
	209		− 132	0.31	0.23–0.34
	267		− 74	0.39	0.27–0.39
	348		+ 107	0.33	
H–17	243	321	− 78	0.35	
	465		+ 144	0.30	0.20–0.30

exceeded that on external osmolality in order to have produced statistically significant changes.

There appears to be a peak in P_w around the isosmolal extracellular concentration of 300 mosm. The regression lines for the points at concentrations greater than and less than 300 mosm are of opposite slope and significantly different from each other (Fig. 5) and from zero, supporting this impression.

In Fig. 6 P_w is plotted as a function of (extracellular osmolality *minus* initial intracellular osmolality). Again the separate regression lines for P_w for swelling and

for shrinking are of opposite slope and significantly different from each other and from zero, suggesting a peak value of P_w at zero flow. We attempted to measure P_w at values of osmolal gradient less than 30 mosm but because the total volume change also becomes small, the error in P_w becomes relatively great, and we were unable to procure more definitive data on this point.

Additional experiments were made in which the major osmotically active species was sucrose, rather than NaCl. The data are summarized in Table II. The regression of P_w on extracellular osmolality is similar to that of the NaCl experiments in Fig. 5, but there is no indication of a maximal value of P_w around 300 mosm.

Human red cells incubated for 1 h with 30 mM fluoride, a potent inhibitor of

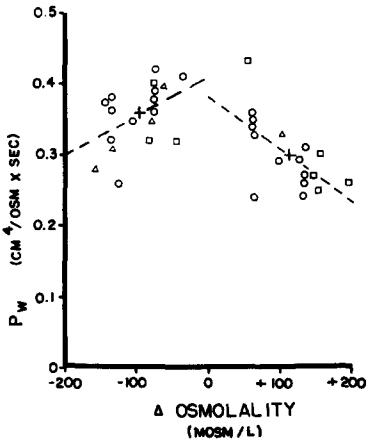


Fig. 6. A graph of P_w *versus* extracellular osmolality *minus* initial intracellular osmolality. Δ indicates an initial osmolality over 300 mosm/l; o indicates an initial osmolality about 300 mosm/l; □ indicates an initial osmolality less than 300 mosm/l. The two crosses are the average points for swelling and shrinking; the dotted lines are the respective separate regressions.

TABLE II

OSMOTIC WATER PERMEABILITY (P_w) OF HUMAN ERYTHROCYTES WHEN THE GRADIENT IS PRODUCED BY SUCROSE

The suspending medium contained 27 mM NaCl, 2.2 mM KH_2PO_4 and 7.8 mM K_2HPO_4 and between 195 and 240 mM sucrose, depending on the final osmolality desired.

Blood sample	*Initial osmolality*		P_w ($cm^4/osm \cdot sec$)	*Range* ($cm^4/osm \cdot sec$)
	Extracellular (*mosm/l*)	*Intracellular* (*mosm/l*)		
H–8	173	342	0.40	0.30–0.40
	254		0.27	
	398		0.23	
	392		0.24	
H–16	197	286	0.35	
	225		0.31	0.21–0.31
	321		0.25	
H–18	202	303	0.42	0.35–0.42
	398		0.28	

glycolysis, had an average P_w as much as one-third less than cells incubated in the absence of this anion.

Osmotic permeability of horse red cells

P_w of horse red cells, originally equilibrated with a 300 mosm/l solution, 135 mM NaCl and 10 mM phosphate buffer (pH 7.5) and 25° was 0.25, 0.22, 0.21 and 0.15 cm⁴/osm·sec at initial extracellular osmolalities of 161, 226, 365, and 433 mosm/l, respectively. $P_w = 0.285 - 0.00026$ (concentration is mosm/l), a similar correlation as for human cells, although the data are much more limited. Average cell volume was considered 53 μ^3 and surface area 141 μ^2, according to the formula of EMMONS[28]. *RW* was 0.50. Change in light transmission was linearly related to the hematocrit.

Osmotic permeability of nucleated red blood cells

P_w for the red blood cells of five eels was 0.052 ($N = 3$) for exosmosis and 0.057 ($N = 2$) for endosmosis at pH 7.8 in the extracellular fluid and 0.060 for both exosmosis and endosmosis (N = 2) at pH 5.9, all in cm⁴/osm·sec. The cells were originally suspended in the same buffer as for human cells with an osmolality of about 300 mosM. There was no significant correlation between P_w and the extracellular osmolality, nor any striking effect of pH. The volume of the cells was 189 μ^3 at pH 7.8. The surface area for a prolate spheroid resembling the eel red cell, with an axial ratio of 3:1 was calculated to be 188 μ^2. P_w tended to increase with time during a change in volume so that we cannot place the same reliance on these results as we can on the data for human red cells, but there is no doubt but that it is about 5 times greater in human than in eel cells.

P_w of the nucleated red blood cells of the domestic chicken was 0.072 cm⁴/osm·sec in one swelling experiment and 0.026 cm⁴/osm·sec in one shrinking experiment. In contrast to the results in eel cells, the volume function increased linearly with time. In calculating P_w it was assumed that the surface area: volume was the same as for eel cells. *RW* was 0.62.

DISCUSSION

Effect of external osmolality on osmotic permeability

RICH *et al.*[29] have reported similar measurements of P_w in human and dog red cells at room temperature using a stopped-flow rapid-reaction apparatus of different design than ours which determined light scattered at 90° rather than that transmitted. Replotting the individual points* from their published graphs, the linear regression of P_w on extracellular osmolality had a slope of −0.001 cm⁴/osm·sec, about three times steeper than our results. P_w at 420 mosm/l was lower, 0.22 cm⁴ per osm·sec as compared with our value of 0.30 cm⁴/osm·sec, while at 200 mosm/l P_w was 0.45 cm⁴/osm·sec as compared with our value of 0.38 cm⁴/osm·sec. RICH *et al.*[29] found a significant correlation between P_w and extracellular osmolality of the form

$$\log P_w = A - B/(\text{extracellular osmolality}) \quad (3)$$

When they (a) decreased the initial intracellular osmolality about 50 mosm/l or (b) increased it to 490 mosm/l, by ingeniously loading the cells with creatinine, which

* These values of P_w presumably include a correction for the presence of a stagnant layer at the cell surface.

was impermeable over the duration of the rapid volume changes, they found that P_w fell on a line determined by Eqn. 3. From this they concluded that intracellular osmolality had little influence on P_w. It must, however, be noted that if P_w is considered a linear function of extracellular osmolality (as in Fig. 5), and there would appear to be as much justification for this as for Eqn. 3, an influence of intracellular osmolality on P_w cannot be ruled out completely on the basis of their data. In support of the conclusion of RICH *et al.*[29] is our finding that P_w is constant during a volume change, although intracellular osmolality changes from 36 to 200 mosm/l (see Table I).

We conclude that P_w increases as extracellular osmolality decreases, that under achievable experimental conditions this means that P_w is greater during inflow than outflow, and that the fundamental mechanism of this phenomenon is most likely dependent on extracellular osmolality, although a residual effect of intracellular concentration cannot be ruled out. The experiments with sucrose (Table II) suggest that it is osmolality and not ionic strength that is of major importance in determining P_w.

Rectification of water flow through membranes, defined as a variation in water permeability with variation in direction of the transmembrane concentration gradient, has been reported previously for arbacia eggs[30], mammalian leucocytes[31], and Valonia[32], to give but a few examples. It is relatively easy to demonstrate that a membrane composed of two layers, where the permeability of each layer is a function of the osmolality of the fluid phase bounding its free surface, but where this function is not the same for the two layers, will exhibit water permeability dependent on ambient osmolality. The basic mechanism by which the osmolality of the suspending fluid alters red cell P_w is not known, but it is tempting to assume that a decrease in fluid osmolality leads to a greater hydration of the local membrane permitting the freeer passage of water molecules.

Unstirred layers

Theoretically there is a stagnant or unstirred boundary layer of fluid of some infinite thickness on the cell surfaces, even during mixing or passage down the observation tube of a continuous-flow rapid-reaction apparatus under turbulent flow conditions. A diffusion barrier of this nature might be rate limiting in the measurements of P_w and its possible presence was an early concern in measurements of rapid exchanges of red cells, particularly gases, in this laboratory and elsewhere[10,11,33]. There have accumulated a series of observations and arguments setting a maximum on the importance of this hypothetical stagnant layer, of which we mention some pertinent.

(1) The cell suspensions we used were approx. 5 % cells by volume. With a red cell volume[26] of 87 μ^3, corresponding to a sphere of 2.8 μ radius, each cell would on the average have a sphere of suspending fluid of total radius 2.8 $(20)^{1/3} = 8$ μ around it. Thus the maximum average distance from the cell surface to the surface of this sphere is approx. 5 μ and represents a maximum stagnant layer thickness from simple geometric considerations.

(2) When a 1:20 suspension of deoxygenated human red cells is mixed with a buffer solution containing 150 mm Hg of dissolved O_2 in a continuous-flow rapid-reaction apparatus and a p_{O_2} electrode used to follow the changes in O_2 concentration with time, the extrapolated intercept at time zero is within 1.5 mm Hg

of the value calculated on the assumption that complete mixing of O_2 has taken place instantaneously[34]. This works out to mean that the suspension is better than 98 % mixed, by convection and/or diffusion, within 0.001 sec of leaving the mixing chamber. Neglecting the volume of the cells entirely, this would represent a shell of fluid less than 0.8 μ thick around each cell.

(3) Once flow has stopped in the reaction apparatus we might expect that a stagnant layer will establish itself, possibly slowing up later stages of the exchanges with cells. This possibility was a primary consideration in testing the validity of the use of a stopped-flow rapid-reaction apparatus in the measurement of gas exchange rates in red cells. The exchange of O_2 with red cells with a half time of about 0.03 sec, a much faster process than exchange of water, has the same rate in a stopped-flow rapid-reaction apparatus as in a spectrophotometric continuous-flow rapid-reaction apparatus[11,16]. In the latter turbulent flow conditions exist in the observation tube, which while presumably not as efficient in maintaining mixing as a four-jet mixing chamber, should be significantly better than no flow.

(4) It is possible to calculate the diffusion resistance of the red cell 'membrane' from measurements of the rate of oxyhemoglobin formation in cell suspensions as compared with that in hemoglobin solutions[11,35–37]. We obtain a maximal thickness of the 'membrane', which includes any stagnant layer, equivalent to 1.4 μ of saline.

(5) Assuming a stagnant layer exists throughout the mixing process, the most extreme case, the time required for 'quasi steady state' water diffusion to be established through it is similar to the classical problem of the time required for permeation through a membrane to become steady, for which solutions are available (ref. 38, p. 44; ref. 39, p. 47). Making reasonable simplifying assumptions the approximate time required for steady-state conditions to become established within the stagnant layer can be calculated from the relation, $t = 0.45$ diffusion coefficient/thickness^2 (ref. 39, p. 49). These calculations show that it takes only 0.18 msec if the stagnant layer is 1 μ thick and 4.5 msec if the layer is 5 μ thick. We conclude that even if a stagnant layer of 1 μ thickness existed it would produce: (A) a transient distortion of the initial portion of the light transmission curve which would disappear before 0.18 msec and which could not be detected experimentally; followed by (B) a nearly constant retardation of the process which would be distinguishable from a 6 % reduction in P_w. SHA'AFI *et al.*[12] have interpreted their measurements of light scattering changes in a stopped-flow rapid-reaction apparatus to mean that there is a 5.5-μ stagnant layer around the red cells. Their overestimation arises from the use of a solution of the diffusion equation for the transient in a semi-infinite layer with constant boundary conditions (ref. 39, Eqns. 4, 17) substituted in an equation where the boundary conditions varied with time.

Water permeability of nucleated red blood cells

P_w of eel cells is of particular interest because of its importance in the mechanism of O_2 secretion by the swim bladder[40,41]. Both eel and chicken erythrocytes have membrane-bounded nuclei and other subdivisions of their volume[42] which might be expected to produce a more complicated water exchange process. However, it appears unlikely that there is any other explanation for their low P_w except a real decrease in water permeability of their cell membranes.

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