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PERTURBATION OF RED CELL VOLUME: CONSTANCY OF MEMBRANE TRANSPORT PARAMETERS FOR CERTAIN SLOW PENETRANTS

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SUMMARY

Following the "perturbation" approach of FARMER AND MACEY (R. E. L. FARMER AND R. I. MACEY, *Biochim. Biophys. Acta*, 196 (1970) 53) a method is described for measuring the concentration dependence of the membrane transport parameters (L_p, σ and ω) for a slowly penetrating non-electrolyte in intact red cells. The KEDEM–KATCHALSKY equations (O. KEDEM AND A. KATCHALSKY, *Biochim. Biophys. Acta*, 27 (1958) 229) are used to describe the transport kinetics. The mathematical solution consists of two independent exponential processes. The first describes a rapid osmotic adjustment of the cell and depends only upon L_p and σ. The second describes a slower penetration of permeant and depends upon σ and ω. Time constants of the two exponentials are expressed in terms of the transport parameters and external concentrations. Dependence of the parameters on concentration was obtained by varying the solution composition and photometrically measuring time constants of cell swelling and shrinking. Results with glycerol and ethylene glycol in beef erythrocytes indicate that L_p, σ and ω are independent of cell volume, salt osmolality and penetrant concentration over a wide range. Rectification of osmotic flow ($L_{p_{in}}$ greater than $L_{p_{out}}$) was observed, but σ and ω were not strongly dependent upon the direction of flow.

INTRODUCTION

In a previous paper[1] we have discussed the use of small concentration gradients or "perturbations" to measure membrane transport parameters. By way of brief review, the "perturbation" method has several noteworthy advantages. (1) It permits measurement of parameters over a wide range of final concentrations, allowing a detailed investigation of concentration dependence. (2) Restriction to small gradients permits use of the KEDEM–KATCHALSKY equations[2-4] in the analysis of the transport kinetics. (3) Volume perturbations are exponential in nature, and the exponential time constants are direct functions of the transport parameters. (4) All of the desired information lies in the time constants, therefore volume calibration curves are not required, and problems of initial mixing are reduced. We used this technique to obtain osmotic filtration coefficients (L_p) for beef and human erythrocytes suspended in buffered salt solutions of various tonicities. The measured coefficients, while indepen-

dent of final tonicity, were not independent of the direction of osmotic flow, *i.e.* we found a rectification of osmotic flow.

In the present paper the investigation of concentration dependency has been extended to the parameters associated with solute transport: the reflection coefficient (σ) and the permeability coefficient (ω). In particular, we have chosen slow, passive, non-electrolyte penetrants because the mathematical analysis is simplified and the experimental approach is straightforward. The treatment of rapid penetrants will be presented in a subsequent paper.

Our results with glycerol and ethylene glycol transport in beef red cells indicate that for such non-facilitated penetrants all three parameters (L_p, σ, ω) are essentially independent of penetrant concentration over a wide range (up to 1.0 M). Rectification of osmotic flow was again observed and this phenomenon also appeared independent of penetrant concentration. Reflection coefficients and permeability coefficients did not appear to depend strongly upon direction of flow.

CALCULATIONS

Beginning with the KEDEM–KATCHALSKY equations[2] for a non-electrolyte penetrant, a second-order system of equations has been constructed to describe the transport kinetics of a cell following a sudden change in composition of the suspending medium. For a slow penetrant the system reduces to two, independent, first-order subsystems. The first subsystem describes a rapid osmotic adjustment of the cell. This adjustment is independent of ω and contains information about L_p and σ. The second subsystem describes the slower penetration of solute which is independent of L_p. This slower process contains information about ω and σ. For small changes in composition these subsystems can be linearized by a Taylor's series expansion. The mathematical solutions to the linearized subsystems show that in each case cell volume changes exponentially with time. The exponential time constants are functionally related to the transport parameters and to the experimental conditions.

In terms of cell volume (V), penetrant content (S), and osmotic pressures (π_m for impermeable solutes, π_s for permeable), the KEDEM–KATCHALSKY equations can be written (primes denote intracellular quantities)

$$\frac{dV}{dt} = k_1[\pi'_m + \sigma\pi'_s - (\pi_m + \sigma\pi_s)] \quad (1)$$

$$\frac{dS}{dt} = (1 - \sigma)\bar{\pi}_s \frac{dV}{dt} + k_2(\pi_s - \pi'_s) \quad (2)$$

where t is time (sec) and σ is the permeant reflection coefficient (unitless). $\bar{\pi}_s$ is a mean value of penetrant osmotic pressure defined by $(\pi_s - \pi'_s)/\ln(\pi_s/\pi'_s)$. Eqn. 1 describes the volume flow into the cell and was used for measurement of L_p under the condition $\pi_s = \pi'_s = 0$ (ref. 1). Eqn. 2 describes the net flow of penetrant into the cell.

Let V_{iso} represent the cell volume at isotonicity (cm^3) and C_{iso} the isotonic concentration (osmoles·cm^{-3}). RT is the universal gas constant times absolute temperature (dyne·cm·$mole^{-1}$). Then for V in units of V_{iso} ($V = 1$ at isotonicity), π_m and π_s in units of RTC_{iso} ($\pi_m = 1$ at isotonicity, π_s in the same units), and S in units of $V_{iso}C_{iso}$ ($S = 1$ when the amount of internal penetrant equals the equivalent number

of osmoles of non-penetrant in a unit volume V_{iso} of solution), the coefficients k_1 and k_2 (each in units of sec^{-1}) are

$$k_1 = RTL_pAC_{iso}/V_{iso} \tag{3}$$

$$k_2 = RT\omega A/V_{iso} \tag{4}$$

where L_p is the osmotic filtration coefficient ($cm^3 \cdot dyne^{-1} \cdot sec^{-1}$), ω is the permeability coefficient of the permeant ($moles \cdot dyne^{-1} \cdot sec^{-1}$) and A is the area of the cell membrane (cm^2).

At equilibrium $\pi_m = \pi'_m$ and the following empirical relation between π'_m and V holds

$$\pi'_m = \frac{1 - b}{V - b} \tag{5}$$

where b is independent of cell volume. The term $V-b$ is related to the water volume of the cell but is not identical to it[5,6]. We also have $\pi_s = \pi'_s$ at equilibrium and

$$\pi'_s = \frac{S}{V - a} \tag{6}$$

where a is constant and independent of cell volume. For non-electrolytes $V-a$ represents 90–100 % of the isotonic cell water volume[7,8]. We assume that Eqns. 5 and 6 also hold for small perturbations around the state of equilibrium. Using these, Eqns. 1 and 2 become

$$\frac{dV}{dt} = k_1\left[\frac{1 - b}{V - b} + \frac{\sigma S}{V - a} - (\pi_m + \sigma\pi_s)\right] \tag{7}$$

$$\frac{dS}{dt} = (1 - \sigma)\bar{\pi}_s\frac{dV}{dt} + k_2\left(\pi_s - \frac{S}{V - a}\right) \tag{8}$$

The assumptions which have gone into the derivation of these equations include uniform solutions, no hydrostatic pressure gradient, membrane interior in steady state, and negligible volume fraction of penetrant. In addition, we assume constant external concentrations and constant membrane area. For slow penetrants the general system (Eqns. 7 and 8) reduces to two independent subsystems.

Rapid subsystem

When first exposed to a new environment the cells undergo a rapid osmotic adjustment. This rapid osmotic process is independent of ω because it is essentially complete before a significant amount of slow penetrant can diffuse across the membrane. The diffusion of penetrant is represented by the second term in Eqn. 8. In a description of the rapid osmotic process this term can be ignored. We then have the rapid subsystem consisting of Eqn. 7 and the modified Eqn. 8

$$\frac{dS}{dt} = (1 - \sigma)\bar{\pi}_s\frac{dV}{dt} \tag{9}$$

After completion of the rapid osmotic adjustment the permeable solute levels may be unbalanced. (This is the driving force for the slower diffusion of solute.) In

the interests of simplifying the analytical treatment and of reducing problems in data processing, experimental conditions (external concentrations) can be chosen so that the remaining solute imbalance is small. Then the final volume of the rapid subsystem is essentially the same as the system equilibrium volume (found by setting $dV/dt = 0$ and $dS/dt = 0$ in Eqns. 7 and 8). The desired conditions can be found as follows. First integrate Eqn. 9 from $t = 0$ to $t = \infty$.

$$\Delta S' = (1 - \sigma)\pi_s \Delta V' \tag{10}$$

where $\Delta V'$ represents the increase in cell volume, and $\Delta S'$ represents the corresponding gain in permeable solute inside the cell following completion of the initial osmotic transient. Since $\Delta S'$ and $\Delta V'$ are required to be small, $\bar{\pi}_s$ has been assumed constant and has been replaced by π_s. The overall system changes ΔS and ΔV can be obtained from initial and final equilibrium conditions. Final equilibrium (final state) is represented by V_∞ and S_∞, the solution composition being π_m and π_s. From Eqns. 5 and 6 we have

$$V_\infty = b + (1 - b)/\pi_m \tag{11}$$

$$S_\infty = (V_\infty - a)\pi_s \tag{12}$$

Initial conditions (initial state) are, by definition of ΔV and ΔS, characterized by $V_0 = V_\infty - \Delta V$ and $S_0 = S_\infty - \Delta S$. Letting $\Delta\pi_m$ and $\Delta\pi_s$ represent the perturbing osmotic pressures, then the solution composition before mixing is $\pi_m - \Delta\pi_m$ and $\pi_s - \Delta\pi_s$. From Eqns. 5 and 6 we have

$$V_0 = V_\infty - \Delta V = b + (1 - b)/(\pi_m - \Delta\pi_m) \tag{13}$$

$$S_0 = S_\infty - \Delta S = (V_\infty - \Delta V - a)(\pi_s - \Delta\pi_s) \tag{14}$$

From these initial and final conditions we get (for small changes)

$$\Delta V = -(1 - b)\pi_m^{-2}\Delta\pi_m \tag{15}$$

$$\Delta S = \pi_s \Delta V + \left(1 + \frac{b - a}{1 - b}\pi_m\right)(1 - b)\pi_m^{-1}\Delta\pi_s \tag{16}$$

Setting $\Delta S' = \Delta S$ and $\Delta V' = \Delta V$ and substituting Eqns. 15 and 16 into Eqn. 10 gives the desired experimental conditions for elimination of the remaining permeable solute gradient.

$$\sigma\pi_m^{-1}\Delta\pi_m = \left(1 + \frac{b - a}{1 - b}\pi_m\right)\pi_s^{-1}\Delta\pi_s \tag{17}$$

To make this calculation one needs values for a and b and an estimate for σ.

The kinetic behavior of the perturbed subsystem is found by expanding Eqn. 7 in a Taylor's series about equilibrium (V_∞, S_∞). Since $V - V_\infty$ and $S - S_\infty$ are small, we may neglect the higher order terms.

$$\frac{dV}{dt} = -k_1\left[\frac{(1 - b)}{(V_\infty - b)^2} + \frac{\sigma S_\infty}{(V_\infty - a)^2}\right](V - V_\infty) + \frac{k_1\sigma}{(V_\infty - a)}(S - S_\infty) \tag{18}$$

But from Eqn. 9

$$S - S_\infty = (1 - \sigma)\pi_s(V - V_\infty) \tag{19}$$

Using this and Eqns. 11 and 12

$$\frac{dV}{dt} = -\frac{k_1\pi_m^2}{(1-b)}\left[1 + \sigma^2\left(1 + \frac{b-a}{1-b}\pi_m\right)^{-1}\frac{\pi_s}{\pi_m}\right](V - V_\infty) \tag{20}$$

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

$$V = V_\infty - \Delta V\, e^{-t/\tau_-} \tag{21}$$

where τ_- is the exponential time constant defined by

$$\frac{1}{\tau_-} = \frac{k_1\pi_m^2}{(1-b)}\left[1 + \sigma^2\left(1 + \frac{b-a}{1-b}\pi_m\right)^{-1}\frac{\pi_s}{\pi_m}\right] \tag{22}$$

Thus for constant π_m and increasing π_s it is apparent that a plot of $\tau_-^{-1}\pi_m^{-2}$ against the ratio π_s/π_m will give a straight line if L_p and σ are constant. The slope of the line will be proportional to σ^2. The dependence upon the square of the reflection coefficient is especially useful when values of σ are near unity. Moreover, when $\pi_s = 0$ the time constant should be a linear function of the inverse square of the final tonicity π_m^{-2}. This has already been shown[1].

Slow subsystem

If Eqn. 17 has been satisfied, there will be no slow solute movement following the rapid osmotic adjustment of the cell. Now let us assume that Eqn. 17 was not satisfied and focus our attention on the slow equilibration of solute. During this slow process the cells are considered to be essentially at osmotic equilibrium, a condition which is expressed by setting $dV/dt = 0$ in Eqn. 7. Thus, the slow subsystem is described by Eqn. 8 and the modified Eqn. 7

$$0 = \frac{(1-b)}{(V-b)} + \frac{\sigma S}{(V-a)} - (\pi_m + \sigma\pi_s) \tag{23}$$

We find it desirable to adjust the experimental conditions so that the magnitude of the rapid osmotic transient is small. This permits the full magnitude of the small volume change to be related to the slow penetration of solute. The appropriate constraint can be found from Eqn. 23 using final and initial conditions from Eqns. 11, 12, 13 and 14.

$$\Delta\pi_m + \sigma\Delta\pi_s = 0 \tag{24}$$

The constraint is simply that the effective osmotic pressure of the external solution must be unchanged. Elimination of the rapid osmotic transient has been used previously as an experimental criterion for the determination of non-electrolyte reflection coefficients in human red cells[9].

The time course of the perturbed subsystem can be found by differentiating Eqn. 23 to get dS/dt and substituting this into Eqn. 8. Using Eqn. 23 to eliminate S gives

$$\left[\frac{(1-b)}{(V-b)}\cdot\frac{(b-a)}{(V-b)}+\pi_m+\sigma(\pi_s-\bar{\pi}_s)+\sigma^2\bar{\pi}_s\right]\frac{dV}{dt}=k_2\left[\frac{(1-b)}{(V-b)}-\pi_m\right] \quad (25)$$

Expanding in a Taylor's series about the equilibrium volume V_∞ and neglecting higher order terms gives

$$\frac{dV}{dt}=-\frac{k_2\pi_m}{(1-b)}\left(1+\frac{b-a}{1-b}\pi_m+\sigma^2\frac{\pi_s}{\pi_m}\right)^{-1}(V-V_\infty) \quad (26)$$

The solution to this equation is again of exponential form

$$V=V_\infty-\Delta V\,e^{-t/\tau_+} \quad (27)$$

where τ_+ is the exponential time constant for the slow subsystem. We can then write

$$\tau_+=\frac{(b-a)}{k_2}+\frac{(1-b)}{k_2\pi_m}\left(1+\sigma^2\frac{\pi_s}{\pi_m}\right) \quad (28)$$

So if we plot τ_+ *vs.* π_s/π_m while holding π_m constant, a straight line relationship will be obtained provided σ and ω are constant (independent of π_s). The slope will be proportional to $\sigma^2\omega^{-1}$ and the τ_+ intercept to ω^{-1}. A second kind of plot is also suggested. For π_s/π_m constant a plot of τ_+ against π_m^{-1} should give a straight line. The slope will be proportional to $(1+\sigma^2\pi_s/\pi_m)\,\omega^{-1}$, and the τ_+ intercept to $(b-a)\,\omega^{-1}$. Notice again that the square of the reflection coefficient appears in the time constant. This is especially useful when values of σ are near unity.

EXPERIMENTAL PROCEDURE

Beef blood was collected and defibrinated at a local slaughterhouse. It was then filtered through cheesecloth and stored at about 2° until used. Hematocrit (International Model MB Micro-capillary Centrifuge), osmolality (Fiske Mark III Osmometer) and cell counts of the blood were routinely determined. Red cell volume was calculated using hematocrit and cell count with no correction for trapped plasma. Cell area was estimated using a right circular cylinder approximation[10]. The quantity b (Eqn. 5) was determined from a plot of hematocrit *vs.* inverse tonicity using the solutions described below. A value for a (Eqn. 6) was estimated from data on human red cells[5,8] allowing for the larger fractional water content of the beef cells[11].

The isotonic salt solution used for preparing cell suspensions consisted of (all Cl^- salts) 133 mM Na^+, 5.4 mM K^+, 2.6 mM Ca^{2+}, 1.8 mM Mg^{2+}, 32 mM Tris. Its pH was about 7.4, and its osmolality was near 300 mosmoles/kg. Hypertonic and hypotonic solutions contained the same relative proportion of salts. Glycerol or ethylene glycol were added to make the desired non-electrolyte concentrations.

The photometric apparatus has been previously described[1]. Basically it measures the change in turbidity of a cell suspension following a sudden change in solution composition. 5.0 ml of a 1.0 % suspension of beef cells is pipetted into the cuvette of the apparatus. After establishing a short baseline, 0.5–0.6 ml of an appropriate injection solution is injected by solenoid into the cuvette, and the resulting perturbation of cell volume is recorded as a change in turbidity. The suspension is withdrawn from

the cuvette and its final osmolality measured. Time constants of cell swelling and shrinking are extracted from the recorded data.

The experiments were of two types, suggested by the forms of Eqns. 22 and 28. The first type was "constant-π_m", *i.e.* suspension and injection solutions were chosen so that the final salt tonicity was always constant. The penetrant level π_s was varied. For rapid exponentials the quantity $\tau_-^{-1}\pi_m^{-2}$ was plotted against the osmotic ratio π_s/π_m (see Eqn. 22), and for slow exponentials $\tau_+\pi_m$ was plotted *vs.* π_s/π_m (see Eqn. 28).

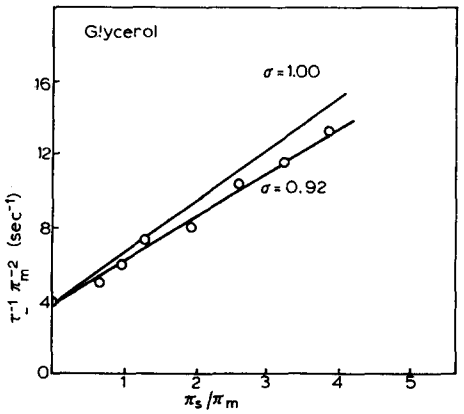

Fig. 1. Showing linearity of the modified inverse time constant $\tau_-^{-1}\pi_m^{-2}$ as the glycerol level (osmotic ratio π_s/π_m) is increased. A non-linear relationship would imply a dependence of L_p and/or σ on glycerol level. From the ordinate intercept the outward filtration coefficient can be calculated to be $RTL_{p_{out}} \simeq 0.40$ cm^4/osmoles per sec. From the slope a value for the reflection coefficient of glycerol is obtained: σ (glycerol) = 0.92 (shrinking). Experimental details are shown in Table I. The straight line fitting the experimental points in this graph (and in all following) has been determined by the method of least squares.

TABLE I

DETAILS OF CONSTANT-π_m OSMOTIC SHRINKING EXPERIMENTS WITH INCREASING LEVELS OF GLYCEROL

Beef red cells (B 1/30 LM) were previously equilibrated at an initial state defined by the solution composition *Before injection* (c_m, salt concentration; c_s, non-electrolyte concentration). Upon injection of the *Injection solution* the cells rapidly approached a final state defined by the *After injection* solution composition. (Injection solutions were prepared so that little or no gradient of penetrant remained after the rapid osmotic shrinking.) The time course of the volume change was exponential in nature with time constant τ_-. Time constants τ_-, salt tonicities π_m, and osmotic ratios π_s/π_m were used to make the plot of Fig. 1. The cells had been stored for 3 days. Isotonicity was 288 mosmoles/kg. The temperature was 25°.

Expt. No.	*Before injection* *		*Injection solution* *		*After injection* *		π_m	π_s/π_m	τ_- *(sec)*
	c_m	c_s	c_m	c_s	c_m	c_s			
1	236	0	460	0	261	0	0.91	0.00	0.308
2	236	155	460	291	261	169	0.91	0.65	0.248
3	223	218	470	466	249	244	0.86	0.98	0.224
4	223	291	460	581	248	322	0.86	1.30	0.189
5	223	436	460	872	248	482	0.86	1.94	0.171
6	223	582	460	1162	248	643	0.86	2.60	0.132
7	223	727	460	1452	248	804	0.86	3.24	0.117
8	223	873	460	1747	248	965	0.86	3.89	0.102

* All concentrations given in mosmoles/l.

The second type of experiment was "constant-π_s/π_m" suggested by Eqn. 28. In this case concentrations in the solutions were adjusted so that the ratio π_s/π_m remained constant as π_m was varied. The slow time constant τ_+ was plotted against π_m^{-1}. In rapid exponential experiments concentrations were chosen so that Eqn. 17 would be approximately satisfied. Slow exponential experiments were designed according to Eqn. 24. Small deviations from these conditions caused no problem in obtaining the proper time constants.

RESULTS

Eqn. 22 predicts that, if the transport parameters are constant, a plot of $\tau_-^{-1}\pi_m^{-2}$ *vs.* π_s/π_m for constant π_m should be a straight line. Details of a series of (near) constant-π_m experiments are shown in Table I. Time constants of rapid osmotic shrinking (τ_-) were measured and plotted (Fig. 1) against π_s/π_m as $\tau_-^{-1}\pi_m^{-2}$. Use of the quantity $\tau_-^{-1}\pi_m^{-2}$ minimizes effects of small variations in final tonicity. It is seen from Fig. 1 that a straight-line relationship is indeed obtained for glycerol. Moreover,

TABLE II

ADDITIONAL CONSTANT-π_m EXPERIMENTS WITH INCREASING LEVELS OF GLYCEROL AND ETHYLENE GLYCOL

Beginning at an initial state defined by the solution composition *Before injection* beef red cells (B 3/13 LM) were subjected to either a small swelling or a small shrinking. In each case the final state was defined by the solution composition *After injection*. Experiments 1–7 were glycerol swelling (blood stored for 1 day). Experiments 8–14 were ethylene glycol shrinking (2-day stored blood). Experiments 15–21 were glycerol shrinking (2-day stored blood). Isotonicity was 319 mosmoles/kg, and the temperature was 25°. Results were plotted in Fig. 2.

Expt. No.	*Before injection**		*Injection solution**		*After injection**		π_m	π_s/π_m	τ_- (*sec*)
	c_m	c_s	c_m	c_s	c_m	c_s			
1	331	0	0	0	294	0	0.921	0.00	0.241
2	331	137	0	0	294	124	0.921	0.42	0.190
3	331	206	0	0	294	187	0.921	0.64	0.162
4	331	274	0	0	294	249	0.921	0.85	0.143
5	331	411	0	0	294	373	0.921	1.27	0.127
6	331	548	0	0	294	499	0.921	1.69	0.110
7	331	685	0	0	294	621	0.921	2.12	0.098
8	314	0	628	0	352	0	1.102	0.00	0.260
9	314	158	628	300	352	172	1.102	0.49	0.236
10	314	236	628	450	352	258	1.102	0.73	0.213
11	314	315	628	600	352	344	1.102	0.98	0.194
12	314	472	628	900	352	515	1.102	1.46	0.166
13	314	630	628	1200	352	688	1.102	1.95	0.146
14	314	789	628	1500	352	860	1.102	2.44	0.127
15	331	0	628	0	362	0	1.134	0.00	0.257
16	331	137	628	291	362	156	1.134	0.43	0.224
17	331	206	628	436	362	246	1.134	0.68	0.200
18	331	274	628	581	362	310	1.134	0.86	0.171
19	331	411	628	872	362	490	1.134	1.35	0.143
20	331	548	628	1162	362	646	1.134	1.78	0.130
21	331	685	628	1455	362	805	1.134	2.22	0.120

* All concentrations given in mosmoles/l.

from the ordinate intercept of 3.7 sec^{-1}, a value of $RTL_{p_{out}} = 0.40$ cm^4/osmoles per sec can be calculated for these cells ($b = 0.42$, $V_{iso}A^{-1} = 0.55 \cdot 10^{-4}$ cm). The reflection coefficient σ for glycerol is calculated from the slope of the line to be about 0.92 ($a = 0.24$). For comparison a line is drawn for which $\sigma = 1$.

Additional constant-π_m experiments on blood from a second animal are described in Table II. In these experiments (glycerol shrinking, glycerol swelling and ethylene glycol shrinking) the osmotic ratio extends up to $\pi_s/\pi_m = 2.5$. The results are plotted in Fig. 2. The data obey straight-line relationships agreeing again with Eqn. 22 assuming constant parameters. The effect of flow rectification is shown clearly. The filtration coefficients can be calculated from the two ordinate intercepts. The inward filtration coefficient $RTL_{p_{in}} = 0.48$ cm^4/osmoles per sec, while $RTL_{p_{out}} = 0.28$ cm^4/osmoles per sec ($b = 0.43$, $V_{iso}A^{-1} = 0.53 \cdot 10^{-4}$ cm). Reflection coefficients are calculated from the slopes. For glycerol, σ is slightly higher during swelling than during shrinking. A mean value is 0.91 ($a = 0.25$). The reflection coefficient for ethylene glycol is calculated to be 0.81. Swelling experiments with the same cells a day later gave $RTL_{p_{in}} = 0.45$ cm^4/osmoles per sec, $\sigma = 0.83$ for ethylene glycol and $\sigma = 0.96$ for glycerol.

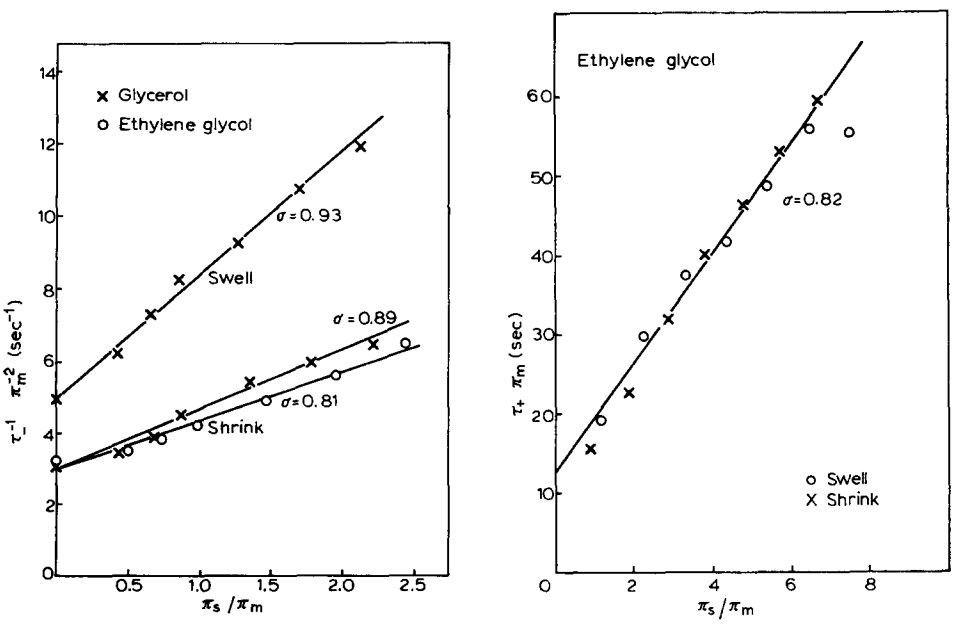

Fig. 2. Results of additional osmotic experiments with increasing levels of glycerol and ethylene glycol. Again there is a linear dependence of the modified inverse time constant $\tau_-^{-1}\pi_m^{-2}$ upon the osmotic ratio π_s/π_m. Note the directional effect of osmotic flow rectification. From the two ordinate intercepts the inward and outward filtration coefficients are calculated to be 0.48 cm^4/osmoles per sec and 0.28 cm^4/osmoles per sec, respectively. From the slopes the reflection coefficients are: σ (glycerol) = 0.93 (swelling), σ (glycerol) = 0.89 (shrinking) and σ (ethylene glycol) = 0.81 (shrinking). See Table II for experimental details.

Fig. 3. Showing linearity obtained in constant-π_m experiments (Table III) with ethylene glycol penetration. Time constants of penetration are plotted in a modified form ($\tau_+\pi_m$) against the osmotic ratio π_s/π_m. A dependence of ω on ethylene glycol level would lead to non-linearity. The following parameters can be calculated for ethylene glycol from the slope and intercept of the line: σ (ethylene glycol) = 0.82 and $RT\omega = 2.9 \cdot 10^{-6}$ cm/sec at 25°.

In the case of slow solute penetration the time constant τ_+ is described by Eqn. 28. This equation predicts that, for constant parameters and constant π_m, a plot of τ_+ *vs.* π_s/π_m should be a straight line. Constant-π_m experiments (swelling and shrinking) are described in Table III. The time constants of ethylene glycol penetration were plotted (Fig. 3) *vs.* π_s/π_m in the form $\tau_+\pi_m$. The data support a linear dependence of τ_+ upon π_s/π_m. The best fit through the points gave $\sigma = 0.82$ and

TABLE III

CONSTANT-π_m EXPERIMENTS INVESTIGATING THE SLOW PENETRATION OF ETHYLENE GLYCOL

Beef erythrocytes (B 5/9 LM) were equilibrated at increasing levels of ethylene glycol (c_s) while the salt level (c_m) was held constant. Injection solutions were chosen so that the initial osmotic transient was small. Time constants of penetration were plotted in Fig. 3 in the modified form ($\tau_+\pi_m$) *vs.* the osmotic ratio π_s/π_m. In Experiments 1–7 the cells were swelling (blood stored for 1 day). In Experiments 8–14 the cells were shrinking (2-day stored blood). Isotonicity was 322 mosmoles/kg, and the temperature was 25°.

Expt. No.	*Before injection* *		*Injection solution* *		*After injection* *		π_m	π_s/π_m	τ_+ (*sec*)
	c_m	c_s	c_m	c_s	c_m	c_s			
1	311	297	0	594	283	324	0.879	1.14	21.8
2	311	594	0	891	283	621	0.879	2.20	34.1
3	311	891	0	1188	283	918	0.879	3.24	42.9
4	311	1188	0	1484	283	1216	0.879	4.30	47.4
5	311	1484	0	1781	283	1513	0.879	5.35	55.3
6	311	1781	0	2080	283	1809	0.879	6.39	63.3
7	311	2080	0	2380	283	2107	0.879	7.45	62.8
8	280	297	525	0	302	272	0.938	0.90	16.1
9	280	594	525	297	302	567	0.938	1.88	23.7
10	280	891	525	594	302	860	0.938	2.85	33.2
11	280	1188	525	891	302	1155	0.938	3.82	41.9
12	280	1484	525	1188	302	1448	0.938	4.80	48.4
13	280	1781	525	1484	302	1741	0.938	5.76	55.3
14	280	2080	525	1781	302	2038	0.938	6.75	62.0

* All concentrations given in mosmoles/l.

$RT\omega = 2.9 \cdot 10^{-6}$ cm/sec ($b = 0.46$, $a = 0.27$, $V_{iso}A^{-1} = 0.52 \cdot 10^{-4}$ cm). The position of the line is rather insensitive to the values of a and b and most sensitive to the value of the permeability coefficient ($RT\omega$). Little or no solute flow rectification is observed.

Eqn. 28 also predicts that, for constant π_s/π_m, a plot of the slow time constant τ_+ *vs.* the inverse tonicity π_m^{-1} should give a straight line. Constant-π_s/π_m experiments with ethylene glycol are described in Table IV. Fig. 4 shows the results of these experiments. Again, agreement with linearity is observed. At low π_s/π_m the fit becomes more insensitive to the value of σ. We may assume a reasonable value for σ and calculate $RT\omega$ from the slopes. The quantity a can then be calculated from the intercepts, which are very sensitive to the difference between a and b. Assuming $\sigma = 0.83$ the average value of the ethylene glycol permeability coefficient is $RT\omega = 4.6 \cdot 10^{-6}$ cm/sec at 26° ($b = 0.44$, $V_{iso}A^{-1} = 0.55 \cdot 10^{-4}$ cm). A small apparent rectification is observed with swelling slower than shrinking by about 18 %. From the intercepts a value of $a = 0.25$ is calculated. This value agrees with that used in previous calculations.

TABLE IV

DETAILS OF CONSTANT-π_s/π_m EXPERIMENTS INVESTIGATING THE PROPERTIES OF ETHYLENE GLYCOL PENETRATION

Beef erythrocytes (B 5/15 LM) were equilibrated in a series of solutions in which the salt concentration (c_m) was varied. The ethylene glycol level (c_s) was adjusted so that the osmotic ratio π_s/π_m was constant. Perturbations were designed so that the initial osmotic transient was small. The time constants of penetration τ_+ were measured and plotted *vs.* π_m^{-1} in Fig. 4. The cells in Experiments 1–6 were swelling, and in Experiments 7–14 they were shrinking. Isotonicity was 300 mosmoles/kg, and the temperature was 26°. The blood had been stored for 1 day.

Expt. No.	*Before injection* *		*Injection solution* *		*After injection* *		π_m	π_s/π_m	τ_+ (*sec*)
	c_m	c_s	c_m	c_s	c_m	c_s			
1	297	456	0	825	270	498	0.90	1.84	21.2
2	330	510	0	894	300	550	1.00	1.83	19.7
3	396	609	0	1092	360	670	1.20	1.86	16.6
4	495	759	0	1388	450	786	1.50	1.75	13.1
5	660	1020	0	1817	600	1086	2.00	1.81	11.3
6	775	1200	0	2110	708	1282	2.36	1.81	9.8
7	264	562	495	272	285	547	0.95	1.92	16.8
8	297	643	594	272	324	617	1.08	1.90	15.0
9	330	732	726	238	366	671	1.22	1.84	13.8
10	363	813	825	238	405	756	1.35	1.87	12.3
11	396	902	957	204	446	825	1.49	1.85	12.1
12	429	991	1090	136	489	931	1.63	1.91	11.0
13	461	1088	1253	68	534	990	1.78	1.86	9.8
14	495	1173	1320	0	570	1059	1.90	1.85	9.0

* All concentrations given in mosmoles/l.

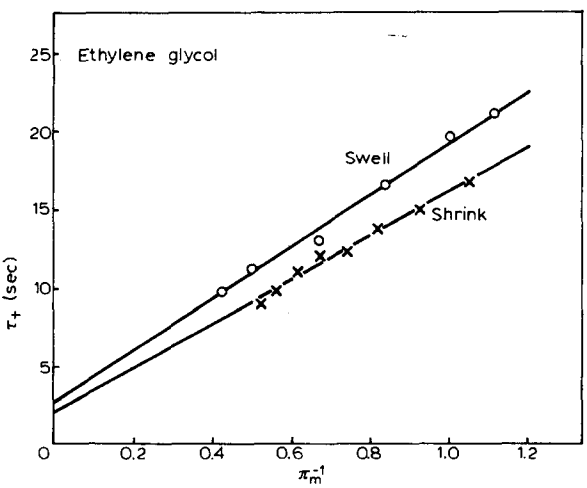

Fig. 4. Showing linearity in constant-π_s/π_m experiments (Table IV) with ethylene glycol penetration. Time constants of penetration τ_+ are plotted against the inverse salt tonicity π_m^{-1}, which determines the size of the cell. A dependence of ω on cell volume or on salt tonicity would cause a deviation from linearity. Assuming $\sigma = 0.83$, the slopes of the lines give a mean permeability coefficient of $RT\omega = 4.6 \cdot 10^{-6}$ cm/sec at 26°. From the τ_+ intercepts is calculated a mean value for a of 0.25. This value agrees well with that used in previous calculations.

The permeability coefficient of glycerol was measured on blood from one animal. It was found to be about $2 \cdot 10^{-7}$ cm/sec at 25° ($a = 0.25$, $b = 0.42$, $V_{iso}A^{-1} = 0.55 \cdot 10^{-4}$ cm).

DISCUSSION

Constancy of transport parameters

It has been previously reported that the osmotic filtration coefficient for beef erythrocytes, whether inward or outward, is independent of cell volume and medium (salt) osmolality[1]. This property of L_p was also observed with the cells used in these experiments. Further, the slow penetration data of Fig. 4 indicate that σ and ω for ethylene glycol are also independent of cell volume and salt osmolality. (A non-constant σ or a non-constant ω would cause a deviation from linearity.) That the permeability of ethylene glycol is independent of cell volume agrees with the work of MACEY AND TOLBERG[12] who found that glycerol permeability is also independent of beef cell volume. Such results are not consistent with the suggestion that the red cell membrane responds osmotically to anisotonic solutions by changing the size of its aqueous channels[13,14]. In fact it appears that the membrane is osmotically stable over the range of tonicities used.

Further evidence of osmotic stability is shown in the rapid osmotic experiments of Figs. 1 and 2. These plots are linear with increasing penetrant levels up to 3.5 times isotonic. The linearity implies that L_p and σ (ethylene glycol and glycerol) are independent of penetrant (non-electrolyte) level. In addition, the slow penetration experiment of Fig. 3 shows linearity as the ethylene glycol level is increased up to 5.5 times isotonic. This implies that ω, too, is independent of penetrant level. So for these solutes the three parameters appear to be constant, *i.e.* independent of cell volume, salt osmolality and penetrant (non-electrolyte) concentration.

Transport parameters and direction of flow

Rectification of water flow across the membrane of beef red cells has been previously described[1]. This property appears in the constant-π_m experiments of Fig. 2 where the rectification ratio (corrected for second-order effects) is about 1.56. The same experiments show a slight dependence of the glycerol reflection coefficient on the direction of water flow. A smaller effect was noted for ethylene glycol using results of additional swelling experiments. We do not believe that this small dependence is significant because it falls within our estimate of experimental error (5 %). The data are not consistent with the suggestion that rectification may be caused by a change in the radius of a uniform set of aqueous pores. This would require σ to be considerably smaller during swelling than during shrinking. The presence of a non-uniform set of pores might be plausible. The data do not rule out the possibility that the availability of aqueous channels may be controlled by the direction of flow. Such a hypothesis is consistent with the suggestion that hemoglobin may partially screen the membrane surface during outward flow of water.

Rectification of solute flow, if it exists, must be small. In Fig. 3 the net inflow of ethylene glycol is very nearly equal to its outflow under the same gradient, and no rectification is observed. In Fig. 4 a small ratio of 0.85 is observed with inflow being slower by about 18 %. This non-unity ratio may have been caused by inadequate

temperature control in the cuvette. (A 1° change can cause a 10 % change in the permeability coefficient since $\Delta H^{\ddagger} \approx 16.4$ kcal/mole for ethylene glycol in beef red cells[15].) Thus, considering experimental error, rectification of solute flow has not been established for slow penetrants.

Osmotic flow vs. solute penetration for the measurement of σ and ω

The experiments which have been described each have certain advantages and disadvantages when they are to be used for measurement of the penetrant transport parameters σ and ω. The rapid osmotic flow, constant-π_m experiments shown in Figs. 1 and 2 and described by Eqn. 22 are useful for determination of σ. Data processing is simplified by the linearity of the plot of the modified inverse time constant $\tau_-^{-1}\pi_m^{-2}$ *vs.* solute level π_s/π_m when L_p and σ are constant. In addition, the slope is found to be proportional to the square of σ giving increased sensitivity for values of σ near unity. On the other hand there is the problem of experimental "scatter" of points. This scatter makes it necessary to have many data points in order to get a good curve fit. Secondly, one needs values for both b and a to calculate σ. A value for b can be obtained from hematocrit *vs.* inverse tonicity experiments. (A mean value for beef red cells is 0.45.) A value for a can be estimated by assuming that practically all of the intracellular water is available for non-electrolyte solution. This assumption has been verified in the case of sugars for human erythrocytes[7]. Ethylene glycol and glycerol, however, may dissolve in only 96 % of the cell water[8]. The human red cell has about 72 % water (by vol.)[5], but the beef red cell may contain up to 4 % more water[11]. Based on these figures we estimate a to be about 0.26 for the beef red cell.

The slow penetration experiments of Figs. 3 and 4 (described by Eqn. 28) are both useful for the determination of the permeability coefficient ω. Perhaps the more versatile of the two is the constant-π_m experiment of Fig. 3. Here again one needs values for b and a although the results are not very sensitive to the choice of these values. From the ordinate intercept one can calculate ω. From the slope, σ can be obtained. Again, σ enters into the calculations as σ^2, increasing the sensitivity to values of σ near unity. However, as seen in Fig. 3, there may be an appreciable "scatter" of points hindering curve fitting.

The constant-π_s/π_m experiment of Fig. 4 can serve to check the estimated value of a. For this purpose it is perhaps best to choose a small ratio of π_s/π_m. In interpreting the linear plot which is obtained one needs an approximate value for σ (from previous experiments). The quantity $\sigma^2\pi_s/\pi_m$ is added to unity, therefore errors in σ are not so important as long as π_s/π_m is small. Then from the slope one can calculate ω and from the ordinate intercept determine a.

Comparison of results

The measured permeability coefficients for glycerol and ethylene glycol are in accord with those in the literature. The value of $2 \cdot 10^{-7}$ cm/sec for glycerol represents the result of measurements on blood from only one animal. However, it compares favorably with the value of $0.9 \cdot 10^{-7}$ cm/sec (at 25°) calculated from constant cell volume results at 31° (ref. 12). The value of $38 \cdot 10^{-7}$ cm/sec for ethylene glycol is an average for two animals. This value compares with $24 \cdot 10^{-7}$ cm/sec which was calculated from hemolysis times at 20° (ref. 16).

On the basis of a "pore" model of the cellular membrane in which non-lipid-

soluble non-electrolytes penetrate *via* small aqueous channels, it is perhaps not surprising to find that ethylene glycol penetrates the membrane more rapidly than glycerol. Ethylene glycol is the smaller molecule, having an estimated radius of 2.24 Å while glycerol is about 2.74 Å (ref. 9). Therefore, ethylene glycol should pass more readily through small "pores' in the membrane. This reasoning is supported by the average measured values of σ: 0.92 for glycerol and 0.82 for ethylene glycol. The lower value for ethylene glycol suggests that it encounters less frictional resistance during passage through the "pores". Data for the human erythrocyte membrane, through which both solutes pass much more rapidly[15], support this line of reasoning. The reported reflection coefficients of 0.88 for glycerol and 0.63 for ethylene glycol[9] are lower than their respective values in beef erythrocytes.

On the other hand, ethylene glycol is more lipid-soluble than glycerol having an olive oil–water partition coefficient of $4.9 \cdot 10^{-4}$ while that for glycerol is $0.7 \cdot 10^{-4}$ (ref. 9). So a lipid pathway can also account for the higher permeability coefficient of ethylene glycol. Experiments with inhibitors of passive permeability (in particular of L_p) in human red cells have shown that such non-aqueous pathways are probably dominant in the membrane of the red cell[17].

GOLDSTEIN AND SOLOMON[9] have suggested an equivalent pore radius of 4.2 Å for the membrane of the human erythrocyte. This value, they say, is in agreement with values of σ for glycerol (0.88) and for ethylene glycol (0.63) on the basis of molecular size. VILLEGAS *et al.*[13], using a different technique, suggest that the membrane of the beef erythrocyte has an equivalent pore radius of 4.1 Å, almost identical to that for human. Following the GOLDSTEIN AND SOLOMON[9] argument one would expect the reflection coefficients for beef red cells to be very near those for human, *i.e.* 0.88 for glycerol and 0.63 for ethylene glycol. The values reported here of 0.92 and 0.82, respectively, suggest that the "equivalent pore radius" for beef erythrocytes may be significantly smaller than 4.1 Å.

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