Red Cell Membrane Permeability Deduced from Bulk Diffusion Coefficients

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ABSTRACT The permeability coefficients of dog red cell membrane to tritiated water and to a series of [^{14}C]amides have been deduced from bulk diffusion measurements through a "tissue" composed of packed red cells. Red cells were packed by centrifugation inside polyethylene tubing. The red cell column was pulsed at one end with radiolabeled solute and diffusion was allowed to proceed for several hours. The distribution of radioactivity along the red cell column was measured by sequential slicing and counting, and the diffusion coefficient was determined by a simple plotting technique, assuming a one-dimensional diffusional model. In order to derive the red cell membrane permeability coefficient from the bulk diffusion coefficient, the red cells were assumed to be packed in a regular manner approximating closely spaced parallelopipeds. The local steady-state diffusional flux was idealized as a one-dimensional intracellular pathway in parallel with a one-dimensional extracellular pathway with solute exchange occurring within the series pathway and between the pathways. The diffusion coefficients in the intracellular and extracellular pathways were estimated from bulk diffusion measurements through concentrated hemoglobin solutions and plasma, respectively; while the volume of the extracellular pathway was determined using radiolabeled sucrose. The membrane permeability coefficients were in satisfactory agreement with the data of Sha'afi, R. I., C. M. Gary-Bobo, and A. K. Solomon (1971. *J. Gen. Physiol.* **58**:238) obtained by a rapid-reaction technique. The method is simple and particularly well suited for rapidly permeating solutes.

INTRODUCTION

It has long been known that diffusion coefficients of various molecules through tissues are less than through water (Krogh, 1919; Kety, 1951). It has also been appreciated that cell membrane, acting as a barrier to free diffusion, is partially responsible for this decrease in the diffusion coefficients (Wright, 1934; Chinard et al., 1969). Therefore, one might hope to deduce cell membrane permeability to a substance from a measurement of the bulk diffusion coefficient of the substance through a "tissue" made up of such cells. In this communication, we report a method for the deduction of the membrane perme-

ability of dog red cells to tracer molecules from bulk diffusion measurements through packed red cells and through hemoglobin and plasma solutions. Mammalian red cells were chosen as the isolated cell type for this study primarily because of the extensive literature concerning the transport of substances across this cell membrane (Whittam, 1964; Naccache and Sha'afi, 1973; Solomon, 1972). The relatively high permeability of the red cell membrane to water and many small molecules has necessitated the use of rapid-reaction techniques for the measurement of these membrane permeability coefficients (Paganelli and Solomon, 1957; Sidel and Solomon, 1957; Sha'afi et al., 1970). These methods require complex equipment and skilled operation in order to obtain satisfactorily reproducible data. In contrast, the present method requires relatively simple equipment and application, and the results are consistently reproducible. The experimental technique is similar to that employed by Gary-Bobo et al. (1971) for the measurement of water diffusion through lipid-water phases, but uses a modification of the gel slicing technique employed by Schantz and Lauffer (1962) in their determination of diffusion coefficients in agar gel.

EXPERIMENTAL METHOD

Fresh blood (approximately 5 ml) was obtained from healthy donor dogs by venipuncture using a syringe containing heparin (0.1 ml; 10,000 USP U/ml) and subjected to a preliminary centrifugation at 450 *g* for 5 min. The plasma and buffy coat were removed with a Pasteur pipet and the lower portion of the cell fraction (ca. 80% hematocrit) was used immediately for the diffusion experiment. 0.5 ml of this concentrated cell suspension was injected with 40 μCi radiolabeled sucrose in isotonic buffer and mixed on a vortex mixer for 2 min. The cell suspension with radiolabeled sucrose was drawn into 0.86-mm internal diameter polyethylene tubing (Intramedic PE 90, Clay Adams, Inc., Div. of Becton, Dickinson & Co., Parsippany, N. J.) by means of a syringe. The tubing was then cut into several appropriate lengths and each length was plugged at one end with clay. Each length was placed with the clay end first inside a glass 100-μl micropipet (Clay Adams, cat. no. 4625) which had been heat sealed at one end and broken approximately 7.5 cm from the sealed end. This process was repeated with the cell suspension not containing the radiolabeled sucrose. All the tubes were then placed into a microhematocrit centrifuge (I.E.C. model MB) and centrifuged at 22,500 *g* for 10 min. After centrifugation, each tube was removed from the glass support and sliced with a razor blade just above the plasma-red blood cell interface and also approximately 0.5 cm below the plasma-red cell interface as indicated in Fig. 1. The portion of the tube containing the plasma supernatant (section A, Fig. 1) was placed on the platform of a Mickle gel slicer (Brinkmann Instruments, Inc., Westbury, N. Y.), frozen with dry ice (solid CO_2), and cut into five uniform slices; the middle portion (section B, Fig. 1) was discarded, while the remaining portion (section C, Fig. 1) containing the packed cell column was used for the diffusion experiment.

Each diffusion tube was pulsed at its open end with radiolabeled solute (approximately 1 μCi of radiolabeled solute added with a 1-μl syringe). After 2 min of contact,

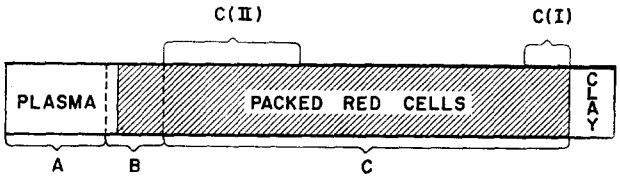


FIGURE 1. Schematic representation of the diffusion tube. Protocol for slicing the polyethylene tube containing packed red cells after centrifugation: section A, plasma supernatant, retained for extracellular volume determination; section B, plasma supernatant-red cell interface region, discarded; section C, packed red cell column, used in the diffusion experiment. Section C(I), used for extracellular volume determination, and C(II), employed for measurement of the tracer diffusion coefficient.

the tracer droplet was removed with blotting paper, and the open end of the tube sealed with Parafilm. Each tube was taped to a separate plastic tray which was then placed in an incubator. The trays were manufactured to fit over the platform of the slicing machine, and were used in order to minimize handling of the diffusion tubes. Diffusion was allowed to proceed for several hours (usually 3–4 h), afterwards each tray was removed from the incubator and mounted upon the platform of the gel slicer. The diffusion tube was frozen with a stream of evaporating liquid Freon, and the steel blade of the slicing machine was also cooled. Extracellular volumes were routinely determined in each of the tracer diffusion runs by slicing the diffusion tube at a distance from the pulsed end beyond which the tracer had not diffused. In order to reduce the possibility of radioactive contamination the following slicing procedure was adopted. A new blade was used for each diffusion tube. The polyethylene tube was positioned under the blade at section C(I), Fig. 1 approximately 1 cm from the clay-sealed end, and five slices of 0.2–1.0-mm thickness taken for counting. The slicing platform was repositioned and the diffusion tube sectioned from the pulsed end into 15 slices of 0.2–1.0-mm thickness for a distance of 0.3–1.5 cm (section C (II), Fig. 1). The tracer molecules and radiolabeled sucrose were labeled with different radioisotopes. The double labeled slices were counted on two channels of a Nuclear-Chicago liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill., model 6848) and corrected for crossover and background using the data from the diffusion tubes without radiolabeled sucrose.

In a typical experiment with [^{3}H]sucrose (Amersham/Searle Corp., Arlington Heights, Ill.) as the extracellular marker and [^{14}C]formamide as the tracer, the 15 slices in section C(II) were counted for ^{14}C activity (cpm). The cpm were plotted semilogarithmically against x^2 (x = distance from the pulsing point), and the slope of the plot measured in order to determine the diffusion coefficient (see Theoretical Analysis). The extracellular volume was calculated as follows: The five slices from section C(I) of the diffusion tube containing the [^{3}H]sucrose were counted, background subtracted, averaged, and denoted, $[^3H]_{pellet}$. The supernatant portion (section A, Fig. 1) was sliced into five equal lengths and similarly counted, background subtracted, averaged, and denoted, $[^3H]_{plasma}$. The ratio of the extracellular volume V_1 to the total volume V was given by the expression

$$V_1/V = [^3H]_{pellet}/[^3H]_{plasma}.$$

Before adopting this standard procedure to determine the extracellular volume in a diffusion experiment, it was confirmed that there was little change of the extracellular volume with the distance along the packed red cell column after centrifugation. This was accomplished by slicing several tubes containing [^{3}H]sucrose along their entire length and measuring the radioactivity in each slice as a function of the distance from the plasma-red cell interface with the observation that the radioactivity was essentially uniform. The approximate constancy of extracellular volume per 1-mm slice as thus determined is a statistical constancy, averaged over the volume of the slice. The extracellular volume around each of the approximately 10^7 cells in each slice might well vary. The extent of this variation appears small, however, from electron microscopic observation of the packed cells (Fig. 7).

No special precautions, other than an isotonic environment at all stages, were taken to avoid hemolysis of the red cells. Electron microscopic observations (Fig. 7) did not reveal appreciable hemolysis.

Diffusion measurements through concentrated hemoglobin solutions were carried out using the bulk diffusion method described above for the packed red cells. Fresh blood from healthy donor dogs was heparinized and the plasma and buffy coat removed after low speed centrifugation. The red blood cells were lysed in 15 vol of distilled water and the ghosts removed by high speed centrifugation. The hemoglobin (Hb) in the supernatant was lyophilized and stored at −20°C. The protein solution used in the diffusion experiments was prepared by the addition of water to the lyophilized hemoglobin, usually to obtain a concentration of 33 g Hb/100 ml. This reconstituted intracellular hemoglobin solution was drawn into the polyethylene tubing, which was then cut into the appropriate lengths for tracer diffusion. Each diffusion tube was sealed at one end with clay, pulsed at the open end with tracer, sealed with Parafilm, taped to a plastic tray, then stored in an incubator for an appropriate diffusion period. The protocol for slicing and determination of the diffusion coefficient was similar to that described for the packed red cells. A similar method was adopted in order to measure the tracer diffusion coefficients in plasma. A 2% gel made from commercial gelatine was used as a support matrix inside the diffusion tube. The solutions were prepared by dissolving the gelatine in fresh dog plasma; reference gels consisted of 2% gelatine dissolved in isotonic buffer. It was also found possible to employ this capillary method to measure the free diffusion coefficients of radiolabeled substances in water or plasma solutions, without the gel being present. A smaller internal diameter tubing (ID = 0.25 mm, BD PX 010) was used for these experiments in order to reduce the influence of convection on the measured diffusion coefficients. Before pulsing, the capillary tubes were allowed to reach thermal equilibrium inside the incubator. All diffusion experiments were carried out at 20 ± 0.2°C.

THEORETICAL ANALYSIS

The experimental conditions are assumed to correspond to one-dimensional semi-infinite diffusion through a homogeneous medium with the initial condition of an impulse deposition of tracer of amount per cross-sectional area m_o(cpm/cm^2) at $x = 0$ and $t = 0$. The theoretical solution for such diffusion is given by Crank (1957).

$$c(x,t) = m_o(\pi Dt)^{-\frac{1}{2}} \exp(-x^2/4Dt), \quad (1)$$

where $c(x, t)$, cpm/cm^3, is the concentration of tracer at distance x(cm) and time t(s), and D(cm^{2}s^{-1}) is the diffusion coefficient through the medium. The logarithm of Eq. 1 is

$$\ln c = \ln m_o(\pi Dt)^{-\frac{1}{2}} - x^2/4Dt. \quad (2)$$

A semilogarithmic plot of c against x^2 is therefore a straight line of slope $1/4Dt$ which yields D, since t is known. In the earlier experiments, the activity in successive slices, minus the background activity in similar slices several centimeters distant from the pulsed end, was plotted semilogarithmically against $(n - \frac{1}{2})^2$ where n was the ordinal number of the slice. A straight line was drawn by eye through the data points and the half-distance squared $\chi^2_{1/2}$ was determined by inspection. Substituting $x = 0.1\delta(n - \frac{1}{2})$ and $t = 3{,}600t_d$ into Eq. 2, where δ is the slice thickness in milimeters and t_d is the diffusion time in hours, gives

$$\frac{0.69315}{\chi^2_{1/2}} = \frac{(0.1\delta)^2}{4 \times 3{,}600Dt_d}, \quad (3)$$

from which

$$D = 0.10019(\delta^2\chi^2_{1/2}/t_d) \times 10^{-5} \text{ cm}^2\text{s}^{-1}. \quad (4)$$

In the later experiments, a computer program[1] was written to obtain the slope of the line of regression of ln c against $(n - \frac{1}{2})^2$ from the data points. In all the results given, the data were sufficiently well correlated so that the manual method was equivalent to the computer method. By these methods, the diffusion coefficients D through packed cells, D_2 through hemoglobin solutions, and D_1 through plasma solutions were determined.

In order to derive the red cell membrane permeability coefficient P_o(cm s^{-1}) from the measured diffusion coefficients, a local steady-state model was assumed. This assumption is justified because the local or intracellular diffusion distance is of order $x = 10 \times 10^{-4}$ cm with a local diffusional equilibration time of order $x^2/D \sim 10^{-6}/10^{-6} = 1$ s. The global, or bulk diffusion, distance is of order 1 cm with a global diffusion equilibration time of order $1/10^{-5} \gtrsim 10^5$ s $= 28$ h. Thus, the local diffusion flux pattern may be considered to be steady state, with the diffusion flux and driving force (concentration gradient) changing slowly with time. A more precise model calculation of this argument is given by Colton et al. (1970). The red cells are assumed packed in a regular manner approximating closely spaced parallelopipeds (Fig. 2). The local

[1] Details of the computer programs, for use on the Wang Model 600 calculator, are available on request from the authors.

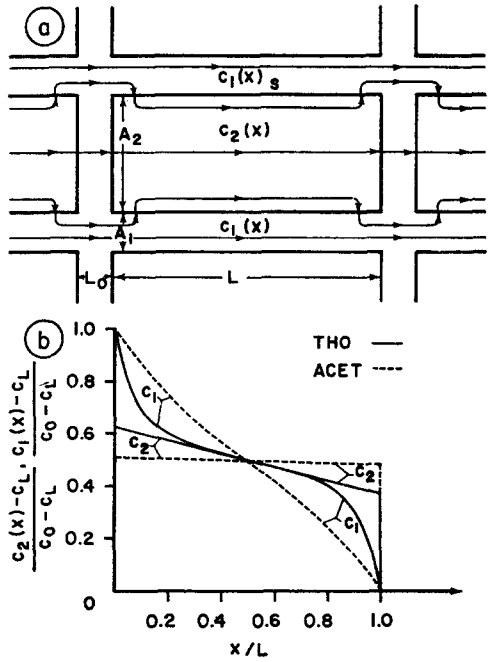


FIGURE 2. Local steady-state one-dimensional model for packed cells. (a): schematic cell geometry and diffusional flux lines. (b): variation of fractional extracellular concentration (subscript 1) and fractional intracellular concentration (subscript 2) with distance, for THO (solid line) and acetamide (dashed line) as calculated from Eqs. 33 *a*, 34 *a* using data in Table IV.

steady-state flux pattern is idealized as a one-dimensional intracellular pathway in parallel with a one-dimensional extracellular pathway. Permeability exchange occurs within the series pathway and between the two pathways. Analysis of this model (Appendix) gives the relation

$$\frac{DA}{D_2A_2} = \frac{1+r}{1+z}, \tag{5}$$

where

$$z = 2[\Pi_o(1+r) + r\alpha \coth \alpha/2]^{-1}, \tag{6}$$

$$\alpha = [ps\Pi_o(1+r^{-1})]^{1/2}, \tag{7}$$

$$p = P/P_o, \tag{8}$$

$$s = S/A_2, \tag{9}$$

$$\Pi_o = P_oL/D_2, \tag{10}$$

$$r = D_1A_1/D_2A_2. \tag{11}$$

D = "packed cell" diffusion coefficient, $cm^2\ s^{-1}$,
D_1 = extracellular diffusion coefficient, $cm^2\ s^{-1}$,
D_2 = intracellular diffusion coefficient, $cm^2\ s^{-1}$,
A_1 = extracellular cross-sectional area, cm^2,
A_2 = intracellular cross-sectional area, cm^2,
L = end-to-end distance of red cell in diffusion direction, cm,
P_o = end face permeability of red cell membrane, $cm\ s^{-1}$,
P = side face permeability of red cell membrane, $cm\ s^{-1}$,
S = side face exchange area of red cell surface, cm^2.

Eqs. 5–11 relate the nondimensional parameter DA/D_2A_2 to the nondimensional parameters Π_o, p, s, and r. The assumptions $p = 1$ and $s = 8$ were made (see Discussion). Physiologic measurement of extracellular space enabled assumption of A_1/A_2. Measurement of the three diffusion coefficients D, D_1, and D_2 then enables solution of Eqs. 5–11 for Π_o. This solution was carried out with the aid of a computer program.[1] For a given value of L (see Discussion), the absolute value of the membrane permeability could finally be determined. Eq. 5 with $p = 1$ is plotted as DA/D_2A_2 against Π_o for various r and for $s = 0$ and 8 in Fig. 3.

The abscissa in Fig. 3 almost equals D/D_2 since $A/A_2 \sim 1.01$ in the present experiments. Fig. 3 may therefore be regarded as a plot of P_oL in units of D_2 against packed cell diffusion coefficient D in units of D_2. In general P_oL increases with D. The parameter $r = D_1A_1/D_2A_2$ is a measure of the diffusion flux through the extracellular pathway relative to the flux through the extracellular-intracellular series pathway. As r increases, for given permeability P_o, the bulk diffusion coefficient D increases. For a given D, the permeability P_o

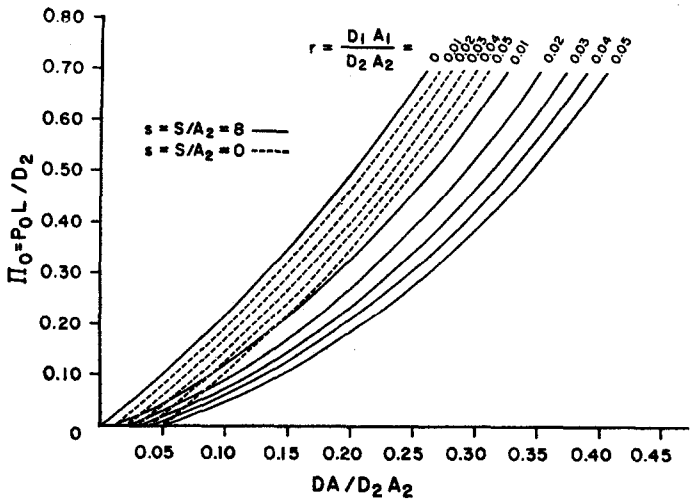


FIGURE 3. Calculated variation of Π_o with DA/D_2A_2 for various values of r and $s = 8$ (solid lines) and 0 (dashed lines). For explanation of symbols and discussion, see text.

decreases as r increases. This variation is sensitive and motivated the present close packing arrangement and the measurement of extracellular volume in each experiment. The parameter $s = S/A_2$ is a measure of the diffusion flux which enters the cell through the side faces of area S compared to the flux that enters through the end face, of area A_2. No sidewise entry corresponds to $s = 0$. The effect of sidewise entry $s = 8$ is, for given permeability P_o, to increase the bulk diffusion coefficient D. Effectively a larger cross-sectional area is available for solute diffusion. For a given D the $s = 8$ assumption yields a substantially lower P_o (see Discussion).

RESULTS AND DISCUSSION

Diffusion in Packed Red Cells

Fig. 4 shows typical experimental data for the diffusion of tritiated water and ^{14}C-labeled low molecular weight amides through packed dog red cells. Similar data have been obtained for the diffusion of these tracers through concentrated solutions of hemoglobin and also through dog plasma and isotonic buffer with and without a gel medium. In all the results presented, the linear correlation coefficients of the semilogarithmic plots of c against x^2 were very close to unity. These plots are denoted the linear plots. Occasionally, initially

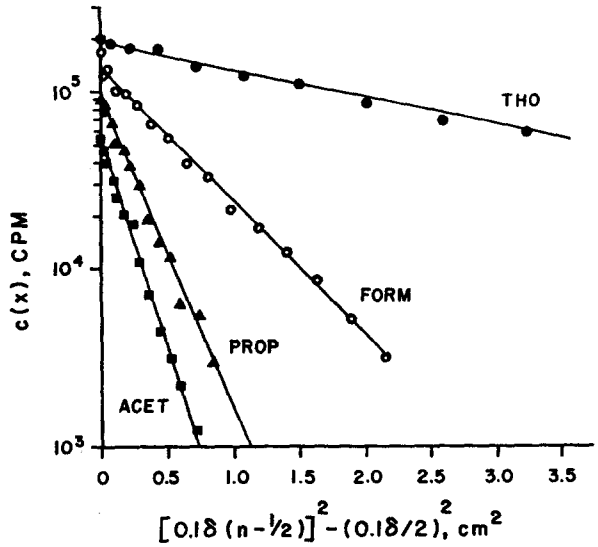


FIGURE 4. Typical semilogarithmic plots of the radioactivity (cpm) as a function of $[0.1\ \delta\ (n - \frac{1}{2})]^2 - [0.1\ \delta/2]^2$, where δ is the slice thickness in millimeters, and n is the ordinal number of the slice. ● Tritiated water, t_d = 6.00 h, $D = 0.294 \times 10^{-5}$ cm^2 s^{-1}, $V_1/V = 0.010$, ○ [^{14}C]formamide, t_d = 6.17 h, $D = 0.0578 \times 10^{-5}$ cm^2 s^{-1}, $V_1/V = 0.0140$, ▲ [^{14}C]propionamide t_d = 6.25 h, $D = 0.0245 \times 10^{-5}$ cm^2 s^{-1}, $V_1/V = 0.0119$, ■ [^{14}C]acetamide, t_d = 6.00 h, $D = 0.0230 \times 10^{-5}$ cm^2 s^{-1}, $V_1/V = 0.0091$.

curvilinear plots were obtained with the slope decreasing with increasing x. The initial curvature could be eliminated, however, by replotting the data with the first sample arbitrarily taken as (say) the third sample ($n = 3$, rather than $n = 1$, in the expression $x = 0.1\delta(n - \frac{1}{2})$). The diffusion coefficients calculated from these linearized plots were found to be in agreement with the values obtained from the linear plots. The initial curvature was, therefore, ascribed to the squeezing out of a portion of the pulsed end of the red cell column upon freezing. These experiments were not further considered. The diffusion coefficients (mean ± SE) computed from the linear plots are given for each tracer in the packed red cell system in Table I. The small standard errors associated with these diffusion coefficients reflect the reproducibility of the data. Each of the packed cell diffusion coefficients (Table I) was determined from a red cell preparation in which the extracellular volume was close to 1% of the

TABLE I

DIFFUSION COEFFICIENTS IN PACKED RED CELLS

Solute	Molar volume	D
	$cm^3\ mol^{-1}$	$10^{-5}\ cm^2\ s^{-1}$
Tritiated water	20.0	0.282 ± 0.006* (29)‡
[^{14}C]Formamide	41.5	0.0599 ± 0.0012 (16)
[^{14}C]Acetamide	61.2	0.0209 ± 0.0014 (18)
[^{14}C]Propionamide	72.1	0.0219 ± 0.0022 (8)

* SEM.

‡ Number of experiments.

total volume. Preliminary trials revealed that the diffusion of radiolabeled solutes through the packed cell preparation varied with the degree of packing, which was determined largely by the time of centrifugation. At short centrifugation times, less than 1 min, the extracellular volume was greater than 3%, and the diffusion coefficients varied widely between the values for the more tightly packed preparations and the diffusion coefficients for free diffusion in plasma. However, between 1- and 5-min centrifugation, there was a linear decrease in the value of the extracellular volume from approximately 3% to 1%, and a concomitant decrease in the packed cell diffusion coefficients occurred. Above 5-min centrifugation, the extracellular volume did not change appreciably, and reproducible values of the bulk diffusion coefficients were obtained (Table I). Chien et al. (1965) have employed [^{131}I]serum albumin in order to measure the amount of plasma trapped in packed red cells in microhematocrit determinations. For dog red cells, after 5-min centrifugation at 15,000 g, the volume occupied by the red cells was 0.97 ± 0.02 of the total packed volume, which is compatible with the present data.

Borun et al. (1957) have found that the density of red cells increases with

age, and that cell populations with different mean ages can be isolated on the basis of this density gradient. Upon centrifugation, the older cells tend to accumulate towards the bottom of the packed cell column. The preliminary centrifugation of whole blood employed in the present studies in effect fractionated the red cells according to age. The older cells, taken from the bottom of the centrifuge tube were used for the diffusion studies. A further fractionation of the red cells was achieved by the removal of section B (Fig. 1) of packed red cell column after the second centrifugation in the polyethylene tubing, and before pulsing the remaining red cell column, section C (Fig. 2), with the radiolabeled solute. The diffusion studies reported in this paper were carried out, therefore, on the older red cells from the whole blood preparations. As red cells age in vivo, their ionic composition changes (Joyce, 1958 and Keitel et al., 1955). Generally, enzyme activities are higher in younger cells and decrease with cell age (Oski, 1970). In addition Westerman and coworkers (1963) have reported in vivo aging to be associated with a parallel loss of total lipid, cholesterol, and phospholipid from human red cells. It is possible that changes in the membrane permeability also occur with cell age, and permeability measurements on unfractionated red cells would, therefore, yield an average permeability coefficient for the red cell population. This is usually the case for the "free floating cell" methods employed by Solomon and associates (Paganelli and Solomon, 1957; Sidel and Solomon, 1957; Sha'afi et al., 1970). The reproducibility of the experimental data in the present method (Table I) may be partly attributable to the uniformity of the mean age of the red cell preparations under investigation.

Diffusion in Concentrated Hemoglobin Solutions

The diffusivity ratio of tritiated water in a 33% Hb solution to tritated water in water was found to be 0.50 ± 0.03; the corresponding diffusivity ratios for the [^{14}C]amides were in the range 0.42–0.49 (Table II). The study of solute diffusion in concentrated solutions of hemoglobin has dealt mainly with the problem of oxygen transport, e.g., Klug et al. (1956), Goldstick and Fatt

TABLE II

DIFFUSION COEFFICIENTS IN CONCENTRATED HEMOGLOBIN SOLUTIONS

Solute	33 g Hb/100 ml (D_3)	45 g Hb/100 ml (D_{45})
	10^{-5} cm^2 s^{-1}	
Tritiated water	1.06 ± 0.05* (6)‡	0.749 ± 0.008 (2)
[^{14}CFormamide	0.694 ± 0.042 (6)	0.440 ± 0.020 (2)
[^{14}C]Acetamide	0.536 ± 0.028 (6)	0.355 ± 0.009 (2)
[^{14}C]Propionamide	0.409 ± 0.044 (4)	0.236 ± 0.027 (2)

* SEM.

‡ Number of experiments.

(1970), and Keller and Friedlander (1966). In each of these studies, slightly lower diffusivity ratios have been obtained than the ratios found in the present study of the diffusion of tritiated water and [^{14}C]amides through concentrated hemoglobin solutions. This does not necessarily imply a conflict with the literature, since it is unlikely that either the diffusion of tritiated water or the low molecular weight amides through concentrated hemoglobin solutions is directly comparable to oxygen diffusion in this system. Goldstick and Fatt (1970) have found that the diffusivity of oxygen in solutions of bovine serum albumin is higher than in hemoglobin solutions. At 33% protein concentration, the corresponding diffusivity ratios were approximately 0.46 and 0.37, respectively. The former value, which probably represents a true diffusivity ratio without the complication of simultaneous chemical reaction occurring during diffusion, lies within the range reported above for the amides. A similar comparison can be made for the diffusivities in 45% protein solutions. The present study yielded a diffusivity ratio of 0.35 for tritiated water in a 45% Hb solution, while the corresponding values for the [^{14}C]amides were in the range 0.24–0.32. Goldstick and Fatt (1970) have reported diffusivity ratios for oxygen in 45% albumin and 45% Hb solutions of 0.32 and 0.23, respectively.

Diffusion in Plasma Solutions

The free diffusion of tritiated water and the [^{14}C]amides in water has been studied, respectively, by Wang et al. (1953) and Gary-Bobo and Weber (1969), using the capillary cell method designed by Wang (1951). The diffusion coefficients in water for these substances, obtained by the present bulk diffusion method, compare favorably with the above literature values (Table III, column 5). There was no significant reduction in the diffusion coefficients of these substances in a 2% gelatin gel in water (Table III, column 4). Similarly, no significant restriction of diffusion of small molecules in a 1.5% agar gel has been reported by Schantz and Lauffer (1962). In contrast, a decrease in the

TABLE III

DIFFUSION COEFFICIENTS IN PLASMA AND IN WATER WITH AND WITHOUT A 2% GEL MATRIX

Solute	Plasma (D_1)	Plasma-gel	Water-gel	Water
	10^{-5} cm^2 s^{-1}	10^{-5} cm^2 s^{-1}	10^{-5} cm^2 s^{-1}	10^{-5} cm^2 s^{-1}
Tritiated water	1.63 ± 0.09* (7)‡	1.99 ± 0.015 (3)	2.12 (1)	2.12§
[^{14}C]Formamide	0.996 ± 0.08 (7)	1.25 ± 0.064 (3)	1.58 (1)	1.48‖
[^{14}C]Acetamide	0.936 ± 0.027 (5)	0.842 ± 0.021 (2)	1.03 (1)	1.10‖
[^{14}C]Propionamide	0.787 ± 0.019 (6)	0.847 ± 0.044 (3)	1.00 (1)	0.98‖

* SEM.

‡ Number of experiments.

§ Wang et al. (1953).

‖ Gary-Bobo and Weber (1969).

diffusion coefficients was observed in both the plasma and plasma-in-gel systems (Table III, columns 2 and 3). The diffusivity ratio for tritiated water in plasma compared with water, calculated from the data in Table II was 0.77 ± 0.05; the mean diffusivity ratio for three [^{14}C]amides was 0.78 ± 0.04. A diffusivity ratio of 0.81 for urea and similar values for several organic solutes in plasma with respect to isotonic saline at 37°C have recently been reported by Colton et al. (1970). The protein concentration in dog plasma was determined to be approximately 4.5 g/100 ml with 61% of the protein as albumin (P. Chowdhury, personal communication). Connor and Gainer (1970) have measured the diffusion of dextrose as a function of albumin concentration in fabricated plasma at 25°C. The diffusivity ratio predicted from their data for the above albumin concentration would be approximately 0.76, in close agreement with the observed values.

Freezing Expansion Correction for Measured Diffusion Coefficients

The experimental value of $\delta(n - \frac{1}{2})$ which is used in the determination of the diffusion coefficient is subject to a slight correction due to the expansion of the diffusion column upon freezing, before slicing and counting for radioactivity. The degree of expansion of the diffusion system was measured directly in the polyethylene tubing for each of the systems studied. The expansion factors measured were: 6.25 ± 0.16% for the packed red cell system; 7.74 ± 0.20% for the plasma solution; 5.78 ± 0.13% for the 33% Hb solution, and 3.51 ± 0.10% for the 45% Hb solution, respectively. The values of the diffusion coefficients listed in Tables I, II, and III have been corrected for the expansion phenomenon according to the following scheme:

$$D \text{ (corrected)} = (0.9375)^2 D \text{ (uncorrected—packed red cells)}$$
$$D \text{ (corrected)} = (0.9226)^2 D \text{ (uncorrected—plasma solution)}$$
$$D \text{ (corrected)} = (0.9422)^2 D \text{ (uncorrected—33 \% Hb solution)}$$
$$D \text{ (corrected)} = (0.9649)^2 D \text{ (uncorrected—45 \% Hb solution)}$$

The corrected mean values of the diffusion coefficients in plasma, 33% Hb solution and packed red cell preparations are plotted in Fig. 5 against the molar volumes of the diffusing substances. The molar volumes were obtained from Sha'afi et al. (1971) and are listed in Table I. A similar correlation between diffusion coefficients of small hydrophilic solutes and molar volume has been presented by Solomon (1972), based on the diffusion data in a water-swollen dextran gel obtained by Horowitz and Fenichel (1964).

Deduction of Red Cell Permeability

The permeability of the red cell membrane to tritiated water and the [^{14}C]amides may be deduced from the bulk diffusion data following the argument developed in the Theoretical Analysis and Appendix. An example of the deter-

mination of the nondimensional parameter Π_o, for each of the diffusing substances is illustrated in Table IV. Each set of experimental data consists of the bulk diffusion coefficient D and the corresponding relative extracellular volume V_1/V in the packed red cell column. The diffusion coefficients D_1 and D_2 are taken from Tables II and III, respectively. D_1 was assumed to be the mean value for the diffusion coefficient in plasma without the gel matrix (Table II); D_2 was taken as the diffusion coefficient in the 33% Hb solution (Table III). In order to relate the experimentally determined relative extracellular volume V_1/V to the relative cross-sectional area A_1/A_2 (Fig. 2), the following relationship was used

$$\frac{A}{A_2} = 1 + \frac{A_1}{A_2} = 1 + \frac{V_1}{V}\left(1 - \frac{V_1}{V}\right)^{-1}\left(\frac{L_1}{L_2}\right)^{-1},$$

where L_1/L_2 is a tortuosity factor given by the ratio of extracellular diffusion

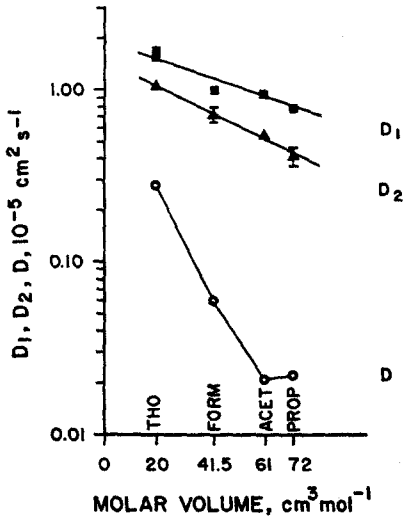


FIGURE 5. Variation of diffusion coefficients (mean ± SE) with the molar volume of the diffusing substances. Diffusion medium: ○ packed red cells; ■ plasma; ▲ 33 g Hb/100 ml.

TABLE IV

CALCULATIONS OF DOG RED CELL PERMEABILITY FROM AVERAGED DIFFUSION DATA

Solute	D	D_2	D_1	V_1/V	DA/D_2A_2	$\frac{\tau D_1A_1}{D_2A_2}$	$\frac{\Pi_o}{P_oL/D_2}$
		10^{-5} cm^2 s^{-1}					
Tritiated water	0.282	1.06	1.63	0.0111	0.269	0.0173	0.457
[^{14}C]Formamide	0.0599	0.694	0.996	0.0166	0.0877	0.0242	0.0678
[^{14}C]Acetamide	0.0209	0.536	0.936	0.0109	0.0394	0.0192	0.0189
[^{14}C]Propionamide	0.0219	0.409	0.787	0.0113	0.0542	0.0220	0.0312

length to intracellular diffusion length. The parameters DA/D_2A_2 and r were calculated from the values in columns 2–5, Table IV, and used to compute the corresponding Π_o for $p = 1$ and $s = 8$ (Theoretical Analysis). Each set of diffusion and extracellular volume measurements yielded a single value of Π_o. The value of D_2 for each solute was used to determine the product P_oL in Table V. The expression $P_oL/8.5 \times 10^{-4}$ listed in Table V was calculated in order to compare the present data with the permeability coefficient P_{sgs} obtained by Sha'afi et al. (1971). Both permeability parameters are expressed in units of cm s^{-1}. The diffusion length of 8.5 μm was chosen in order to obtain a best fit for tritiated water and the solutes formamide and propionamide. Both parameters are plotted as a function of the molar volume of the diffusing solute in Fig. 6. The agreement between the present data and the literature values is

TABLE V

COMPARISON OF DOG RED CELL PERMEABILITY WITH LITERATURE DATA

Solute	P_oL	$P_oL/8.5 \times 10^{-4}$	P_{sgs}*
	10^{-5} cm^2 s^{-1}	10^{-5} cm s^{-1}	10^{-5} cm s^{-1}
Tritiated water	0.484 ± 0.0234‡ (25)§	569 ± 27.5	560
[^{14}C]Formamide	0.0472 ± 0.0028 (16)	55.5 ± 3.3	55.5 ± 12.1‡ (4)§
[^{14}C]Acetamide	0.0101 ± 0.0014 (18)	11.9 ± 1.6	21.7 ± 4.8 (2)
[^{14}C]Propionamide	0.0128 ± 0.0034 (8)	15.1 ± 4.0	16.9 ± 2.4 (5)

* Values of Sha'afi et al. (1971).

‡ SEM.

§ Number of experiments.

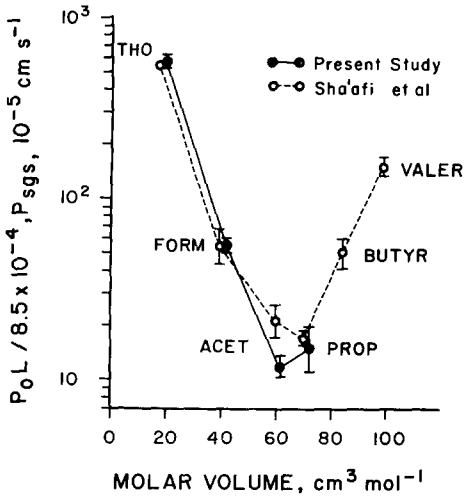


FIGURE 6. Variation of red cell permeability with the molar volume of the test substance. ● $P_oL/8.5 \times 10^{-4}$ permeability parameter deduced from the packed cell diffusion coefficient and the diffusion coefficients through plasma and concentrated hemoglobin solutions. ○ permeability coefficients P_{sgs} of Sha'afi et al. (1971).

most satisfactory, and the slight discrepancy in the value for acetamide may be attributable to the limited data ($n = 2$) obtained by Sha'afi et al. (1971) for this substance (Table V).

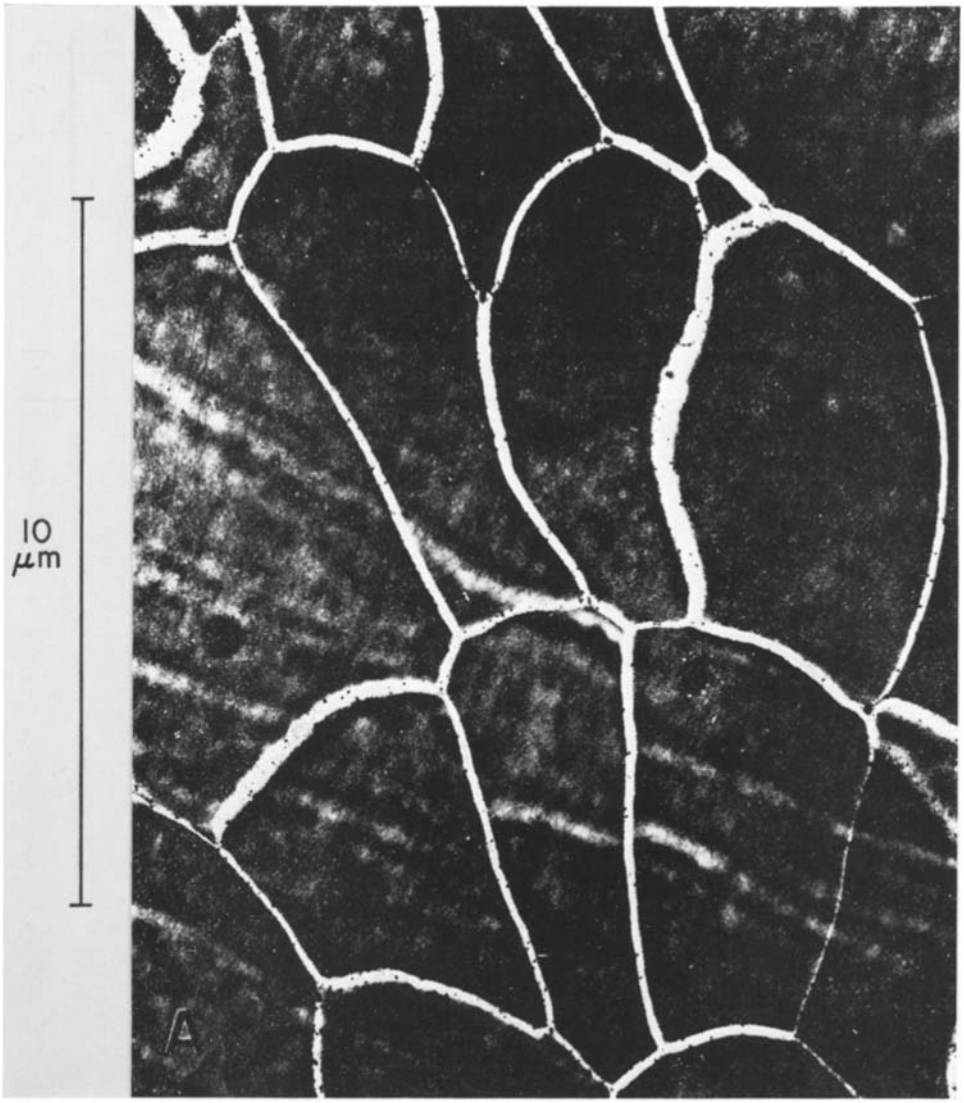
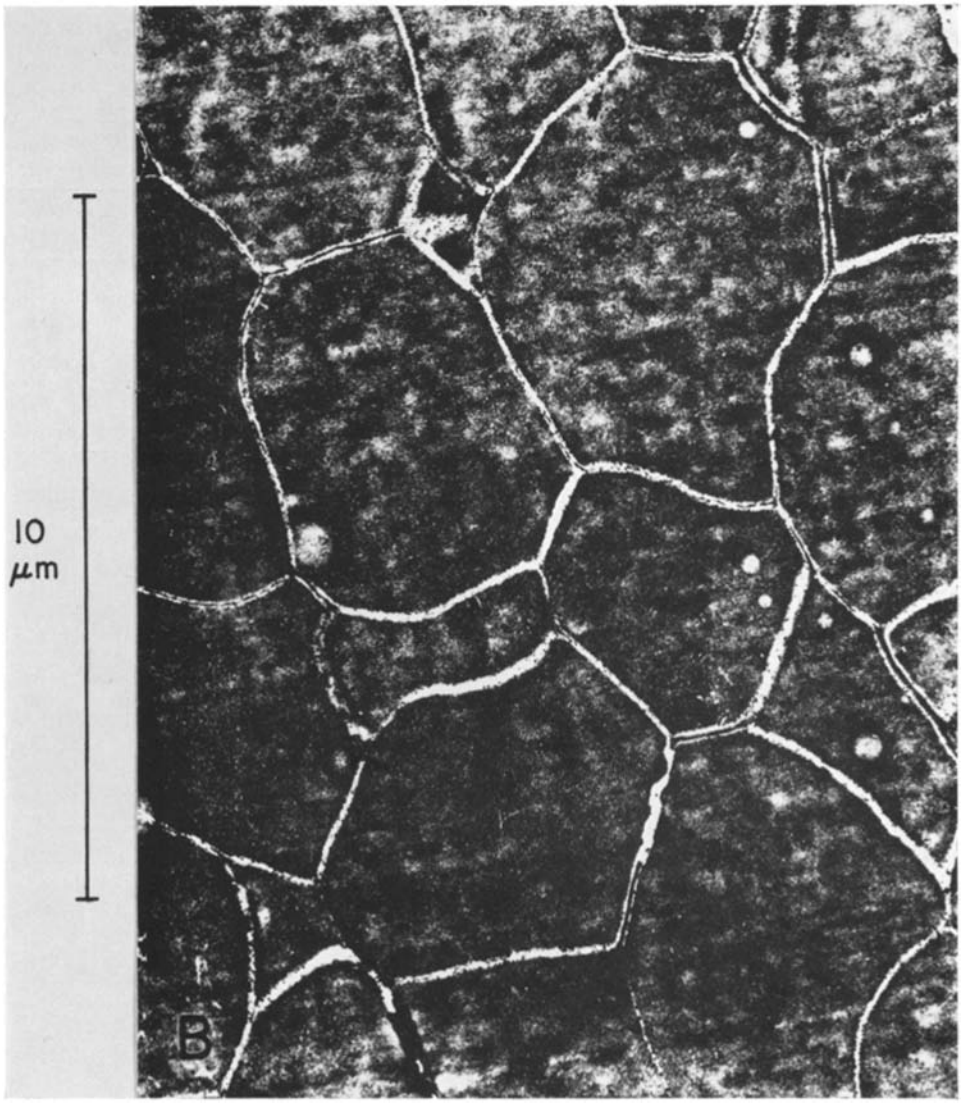


FIGURE 7. (A, B) Electron micrographs of packed red cells (× 14,500). 1-mm slices of the diffusion tube containing packed red cells were fixed in 5% glutaraldehyde, postfixed in osmium tetroxide, and embedded in Epon. Sections were stained with uranyl acetate and lead citrate and examined in a Philips EM 300 (Philips Electronic Instruments, Mount Vernon, N. Y.) operated at 60 kV (courtesy of Dr. P. Farnsworth).

Assumptions

DIFFUSION LENGTH, L Justification for using 8.5 μm for the diffusion length L is provided by the evidence that upon centrifugation of the red cells in the polyethylene tubing, a preferred orientation occurs in the direction of the centrifugal field. Fig. 7 illustrates two typical sections, as observed by electron microscopy, through a packed red cell column. The plane of the longitudinal section shown in Fig. 7 A was parallel to the long axis of the packed cell column, while the plane of the cross section (Fig. 7 B) was perpendicular to this



direction. The longitudinal sections usually showed an orientation of the red cells which appeared as elongated parallelopipeds, whereas, in the cross sections, the red cells were more cuboidal in shape and displayed no preferential orientation. These results suggested that the packed red cells were aligned parallel to the long axis of the red cell column, with the longer cell dimension in the direction of the diffusion.[2] In view of this indicated geometry of the packed red cells, the value $L = 8.5$ μm used in the analysis, which is slightly larger than the mean diameter of 7 μm of the dog red cell, reported by Altman and Dittmer (1971), is considered reasonable.

CHOICE OF $s = S/A_2$ Rows 1 and 2 of Table VI list the calculated values of $P_oL/8.5 \times 10^{-4}$ for the conditions $s = 8$ and 0, respectively. It is apparent

TABLE VI

CALCULATED DEPENDENCE OF THE PERMEABILITY ON THE AREA ORIENTATION, TORTUOSITY, INTRACELLULAR HEMOGLOBIN CONCENTRATION, AND ANISOTROPIC PERMEABILITY

s S/A_2	L_1/L_2	[Hb%]	P/P_o	THO	Form	Acet	Prop
				$P_oL/8.5 \times 10^{-4}$, 10^{-5} cm s^{-1}			
8	1	33	1	569	55.5	11.9	15.1
0	1	33	1	840	111	26.0	32.1
8	2	33	1	659	75.1	19.5	22.7
8	1	45	1	681	58.0	12.0	15.4
8	1	33	0.5	647	71.7	16.1	20.1

that the permeability values for $s = 0$ are approximately twofold larger than the values for $s = 8$, which have been shown to be in substantial agreement with the literature values (Table V). A side face exchange area of the red cell eight times the end area is not unreasonable, considering the appearance of the packed cells in the longitudinal section (Fig. 7 A). This ratio would correspond to a cylindrical cell of length twice the end face diameter.

TORTUOSITY FACTOR, L_1/L_2 The dependence of the calculated permeability parameter $P_oL/8.5 \times 10^{-4}$ on the value of L_1/L_2 is illustrated in rows 1 and 3 of Table VI. For a twofold increase in the tortuosity, there is less than a 20% increase in the calculated permeability coefficient to tritiated water, but a 60% increase in the permeability to acetamide is predicted. This result would be anticipated on a priori grounds, since an increased tortuosity should have a more pronounced affect on the less permeable solutes which are forced to follow the extracellular pathway through the red cell column. This reason-

[2] Dr. P. Farnsworth and coworkers have carried out extensive measurements on these preparations and have confirmed that there is an orientation of the red cells and a concomitant change in cell shape upon centrifugation (private communication). We thank Dr. Farnsworth and coworkers for this figure and for communication of similar results.

ing suggests using a tortuosity factor L_1/L_2 which increases as the permeability decreases. The data in Table VI predict that, upon increasing the tortuosity factor for acetamide and propionamide, the minimum in the experimental curve in Fig. 6 becomes less well defined.

ESTIMATION OF D_2 The hemoglobin content of the dog red cell has been quoted as 33 g Hb/100 ml red blood cells (RBC) by Altman and Dittmer (1971). The diffusion coefficient D_2 used in the diffusion analysis has been identified with an experimental value for a reconstituted solution of hemoglobin at this bulk concentration. Gary-Bobo and Solomon (1968) have shown that the water in the red cell behaves like solvent water with regard to nonelectrolytes, and differs in no quantitatively important respect from bulk water in free solution. Accordingly, the present assumption that the internal milieu of the red cell may be represented by a bulk solution containing 33% g Hb/100 ml appears reasonable. Tacit in this assumption is that the red cell membrane does not have a significant effect on the internal water structure or Hb distribution in the cell. The diffusion analysis reveals that for a given D and D_1, the calculated membrane permeability is fairly insensitive to the value of D_2. Row 4 (Table VI) lists the predicted membrane permeabilities corresponding to a red cell containing an internal hemoglobin solution at 45% concentration. These values are negligibly higher for the amides and less than 20% higher for the most permeable solute, tritiated water.

THE ASSUMPTION $P_o = P$ In view of the anisotropic orientation of the packed red cells, it is conceivable that the end face permeability P_o might differ from the side face permeability P. The assumption $P/P_o = 0.5$ gives the results in row 5 of Table VI. The assumption $P/P_o = 2$ gave similar but slightly greater increments in the opposite direction. At present it is not known how real this effect might be.

CONCLUSION

The present method of deducing cell membrane permeability from a measured bulk diffusion coefficient through the packed cells provides a permeability which is an average of the "outside-to-inside" permeability and the "inside-to-outside" permeability. Rapid reaction free floating cell methods can be arranged in principle to yield either permeability. The quantitative agreement between the present permeabilities and the outside-to-inside permeabilities of Vieira et al. (1970) and Sha'afi et al. (1971) suggests permeability symmetry of the red cell membrane to the present test solutes, although this agreement might also result from an asymmetrical membrane permeability and/or systematic errors in either method. The present method "slows down" the permeability process by putting many membranes in series. The diffusion within and

around the cells represents "unstirred layer" effects which are corrected for by a model assuming simplified geometry, and measured diffusion coefficients through appropriate solutions. Because of the slowing-down effect the present method works best for rapidly permeating solutes, such as tritiated water. These are just the solutes most difficult to measure by free floating cell methods. As such, the present method should complement existing methods for determining cell membrane permeability. Where a choice between the present and existing methods exists, the present method commends itself by its simplicity.

APPENDIX

Steady-State Diffusion Model

W. PERL

The red cells are assumed packed into a regular parallelopiped arrangement (Fig. 2 *a*). One-dimensional diffusion in the x direction is assumed, together with first order permeability exchange of indicator across the end and side faces of the membrane. The necessity of including exchange across the side faces of the membrane is shown in Fig. 2 *b*. The extracellular indicator concentration $c_1(x)$ decreases monotonically from the $x = 0$ face of the cell to the $x = L$ face of the cell. The intracellular indicator concentration $c_2(x)$ decreases "discontinuously" across the $x = 0$ face of the cell, then decreases monotonically from $x = 0$ to $x = L$, then decreases discontinuously across the $x = L$ face of the cell. In the region where $c_1(x) > c_2(x)$ there will be an inward diffusive flux of indicator. In the region where $c_1(x) < c_2(x)$ there will be an outward diffusive flux of indicator. Steady-state mass balance of indicator in an extracellular volume element of thickness dx at x is

$$- D_1 A_1 \frac{d^2 c_1}{dx^2} + \frac{PS}{L}(c_1 - c_2) = 0\,, \tag{1 a}$$

and in an intracellular volume element of thickness dx at x is

$$- D_2 A_2 \frac{d^2 c_2}{dx^2} + \frac{PS}{L}(c_2 - c_1) = 0, \tag{2 a}$$

where $c_1(x)$ and $c_2(x)$ (cpm/cm^3 water) are, respectively, the concentrations of indicator at distance x in extracellular water and in intracellular water, and the other quantities have been defined in the text. Eqs. 1 *a* and 2 *a* are written in non-dimensional form as

$$d^2c_1/dX^2 - \alpha_1^2(c_1 - c_2) = 0, \tag{3 a}$$

and

$$d^2c_2/dX^2 - \alpha_2^2(c_2 - c_1) = 0, \tag{4 a}$$

where $X = x/L$ and $\alpha_1^2 = PSL/D_1A_1$, $\alpha_2^2 = r\alpha_1^2$. The boundary conditions for extracellular indicator are that the indicator concentrations at $X = 0$ and $X = 1$ are continuous, or

$$c_1(0) = c_o, \tag{5 a}$$

and

$$c_1(1) = c_L. \tag{6 a}$$

The boundary conditions for intracellular indicator are that the intracellular indicator flux j_2 (amount per second) is continuous across the cell membrane at $X = 0$ and at $X = 1$, or,

$$j_2(0) = -\frac{D_2A_2}{L}\left(\frac{dc_2}{dX}\right)_0 = P_oA_2[c_o - c_2(0)], \tag{7 a}$$

$$j_2(1) = -\frac{D_2A_2}{L}\left(\frac{dc_2}{dX}\right)_1 = P_oA_2[c_2(1) - c_L], \tag{8 a}$$

where P_o (centimeters per second), the permeability coefficient of the end portions of the cell membrane, is not necessarily equal to P. The problem is, to calculate the bulk diffusion coefficient D defined by

$$D = \frac{(L + L_o)j}{A[c_1(-L_o) - c_L]}, \tag{9 a}$$

where

$$j = j_1 + j_2 = -D_1A_1(dc_1/dx) - D_2A_2(dc_2/dx), \tag{10 a}$$

is the total indicator flux, A is the total cross-sectional area and $c_1(-L_o)$ is the intracellular indicator concentration at emergence from the cell preceding the one under consideration. Since the end-to-end distance L_o between cells is of order 0.01 L, then L_o may be neglected in Eq. 9 a and therefore also $c_1(-L_o) \approx c_1(0) \equiv c_o$. Eq. 9 a then becomes

$$DA/L = j/(c_o - c_L). \tag{11 a}$$

Adding Eqs. 1 a and 2 a, integrating twice and using Eq. 10 a gives

$$D_1A_1c_1(X) + D_2A_2c_2(X) = -iLX + C, \tag{12 a}$$

where C is a constant of integration. Elimination of $c_2(X)$ from Eq. 3 a by Eq. 12 a gives

$$\frac{d^2c_1}{dX^2} - \alpha^2c_1 - \frac{\alpha_1^2jL}{D_2A_2}X + \frac{\alpha_1^2C}{D_2A_2} = 0, \tag{13 a}$$

where $\alpha^2 = \alpha_1^2 + \alpha_2^2$ (see text Eq. 7). The solution of Eq. 13 *a* is of the form

$$c_1(X) = G + BX + Ee^{\alpha X} + Fe^{-\alpha X}. \tag{14 a}$$

Substitution of Eq. 14 *a* into Eq. 13 *a* and equating corresponding coefficients gives,

$$B = -jL/(D_1A_1 + D_2A_2), \tag{15 a}$$

and

$$G = C/(D_1A_1 + D_2A_2). \tag{16 a}$$

The boundary conditions, Eqs. 5 *a*, 6 *a* applied to Eq. 14 *a* give, respectively

$$G + E + F = c_o, \tag{17 a}$$

and

$$G + B + e^{\alpha}E + e^{-\alpha}F = c_L. \tag{18 a}$$

The boundary conditions, Eqs. 7 *a*, 8 *a* applied to Eqs. 12 *a*, 14 *a* yield, respectively, after some reduction,

$$G - \Pi_o^{-1}B - a_-E - a_+F = c_o, \tag{19 a}$$

and

$$G + (1 + \Pi_o^{-1})B - a_+e^{\alpha}E - a_-e^{-\alpha}F = c_L, \tag{20 a}$$

where

$$a_+ \equiv r(1 + \alpha\Pi_o^{-1}), \tag{21 a}$$

and

$$a_- \equiv r(1 - \alpha\Pi_o^{-1}), \tag{22 a}$$

r, Π_o are defined in text Eqs. 10, 11. Solution of the simultaneous Eqs. 17 *a*–20 *a* for G, B, E, F gives $c_1(X)$ by Eq. 14 *a* and $c_2(X)$ by Eqs. 12 *a*, 15 *a*, 16 *a*. The macroscopic diffusion coefficient D, Eq. 11 *a*, which is all that is needed for present purposes, is obtained en route to the complete solution. Subtraction of Eqs. 17 *a* and 19 *a* and subtraction of Eqs. 18 *a* and 20 *a* give two simultaneous equations for E/B, F/B which yield

$$E/B = [(1 + a_+) + (1 + a_-)e^{-\alpha}]/\Pi_o \det, \tag{23 a}$$

$$F/B = -e^{\alpha}E/B, \tag{24 a}$$

and

$$\det = (1 + a_+)^2e^{\alpha} - (1 + a_-)^2e^{-\alpha}. \tag{25 a}$$

Subtraction of Eqs. 17 *a* and 18 *a*, or equivalently, subtraction of Eqs. 19 *a* and 20 *a* yields

$$(c_o - c_L)/(-B) = 1 + 2(e^{\alpha} - 1)E/B. \tag{26 a}$$

Eqs. 11 *a* and 15 *a* give

$$DA/D_2A_2 = -(1 + r)B/(c_o - c_L). \tag{27 a}$$

Eqs. 26 *a*, 27 *a* give

$$\frac{DA}{D_2A_2} = \frac{1 + r}{1 + 2\,(e^{\alpha} - 1)\,E/B}, \tag{28 a}$$

which, after substitution of Eqs. 23 *a*–25 *a* and some reduction, yields text Eqs. 5, 6.

For $S/A_2 \to 0$, sidewise entry becomes negligible relative to end face entry and text Eq. 5 reduces to

$$DA/D_2A_2 = (1 + 2\Pi_o^{-1})^{-1} + r. \tag{29 a}$$

Eq. 29 *a* expresses the extracellular diffusional conductance pathway in parallel but not interacting with the cellular pathway composed of two membrane resistances in series with the intracellular diffusion resistance, thus

$$\frac{DA}{L} = \frac{D_1A_1}{L} + \left(\frac{L}{D_2A_2} + \frac{2}{P_oA_2}\right)^{-1}, \tag{30 a}$$

which is equivalent to Eq. 29 *a*. For $S/A_2 \to \infty$ text Eq. 5 reduces to

$$\frac{DA}{D_2A_2} \to \frac{1 + r}{1 + (2/r\alpha)} \to 1 + r\,. \tag{31 a}$$

The second limiting form of Eq. 31 *a* results from $\alpha \to \infty$ by text Eq. 7. This form corresponds to

$$\frac{DA}{L} = \frac{D_1A_1}{L} + \frac{D_2A_2}{L}, \tag{32 a}$$

which represents the extracellular diffusional conductance in parallel with the intracellular diffusional conductance, both end and side membrane having no effect in this limit. The solutions for $c_1(X)$, $c_2(X)$ are obtained from Eqs. 14 *a*, 12 *a* and the subsequent equations as

$$\frac{c_1(X) - c_L}{c_o - c_L} = \tfrac{1}{2} + \left(\frac{\tfrac{1}{2} - X}{1 + z}\right) + \frac{z}{2(1 + z)}\,\frac{\sinh \alpha(\tfrac{1}{2} - X)}{\sinh \tfrac{1}{2}\alpha}, \tag{33 a}$$

and

$$\frac{c_2(X) - c_L}{c_o - c_L} = \tfrac{1}{2} + \left(\frac{\tfrac{1}{2} - X}{1 + z}\right) - \frac{rz}{2(1 + z)}\,\frac{\sinh \alpha(\tfrac{1}{2} - X)}{\sinh \tfrac{1}{2}\alpha}, \tag{34 a}$$

which are antisymmetric with respect to $X = \tfrac{1}{2}$, ordinate $= \tfrac{1}{2}$ (Fig. 2 *b*).

The authors thank Dr. Per Sejrsen for a helpful discussion and Mr. Balvant C. Patel and Mr. John Fahey for technical assistance. Thanks are also due to Mr. George Kalonaros and Leo Waivers of the Stevens Institute of Technology, Hoboken, N. J., who contributed to this project under the aegis of the Undergraduate Projects in Technology and Medicine (UPTAM) program.

Dr. Perl thanks Dr. William R. Redwood for stimulating discussions which led to the formulation of the model in the Appendix.

This study was supported in part by USPHS, NIH Grants HL-12974, HL-12879, and GM-18697 and the UPTAM program.

Received for publication 3 May 1974.

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