Effect of Osmolality on the Hydraulic Permeability Coefficient of Red Cells

G. T. RICH, R. I. SHA'AFI, A. ROMUALDEZ, and A. K. SOLOMON

From the Biophysical Laboratory, Harvard Medical School, Boston, Massachusetts 02115. Dr. Rich's present address is the School of Biological Sciences, University of East Anglia, Norwich, England

ABSTRACT The osmotic water permeability coefficient, L_p, for human and dog red cells has been measured as a function of medium osmolality, and found to depend on the osmolality of the bathing medium. In the case of human red cells L_p falls from 1.87×10^{-11} cm³/dyne sec at 199 mOsM to 0.76×10^{-11} cm³/dyne sec at 516 mOsM. A similar decrease was observed for dog red cells. Moreover, L_p was independent of the direction of water movement and the nature of the solute used to provide the osmotic pressure gradient; it depended only on the final osmolality of the medium. Furthermore, L_p was not affected by pH in the range of 6 to 8 nor by the presence of drugs such as valinomycin (1×10^{-6} M) and tetrodotoxin (3.2×10^{-6} M). The instantaneous nature of the response to changes in external osmolality suggests that the hydraulic conductivity of the membrane is controlled by a thin layer at the outer face of the membrane.

INTRODUCTION

It has long been known that the permeability of single cells depends upon the osmolality of the medium in which the measurements have been made (Lucké and McCutcheon (1)). For a given driving force, cell swelling measured under hypotonic conditions appeared to be much faster than cell shrinking measured under hypertonic conditions. Dainty and Hope (2) have pointed out that this directional effect of water movement may be only apparent due to a "sweeping away" effect associated with the presence of an unstirred layer around the cell surface. A complex dependence of permeability properties on the osmolality of the bathing solutions has been reported in systems with more than a single series barrier such as the gall bladder studied by Diamond (3). It was therefore decided to study the basic mechanism in a simple system in order to determine the direct effect of osmolality on water permeability. In mammalian red cells there is only a single limiting membrane and the unstirred layer has been shown by Sha'afi, et al. (4) to have a negligible effect on the hydraulic permeability coefficient, L_p. The present results indicate that L_p is

independent of the direction of water movement and of the volume of the red cells; L_p is determined only by the final osmolality of the system. The effect of the medium pH on L_p was also investigated, as was the addition of drugs such as valinomycin which are known to affect the cation permeability of cellular membranes.

EXPERIMENTAL PROCEDURE

Immediately before an experiment human blood was drawn from healthy male or female donors by venipuncture. Dog blood was drawn from anesthetized dogs (Nembutal, 30 mg/kg) by heart puncture or from the femoral vein. Heparin (1000 units/ml, 4 ml/liter) was used to prevent clotting. The blood was diluted in a buffered saline medium to form a suspension of 3% by volume of whole blood. The isosmolal medium had the following composition: NaCl, 117.8 mM; $MgCl_2$, 0.5 mM; $CaCl_2$, 1.2 mM; KCl, 4.4 mM; Na_2HPO_4, 1.7 mM; NaH_2PO_4, 4.2 mM; Na_2CO_3, 13.5 mM, and was aerated with 5% CO_2-95% air to bring it to pH 7.4. The osmolality of this buffer was 290 ± 5 mOsM, as measured by freezing point depression (Osmometer model G-62, Fiske Associates, Inc., Bethel, Conn.). Hyper- and hyposmolal solutions were prepared from the same buffer by changing the NaCl concentration alone.

Hydraulic permeability coefficients were measured in the stop-flow apparatus previously described (4). Red cell suspensions were rapidly mixed with anisosmolal media, and 90° scattered light was used to measure the changes in cell volume. To calibrate the volume changes, the initial and final volumes of the cells were determined by equilibrium hematocrit measurement.

In the initial experiments the red cells in isosmolal buffer were mixed with hyper- or hyposmolal solutions to give shrinking and swelling curves with different relative volume changes. In further experiments of a similar kind the initial volume of the cell was altered by preequilibration in anisosmolal solutions. In both of these types of shrinking experiments the final osmolality of the medium did not exceed 1.5 times the isosmolal concentration in order to keep within the range in which the intensity of the scattered light signal depended linearly on cell volume.

The osmolality range could be extended beyond the usual limits by using a permeant solute which did not cross the cell membrane during the 1 sec experimental period. At room temperature creatinine penetrates the human red cell slowly with a halftime of about 2.5 hr (5). Although there is no evidence for facilitated transport, creatinine penetration has a high temperature coefficient, and at 37°C it equilibrates rapidly. Human red cells were therefore equilibrated with 50 or 100 mOsM creatinine at 37°C for 2 hr. After cooling to room temperature the suspensions were mixed in the stop-flow apparatus with hyperosmolal solutions containing identical concentrations of creatinine. Hematocrit measurements confirmed that at room temperature the red cell membrane may be considered to be effectively impermeable to creatinine over the time course of the shrinking experiment, and that a period of 2 hr at 37°C was sufficient for complete equilibration of creatinine across the red cell membrane. This method of extending the osmolality range made it possible to retain the linear relationship between cell volume and scattered light. However, it is only valid if creatinine itself has no effect on the red cell beyond that expected from the rise in

osmolality. The absence of any volume changes induced by creatinine was demonstrated by comparing hematocrit measurements and wet to dry weight ratios of cells equilibrated at 37°C in isosmolal buffer with and without creatinine; the cell volume change in two experiments was less than 1% with up to 100 mOsM creatinine. This and the consistency of the hydraulic permeability coefficients measured in NaCl alone and with creatinine indicates that this is an acceptable method of extending the osmolality range.

RESULTS

Cell volume is related to the osmotic pressure difference according to the following equation, initially given by Jacobs (6)

$$dV_c/dt = L_pA[\pi_i - RTC_m] \quad (1)$$

in which: V_c is the cell volume, which under isosmolal conditions is taken as 87×10^{-12} cm³ for the human and 68×10^{-12} cm³ for the dog (see references 4 and 7). L_p is the hydraulic permeability coefficient expressed in cm³/dyne sec. R and T have their usual meanings (the osmotic permeability coefficient, P_w, which is expressed in cm⁴/osmole sec, is equal to RTL_p). The medium concentration, C_m, is given in terms of osmole/cm³, and has been corrected for the osmotic coefficient of the solutes. Since the hematocrit reading is very low, C_m is effectively constant during the swelling and shrinking experiments. π_i is the osmolality of the cell and π_m that of the medium. π_i may be determined from the cell volume, which is routinely measured by the hematocrit method as a function of $1/\pi_m$ in an equilibrium experiment for each blood. The linear relationship observed between V_c and $1/\pi_m$ is very helpful in interpolating between the measured points, but as pointed out by Gary-Bobo and Solomon[1] the slope of the line does not give any information about the osmotic properties of the cell water. In equation 1, as used here and in previous papers from this laboratory, the slope serves only to relate the osmolality of the cell to the measured cell volume.

The cell surface area, A, is 1.67×10^{-6} cm² for the human and 1.24×10^{-6} cm² for the dog under isosmolal conditions (see references 4 and 7). Jacobs (8) has pointed out that the human red cell area remains constant over a wide range of volumes as the cell swells and Sha'afi et al. (4) have given arguments which support this assumption when human cells shrink up to 17%, corresponding to the smallest volume in these experiments. They have also computed that the maximum possible area change on shrinking by this amount would be of the order of 10%, which is not large compared to the observed effect on L_p. The present experimental results give further evidence on this point, which will be discussed in a later section.

[1] Gary-Bobo, C. M., and A. K. Solomon. 1968. Properties of hemoglobin solutions in red cells. *S. Gen. Physiol* **52**: 825.

Effect of Medium Osmolality on L_p

Fig. 1 shows a typical experiment in which dog red cells initially placed in isosmolal buffer were shrunken in a 420 mOsM medium. L_p was determined by the method of the least squares using a computer program which utilized the data points between 100 and 450 msec. The technique for curve fitting and the reason for the choice of this particular time interval have been discussed by Sha'afi et al. (4).

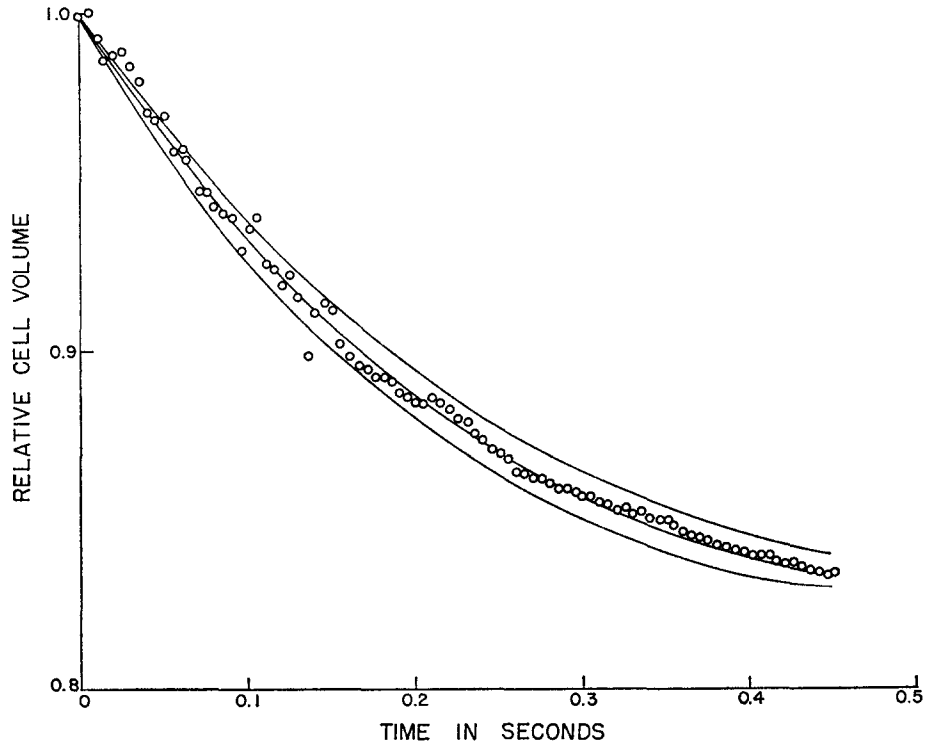

FIGURE 1. Typical cell shrinking curve for dog red cells. The central line corresponds to the least squares computed curve for $L_p = 1.35 \times 10^{-11}$ cm³/dyne sec; the upper and lower curves have been drawn for L_p's that differ by plus and minus 10%, 1.21, and 1.48×10^{-11} cm³/dyne sec.

Fig. 2 shows the effect of the the final equilibrium osmolality on L_p in all the experiments on human and dog red cells. As the osmolality is increased L_p decreases significantly, so that swelling may be up to two times faster than shrinking. The L_p's measured in the presence of creatinine in Fig. 2 are consistent with those in which the cells shrank in NaCl. The results of the previous experiments of Sha'afi et al. (4) in man and those of Rich et al. for dogs (7) are indicated by full circles in the figure. It can be seen that these data are

entirely consistent with the present results. The values for L_p under isosmolal conditions, taken from Fig. 2, are 1.27 × 10^{-11} cm³/dyne sec for man and 1.95 × 10^{-11} cm³/dyne sec for the dog. In our previous experiments, the osmolality range covered was insufficient to show a consistent variation of L_p with osmolality that was greater than the random errors of analysis.

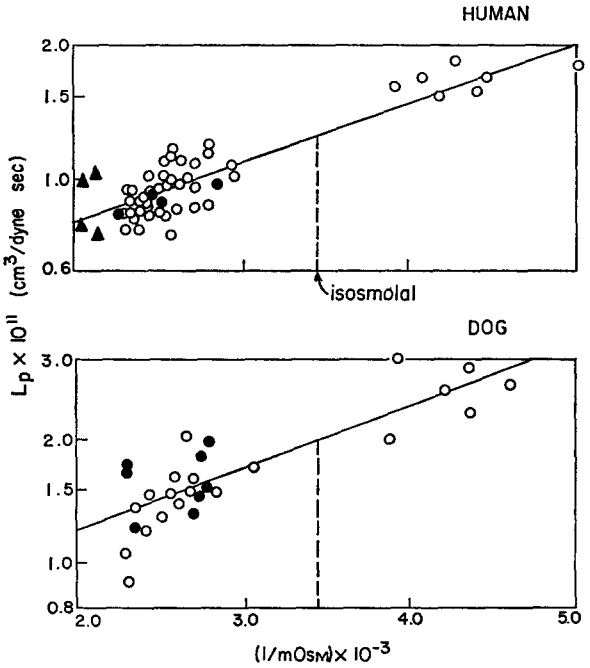

FIGURE 2. ln L_p as a function of $1/\pi_m$ for all the experiments in both human and dog red cells. The lines have been drawn by least squares. In both cases the filled circles give the results obtained in previous studies in this laboratory, for humans by Sha'afi et al. (4), and for dogs by Rich et al. (7). In the case of the human red cells, the triangles denote the results of the experiments in which creatinine was added.

There was a rather wide variation of L_p among different dogs when measured under the same conditions as can be seen from the results presented in Table I. However, in any one sample of blood the observed trend of L_p with osmolality was consistent, both for human and dog cells as shown in Fig. 3.

The change in L_p appeared to be very rapid, and to be essentially complete within at least 50 msec. The data in Fig. 1 are for an experiment in which L_p shifts by 30% from an initial isosmolal value of 1.95 × 10^{-11} cm³/dyne sec to a final value of 1.35 × 10^{-11} cm³/dyne sec. Fig. 1 gives the envelope for changes in L_p of ±10% from the final least squares value, and it is clear that virtually all the data points after the first 50 msec are contained well within

these limits. There is no indication of any progressive change in L_p with time after the first 50 msec. This conclusion has also been confirmed by least squares analysis, as shown in Table II which compares the least squares L_p for the first 50 msec with the value determined at later time intervals. Though the resolution in the shrinking experiments is, characteristically, very much better than in the swelling experiments, a similar rapid adjustment obtains for the swelling experiments, as shown in Fig. 4 and Table II. In this case there is a

TABLE I

VARIATION IN L_p AMONG DIFFERENT DOGS

Dog	Exp	Date	Equilibrium osmolality of medium relative to isosmolal	$L_p \times 10^{11}$	Residual sum of squares*
			$mOsm/(mOsm)_{iso}$	$cm^3/dyne\ sec$	
A	56	6/2/66	1.34	1.95	1×10^{-3}
	73	7/13/66	1.36	1.95	5×10^{-4}
	76	7/19/66	1.31	1.78	—
B	72	7/12/66	1.27	1.28	2×10^{-3}
	77	7/19/66	1.31	1.42	4×10^{-4}
C	80	10/20/66	1.25	1.10	1×10^{-3}
D	81	10/21/66	1.25	1.52	1×10^{-3}

* The program for the computer gives the residual sum of the squares of the differences between the predicted values of cell volume, (obtained from the chosen L_p and equation 1) and the observed volume at each point. In each computation there are 71 points, so that the average residual sum of the squares of 9.8×10^{-4} corresponds to a mean error of 0.004 in relative cell volume. This figure may be compared with volume shifts in typical experiments of 0.1 to 0.15. In a random series of eight experiments on human red cells the average value for the residual sum of the squares is 23.7×10^{-4} corresponding to an average mean error of 0.006 in cell volume.

50% change in L_p ; L_p reaches its final value within 50 msec and does not shift more than 10% over the entire 450 msec time course. Since the internal osmolality is changing continuously over the 450 msec time interval whereas the external osmolality remains constant, these experiments indicate that L_p is determined primarily by the external medium and that the internal environment plays a negligible role.

Absence of Directional Preference for Water Movement

The second point of interest is the possibility of a directional preference of water movement in red cells. That is, does the fact that swelling is faster than shrinking merely reflect the equilibrium osmolality, or is swelling always the

faster process? In order to answer this question an experiment was designed in which samples of cells from a single donor were initially either swollen or shrunken prior to the determination of L_p. Two experiments of this kind were performed on both dog and human red cells, and the results of one of each

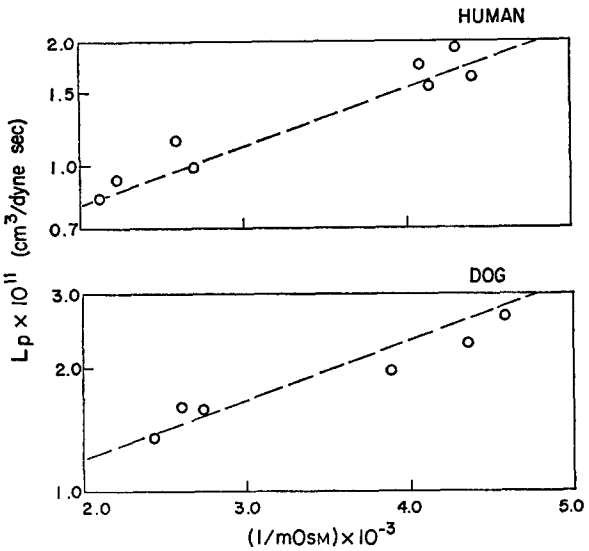

FIGURE 3. ln L_p as a function of $1/\pi_m$. In this case the results of single experiments with human red cells and with dog red cells are given. The dashed lines are the least squares lines for all the experiments taken from Fig. 2.

TABLE II

TIME COURSE OF L_p ADJUSTMENT

Time	$L_p \times 10^{11}$	
	Shrink (medium osmolality, 420 mOsM)	Swell (medium osmolality, 230 mOsM)
	cm³/dyne sec	*cm³/dyne sec*
Computed initial value (from Fig. 2; initial osmolality, 290 mOsM)	1.95	1.95
Least squares value for time interval		
0–50 msec	1.16	2.98
50–100 msec	1.34	3.30
100–450 msec	1.35	2.92

are given in Table III. In order to facilitate comparison, the results at the several osmolalities were each referred to a calculated value for the same osmolality obtained from the least squares lines in Fig. 2. The ratios of the calculated to the measured L_p's are given in columns 5 and 6. The average ratio for the swelling experiments was compared to that for the shrinking

experiments. In the case of human cells, the ratios are close to 1.0 and comparison of columns 5 and 6 shows that the difference between swelling and shrinking is 6%. This would appear to be in the range of the normal variability characteristic of such experiments. In the case of the dog, variations from animal to animal are appreciable (see Table I), so it is not surprising that the mean ratio in this experiment is 0.84 instead of 1.0. The difference in L_p

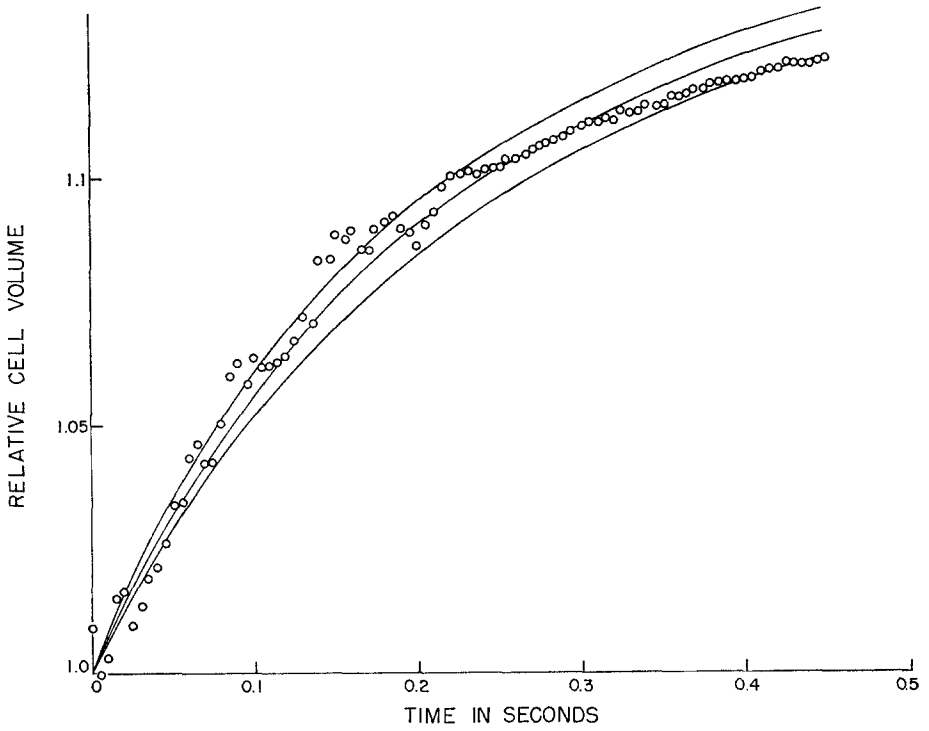

FIGURE 4. Typical cell swelling curve for dog red cells. The central line corresponds to the least squares computed curve for $L_p = 2.92 \times 10^{-11}$ cm³/dyne sec, and the upper and lower curves have been drawn for L_p's that differ by plus and minus 10%, 3.21, and 2.63×10^{-11} cm³/dyne sec.

between swelling and shrinking is about 12%, and is in the opposite direction to the difference of 6% observed in human red cells. We may conclude that the dependence of L_p on the direction of water movement is small, if real, and is in any case below the limits of resolution of the present methods. The same is true of the effect of initial cell volume on L_p, since this parameter also depends on the initial osmolality.

Effect of Medium Constituents on Hydraulic Permeability

Phosphate buffer was used to study the effect of pH on L_p. The buffer had the following composition at pH 7.4: NaCl, 130 mM; $MgCl_2$, 0.16 mM; KCl,

5.0 mM; $CaCl_2$, 0.4 mM; Na_2HPO_4, 5.2 mM; NaH_2PO_4, 0.8 mM. The osmolality, as measured by freezing point depression, was 280 ± 5 mOsM. In four experiments L_p at pH 7.4 in the phosphate buffer was (1.03 ± 0.1) × 10^{-11} cm³/dyne sec as compared with (1.02 ± 0.1) × 10^{-11} cm³/dyne sec for the bicarbonate.

In six experiments L_p for human red cells was found to be independent of

TABLE III

EFFECT OF DIRECTION OF WATER FLUX ON L_p

Initial cell osmolality	Final medium osmolality	$L_p \times 10^{11}$		$\frac{L_{p(calc)}}{L_{p(meas)}}$	
		Measured	Calculated	Swell	Shrink
mOsm	*mOsm*	*cm³/dyne sec*	*cm³/dyne sec*		
Human					
250	202	2.20	2.10	0.95	
	330	1.10	1.22		1.10
	233	1.62	1.68	1.04	
290	207	2.00	2.02	1.01	
	396	0.93	0.95		1.02
	234	1.67	1.68	1.00	
Average				1.00	1.06
Dog					
237	290	2.39	1.95		0.82
	322	2.16	1.75		0.81
	335	2.12	1.69		0.80
288	230	2.92	2.64	0.90	
	376	2.05	1.50		0.73
373	271	2.25	2.10	0.93	
	288	2.24	1.96	0.86	
Average				0.90	0.79

pH in the range from pH 6 to 8; the value of L_p at pH 6.0 was (0.99 ± 0.1) × 10^{-11} cm³/dyne sec as compared with (1.05 ± 0.1) × 10^{-11} cm³/dyne sec at pH 8.0. It had been hoped that some singularities would appear so that we might obtain information about the effect of ionizable protein groups on permeability and membrane structure, but it is not altogether surprising that L_p is constant between pH 6 and 8 since most of the amino acid pK's lie outside this narrow range. Furthermore, the mobility, and the external charge on the red cell membrane which are largely ascribed to sialic acid, also remain virtually constant over this pH range, as shown by Eylar et al. (9).

A series of experiments were carried out to study the effect of the ionic strength of the medium by replacing increasing amounts of NaCl with sucrose. Variation of the ionic strength from 148 mmolal to 238 mmolal did not appear to cause any consistent change in L_p as can be seen from the data in Table IV. In another experiment 252 mOsM of NaCl was replaced with NaBr. There was no change in L_p (0.72 and 0.73 $\times$ 10^{-11} cm^3/dyne sec). All of the results of these several sets of experiments are consistent with the view that it is the osmolality of the medium, rather than any specific detail of its chemical composition that is responsible for the effects on L_p shown in Fig. 2.

Gary-Bobo and Solomon[1] have shown that there is a total chloride shift of 10–12 mEq/liter red cell concomitant with changes of cell volume over the osmolality ranges that we have used. The sensitivity of the stop-flow apparatus

TABLE IV

EFFECT OF SUCROSE SUBSTITUTION ON L_p IN HUMAN RED CELLS

Medium			Ionic strength	$L_p \times 10^{11}$
NaCl	Sucrose	Total		
mOsm	*mOsm*	*mOsm*	*mmolal*	*cm³/dyne sec*
464	0	464	238	0.83
433	31	464	222	0.82
350	0	350	181	1.01
290	60	350	151	1.16
389	0	389	200	1.06
285	100	385	148	1.01

is not expected to detect such changes, which should be occurring simultaneously with the water movement, since the halftime for chloride exchange in human red cells has been shown to be 0.2 sec by Tosteson (10). Gary-Bobo and Solomon have shown that the direction of the chloride shift is reversed at the hemoglobin isoelectric point (pH 6.95), so that a comparison of the detailed time course of shrinking at pH 6 and pH 8 should show whether there is any possibility of detecting the effect. However, a close comparison reveals no apparent difference in the time course of shrinking over this pH range. Therefore, the chloride shift is of negligible importance in the determination of L_p by our method.

Effect of Tetrodotoxin and Valinomycin

There are a number of compounds that produce selective actions on sodium and potassium transport across the membrane. We have selected two of these, tetrodotoxin and valinomycin, for further study. Tetrodotoxin selectively

blocks the sodium conductance in nerve and muscle fibers (11) and thus effectively inhibits the action potential with no change of resting potential. Current theory considers the channels for sodium conductance to be specific for the sodium ion; we would not therefore expect any tetrodotoxin effect on L_p, and none has been found. The results are given in Table V which covers the range of 10^{-6} to 10^{-7} M, a concentration range which has been effective in blocking action potential, when applied to the bathing medium of lobster axons (12). Though it is not to be expected that our findings may be extrapolated to nerve, the present observation is consistent with the current view that the sodium conduction channels are primarily specialized for sodium carriage.

TABLE V

EFFECT OF TETRODOTOXIN AND VALINOMYCIN ON L_p IN HUMAN RED CELLS*

	Concentration	$\frac{L_{p(drug)}}{L_{p(control)}}$
	M	
Tetrodotoxin	0	1.00
	2×10^{-7}	1.03
	4×10^{-7}	1.04
	8×10^{-7}	0.96
	1.6×10^{-6}	0.95
	3.2×10^{-6}	0.95
Valinomycin	0	1.00
	3×10^{-7}	1.00
	1×10^{-6}	1.06
	1×10^{-6}	1.00

* We have also found that amphotericin B at a concentration of 1.5 mg/liter suspension has no effect in conformity with the observations of Hempling on ascites tumor cells (18).

The interest in valinomycin arises from its demonstrated efficacy in promoting massive potassium-hydrogen exchange in mitochondria (13). Recently, Tosteson et al. (14) have shown that valinomycin at 10^{-7} M, can also produce a 10- to 20-fold increase in net potassium diffusion in both HK and LK sheep red cells, and that this increase is independent of ouabain inhibition. Valinomycin also induces selective potassium permeability in black lipid membranes (15). It is thought that this cyclic depsipeptide exerts its effect either by acting as a carrier for the hydrated potassium ion or by forming potassium specific channels. The experiments reported in the bottom of Table V show that valinomycin does not have any effect on red cell hydraulic permeability. We conclude either that valinomycin induced potassium permeability does not use channels available for water, or that if valinomycin does form such channels, their number is small compared to the number of water

pathways. In this context, it is of interest that Cass and Finkelstein (16) have recently shown that the excitability inducing material of Mueller et al. (17) has no effect on either hydraulic or diffusion permeability coefficients in black lipid membranes. This unpurified protein lowers the membrane electrical resistance by 500–1000 fold, an effect which has been attributed to increased permeability to cations. The results with tetrodotoxin and valinomycin in the red cell, in conjunction with those on the black lipid membranes, are consistent with the view that cations travel by specialized pathways whose impedance can be controlled by specific reagents that have little or no effect on the generalized permeability properties of the membranes.

DISCUSSION

A dependence of L_p on osmolality has been found in many other investigations of the permeability properties of the membrane bounding living cells. For example, Kamiya and Tazwa (19) found that the outward movement of water in *Nitella flexilis* was less than half of the inward movement. Dainty and Hope (2) obtained similar results for *Chara* after taking the unstirred layers into account. This latter effect was explained by Dainty and Ginzburg (20) who postulated that the membrane was more hydrated during endosmosis because the osmolality was lower. In this connection, Hartley (21) has suggested that the diffusion coefficient of water through polymers should be strongly dependent on the degree of hydration of the polymer and Lagos and Kitchener (22) have demonstrated that increased hydration increases the diffusion of nonelectrolytes along thin strips of ion exchange resin.

Our results are entirely consistent with such a mechanism. In the case studied by Lagos and Kitchener, the increase in the nonelectrolyte diffusion coefficient is attributed to geometrical factors. The swelling of the resin structure leads to an increase in the size of the water pathways. In the case of the red cell membranes, increased hydration might act either by swelling equivalent pores, or possibly by some generalized decrease in the hindrance offered by the entire fabric of the membrane.

The fact that L_p does not depend on initial osmolality, initial cell volume, or direction of water movement suggests that, unless there is a fortuitous cancellation of errors, the assumption that the red cell area is constant is correct. Additional experiments have confirmed this point. For example, human red cells of normal volume were swollen in 234 mOsM buffer; this experiment was compared to one in which cells swollen to 1.6 times normal volume were shrunk in the same medium. The L_p's were identical: 1.67×10^{-11} and 1.64×10^{-11} cm³/dyne sec.

The most important aspect of our study is the experimental demonstration that there is no apparent rectification of water movement in a system composed of a single cellular membrane in which unstirred layer effects are

unimportant. The osmotic permeability coefficient is set by the final osmolality of the system and is essentially independent of the direction of water movement. Thus, though there is not yet enough information to specify the exact molecular mechanism within the membrane, the hydraulic conductance of the membrane appears to conform to simple physical laws.

The independence of L_p of the direction of water flow is consistent with the presence of a single rate-limiting barrier for water movement since two rate-limiting barriers in series can often lead to rectification of water flow as discussed theoretically by Patlak, Goldstein and Hoffman (23) and shown experimentally by Ogilvie, McIntosh, and Curran (24) in the case of cellulose membranes.

Since a single value of L_p fits each swelling and shrinking curve, any change in the cell that affects L_p must be effective within a time of the order of 50 msec or less. Such a rapid response means that the rate-limiting barrier is at the outer face of the membrane. During the volume shift there is an osmotic pressure gradient across the cell membrane and the water activity of the interior milieu only reaches that of the outer face after more than 0.5 sec have elapsed. Thus it appears that the permeability characteristics of the inner facing membrane differ from those at the outer face with respect to the passage of water under an osmotic pressure gradient. An extension of this argument to the region between the two faces suggests that a thin external skin responds instantaneously to the osmolality of the environment and thereby controls the hydraulic permeability coefficient. From this we infer that the inner and outer facing surfaces of the red cell membranes are not symmetrical but are characterized by significant differences in molecular fine structure.

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