Tracer Determinations of Human Red Cell Membrane Permeability to Small Nonelectrolytes

DAVID SAVITZ and A. K. SOLOMON

From the Biophysical Laboratory, Harvard Medical School, Boston, Massachusetts 02115. Dr. Savitz' present address is Reynolds Memorial Hospital, Winston-Salem, North Carolina 27101. Please send all reprint requests to the Biophysical Laboratory, Harvard Medical School, Boston, Massachusetts 02115.

ABSTRACT A flow system has been used to determine the permeability of human red cell membranes to four small nonelectrolytes labeled with ^{14}C. The permeability coefficients, ω, in units of mol dyne^{-1} sec^{-1} $\times$ 10^{15}, are: ethylene glycol, 6; urea, 13; formamide, 22; and methanol, 131. The values for urea and formamide are in good agreement with values obtained by Sha'afi, Gary-Bobo, and Solomon by the minimum method. The unusually high value for ω for methanol is ascribed to its solubility in the red cell membrane since its ether: water partition coefficient is 0.14, higher by more than an order of magnitude than the ether:water partition coefficient for water. The other three solutes are hydrophilic and are characterized by values of ω which behave consistently with those of other hydrophilic amides and ureas. The values of ω for the three hydrophilic solutes measured are also consistent with an equivalent pore radius of about 3.5 A in agreement with previous estimates made on the basis of other types of studies.

Classical measurements of red cell permeability (1) have relied on hemolysis as the end result of nonelectrolyte penetration, so that the kinetics of hemolysis have been superimposed on those of the nonelectrolyte flux. Recently Sha'afi et al. (2) have developed a method of measuring the permeability coefficient of urea from the minimum volume reached when suspensions of red cells are placed in an isosmolar solution containing urea at concentrations of the order of 0.3 M. In the present experiments, the urea permeability of human red cells has been measured directly in vitro using ^{14}C as a tracer. In order to determine the time course of this rapid exchange, a flow method was used which followed closely the method of Paganelli and Solomon (3). The same method was used to measure the permeability coefficients of three other small nonelectrolytes.

EXPERIMENTAL METHOD

A suspension of cells is injected into the mixing chamber of a rapid flow system where it is mixed with isosmolar buffer containing urea-^{14}C or other labeled nonelectrolytes. The mixture flows down an observation tube, under a hydrostatic pressure gradient and the extracellular fluid is sampled, at intervals along the tube, through Millipore filters which red cells cannot pass. The samples of labeled filtrate, each representing a certain time of exchange, are analyzed for their ^{14}C content, and a kinetic curve is plotted showing the fall in concentration of urea-^{14}C as a function of time. The reagent containers, mixing chamber, filtration chamber, and sampling pipettes used in these experiments were identical to those of Paganelli and Solomon except that the filtration chamber was extended to a length of 44 cm and contained seven collecting ports.

All experiments were carried out at room temperature ($\approx$21°C) on heparinized blood freshly drawn from healthy adult human beings. About 500 ml of blood were lightly centrifuged and the bulk of the plasma removed. Isosmolar phosphate—bicarbonate buffer, whose composition is given by Paganelli and Solomon (3), was added to give an enriched cell suspension of hematocrit 0.60-0.65. The buffer was altered by the addition of 4.3 mM urea (or suitable concentrations of other nonelectrolytes) with appropriate adjustment of NaCl concentration to maintain isosmolarity with the plasma. In the urea experiments, the enriched cell suspension was allowed to exchange with buffer of the same composition to which urea-^{14}C (about 0.08 mCi mg^{-1}) had been added at a concentration of about 2 mg liter^{-1}.

The flow of buffer and cell-enriched suspension was maintained by the application of a pressure of 75 psi, sufficient to maintain a linear flow rate in the filtration chamber of 720–850 cm sec^{-1}. The experimental points thus represented exchange periods between about 4 msec (the sample from the first filtration port) and 60 msec (the sample from the last port).

A small amount of hemolysis occurred. The degree of hemolysis was estimated by measurement of the optical density of hemoglobin in the filtrates and appropriate corrections applied to the observed counting rates. These corrections were usually under 1 %.

^{14}C-labeled urea, methanol, ethylene glycol, and formamide were obtained from the New England Nuclear Corporation (Boston, Mass.). Radioactivity was estimated in a liquid scintillation counter (model 314 SC, Packard Instrument Co., Inc., La Grange, Ill.) by diluting weighed samples of 25–75 mg size in glass vials with a standard mixture of toluene, methanol, dioxane, and naphthalene. All samples were counted to at least 10^4 counts to minimize counting errors.

As an over-all check on the performance of the apparatus used for the exchange experiments, a simulated experiment was performed in which the buffer contained ^{24}NaCl instead of ^{14}C-labeled nonelectrolyte. Over the 60 msec time scale of the experiment there should be no exchange of ^{24}Na between extracellular and intracellular fluids. This was found to be the case, as the count rate in each filtrate was the same and equal to the count rate (corrected for dilution in the mixing chamber) in the initial buffer.

RESULTS AND DISCUSSION

The mathematical treatment is based on the assumption that the total concentration (labeled plus unlabeled) of nonelectrolyte is approximately the same in both compartments and thus relatively constant during the exchange. In the case of urea, this condition was approximated by the use of buffer containing urea at a concentration of 4.3 mM. The urea concentration of normal blood lies between 2.9 and 6.5 mM. In the preparation of the enriched cell suspension, buffer containing 4.3 mM urea was added in a volume at least 1.5 times the volume of the remaining plasma and time allowed for the added urea to equilibrate between cellular and extracellular water. Furthermore at time zero the extracellular fluid of the cell-enriched suspension was diluted in the mixing chamber with buffer containing 4.3 mM urea. Calculation from sample data leads to an estimate of the outer limits for the difference in urea concentration between extracellular and cellular fluid after mixing; i.e., at the beginning of the urea-^{14}C exchange. If the urea concentration in the blood is 2.9 mM, the ratio of cell urea concentration to plasma concentration is 3.2 mM:4.1 mM; if the blood concentration is 6.5 mM, the ratio is 5.9 mM:4.7 mM.

None of the other nonelectrolytes studied is a normal constituent of the blood. In these cases carrier was added to make sure that the ratio of unlabeled to labeled concentration was $>100:1$. The total concentration of added nonelectrolyte was 0.5–1 mM which does not make an appreciable contribution to total osmolality. The nonelectrolytes were added to the buffer alone, so that these experiments were made in the presence of a net solute flux but with negligible solvent flux. DiPolo, Sha'afi, and Solomon (4) have studied the effect of net solute flow on the permeability coefficient of ethylene glycol in a nonporous cellulose acetate membrane. They found that an ethylene glycol concentration gradient of 0.1 M had a negligible effect on the permeability coefficient in the absence of net osmotic solvent flow.

The equation for diffusion (see references 3 and 5) between two well-stirred compartments is

$$dP/dt = -k(p - q) \tag{1}$$

in which P is the total radioactivity (cpm) in the extracellular compartment and p and q are the cpm per ml of H_2O in the extracellular and cell compartments. k is a coefficient related to the permeability coefficient, $\omega(\omega RT = P_d$, the membrane diffusion permeability coefficient in cm/sec). In order to integrate the equation the assumptions are made that both compartments are of constant volume and that both are well-stirred. The absence of an osmotic gradient serves to keep the compartment volumes constant. Evidence for the

absence of an unstirred layer in apparatus of this design has been given by Sha'afi et al. (6). The solution to the equation is

$$(p/p_\infty - 1) = (p_0/p_\infty - 1)e^{-kp_0t/v_qp_\infty} \quad (2)$$

in which the subscripts 0 and ∞ refer to zero time and infinite time, t is time, and v_q is the cell water volume. Fig. 1 shows a semilogarithmic graph of $(p/p_\infty - 1)$ against time for the data for a single experiment. p_∞ is measured experimentally and p_0 is determined by extrapolation from the data. The data plotted in this manner fall on a straight line between 2 and 60 msec, consistent with the postulated two-compartment kinetics over this time period. The half-time of the exchange may be obtained by setting $p = (p_0 + p_\infty)/2$, and $t = t_{1/2}$ in equation 2. Thus, $t_{1/2} = -0.693/S$, in which S is the slope of the line in Fig. 2.

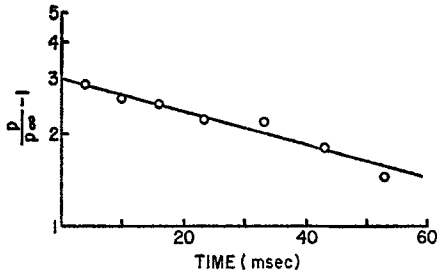

FIGURE 1. Time course of urea-^{14}C diffusion into human red cells.

The rate of exchange of urea-^{14}C across the human red cell under isosmolar conditions at 21°C was measured in seven samples of blood drawn from normal adults. The results for urea are given in Table I. Table II contains the data obtained with methanol, ethylene glycol, and formamide. For these computations the area of the red cell is taken as 155×10^{-8} cm^2, and the water content of a single red cell as 63×10^{-12} cm^3 (7). In the concentration range used in these experiments tracer diffusion may be taken as a reliable index of the diffusion of the unlabeled species, as discussed by Curran, Taylor, and Solomon (8).

Two of the permeability coefficients determined by the tracer method have also been determined by the minimum method; the agreement between the two determinations is surprisingly good as has been previously discussed (2). In the case of urea, the present value of 13×10^{-15} mol dyne^{-1} sec^{-1} is to be compared with the minimum method value of 15×10^{-15} mol dyne^{-1} sec^{-1}. In the case of formamide the comparative values are 22 and 18×10^{-15} mol dyne^{-1} sec^{-1}.

The value of the permeability coefficient for methanol is very high, es-

sentially equal to the water permeability coefficient (9) of 136 × 10^{-15} mol $dyne^{-1}$ sec^{-1}. The value for the ether-water partition coefficient, k_{ether}, for methanol of 0.14 is higher by more than one order of magnitude than the value of k_{ether} for water itself which is 0.003.[1] Thus methanol is in the class of lipophilic molecules and presumably permeates the red cell primarily by diffusion through the membrane fabric. It is interesting that of all the molecules whose permeation into *Chara* was studied by Collander (10), methanol

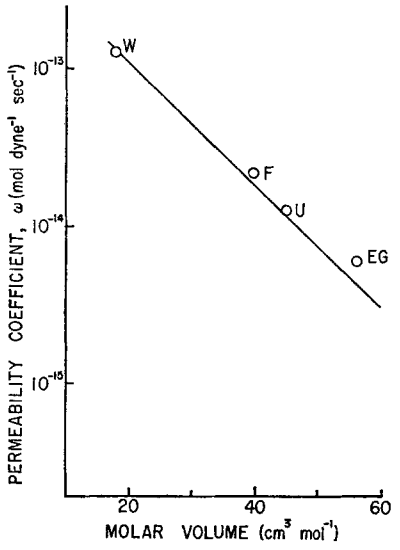

FIGURE 2

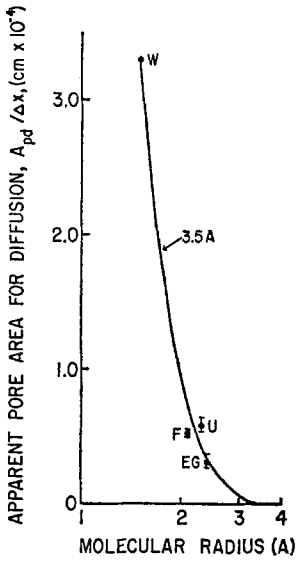

FIGURE 3

FIGURE 2. Permeability coefficient of hydrophilic solutes as a function of molar volume. The symbols are: water, *W*; formamide, *F*; urea, *U*; and ethylene glycol, *EG*. The permeability coefficients for the three solutes are taken from Table II; that for water from reference 9. The line is that given in Fig. 3 of footnote 1.

FIGURE 3. $A_{pd}/\Delta x$, apparent pore area for diffusion divided by path length through the membrane (see references 12 and 13), as a function of solute molecular radius. The curve has been drawn from Renkin's equations for an equivalent pore radius of 3.5 A.

was found to have the highest permeability coefficient. Values for k_{ether} for ethylene glycol, formamide, and urea (11) are: 0.0053, 0.0014, and 0.00047 which means that these molecules may be treated as hydrophilic solutes, permeating primarily through water-filled channels.

Sha'afi, Gary-Bobo, and Solomon[1] have shown that the logarithm of the permeability coefficient of small hydrophilic solutes is linearly related to the molar volume, which is the molecular weight divided by the crystalline density. The line in Fig. 2 is taken from their study which included measure-

[1] Sha'afi, R. I., C. M. Gary-Bobo, and A. K. Solomon. 1970. Permeability of red cell membranes to small hydrophilic and lipophilic solutes. *J. Gen. Physiol.* **58**:238.

ments of ω for urea, methyl urea, dimethyl urea, formamide, and acetamide. It is apparent that the present figure for ethylene glycol is compatible with their conclusions. The data for formamide and urea must fit the line in Fig. 2 because of the agreement of the permeability coefficients obtained by the

TABLE I
PERMEABILITY COEFFICIENTS FOR UREA IN HUMAN RED CELLS

Experiment	Flow rate	Hematocrit	Last point	Half-time	$\omega \times 10^{15}$
	cm sec^{-1}		*msec*	*msec*	*mol dyne*$^{-1}$ *sec*$^{-1}$
1	845	0.345	16	46	13.1
2	819	0.287	44	39	15.8
3	829	0.278	53	44	14.1
4	783	0.280	56	36	17.6
5	722	0.264	61	70	8.9
6	735	0.243	60	78	7.7
7	829	0.269	53	56	11.5
Average ± SE				53±6	13±1

TABLE II
PERMEABILITY COEFFICIENTS FOR NONELECTROLYTES IN HUMAN RED CELLS

Experiment	Solute	Half-time	$\omega \times 10^{15}$
		msec	*mol dyne*$^{-1}$ *sec*$^{-1}$
8	Ethylene glycol	109	8.0
9	Ethylene glycol	93	8.8
10	Ethylene glycol	189	2.6
11	Ethylene glycol	82	4.8
Average ± SE		118±24	6±1
12	Methanol	6.5	130
13	Methanol	7.0	132
14	Methanol	7.0	132
Average ± SE		6.8±0.2	131±1
15	Formamide	43	21
16	Formamide	41	23
Average		42	22

tracer method with those obtained by the minimum method, as already pointed out.

Alternatively the present results may be treated in terms of the equivalent pore radius which, as discussed by Solomon (12), is in the range of 3.5 to

4.5 A for human red cells. If all the interactions among solute, solvent, and membrane within the equivalent pore may be assigned to steric hindrance, an equation given by Renkin (13) may be used to compute the expected permeability coefficient relative to that of water in terms of the solute radius (computed on a spherical basis) and the equivalent pore radius. When this procedure is followed, the permeability coefficients for formamide, ethylene glycol, and urea are found to fit an equivalent pore radius of 3.5 A, as shown in Fig. 3. The agreement of this figure with the 4.3 A value obtained by a similar method from the reflection coefficient by Goldstein and Solomon (14) indicates the paramount role played by steric hindrance in hydrophilic solute permeation through the human red cell membrane.

It is somewhat surprising that the present results fit so well with an equivalent pore radius of 3.5–4.5 A. For one thing, evidence has been presented by Sha'afi et al. (2) indicating that the human red cell membrane behaves as a series membrane so that the same barrier may not necessarily control both the reflection coefficient and ω. Furthermore, DiPolo, Sha'afi, and Solomon (4) have demonstrated that hydrogen bonding in a homogeneous porous cellulose acetate membrane has a different effect on the reflection coefficient than on the permeability coefficient. Finally, Sha'afi, Gary-Bobo, and Solomon[1] have shown that solute-solvent hydrogen bonding makes an important contribution to ω for small hydrophilic solutes in red cell membranes. All these factors indicate that the equivalent pore radius of the human red cell is an empirical parameter, relatively insensitive to appreciable perturbations arising either from chemical interactions or from pore fine structure. Nonetheless the equivalent pore radius has provided an important conceptual framework in understanding the nature of hydrophilic permeation through biological membranes. The next step in extracting information about the biophysical properties of the membrane will come from a detailed study of the perturbations themselves.

The authors would like to express their thanks to Mr. R. Dooley for the construction of the apparatus. This study has been supported in part by the Atomic Energy Commission Contract AT(30-1)-2453.

Received for publication 12 December 1970.

BIBLIOGRAPHY

1. DAVSON, H., and J. F. DANIELLI. 1952. The Permeability of Natural Membranes. Cambridge University Press. Chap 8.
2. SHA'AFI, R. I., G. T. RICH, D. C. MIKULECKY, and A. K. SOLOMON. 1970. Determination of urea permeability in red cells by minimum method. *J. Gen. Physiol.* **55**:427.
3. PAGANELLI, C. V., and A. K. SOLOMON. 1957. The rate of exchange of tritiated water across the human red cell membrane. *J. Gen. Physiol.* **41**:259.
4. DIPOLO, R., R. I. SHA'AFI, and A. K. SOLOMON. 1970. Transport parameters in a porous cellulose acetate membrane. *J. Gen. Physiol.* **55**:63.
5. SOLOMON, A. K. 1953. The kinetics of biological processes. *Advan. Biol. Med. Phys.* **3**:65.
6. SHA'AFI, R. I., G. T. RICH, V. W. SIDEL, W. BOSSERT, and A. K. SOLOMON. 1967. The

effect of the unstirred layer on human red cell water permeability. *J. Gen. Physiol.* **50**:1377.

7. WHITTAM, R. 1964. Transport and Diffusion in Red Blood Cells. Edward Arnold (Publishers) Ltd., London. 2, 58.
8. CURRAN, P. F., A. E. TAYLOR, and A. K. SOLOMON. 1967. Tracer diffusion and unidirectional fluxes. *Biophys. J.* **7**:879.
9 VIEIRA, F. L., R. I. SHA'AFI, and A. K. SOLOMON. 1970. The state of water in human and dog red cell membranes. *J. Gen. Physiol.* **55**:451.
10. COLLANDER, R. 1937. The permeability of plant protoplasts to non-electrolytes. *Trans. Faraday Soc.* **33**:985.
11. COLLANDER, R. 1949. Die Verteilung organischer Verbindungen zwischen Äther und Wasser. *Acta Chem. Scand.* **3**:717.
12. SOLOMON, A. K. 1968. Characterization of biological membranes by equivalent pores. *J. Gen. Physiol.* **51**(5, Pt. 2):335.
13. RENKIN, E. M. 1955. Filtration, diffusion, and molecular sieving through porous cellulose membranes. *J. Gen. Physiol.* **38**:225.
14. GOLDSTEIN, D. A., and A. K. SOLOMON. 1960. Determination of equivalent pore radius for human red cells by osmotic pressure measurement. *J. Gen. Physiol.* **44**:1.