BBA 75358

PERTURBATION OF RED CELL VOLUME: RECTIFICATION OF OSMOTIC FLOW

ROBERT E. L. FARMER AND ROBERT I. MACEY
Department of Physiology-Anatomy, University of California, Berkeley, Calif. (U.S.A.)
(Received July 21st, 1969)

SUMMARY

A method is described which allows determination of the concentration dependence of the filtration coefficient L_p for membranes of intact cells. After a small, sudden perturbation of solution osmolality the cell volume exponentially approaches a new equilibrium value. The time constant of this osmotic process is measured by turbidity changes and is used to calculate L_p for each set of experimental conditions. Application of this method to beef and human erythrocytes has shown: (a) Values of L_p are in good agreement with those found by other investigators using other techniques. (b) L_p is independent of cell size and osmolality of the suspending medium. (c) An apparent rectification of water flow occurs, inward flow being 40–50 % greater than outward flow for the same osmotic gradient. The two latter results contradict the recent conclusions of RICH *et al.* (*J. Gen. Physiol.*, 52 (1968) 941) but are consistent, nevertheless, with the experimental data reported by these investigators.

INTRODUCTION

In the past ten years it has become common practice to describe the kinetics of membrane transport using principles of irreversible thermodynamics[1]. The goal has been to formulate an unambiguous method for defining and measuring transport parameters. Definition and measurement of parameters has, however, been complicated by the fact that the commonly used, linear, thermodynamic coefficients are implicitly dependent upon the concentrations of solutes and solvent. Since flux measurements are often made in the presence of sizeable concentration gradients, precise definition of the transport parameters is difficult unless the concentration dependence of each linear coefficient is made explicit. Formal integration of the differential flux equations through the membrane often lead to results which are not compatible with the original Kedem–Katchalsky equations[2,3].

These complications can be minimized by the measurement of transport fluxes which result from very small concentration gradients or concentration "perturbations"[4]. If, for example, a particular concentration is abruptly changed from its equilibrium value $c-\Delta c$ to c, measurement of the ensuing flux enables calculation of the transport parameters. The parameters are referenced to a specific concentration which lies within the narrow range of $c-\Delta c$ to c. By changing the final concentration

c, it is possible to extend measurements over a large span of concentrations and hence to obtain an explicit and detailed account of the dependence of the parameters on c.

The purpose of this paper is to apply the perturbation method to the relatively simple case of osmotic water flow in erythrocytes. Later papers will deal with more complicated cases of simultaneous water and solute transfer. It will be seen that there are a number of practical advantages associated with this technique. Analytical treatment is straight forward, data processing is simplified, calibration curves are not required and the usual rigorous requirements of rapid initial mixing are relaxed.

Applying this method to beef and human red cells shows that (1) the filtration coefficient is independent of cell size and osmolality of the suspending medium, (2) an apparent rectification of water flow occurs; inward flow is greater than outward flow for the same osmotic gradient. These findings readily account for the data recently reported by RICH *et al.*[5] who, on the basis of a less detailed study, have proposed an opposite interpretation.

CALCULATIONS

Analysis of the problem begins with the kinetic equation for osmotic water flow in the absence of a permeable solute and in the absence of a hydrostatic pressure gradient. Allowance is made for variations of cell parameters with cell size and osmolality of the suspending medium. For small osmotic gradients the differential equation can be linearized by a Taylor's series expansion. The linear solution predicts that the cell volume changes exponentially with time. The exponential time constant depends upon cell parameters and medium osmolality.

The equation describing the time dependence of cell volume following a sudden, small increase in osmotic pressure can be written:

$$\frac{dV}{dt} = k(V,\Pi)[\Pi'(V)-\Pi] \tag{1}$$

where: V = normalized cell volume ($V = 1$ at isotonicity), Π = normalized external osmotic pressure or final tonicity, assumed constant ($\Pi = 1$ at isotonicity), $\Pi'(V)$ = internal tonicity (a function of cell volume), t = time in sec. The functional dependence of the cell parameters on cell volume and osmotic pressure is written:

$$k(V,\Pi) = RTc_0 L_p(V,\Pi)A(V)/V_0 \tag{2}$$

where: RT = universal gas constant times absolute temperature (dyne·cm·mole^{-1}), $L_p(V,\Pi)$ = osmotic filtration coefficient at volume V and osmotic pressure Π (cm^3·dyne^{-1}·sec^{-1}), $A(V)$ = area of cell membrane at volume V (cm^2), c_0 = isotonic concentration (osmoles·cm^{-3}) and V_0 = isotonic cell volume (cm^3).

The functional dependence of Π' upon V has been found from equilibrium experiments[6,7] to have the following empirical form

$$V = b + (1-b)/\Pi' \tag{3}$$

where b is independent of cell volume. The parameter b is related to the non-water volume of the cell. Its physical significance has been discussed by GARY-BOBO AND SOLOMON[8]. The fractional volume $(1-b)$ might be identified with the apparent cell water at isotonicity. Utilizing Eqn. 3, Eqn. 1 becomes

$$\frac{dV}{dt} = k(V,\Pi)\left[\frac{(1-b)}{(V-b)} - \Pi\right] \tag{4}$$

Let $\Delta\Pi$ represent a small increase in external osmotic pressure and let ΔV represent a corresponding increase in cell volume from the initial volume $V_\infty - \Delta V$ to the final volume V_∞. The kinetics of perturbation can be shown by expanding the right-hand side of Eqn. 4 in a Taylor's series about the final volume V_∞. The expanded form is written

$$\frac{dV}{dt} = \frac{\partial}{\partial V}\left[k(V,\Pi)\left[\frac{(1-b)}{(V-b)} - \Pi\right]\right]_{V_\infty}(V-V_\infty) + \frac{1}{2}\frac{\partial^2}{\partial V^2}\left[k(V,\Pi)\left[\frac{(1-b)}{(V-b)} - \Pi\right]\right]_{V_\infty}(V-V_\infty)^2 + \ldots \tag{5}$$

Using Eqn. 3, with the condition that at $V = V_\infty$, $\Pi' = \Pi$, Eqn. 5 becomes

$$\frac{dV}{dt} = -\frac{\Pi^2}{(1-b)}[k(V_\infty,\Pi)](V-V_\infty) + \frac{\Pi^2}{(1-b)}\left[\frac{k(V_\infty,\Pi)}{(V_\infty-b)} - \frac{\partial k}{\partial V}\Big|_{V_\infty}\right](V-V_\infty)^2 + \ldots \tag{6}$$

For small ΔV, Eqn. 6 may be approximated by

$$\frac{dV}{dt} \simeq -\frac{\Pi^2}{(1-b)}[k(V_\infty,\Pi)](V-V_\infty) \tag{7}$$

The solution to this first-order, linear, differential equation is

$$V \simeq V_\infty - \Delta V \exp\left[-\frac{\Pi^2}{(1-b)}k(V_\infty,\Pi)t\right] \tag{8}$$

where ΔV is found from Eqn. 3 to be approximated by

$$\Delta V \simeq -(1-b)\Pi^{-2}\Delta\Pi \tag{9}$$

The time constant τ of the exponential in Eqn. 8 is clearly

$$\tau = (1-b)\Pi^{-2}[k(V_\infty,\Pi)]^{-1} \tag{10}$$

where $k(V,\Pi)$ is expressed by Eqn. 2.

In red cells L_p and A are commonly assumed constant, *i.e.*, independent of cell volume and medium tonicity[9,10]. With these assumptions, Eqn. 10 becomes

$$\tau = \left[\frac{(1-b)V_0}{RTC_0L_pA}\right]\Pi^{-2} = (\text{Constant})\,\Pi^{-2} \tag{11}$$

It should be noted, however, that the general treatment given above is fully capable of handling and, in fact, testing cases of non-constant L_p and A. For example, DIAMOND[11] found that the resistance to water flow through rabbit gall bladder increased linearly as the mean osmolality of the bathing solutions was raised. Such a membrane property leads to an expression for Eqn. 10 of the form

$$\tau = (\text{Constant})\,\Pi^{-2}[1 + B(\Pi-1)] \tag{12}$$

where $B = 0.74$ is obtained for rabbit gall bladder. RICH *et al.*[5] have postulated a

linear increase of log L_p with increasing Π^{-1} for dog and human red cells. Such a dependence leads to

$$\tau = (\text{Constant})\ \Pi^{-2} \exp\left[D(1-\Pi^{-1})\right] \tag{13}$$

where D is near 1.0.

For spherical cells, in which the surface area increases as the two-thirds power of the volume, one may assume constant L_p as did LUCKÉ *et al.*[12] for Arbacia eggs. The result is

$$\tau = (\text{Constant})\ \Pi^{-2}\left[b + (1-b)\Pi^{-1}\right]^{-2/3} \tag{14}$$

Again for spherical cells, NORTHROP[13] assumed membrane volume to be independent of cell volume (L_p not constant). This leads to the form

$$\tau = (\text{Constant})\ \Pi^{-2}\left[b + (1-b)\Pi^{-1}\right]^{-4/3} \tag{15}$$

EXPERIMENTAL PROCEDURE

Fresh beef blood was obtained at a local slaughterhouse and was defibrinated, filtered and stored at about 2°. Human blood was collected in a plastic bag containing acid–citrate–dextrose. Hematocrit (International Model MB Micro-capillary Centrifuge), plasma osmolality (Fiske Mark III Osmometer) and cell counts of the blood were routinely determined. Red cell volume was calculated from hematocrit and cell count with no correction for trapped plasma. Cell area was estimated using a right circular cylinder approximation[14].

The isotonic salt solution used with beef blood had an approximate final concentration of (all Cl^- salts) 133 mM Na^+, 5.4 mM K^+, 2.6 mM Ca^{2+}, 1.8 mM Mg^{2+}, 32 mM Tris. The solution used with human blood had 178 mM Na^+, 4.7 mM K^+, 154 mM Cl^-, 4.7 mM $H_2PO_4^-$, 12.0 mM HPO_4^{2-}. The pH of each solution was about 7.4, and the osmolalities were near 300 mosM. Hypertonic and hypotonic solutions contained the same relative proportions of salts.

Red cell volume was studied as a function of medium tonicity at osmotic equilibrium for each lot of blood. Micro-hematocrits of cells suspended in solutions of varying tonicities were obtained, and a linear dependence of hematocrit on inverse tonicity[7] was obtained for both beef and human cells. The slope of the best (least mean square error) straight line fit through the experimental points was determined. Dividing this slope by the hematocrit at isotonicity gave the apparent cell water at isotonicity, represented by $(1-b)$ in Eqn. 3. The quantity $(1-b)$ was found to be relatively independent of limited changes in temperature and pH.

Kinetic behavior of the cells was measured photometrically by a modification of the method of ØRSKOV[15] and WILBRANDT[16] (see Fig. 1). The light source (G.E. No. 40, 6–8 V bulb seated in a flashlight reflector assembly) was powered by an adjustable 6.6 V d.c. supply. The photo-detector (1P21 photomultiplier biased at 800 V) received transmitted light passing through the cuvette and then through a red filter (Klett No. KS-62). The photomultiplier load consisted of a parallel resistor–capacitor filter with a 5-msec time constant. An alternate photo-detector (RCA 6953 phototube biased at 70 V) has also been used.

The cell suspension was placed in a 13-mm square cuvette containing a 3/8 inch × 1/8 inch, Teflon-coated, magnetic stirring bar. The stirring bar was driven by a

stirring magnet to speeds of 50–60 rev./sec (speeds below 40 rev./sec began to adversely affect time constant measurements.) Temperature was controlled in the cuvette by circulating water from a water bath through a gold-plated copper coil immersed just below the surface of the cell suspension, yet out of the light path. Temperature was monitored by a thermistor placed in the cuvette.

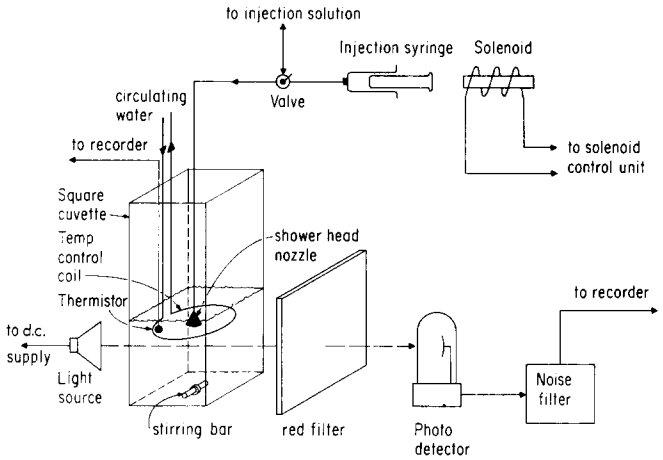

Fig. 1. Simplified diagram of apparatus. See text for description and for details of operation.

The tonicity of the suspension was perturbed by a sudden injection of a small amount of "injection solution" which was either hypo- or hypertonic. The injection was carried out upon electrical command by a solenoid-driven syringe. The solenoid (WesCo Model 2-20-C, 115 V, 60 cycles) was mechanically coupled with an automatic syringe attachment (Scientific Industries S-202-2) containing a 2-ml glass syringe. The unit was adjusted to deliver 0.5–0.6 ml. A line leading from the syringe to the cuvette was terminated in a lucite "shower-head" nozzle positioned just below the surface of the cell suspension. This nozzle created a "spray" of injection solution which facilitated mixing.

Each perturbation experiment was carried out in the following manner. A 1.0 % suspension of beef cells (1.4 % for human cells) and the appropriate injection solutions were brought to thermal equilibrium in a water bath (5–45°). The cuvette was washed several times with the preequilibrated cell suspension to minimize temperature differences, and the injection syringe was manually operated until the injection line was free of air bubbles. 5.0 ml of the cell suspension were then pipetted into the cuvette. After a short baseline was established, 0.5–0.6 ml of the injection solution was injected by solenoid into the cuvette, and the resulting perturbation signal was recorded. The suspension was withdrawn from the cuvette, and its final osmolality was measured in the Fiske osmometer.

RESULTS

Fig. 2 shows a typical series of swelling experiments. Beef erythrocytes, initially in near isotonic solution, were perturbed by the injection of a small quantity

of distilled water. The initial large jump was caused by dilution of the cells. The slowly changing part of the trace represents swelling of the cells and is expected to exponentially approach an asymptote. Injection and mixing are seen to be complete within about 100 msec.

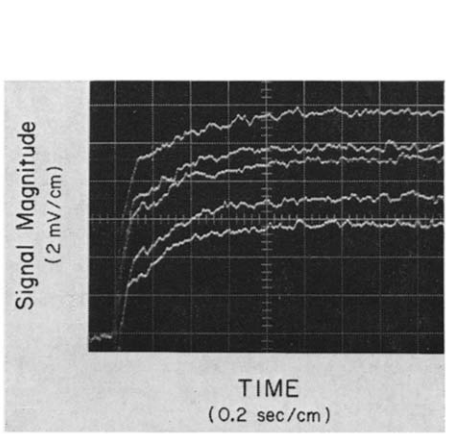

Fig. 2. Oscilloscope record of beef erythrocyte swelling following an injection of distilled water. Cells were initially equilibrated in a saline solution of tonicity 1.06. Final tonicity was about 0.95. The initial large jump up from the baseline (lower left-hand corner of the photograph) was caused by dilution of the cells. The slowly curving portion of the trace represents kinetic swelling of the cells. The baseline was shifted progressively downward to show five consecutive runs.

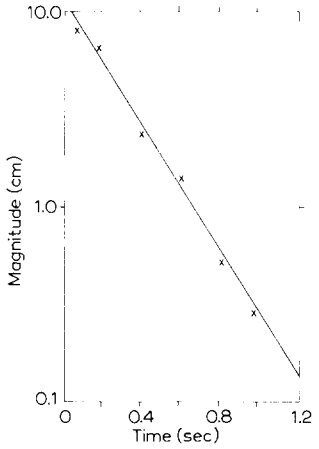

Fig. 3. Semilog plot showing exponential approach to asymptote. Plotted here is the sum of the five runs of Fig. 2. From the slope of the line a time constant of 0.26 sec was obtained.

The exponential prediction is verified in Fig. 3 which represents a semilog plot of the sum of the five runs illustrated in Fig. 2. Individual points were obtained by first measuring the vertical distance of each trace from its corresponding asymptote for a series of times (measured from an arbitrary reference). Distance values obtained for a given time were then summed and plotted as shown. The exponential time constant taken from the slope of the least-squares fit corresponds to τ in Eqn. 10 and allows the value of L_p to be calculated. It should be noted that exponential time constants are independent of initial values, so that this estimate of L_p can be obtained independent of measurements at the instant of injection. In other words, the pertinent data can be extracted even though transient mixing and dilution signals obscure early portions (100 msec) of the trace. Further, the time constant is independent of magnitude scaling factors. Thus, the data can be extracted from Fig. 2 without converting the distance units of the vertical scale to volume units. This is particularly advantageous when measurements are extended over a large range of concentrations where changes can occur in the scaling factors (*e.g.*, by change in refractive index).

If we assume that L_pA is independent of tonicity and cell volume then Eqn. 11 suggests that a plot of τ *versus* Π^{-2} should give a straight line. Marked dependence of L_pA on tonicity or cell volume should show up as a systematic deviation from a straight line. Fig. 4 shows the results of experiments which were performed on beef cells of varying sizes (pre-equilibrated in media of differing osmolalities). The time constants of both swelling and shrinking experiments are plotted *versus* Π^{-2}. Details

of individual experiments are tabulated in Table I. The data indicate that linearity holds within each set of experiments, *i.e.*, for a given direction of flow L_pA seems to be constant over a range of tonicities from 0.75 to 1.6. However, the two sets of experiments possess markedly different slopes. Although L_p appears to be independent of cell size and final tonicity, it appears to be dependent upon the direction of water flow. We express this rectification property by a ratio defined as $L_{p_{in}}/L_{p_{out}}$ where $L_{p_{in}}$ corresponds to cell swelling and $L_{p_{out}}$ corresponds to shrinking. With these particular cells, the measured rectification ratio is found to be about 1.64 with swelling faster than shrinking.

TABLE I

INDIVIDUAL PERTURBATION EXPERIMENTS LEADING TO FIG. 4

The cuvette contained 5.0 ml of a 1.0% suspension of beef erythrocytes initially in equilibrium with a solution of osmolality "initial". About 0.6 ml of a solution of osmolality "injected" was injected into the cuvette. The time constant τ was obtained by a least-square-error exponential fit to the resulting volume change. The measured final osmolality "final" was used to compute the tonicity Π. The plasma osmolality (isotonic) for these cells was 309 mosM, and the temperature was 25°. The last column of this table shows the size of the volume perturbation in percent. The blood was 1 day old in Expts. 1–6 and 2 days old in Expts. 7–18.

Expt. No.	*Expt. type*	*Solution osmolality (mosM)*			Π	τ (*sec*)	ΔV^* (%)
		Initial	*Injected*	*Final*			
1	Swell	270	0	249	0.806	0.337	5.1
2	Swell	316	0	285	0.923	0.218	6.3
3	Shrink	316	631	351	1.137	0.258	5.3
4	Shrink	207	631	266	0.861	0.407	13.3
5	Shrink	270	631	310	1.002	0.315	7.2
6	Shrink	416	631	434	1.402	0.170	2.0
7	Swell	270	0	242	0.784	0.293	7.2
8	Swell	316	0	284	0.920	0.239	6.6
9	Swell	333	0	296	0.959	0.191	7.1
10	Swell	416	0	366	1.184	0.131	7.1
11	Shrink	207	631	262	0.848	0.448	12.6
12	Shrink	270	631	311	1.008	0.302	7.4
13	Shrink	316	631	353	1.142	0.258	5.5
14	Shrink	333	631	366	1.186	0.228	4.7
15	Shrink	416	631	436	1.411	0.160	2.2
16	Shrink	631	942	654	2.12	0.107	1.3
17	Shrink	221	316	251	0.813	0.516	7.3
18	Shrink	207	316	228	0.739	0.567	5.8

* $|\Delta V/V_\infty|$, calculated from Eqns. 3 and 9.

Using the data of Fig. 4, together with Eqn. 11 and measurements of cell parameters at equilibrium, we find that $RTL_{p_{in}} = 0.49$ cm^4·osmole^{-1}·sec^{-1}. With a rectification ratio of about 1.64, the outward filtration coefficient $RTL_{p_{out}}$ is about 0.30 cm^4·osmole^{-1}·sec^{-1}. Using the same technique, the average inward filtration coefficient for blood from 12 different animals was found to be $RTL_{p_{in}} = 0.47 \pm 0.03$

(S.E.) $cm^4 \cdot osmole^{-1} \cdot sec^{-1}$. On 8 of these 12 the rectification ratio was determined, and the average measured value was 1.67 ± 0.04 (S.E.).

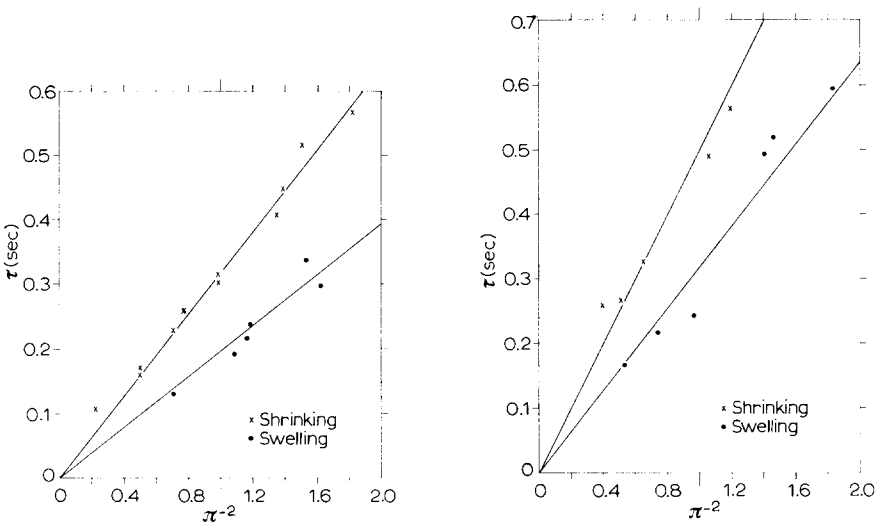

Fig. 4. Dependence of the perturbation time constant upon tonicity (cell size) and direction of flow. Cells are beef erythrocytes at a temperature of 25°. Individual experiments are described in Table I. Note that shrinking time constants are always slower than swelling time constants for the same final tonicity. However, linearity holds within each set of experiments. Using Eqn. 11 and values for cell parameters measured at equilibrium the inward filtration coefficient is calculated to be $RTL_{p_{in}} = 0.49$ $cm^4 \cdot osmole^{-1} \cdot sec^{-1}$. The rectification ratio $L_{p_{in}}/L_{p_{out}}$ is about 1.64.

Fig. 5. Dependence of the time constant τ upon Π^{-2} for human red cells at 25°. Again shrinking is slower than swelling for the same final tonicity. For these particular cells the inward filtration coefficient $RTL_{p_{in}}$ is calculated to be about 0.32 $cm^4 \cdot osmole^{-1} \cdot sec^{-1}$. The rectification ratio $L_{p_{in}}/L_{p_{out}}$ is about 1.58.

Although there is more scatter in the data, human red cells also appear to posses a linear dependence of τ upon Π^{-2} (shown in Fig. 5). Again there is a marked difference between swelling experiments and shrinking experiments implying rectification of flow. For the data of Fig. 5, the measured rectification ratio is found to be about 1.58. From Eqn. 11 and measurements of cell volume, *etc.*, at equilibrium, a value of $RTL_{p_{in}} = 0.32$ $cm^4 \cdot osmole^{-1} \cdot sec^{-1}$ is calculated for these cells. $RTL_{p_{out}}$ is about 0.20 $cm^4 \cdot osmole^{-1} \cdot sec^{-1}$. The average inward filtration coefficients for blood from 5 different subjects was $RTL_{p_{in}} = 0.43 \pm 0.05$ (S.E.) $cm^4 \cdot osmole^{-1} \cdot sec^{-1}$ at 25°. The average measured rectification ratio for three of these was 1.53 ± 0.04 (S.E.).

These values for mammalian cells may be compared with preliminary values for nucleated chicken erythrocytes where $RTL_{p_{in}} = 0.016$ $cm^4 \cdot osmole^{-1} \cdot sec^{-1}$ at 25° has been found. The measured rectification ratio was near 1.18.

Fig. 6 shows the temperature dependence of L_p. This is a semilog plot of $(T\tau\Pi^2)^{-1}$, which is proportional to L_p, *versus* the inverse absolute temperature (T^{-1}). The temperature range is from 10 to 40°. The final tonicity was near 0.9 for all points. The upper line is composed of swelling experiments similar to those discussed before. The lower line is composed of shrinking experiments. From the slopes of these two lines the equivalent heat of activation[17] for water filtration in beef cells is found to

be about 4.0 kcal·mole^{-1} for both swelling and shrinking. For chicken cells a preliminary value of 11.4 kcal·mole^{-1} has been obtained for the swelling process only.

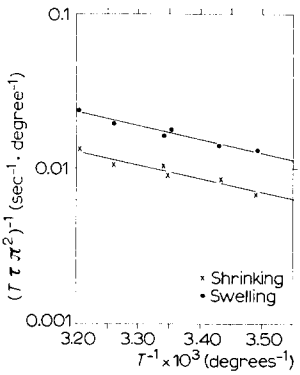

Fig. 6. Temperature dependence of water filtration in beef erythrocytes. Plotted is $(T\tau\Pi^2)^{-1}$, which is proportional to L_p, *vs.* the inverse absolute temperature T^{-1}. The temperature range is from 10 to 40°. In swelling, the cells were initially suspended in about 296 mosM. After injection of 0.6 ml distilled water the final concentration was near 264 mosM ($\Pi = 0.88$). In shrinking, the cells started at about 264 mosM. Injection of 0.6 ml of 495 mosM solution brought the final concentration to 289 mosM ($\Pi = 0.96$). The measured rectification ratio is about 1.74. The heat of activation for the swelling process was about 4.1 kcal·mole^{-1}. For shrinking it was about 3.9 kcal·mole^{-1}.

DISCUSSION

Water transport, cell volume and osmolality

The apparent independence of L_p from cell volume and osmolality, it might be argued, is in agreement with early results of SIDEL AND SOLOMON[18] on human erythrocytes. These investigators found a nearly constant water flow coefficient in osmotic experiments which traversed three different tonicity ranges. On the other hand, VILLEGAS *et al.*[19] reported a decrease in the rate of ^{3}HHO diffusion into the beef red cell with an increase in cell size (decrease in osmolality). They suggested that water enters the cell *via* membrane "pores" whose sizes depend upon the solution tonicity. Now the rate of water diffusion through pores should be approximately proportional to the square of the pore radius while net water flow may be proportional to an even higher power of the radius. Thus, the large dependence on pore size should make net flow a sensitive indicator of changes in pore dimensions. The observed constancy of L_p indicates that no such changes take place as osmolality is varied. Further, using glycerol as a water-soluble "pore probe" MACEY AND TOLBERG[20] found no dependence of glycerol permeability on beef cell volume or tonicity.

Recently, RICH *et al.*[5] have reported a dependence of L_p upon solution osmolality or tonicity for human red cells. The primary basis of this assertion is a scatter-gram consisting of many measurements of log L_p plotted against Π^{-1}. L_p's from swelling experiments (from an isotonic to hypotonic medium) tend to cluster around a value of L_p that is definitely higher than the cluster of L_p's from shrinkage experiments (isotonic to hypertonic medium). Plotted this way, there is an apparent correlation between log L_p and Π^{-1}. Inspection of these results shows that the data is consistent with an alternative interpretation—namely that the value of L_p depends on whether

the cells are swelling or shrinking, *i.e.*, flow rectification. The authors' later arguments that there is no rectification are not compelling. They arise from a very few experiments, and they are based on the assumption *a priori* that L_p depends on osmolality.

Our interpretation is that rectification occurs in both beef and human red cells; we believe that L_p is relatively independent of Π but dependent on direction of flow (swelling or shrinking). We base this on the data of Figs. 4 and 5 which cover a greater range of concentration for both swelling and shrinking than the corresponding data of RICH *et al.*[5]. Finally, the compatibility of both sets of data with this interpretation is illustrated in Fig. 7, where we have replotted typical L_p data of RICH *et al.*[5] together with our own against Π^{-1}. Fig. 8 shows a similar plot for beef red cells using data from Table I.

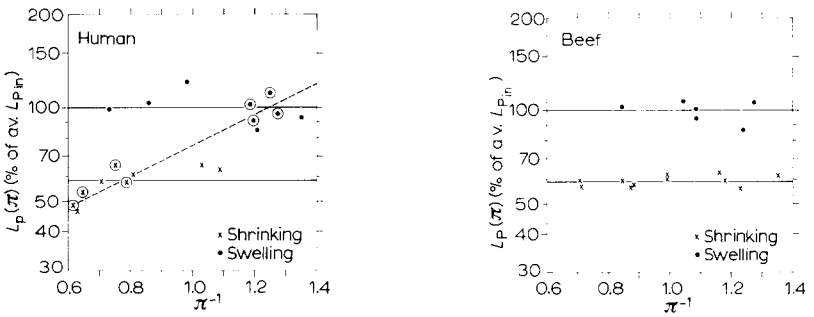

Fig. 7. Plot of L_p against inverse tonicity Π^{-1} for human red cells. The points surrounded by circles have been replotted from RICH *et al.*[5]. The points without circles are our own. Each set of points has been normalized to the average $L_{p_{in}}$ to minimize animal-to-animal variation. The dashed line represents the interpretation of RICH *et al.*[5] in which no rectification is permitted; so L_p depends heavily upon Π. The two solid lines show our interpretation of rectification with $L_{p_{in}}$ (swelling) and $L_{p_{out}}$ (shrinking) relatively constant.

Fig. 8. Plot of L_p against inverse tonicity Π^{-1} for beef red cells. The points are drawn from the data of Table I. The upper solid line represents the average $L_{p_{in}}$ (swelling) at 100%. The lower line represents the average $L_{p_{out}}$ (shrinking) at about 59% of $L_{p_{in}}$. The two values for L_p seem to be reasonably independent of Π.

Rectification of flow

Rectification of water flow is well known in plant cells although its cause remains obscure[21]. DAINTY[21] has cautioned that in some cases unstirred layers can create an apparent flow rectification. The size of unstirred layers around red cells has been studied by SHA'AFI *et al.*[22] who have been able to show that these layers have negligible effect on their determinations of osmotic permeability coefficients. Since our permeability measurements agree closely with those of SHA'AFI *et al.*[22] (see Table II) and since the cell suspensions were subjected to continuous vigorous stirring throughout the measurement, it seems unlikely that unstirred layers play a significant role in our results.

An apparent rectification can result from the first-order analytical approximation used in data processing. Dropping higher order terms of Eqn. 6 accentuates the magnitude of rectification, but an analysis of the second-order correction shows that the error in each L_p determination is less than 5%. The contribution of this second-order effect toward the rectification ratio is near 9%. Therefore, the average measured rectification ratio of 1.67 for beef erythrocytes, when corrected by 9% becomes 1.52.

TABLE II

COMPARISON OF RESULTS OF VOLUME PERTURBATION OF BEEF, HUMAN AND CHICKEN RED CELLS WITH RESULTS OBTAINED BY OTHER METHODS

Also included in this table are results compiled for dog erythrocytes.

Species	*Method*	$(1-b)$	$RTL_{p_{in}}$ *	$RTL_{p_{out}}$ *	*Ratio* $L_{p_{in}}/L_{p_{out}}$	*Source*
Beef	Perturbation	0.54	0.45	0.29	1.52	This paper
	Stop-flow	0.52	—	0.28		RICH *et al.*[24]
	Flow-tube	(0.35 / 0.52**)	(0.28 / 0.42**)		>1.50	VILLEGAS *et al.*[19]
Dog	Stop-flow	—	0.57***	0.36***	1.59***	RICH *et al.*[5]
	Stop-flow	0.57	—	0.36		RICH *et al.*[24]
	Flow-tube	(0.70 / 0.57**)	(0.72 / 0.58**)		>1.61	VILLEGAS *et al.*[19]
Human	Perturbation	0.60	0.41	0.29	1.39	This paper
	Stop-flow	—	0.40***	0.22***	1.82***	RICH *et al.*[5]
	Stop-flow	—	—	0.22(20°)		SHA'AFI *et al.*[22]
	Flow-tube	(0.46 / 0.57**)	(0.23 / 0.29**)		>1.32	SIDEL AND SOLOMON[18]
	Stop-flow	—	0.41	0.24	1.71	BLUM AND FORSTER[25,§]
Chicken	Perturbation	0.46	0.015	0.014	1.07	This paper

* Given in units of $cm^4 \cdot osmole^{-1} \cdot sec^{-1}$. Values located midway between the columns $RTL_{p_{in}}$ and $RTL_{p_{out}}$ were determined from both swelling and shrinking experiments. Temperature is 23–26° except where noted.

** Recalculated using a more acceptable value for $(1-b)$.

*** Reinterpreted allowing for rectification of flow.

§ R. E. FORSTER, personal communication.

The measured ratio of 1.53 for human erythrocytes becomes 1.39. For chicken red cells, the measured rectification ratio of 1.18 is more accurately 1.07, which is very close to unity.

Assuming the observed rectification of water flow is indeed a property of the cell membrane, several possible explanations arise. PATLAK *et al.*[2] for example, have shown that a double-membrane system can lead to flow rectification. A second possibility involves a dependence of membrane structure upon the direction of water flow; in particular, a valve-like action occurring within aqueous channels could lead to a direction-dependent value of L_p by changing the "effective pore radius". One further possibility associates the rectification property with hemoglobin inside the cell. During swelling the inward flow of water may tend to separate the membrane and adjacent layers of hemoglobin (perhaps by force of entry or by electro–osmotic charge unbalance). As a result, a maximal membrane area would be available for transport of water into the cell. On the other hand, in shrinking, the outward flow of water may bring the membrane and adjacent layers of hemoglobin into close contact thus reducing the effective area of the membrane. Since at isotonicity only about 72 % of the cell volume is water[7], one might conjecture that, in shrinking, around 28 % of the cell membrane area is occluded by non-water materials, *e.g.*, hemoglobin. The rectification ratio may then be expected to be about 1/0.72 = 1.4. The value of 72 % cell water,

while it may be dependent to some degree upon temperature[23] is roughly constant, so a rectification ratio which is rather independent of temperature (Fig. 6) is plausible.

Comparison with results from other methods

Table II is a comparison of filtration coefficients which have been determined by several methods. The coefficients determined by perturbation have been corrected by 5 % for second-order effects. They are for the beef erythrocyte $RTL_{p_{in}} = 0.45$ $cm^4 \cdot osmole^{-1} \cdot sec^{-1}$ and $RTL_{p_{out}} = 0.29$ $cm^4 \cdot osmole^{-1} \cdot sec^{-1}$ at 25°. The rectification ratio is 1.52. This means that inward filtration of water is about 52 % faster than outward filtration for the same osmotic gradient. For the human erythrocyte $RTL_{p_{in}} = 0.41$ $cm^4 \cdot osmole^{-1} \cdot sec^{-1}$ and $RTL_{p_{out}} = 0.29$ $cm^4 \cdot osmole^{-1} \cdot sec^{-1}$ at 25°. The rectification ratio is about 1.39. It should be noted that chicken erythrocytes, on the other hand, seem to possess a rectification ratio which is near unity. These cells also possess a filtration coefficient which is slower than those for beef and human cells by more than an order of magnitude.

RICH *et al.*[24] have carefully determined the outward filtration coefficient for beef erythrocytes. Their value of 0.28 $cm^4 \cdot osmole^{-1} \cdot sec^{-1}$ compares favorably with the 0.29 reported here. These authors also claim good agreement with the value of 0.28 found by VILLEGAS *et al.*[19] almost 10 years earlier. However, the latter authors had averaged together both swelling and shrinking data to get their figure. Moreover, in their calculations they used a value for $(1-b)$ of 0.35 which must be judged too low. (Out of 15 determinations the lowest value found here was 0.50; the average, 0.54.) A recalculation of their results based upon a value of 0.52, which was reported by the same laboratory[24], gives a filtration coefficient of 0.42 $cm^4 \cdot osmole^{-1} \cdot sec^{-1}$. Curiously, this is closer to the 0.45 $cm^4 \cdot osmole^{-1} \cdot sec^{-1}$ which is reported here as the inward filtration coefficient. Using this recalculated value of 0.42 and the 0.28 found by RICH *et al.*[24], a minimum rectification ratio can be computed to be 0.42/0.28=1.50.

In exactly the same manner a minimum rectification ratio can be found for dog erythrocytes using data from the same two papers. This ratio is computed to be 0.58/0.36 = 1.61. Further, using data from SIDEL AND SOLOMON[18] and SHA'AFI *et al.*[22], the same computations can be made for the human erythrocyte. The minimum rectification ratio is found to be 0.29/0.22 = 1.32. BLUM AND FORSTER[25] have reported a rectification of flow in the human erythrocyte. They report a ratio of 1.71 in the correct direction (R. E. FORSTER, personal communication), "endosmosis" being greater than "exosmosis" by 71 % for the same osmotic gradient.

If the data of RICH *et al.*[5] are reinterpreted allowing for rectification of flow, the inward filtration coefficient for human red cells can be estimated to be 0.40 $cm^4 \cdot osmole^{-1} \cdot sec^{-1}$. This value agrees well with the 0.41 $cm^4 \cdot osmole^{-1} \cdot sec^{-1}$ obtained by perturbation. The rectification ratio is estimated to be 1.82. The agreement between this value and our value of 1.39, while not particularly outstanding, is certainly adequate to demonstrate that rectification of water flow may occur across the membrane of red cells.

Finally, it is of interest to note that DAINTY[26], upon evaluating the experiments of KAMIYA AND TAZAWA[27], concludes that the plant cell Nitella also exhibits rectification of osmotic flow. This cell has a relatively high inward filtration coefficient ($RTL_{p_{in}}$ is about 0.72 $cm^4 \cdot osmole^{-1} \cdot sec^{-1}$ at 20°), and DAINTY[26] estimates the rectification ratio $L_{p_{in}}/L_{p_{out}}$ to be about 1.55.

ACKNOWLEDGMENTS

The authors would like to gratefully acknowledge the superb technical assistance of Mrs. Frances Johnson. This work was supported in part by NSF Grant GB 11981, in part by AEC Contract AT (11-1)-34 PA No. 136-15, in part by Public Health Service pre-doctoral fellowship F1-GM-30,926 from the National Institute of General Medical Sciences, and in part by a post-doctoral research fellowship from the Bay Area Heart Research Committee.

REFERENCES

1 O. KEDEM AND A. KATCHALSKY, *Biochim. Biophys. Acta*, 27 (1958) 229.
2 C. S. PATLAK, D. A. GOLDSTEIN AND J. F. HOFFMAN, *J. Theoret. Biol.*, 5 (1963) 426.
3 E. H. BRESLER AND R. P. WENDT, *Science*, 163 (1969) 944.
4 R. E. L. FARMER AND R. I. MACEY, *Biophys. Soc. Abstr., 11th Ann. Meeting*, (1967) 6.
5 G. T. RICH, R. I. SHA'AFI, A. ROMUALDEZ AND A. K. SOLOMON, *J. Gen. Physiol.*, 52 (1968) 941.
6 D. A. T. DICK, *Inter. Rev. Cytol.*, 8 (1959) 387.
7 D. SAVITZ, V. W. SIDEL AND A. K. SOLOMON, *J. Gen. Physiol.*, 48 (1964) 79.
8 C. M. GARY-BOBO AND A. K. SOLOMON, *J. Gen. Physiol.*, 52 (1968) 825.
9 M. H. JACOBS, *Biol. Bull.*, 62 (1932) 178.
10 E. PONDER, *Hemolysis and Related Phenomena*, Grune and Stratton, New York, 1948, p. 82.
11 J. M. DIAMOND, *J. Physiol. London*, 183 (1966) 58.
12 B. LUCKÉ, H. K. HARTLINE AND M. MCCUTCHEON, *J. Gen. Physiol.*, 14 (1931) 405.
13 J. H. NORTHROP, *J. Gen. Physiol.*, 11 (1927) 43.
14 W. F. EMMONS, *J. Physiol. London*, 64 (1927) 215.
15 S. L. ØRSKOV, *Biochem. Z.*, 279 (1935) 241.
16 W. WILBRANDT, *Arch. Ges. Physiol.*, 241 (1938) 289.
17 K. J. LAIDLER, *Chemical Kinetics*, McGraw-Hill, New York, 2nd ed., 1965.
18 V. A. SIDEL AND A. K. SOLOMON, *J. Gen. Physiol.*, 41 (1957) 243.
19 R. VILLEGAS, T. C. BARTON AND A. K. SOLOMON, *J. Gen. Physiol.*, 42 (1958) 355.
20 R. I. MACEY AND A. B. TOLBERG, *Biochim. Biophys. Acta*, 120 (1966) 104.
21 J. DAINTY, in R. D. PRESTON, *Advances in Botanical Research*, Vol. 1, Academic Press, New York, 1963, p. 279.
22 R. I. SHA'AFI, G. T. RICH, V. W. SIDEL, W. BOSSERT AND A. K. SOLOMON, *J. Gen. Physiol.*, 50 (1967) 1377.
23 M. H. JACOBS AND A. K. PARPART, *Biol. Bull.*, 60 (1931) 95.
24 G. T. RICH, R. I. SHA'AFI, T. C. BARTON AND A. K. SOLOMON, *J. Gen. Physiol.*, 50 (1967) 2391.
25 R. M. BLUM AND R. E. FORSTER, *Physiologist*, 9 (1966) 141.
26 J. DAINTY, *Protoplasma*, 57 (1963) 220.
27 N. KAMIYA AND M. TAZAWA, *Protoplasma*, 46 (1956) 394.