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Reflection Coefficients of Permeant Nonelectrolytes for Dog and Beef Red Cell Membranes

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Summary. The reflection coefficient, σ, for several small permeant nonelectrolytes was determined for dog and beef red blood cell membranes. Our σ values were considerably higher than those previously reported for dog cells; e.g., our σ_{urea} was 87% higher than the σ_{urea} of Rich, Sha'afi, Barton and Solomon (*J. Gen. Physiol.* **50**: 2391, 1967). Our σ values for urea were only slightly greater in beef cells than previously reported by Farmer and Macey (*Biochim. Biophys. Acta* **290**: 290, 1972). We found that a trend exists when $(1-\sigma)$ is plotted against the log of the permeability coefficient, ω. This observation is also consistent with our previously reported σ data for human red cell membranes (Owen & Eyring, *J. Gen. Physiol.* **66**: 251, 1975). This trend suggests that small hydrophilic molecules interact highly with cell membrane water. The exceptions to this trend were lipophilic molecules, indicating they do not interact with water while penetrating the red cell membrane.

Solute molecules which penetrate membranes do not exert their full osmotic effect, as first reported by Staverman (1951). Reflection coefficients, σ, indicate the ability of a membrane to distinguish between solute and solvent molecules. The expression relating σ to ω, the solute permeability coefficient, is

$$(1-\sigma)=\frac{\omega V_s}{Lp}+\frac{k_s f_{sw}}{f_{sw}+f_{sm}} \tag{1}$$

where V_s is the molar volume, Lp is the osmotic water permeability, k_s is the solute partion coefficient, f_{sw} is the solute-water frictional coefficient and f_{sm} is the solute-membrane frictional coefficient. When $(1-\sigma)$ for a solute is approximately equal to $\omega V_s/Lp$ a lipid permeation mechanism is implied, while a $(1-\sigma)$ greater than $\omega V_s/Lp$ indicates permeation through water-filled channels and the contribution due to volume flow through aqueous pathways is represented by the frictional coefficient term in Eq. (1) (Dainty & Ginzburg, 1963).

Goldstein and Solomon (1960) initially determined σ values for small permeant molecules in human red cell membranes with a rapid flow apparatus. Several years later, Sha'afi, Rich, Mikulecky and Solomon (1970) duplicated Goldstein and Solomon's urea σ value using a stopped-flow apparatus. Basically, the trend in σ previously found by Goldstein and Solomon (1960) and Sha'afi *et al.* (1970) was that rapid permeant molecules, such as urea, acetamide and ethylene glycol, had σ values of about 0.6, while slow permeant molecules such as glycerol, malonamide and propionamide, had σ values of approximately 0.85. By fitting a plot of $(1-\sigma)$ as a function of permeant molecule radius with a theoretical curve from the Renkin (1954) equation, Goldstein and Solomon (1960) postulated the existence of a 4.2 Å equivalent pore radius in human red blood cells.

Rich *et al.* (1967), using a stopped-flow apparatus, determined membrane pore radii of 5.9 Å and 6.2 Å for dog red cells from the P_f (water osmotic permeability): P_d (water diffusion permeability) ratio being greater than unity and from their σ data, respectively.

Villegas, Barton and Solomon (1958), using a rapid flow apparatus, reported a pore radius of 4.1 Å for beef red cells from their P_f/P_d values. Farmer and Macey (1972*a*), using a rapid mixing apparatus, found $\sigma=0.82$ for ethylene glycol in beef red cell suspensions, but Goldstein and Solomon (1960) reported a $\sigma=0.63$ for ethylene glycol and a pore radius of 4.2 Å for human red cells. Also, Farmer and Macey (1972*b*) have reported a $\sigma=0.73$ for urea in beef cells which was greater than either the 0.62 (Goldstein & Solomon, 1960) or 0.55 (Sha'afi *et al.*, 1970) urea σ values previously reported in human red cells. Farmer and Macey (1972*a*) concluded that since their σ values were higher in beef cells than in human cells, it was possible that the pore radius might be smaller in beef cells than in human cells.

Since the consistency of the earlier σ work of Goldstein and Solomon (1960) and Sha'afi *et al.* (1970) with human red cells has been questioned recently by Stein (1967), Forster (1971), Lieb and Stein (1971), Eilam and Stein (1974), Levitt (1974), and Owen and Eyring (1975), we have used a stopped-flow technique to measure σ in dog and beef red cells. Also, Solomon, Milgram, and Kirkwood (1975) have recently reported computer simulated human red cell relative volume *vs.* time curves using the original urea σ of Goldstein and Solomon (1960). Solomon *et al.* (1975) indicated the original urea σ of 0.62 may be underestimated by 10–20%, which would bring their corrected σ closer to a recently reported experimental σ of 0.79 (Owen & Eyring, 1975).

Experimental Methods

The experimental procedure and equipment were the same as previously reported (Owen & Eyring, 1975). Basically, eight volumes of nonelectrolyte solution were rapidly mixed (~5 msec) with one volume of a 0.2 % by volume red cell suspension with a Durrum stopped-flow apparatus. Oscilloscope traces of the amount of 550 nm light scattered at 90° versus time were recorded on Polaroid film, and the initial portion of the traces due to water movement across the cell membrane, which was between approximately 20 and 100 msec, was analyzed by drawing maximum slopes with a straight edge. Since it has been shown (Sha'afi *et al.*, 1970) that light scattering and cell volume change are directly proportional, we used change in scattered light to represent relative change in cell volume.

The value for σ was found by:

$$\sigma = \frac{C_i}{C_s} \tag{2}$$

where C_i is the isotonic red cell buffer concentration and C_s is the extracellular permeant molecule concentration which causes no change in cell volume, when it is mixed with a red cell suspension (Kedem & Katchalsky, 1958; Goldstein & Solomon, 1960; Owen & Eyring, 1975). The concentrations in Eq. (2) are the concentration before mixing in the stopped-flow apparatus, since a straightforward calculation shows σ is independent of permeant and impermeant concentration mixing ratios[1]. The value for C_s was found by plotting the

1 From the Kedem and Katchalsky (1958) volume flow equation

$$J_V = -L_p \Delta\pi_i + L_{pD} \Delta\pi_s \tag{a}$$

where J_V is the volume flow per unit area, L_p is the hydraulic conductivity, π_i is the osmotic pressure due to the impermeant solute, L_{pD} is the cross-coefficient for the volume flow and π_s is the osmotic pressure of the permeable solute. At zero volume change, which is the condition where σ is determined, Eq. (a) becomes:

$$L_p \Delta\pi_i = L_{pD} \Delta\pi_s. \tag{b}$$

Since σ is defined (Kedem & Katchalsky, 1958) as:

$$\sigma = -L_{pD}/L_p \tag{c}$$

substituting Eq. (c) into Eq. (b) yields:

$$\sigma = -\Delta\pi_i/\Delta\pi_s. \tag{d}$$

Since

$$\Delta\pi_i = RT\Delta C_i = RT(C_i^0 - C_i^{\Delta x})$$

and

$$\Delta\pi_s = RT\Delta C_s - RT(C_s^0 - C_s^{\Delta x})$$

Eq. (d) becomes:

$$\sigma = \frac{C_i^{\Delta} - C_i^0}{C_s^0 - C_s^{\Delta x}}. \tag{e}$$

$C_s^{\Delta x}$ is equal to zero at $J_V = 0$, so Eq. (e) is:

$$\sigma = \frac{C_i^{\Delta x} - C_i^0}{C_s^0}. \tag{f}$$

If under the normal stopped-flow operation an equal volume of a red cell suspension is mixed

initial maximum slopes of the light-scattering curves, due to cell water movement when the cells were mixed with hypo- and hypertonic permeant solute concentration, as a function of permeant solute concentration.

The blood was obtained from unmedicated, catheterized, anesthetized healthy dogs or cows, usually from the femoral artery.

Results and Discussion

Determination of σ in Dog Red Cell Suspensions

Fig. 1*A* and 2*A* represent typical light-scattering results from one experiment for acetamide and propionamide, respectively, in dog red cell suspensions. In Fig. 1*B*, the negative values of the slopes of the scattered light curves in Fig. 1*A* were plotted as a function of acetamide concentration. At zero voltage change, which corresponds to zero cell volume change, an acetamide C_s value of 421 milliosmoles (mosm) was obtained. Substituting this value for C_s in Eq. (2) along with $C_i = 320$ mosm gives $\sigma = 0.76$ for acetamide in dog red cells. Similarly, in Fig. 2*B*, a σ of 0.96 for propionamide was obtained with $C_s = 334$ mosm in dog cells. Table 1 compares our σ data from several separate experiments for dog cells with that of Rich *et al.* (1967). Our values were higher for all the solutes we investigated than the earlier results of Rich *et al.* (1967). Also, Rich *et al.* (1967) found similar σ values for acetamide and propionamide whereas ours were considerably different for these two solutes.

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with an equal volume of nonelectrolyte:

$$C_i^0 = C_i^{Ax}/2 \quad \text{and} \quad C_s^0 = C_s^0/2. \tag{g}$$

Eq. (f) becomes:

$$\sigma = \frac{C_i^{Ax}}{C_s^0}. \tag{h}$$

Since $C_i^{Ax} = C_i^0$ before mixing, Eq. (h) is:

$$\sigma = \frac{C_s^0}{C_s^0} \tag{i}$$

or dropping the superscripts:

$$\sigma = \frac{C_i}{C_s} \tag{j}$$

which is equivalent to Eq. (2) in the text.

We modified our syringe ratios to 8:1 instead of 1:1 nonelectrolyte to red cell suspension ratio. This would alter Eq. (g) to

$$C_i^0 = C_i^{Ax}/9 \quad \text{and} \quad C_s^0 = 8\,C_s^0/9. \tag{g'}$$

But substituting Eq. (g') into Eq. (f) gives Eq. (i), indicating, as one would expect, the calculation of σ is independent of the solvent:solute mixing ratio. Therefore, for convenience, σ is found by using the values of C_i and C_s before mixing the two components.

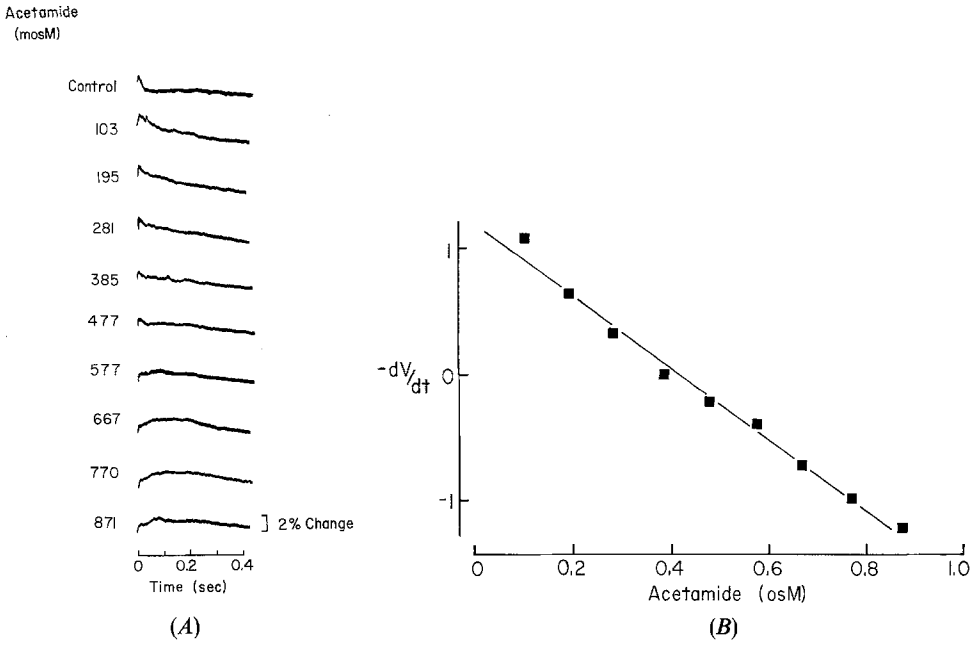

Fig. 1. (*A*) The effect of different acetamide concentrations on the rate of change in intensity of 90° scattered light from a dog red cell suspension, dV/dt, where V is the response of the photomultiplier tube in volts. The light-to-dark voltage was 6 volts and therefore, the 2% calibration mark corresponds to 120 mV. Since red cell volume is indirectly proportional to the amount of 90° scattered light, we use photomultiplier voltage as an index of cell volume, i.e., increases in voltage represent cell shrinking and decreases in voltage represent cell swelling. The control trace represents the rapid mixing of ~8 volumes of isotonic buffer with 1 volume of an isotonic red cell suspension. (*B*) The negative of the slopes of the tangents drawn to the curves in Fig. 1*A* between approximately 0.05 and 0.1 sec plotted as a function of acetamide concentration. The line drawn through the data points is a least-squares line. From Eq. (1), σ in this representative experiment is equal to $320/421 = 0.76$ [*see* Eq. (3)], where 320 mosm is the concentration of the red cell isotonic buffer, C_i, and 421 mosm, C_s, is calculated from extrapolation to zero-voltage (or zero relative volume) change

Fig. 3 is a plot of $(1-\sigma)$ as a function of the log of the permeability coefficient, ω, in dog red cell suspensions. The ω values for the hydrophilic molecules (urea, acetamide, and glycerol) are each from a different source (Rich *et al.*, 1967; Sha'afi *et al.*, 1971; Wessels & Veerkamp, 1973), so the least-squares line through the data points can only be considered an approximate trend. A similar trend was previously reported (Owen & Eyring, 1975) with human red cells for several permeant molecules. The one exception to this trend with dog cells was propionamide, which gave a σ higher than it would be predicted by ω. From Eq. (1) the calculated value for $(1-\sigma)$ agrees closely to the experimental value by

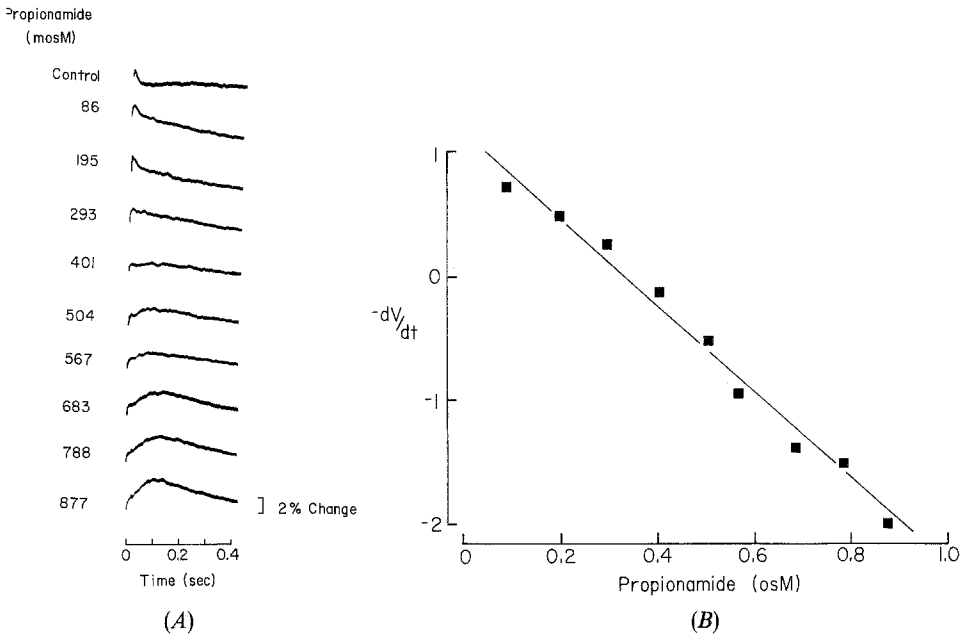

Fig. 2. (*A*) Same as Fig. 1*A*, except propionamide was the permeant molecule. (*B*) Same as Fig. 1*B*, except propionamide was used instead of acetamide and $\sigma = 320/334 = 0.96$

Table 1. Nonelectrolyte reflection coefficients (σ) for dog red cells

Solute	σ [a]	σ [b]	Molecular radius [b]	Permeability coefficient, ω, (10^{-15} moles/dyne sec)
Urea	0.71 ± 0.06 (3)	0.38 ± 0.02 (2)	2.03	25 [b]
Acetamide	0.75 ± 0.03 (3)	0.55 ± 0.05 (2)	2.27	9 ± 2 (2) [c]
Ethylene glycol	1.01 ± 0.02 (4)	0.83 ± 0.02 (2)	2.24	
Propionamide	0.91 ± 0.04 (4)	0.56 ± 0.02 (2)	2.31	7 ± 1 (5) [c]
Glycerol	0.97 ± 0.05 (4)	0.68 ± 0.02 (2)	2.74	0.055 ± 0.008 (3) [d]
Malonamide	1.03 ± 0.03 (3)	0.67 ± 0.02 (2)	2.57	
Glucose	1.05 (1)	0.96	4.2	
NaCl	1.0 (1)			

[a] Data from this paper in terms of mean $\pm$ standard error of the mean and the number of separate experiments is given in parentheses.
[b] Data from Rich *et al.* (1967).
[c] Data from Sha'afi *et al.* (1971).
[d] Data from Wessels and Veerkamp (1973) in terms of mean $\pm$ standard deviation with the number of animals in parentheses.

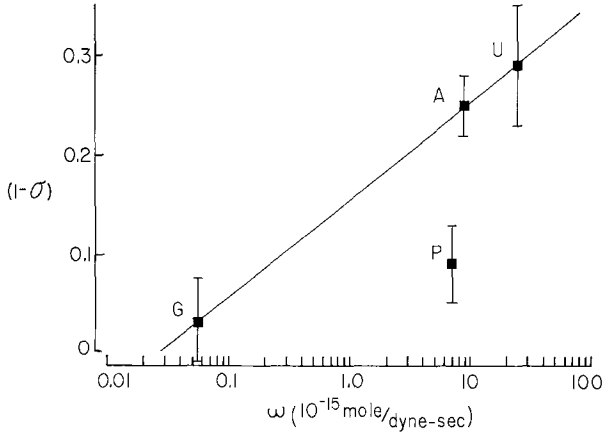

Fig. 3. A plot of the dog σ data in Table 1 of $(1-\sigma)$ as a function of $\log \omega$. (*U*) denotes urea, (*A*) acetamide, (*P*) propionamide, and (*G*) glycerol. The line was drawn by the method of least squares

using only the first term in Eq. (1). This implies propionamide passes through the dog red cell membrane lipids or a separate channel than water, since the second term in Eq. (1) involving the frictional coefficients is not necessary.

Determination of σ in Beef Red Cell Suspensions

Fig. 4*A* shows our red cell light-scattering results in beef red cell suspensions with various concentrations of urea. From Fig. 4*B*, an isosmolar urea concentration of 352 mosm was obtained and substituting this value for C_s in Eq. (2) along with $C_i = 312$ mosm gives $\sigma = 0.89$ for urea in beef red cells. Table 2 shows that our σ data for ethylene glycol and glycerol are also slightly higher than the values reported by Farmer and Macey (1972*a*, *b*). The σ data in Fig. 4 are from a single representative experiment and the σ listed in Table 2 are the mean values from several experiments.

Fig. 5 is a plot of $(1-\sigma)$ for urea, ethylene glycol and glycerol as a function of $\log \omega$. Farmer and Macey's $(1-\sigma)$ values are shown in open circles and our values (Table 2) are shown in solid circles. The line drawn through our data is almost parallel to their data line, but it is shifted downwards due to our higher σ values.

Comparisons Between Dog, Beef, and Human σ

Previously (Owen & Eyring, 1975), we showed that a trend between $(1-\sigma)$ and the $\log \omega$ existed for human red cells. Fig. 6 is a combination

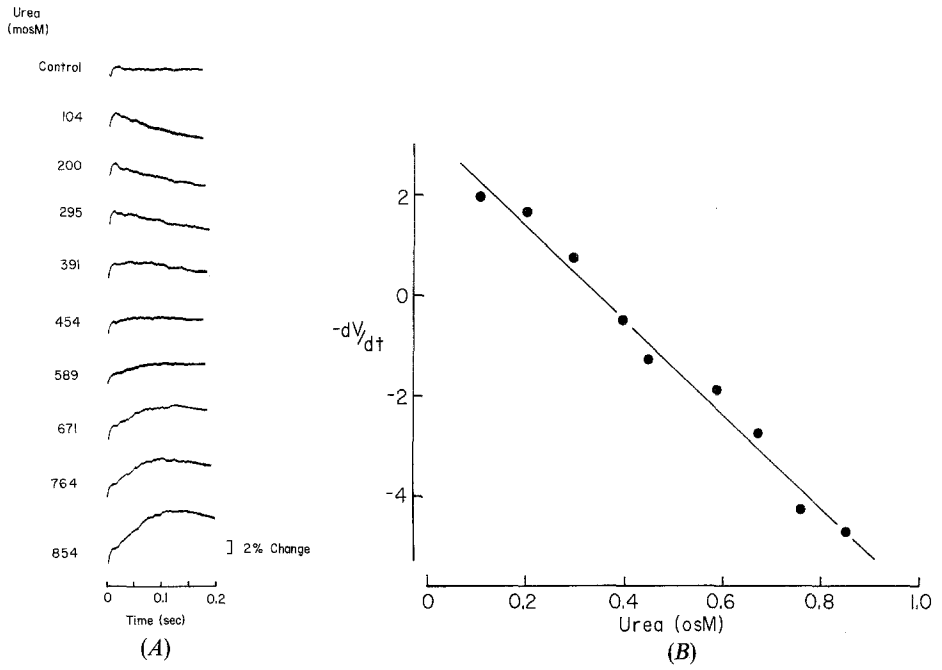

Fig. 4. (*A*) Same as Fig. 1*A*, except urea was the permeant molecule and beef red cells were used instead of dog red cells. (*B*) Same as Fig. 1*B*, except urea and $\sigma = 312/352 = 0.89$ for beef red cells

Table 2. Nonelectrolyte reflection coefficients (σ) for beef red cells

Solute	σ [a]	σ	Permeability coefficient, ω, (10^{-15} moles/dyne sec)
Urea	0.84 ± 0.03 (6)	0.73 [c]	5.5 [c]
Acetamide	0.90 ± 0.03 (4)		
Ethylene glycol	0.97 ± 0.03 (5)	0.82 [b]	0.15 [b]
Propionamide	0.93 ± 0.03 (4)		
Glycerol	0.99 ± 0.04 (5)	0.92 [b]	0.0081 [b]
			0.017 ± 0.0036 (7) [d]
Malonamide	1.05 ± 0.02 (3)		
NaCl	0.96 (1)		

[a] Data from this paper in terms of mean $\pm$ standard error of the mean and the number of experiments is given in parentheses.
[b] Data from Farmer and Macey (1972*a*).
[c] Data from Farmer and Macey (1972*b*).
[d] Data from Wessels and Veerkamp in terms of mean $\pm$ standard deviation with the number of animals given in parentheses.

of all our $(1-\sigma)$ values plotted as a function of $\log \omega$ for dog, beef and human red blood cells. Although the points have large standard errors and are slightly scattered, a definite trend does appear to exist between

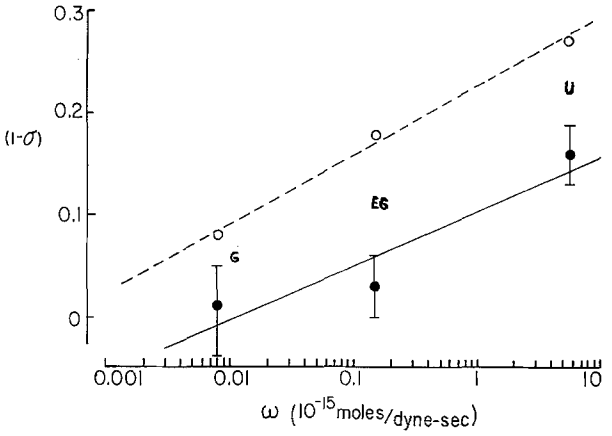

Fig. 5. A plot of $(1-\sigma)$ *vs.* $\log\omega$ for beef red cells. Farmer and Macey's $(1-\sigma)$ values are shown in open circles and our values (Table 2) are shown in solid circles. (*G*) is glycerol, (*EG*) is ethylene glycol, and (*U*) is urea

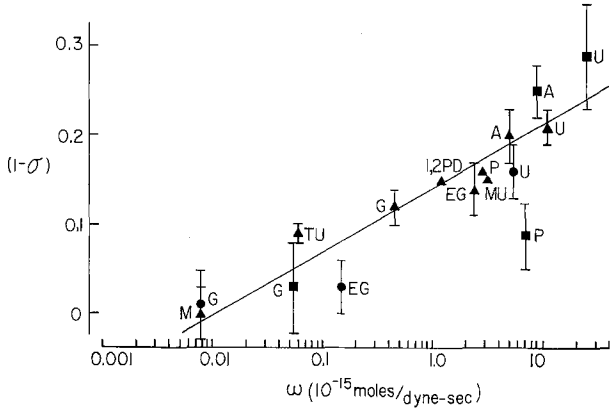

Fig. 6. A plot of $(1-\sigma)$ *vs.* $\log\omega$ for dog cells (■), beef cells (●) and human cells (▲). The dog and beef σ data are from Tables 1 and 2, whereas the human σ data are from previously published work (Owen & Eyring, 1975)

$(1-\sigma)$ and the $\log\omega$ for the three different species. The $(1-\sigma)$ value for propionamide in dog cells and possibly ethylene glycol in beef cells are somewhat aberrant in Fig. 6. Both these molecules are lipophilic, e.g., $k_{\text{ether}}=0.053$ for ethylene glycol (Collander, 1949) and $k_{\text{ether}}=0.013$ for propionamide and $k_{\text{ether}}=0.003$ for water, indicating that these molecules probably penetrate the red cell membrane primarily by a lipid pathway and therefore their $(1-\sigma)$ values would be represented by only the first term in Eq. (1).

Our σ values for dog cells (Table 1) are much higher than those reported by Rich *et al.* (1967). However, our beef σ values (Table 2) are only slightly higher than those reported by Farmer and Macey (1972*a*). These differences in σ values may be attributable to which portion of the light-scattering results is analyzed. For the reasons given previously with σ in human cells (Owen & Eyring, 1975), we chose to ignore the initial ($\sim$20 msec) light-scattering phenomenon which we concluded was not related to osmotic water movement. Briefly, this initial portion of the trace appeared the same in the control as well as with the test solutions, and it, therefore, can be considered an artifact. If Rich *et al.* (1967) relied heavily on the initial light scattering, especially for hypotonic urea solutions, and the dog red cell results looked similar to their human red cell light-scattering data (Owen & Eyring, 1975), then their dog cell σ values would be low. Our $\sigma = 0.97$ for ethylene glycol in beef red cells is considerably greater than the $\sigma = 0.82$ value reported by Farmer and Macey (1972*a*) but it is closer to unity, as predicted by Levitt on the basis of a computer analysis technique (Levitt, 1974). Levitt also suggested that a σ for glycerol should be close to one, which is also in agreement with our 0.99 value in Table 2.

We did our dog and beef σ experiments at a higher sensitivity than our previous human σ experiments. Our controls in Figs. 1*A*, 2*A*, and 4*A*, show a biphasic light-scattering initial phenomenon (BLIP) which was not overly apparent in the human σ work (Owen & Eyring, 1975). It is interesting to note that the BLIP in dog cells (Figs. 1*A* & 2*A*) is the opposite of the BLIP in beef cells (Fig. 4*A*). This may be related to the effects reported by Anderson (1966), that dog red cells transmit more light and beef cells less light when flowing than when stationary. Therefore, upon rapidly stopping the flow of dog cells in the stopped-flow apparatus, they would be expected to transmit less light or scatter more light, since the amount of red cell absorbance and scattered light are directly proportional (Anderson, 1966). The opposite could be predicted with beef cells. When human red cells are mixed with buffer on a similarly expanded scale, the control resembles the dog red cell control (Owen, *unpublished observations*). Human red cell suspensions are also known to behave like dog cells in that they transmit more light while rapidly flowing (Anderson, 1966).

Farmer and Macey (1972*a*, *b*) utilized a perturbation technique to measure σ in beef red cells, so it is difficult to directly compare our two different types of procedures. They found that analyzing swelling data separately from their shrinking data gave slightly higher σ values for nonelectrolytes during swelling, but they concluded that if any solute flow rectifications existed it was very small. Rectification of water flow is thought

to occur in dog, beef and human red cells (Farmer & Macey, 1970), with swelling being as much as 30% greater than shrinking. One implicit assumption in the Goldstein and Solomon zero-time slope method is that red cell swelling and shrinking occur at a similar rate. Otherwise, drawing a single line through the slope from the swelling and shrinking solute solutions would be incorrect (*see* Goldstein & Solomon, 1960, Fig. 3). Analyzing the swelling data apart from the shrinking data is difficult with our stopped-flow results. Since, with a reasonable separation of osmolality values, we can only look at two or three nonelectrolyte concentrations below the C_s concentration, the number of swelling points we can obtain is too small for us to conclude that any rectification of water is occurring. If it occurred one would expect a least-squares line drawn through the swelling and shrinking data to be essentially a composite line drawn through a wedge-shaped plot of points intersecting each other at a $-dV/dt = \text{zero}$, where the steeper half of the wedge would denote swelling and the other half would represent shrinking. Also, C_s would be higher and consequently σ would be calculated to be less from this composite line than if two separate lines were drawn through the swelling and shrinking data points. Our σ values were higher, not lower, than Farmer and Macey's σ, and the data points (Figs. 1*B*, 2*B*, and 4*B*) were randomly scattered around the least-squares line drawn through all points and did not appear to be a wedge-shaped plot of points. Therefore, if rectification of water flow exists in red cells, it did not seem to be so dominant as to preclude measuring σ with the Goldstein and Solomon zero-time method.

Mechanism for Solute Flow Through Red Cell Membranes

The implication in Fig. 6 is that hydrophilic solutes penetrate red cells according to their interaction with membrane water, as manifested by the linear plot of $(1-\sigma)$ as a function of $\log \omega$. Whether one can accurately determine the dimensions of red cell aqueous pathways is doubtful from these σ experiments[2], but an approximation of 3–4 Å for a membrane pore radius would be consistent with the upper limits of permeability of the large hydrophilic nonelectrolytes, such as glycerol and glucose. A similar approximation was utilized by Holz and Finkelstein to determine the pore size of the mystatin-treated thin lipid membranes (Holz & Finkelstein, 1970).

2 It is interesting to note that Levitt (1975) has recently shown by computer analysis that the calculated value for a red cell "equivalent pore radius" is dependent upon which parameter σ, ω and Lp (hydraulic permeability coefficient) is being measured.

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