Permeability of Human Red Cells to a Homologous Series of Aliphatic Alcohols

Limitations of the Continuous Flow-Tube Method

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ABSTRACT Human red cell permeability to the homologous series of methanol, ethanol, n-propanol, n-butanol, and n-hexanol was determined in tracer efflux experiments by the continuous flow tube method, whose time resolution is 2–3 ms. Control experiments showed that unstirred layers in the cell suspension were $<2 \times 10^{-4}$ cm, and that permeabilities $\leq 10^{-2}$ cm s^{-1} can be determined with the method. Alcohol permeability varied with the chain length (25°C): P^{meth} 3.7×10^{-3} cm s^{-1}, P^{eth} 2.1×10^{-3} cm s^{-1}, P^{prop} 6.5×10^{-3} cm s^{-1}, P^{but} $\leq 61 \times 10^{-3}$ cm s^{-1}, P^{hex} 8.7×10^{-3} cm s^{-1}. The permeability for methanol, ethanol, and n-propanol was concentration independent (1–500 mM). The permeability to n-butanol and n-hexanol, however, increased above the upper limit of determination at alcohol concentrations of 100 and 25 mM, respectively. The activation energies for the permeability to methanol, n-propanol, and n-hexanol were similar, 50–63 kJ mol^{-1}. Methanol permeability was not reduced by p-chloromercuribenzene sulfonate (PCMBS), thiourea, or phloretin, which inhibit transport of water or hydrophilic nonelectrolytes. It is concluded (a) that all the alcohols predominantly permeate the membrane lipid bilayer structure; (b) that both the distribution coefficient and the diffusion coefficient of the alcohols within the membrane determine the permeability, and (c) that the relative importance of the two factors varies with changes in the chain length.

INTRODUCTION

It is well known (e.g., Collander and Bärlund, 1933) that the permeability of plasma membranes to small hydrophilic nonelectrolytes is larger than predicted from Overton's rule (1895), which states that the membrane permeability coefficient to a first approximation should be proportional to the oil/water partition coefficient of the permeant solute. Collander and Bärlund (1933) suggested that the relatively larger permeability to the small hydrophilic molecules was determined by so-called sieving properties of the membrane, and Overton's rule should remain valid for lipophilic molecules.

A current controversy deals with the unsettled problem of whether the partition coefficient of a lipophilic solute is the overwhelming determinant for the permeability coefficient of artificial lipid membranes (Finkelstein, 1976; Orbach and Finkelstein, 1980), or whether the permeability coefficient also depends significantly on variations in the intramembraneous diffusion coefficient of the solute (Wolosin et al., 1978).

Human red cells have structurally inhomogenous membranes, caused by the insertion of integral membrane proteins into the lipid bilayer leaflet (Singer and Nicolson, 1972). The relatively large permeability to small hydrophilic nonelectrolytes such as formamide, acetamide, and urea has thus been explained to be caused by diffusion through polar pathways (Sha'afi et al., 1971); for urea, a facilitated transport system has been hypothesized (Macey and Farmer, 1970; Wieth et al., 1974; Brahm, 1983). For lipophilic molecules, on the other hand, one would still expect the permeability to increase with solute lipophilicity if Overton's rule also holds for red cells.

To examine this question, the permeability coefficient of the human red cell membrane was determined for a homologous series of the lipophilic primary alcohols, from methanol through n-hexanol. These alcohols permeate the red cell membrane extremely rapidly. Proper performance of the continuous flow-tube method was therefore of crucial importance, as the flux determinations are limited by the existence of an equivalent unstirred layer adjacent to the membrane. The minimum magnitude of this layer found in the present study, 1.7×10^{-4} cm, sets an upper limit of 10^{-2} cm s^{-1} for permeabilities accessible to experimentation. The results indicate that the permeability to ethanol, n-propanol, and n-butanol can be accounted for by Overton's rule, whereas the methanol permeability is too large and the n-hexanol permeability is too low to be explained by the simple relation between permeability and lipid solubility originally proposed by Overton (1895).

MATERIALS, METHODS, AND CALCULATIONS

Electrolyte Media

n-Butanol and the larger aliphatic alcohols induce cation leaks (Gutknecht and Tosteson, 1970). Therefore, the alcohol permeability experiments were all performed in a potassium medium. The stock medium was 150 mM KCl buffered with 0.5 mM KH$_2$PO$_4$ (pH 7.2 at 25°C). Methanol, ethanol, and n-propanol were added to obtain concentrations of 1, 100, and 500 mM. The upper n-butanol concentration was 250 mM. The hexanol experiments were performed at 1 and 25 mM n-hexanol.

Labeling and Packing of Cells

Human red cells from freshly drawn, heparinized blood were washed once in the appropriate medium and titrated to the desired pH with CO$_2$ or 0.1 N KOH. Subsequently, the cells were washed three times in the medium and resuspended to a hematocrit of ~60%. Isotopes ([^{3}H]methanol, 50 mCi mmol^{-1}; [^{14}C]n-butanol, 1.9 mCi mmol^{-1} [New England Nuclear Corp., Boston, MA]; [^{14}C]n-propanol, 2 mCi mmol^{-1}; [^{14}C]n-hexanol, 5 mCi mmol^{-1} [ICN Pharmaceuticals, Inc., CA]; [^{14}C]ethanol, 51.4 mCi mmol^{-1}; ^{51}CrEDTA, 1 mCi/mg Cr [Amersham Corp., Arling-

ton Heights, IL]) were added to obtain a final concentration of ~0.5–1 μCi/ml cell suspension. After incubation, the cell suspension was divided into a major and a minor portion. Both portions were centrifuged at 50,000 g for 15 min (Sorvall RC-5 centrifuge; DuPont Instruments-Sorvall Biomedical Div., Newtown, CT). The major portion was spun in 10-ml tubes, the supernate and the upper 2–3 mm of cell layers were sucked off, and the remaining packed cells were kept cold in a 5-ml syringe until use. The minor portion was spun in nylon tubes (Funder and Wieth, 1966) for determinations of cell water and activity of tracer in cells and supernatant. The medium trapped between the cells during the packing procedure was 2% (wt wt^{-1}) in the nylon tubes, and 7–8% (wt wt^{-1}) in the 10-ml tubes, determined as the extracellular [^{3}H]inulin or ^{51}CrEDTA space.

Methanol permeability was also determined in cells treated with p-chloromercuribenzene sulfonate (PCMBS; Sigma Chemical Co., St. Louis, MO) and phloretin (ICN K&K Laboratories, Inc., Plainview, NY). The red cells were incubated for 45 min at 38°C in a medium containing 1 mM PCMBS as described elsewhere (Brahm, 1982). Phloretin was dissolved in ethanol and added to the medium to give a final concentration of 0.25 or 0.5 mM (ethanol 0.1% vol vol^{-1}). The cells were equilibrated with the phloretin concentration identical to that of the efflux medium.

Determinations of the Rate of Efflux and the Efficiency of Mixing

All experiments were done with the flow-tube technique. The flow tube was an improved version of a design originally described by Piiper (1964) and modified by Brahm (1977) to measure the rate of efflux of tracer from labeled red cells in a dilute cell suspension. A radioactive-labeled sample of packed erythrocytes is mixed with a nonradioactive medium which is forced into a mixing chamber of small dimensions by means of air pressure. After mixing, the dilute cell suspension moves through the tube with constant linear velocity. At predetermined distances from the mixing chamber, the tube is replaced by filtering units whose walls in part bulge into the tube and consist of a filter (pore diameter 0.4 μm, Shandon Southern Instruments Inc., Sewickley, PA) that ensures a sufficient amount of cell-free filtrates. The constant velocity of flow through the tube of the apparatus implies that a given distance from the mixing chamber is equivalent to the time to run through the distance. Six filtrates were collected simultaneously at well-defined time intervals after the mixing, which initiates the release of radioactivity from the cell sample.

In the present version of the apparatus, the time resolution was increased by' reducing the distance between the filtering units to 1.2 cm, corresponding to time intervals of ~2.5 ms at the largest velocities of flow (~475 cm s^{-1}); i.e., the six samples represent determinations within the first 15–20 ms of the efflux process.

The mixing efficiency was measured by means of the radioactive-labeled, impermeant ^{51}CrEDTA that was trapped between the packed erythrocytes during the preparation procedures. At the subsequent mixing of the cell sample with nonradioactive medium, the radioactivity in the cell-free filtrates is a measure of how rapidly and efficiently the mixing is performed. The importance of unstirred layers was estimated using n-butanol because this alcohol permeates the red cell membrane so rapidly that diffusion through the unstirred layers may partially rate-control the transport of the alcohol.

The permeability coefficient of the red cell membrane to the aliphatic alcohols was determined from the rate of efflux of the radioactive-labeled alcohols under steady state conditions, i.e., where a net flux of tracer proceeds without changing the intra- or extracellular chemical concentrations.

Determinations of Radioactivity and Cell Water Content

Radioactivity of the filtrates and of the cells and medium was determined as previously described (Brahm, 1982). [51]CrEDTA was counted in a γ-scintillation spectrometer (model 3003 Auto-gamma; Packard Instrument Co., Inc., Downers Grove, IL). The cell water content was determined in each experiment by drying samples of red cells to constant weight. A correction was made for the contribution from extracellular trapped medium.

Calculation of Permeability

The rate of tracer efflux was well described by a model with two constant compartments in which the efflux of tracer takes place in a very large compartment because the hematocrit of the cell suspension was very low (<0.6%):

$$\ln(a_t - a_\infty)(a_o - a_\infty)^{-1} = -kt, \tag{1}$$

where a_t and a_∞ are extracellular activities in a sample taken at time t and in a sample taken after isotopic equilibrium has been achieved, respectively, and a_o is the extracellular radioactivity at time zero and represents graphically the (extrapolated) intercept of the tracer washout curve with the ordinate. The magnitude of the intercept depends on (a) the amount of extracellular tracer trapped between the cells during the packing procedure, (b) a time lag caused by the geometry of the mixing chamber, and (c) a temperature-dependent decrease of the intercept for alcohol efflux curves. The decrease of the intercept for methanol efflux curves with temperature was identical to that demonstrated for water efflux and discussed by Brahm (1982). The rate constant, k (s^{-1}), was determined from the slope of the semilogarithmic washout curves by linear regression analysis. The rate constant was converted to a permeability coefficient, P (cm s^{-1}), by multiplication with the ratio of the intracellular solvent volume, V (cm^3), to the membrane area, A (cm^2):

$$P = kVA^{-1} \qquad (cm\ s^{-1}). \tag{2}$$

The ratio VA^{-1} (cm) was determined by drying the cells to constant weight, assuming that 1 g of cell solids equals 3.1×10^{10} normal erythrocytes with a total membrane area of 4.4×10^4 cm^2 (1.42×10^{-8} cm^2 $cell^{-1}$; see Brahm, 1982).

In experiments with butanol and hexanol at 25 mM, the tracer efflux proceeded so rapidly that only one or two filtrates had a radioactivity below the steady state value in the sample collected at the outlet of the flow tube. In these experiments the rate constants were estimated directly from the washout curves as shown in Fig. 3, by assuming first-order kinetics at which 87.5% of the cellular activity has been given off within three half-times, equilibrium for practical purposes being achieved after six half-times ($T^{1/2} = \ln 2\ k^{-1}$, s).

RESULTS AND DISCUSSION

The Continuous Flow-Tube Method

The transport of the short-chained aliphatic alcohols through the red cell membrane is very rapid. Therefore, it must be considered whether the present method is suitable for measuring rapid transport processes. Control experiments included determinations of the nature of flow through the tube, the efficiency of the mixing chamber, and the importance of unstirred layers.

NATURE OF FLOW   It is important that flow through the tube is turbulent

because the ensuing continuous mixing (*a*) facilitates the exposure of the total membrane area for the transport process, (*b*) reduces diffusion barriers of unstirred layers of medium adjacent to the cell membrane, and (*c*) causes a flattened velocity profile of the fluid moving down the tube (Lamb, 1932; Seeger, 1967), so that the filtrates collected during the experiments represent narrow time distributions. Turbulence in aqueous solutions flowing through straight, uniform cylindrical tubes appears above a critical flow velocity (Reynolds, 1884) determined by:

$$\bar{v}_{crit} = Re\eta(R\rho)^{-1} \qquad (\text{cm s}^{-1}), \tag{3}$$

where η (poise) and ρ (g cm^{-3}) are the viscosity and the density of the fluid, respectively, R (cm) is the radius of the tube, and the dimensionless Reynolds' number, Re, is 1,000. R is 0.1 cm in the flow tube. The viscosity and density of the dilute cell suspension (hematocrit $< 0.6\%$) can be considered equal to 0.01 poise and 1 g cm^{-3}, respectively, for aqueous solutions. Thus, Eq. 3 gives a critical flow velocity of 100 cm s^{-1}. Turbulence may, however, occur at Re $<1,000$ and hence at lower flow velocities if the geometry of the tube is nonuniform, as is the case in the present version of the flow tube, because the six filters bulge into the blood stream.

The appearance of turbulence can be detected directly by plotting the flow velocity vs. the pressure difference, ΔP (dyn cm^{-2}). Below the critical velocity, the pressure difference is directly proportional to the flow velocity according to Poiseuille's law for laminar flows:

$$\Delta P(\text{lam}) = 8\bar{v}\eta LR^{-2} \qquad (\text{dyn cm}^{-2}), \tag{4}$$

where the pressure-flow relation in the presence of turbulence should take the form (Whitmore, 1968):

$$\Delta P(\text{turb}) = 0.079 \, \bar{v}^{1.75}\eta^{0.25}LR^{-1.25} \qquad (\text{dyn cm}^{-2}), \tag{5}$$

where L is the length of the tube. The flow pattern in a series of control experiments is shown in Fig. 1, where the flow velocity is plotted as a function of the applied pressure (P$_{appl}$, dyn cm^{-2}) instead of the pressure difference, which is difficult to measure in the narrow flow tube. Calculations (not shown) indicate that under assumption of turbulence, the ratio ΔP(turb) P$_{appl}^{-1}$ remains constant, whereas the ratio ΔP(lam) P$_{appl}^{-1}$ decreases by a factor of 2–3 when assuming laminar flow. The inset in Fig. 1 illustrates the ideal shape of the curve as laminar flow becomes turbulent. This flow-pressure diagram has the advantage over the diagram of flow vs. square root of the applied pressure used by Paganelli and Solomon (1957) because the change from laminar to turbulent flow is more conspicuous. The results indicate that flow becomes turbulent at linear velocities of ~80–85 cm s^{-1} (Reynolds' number of 800–850). This is in accordance with the concept that turbulence appears at a lower Reynolds' number when the geometry of the tube is not uniform. All the experiments in this article were performed at linear flow velocities >250 cm s^{-1}, far above the critical flow velocity.

MIXING EFFICIENCY   The efficacy of several mixing chambers with differ-

ent geometry was studied by Hartridge and Roughton (1923), who concluded that the tangential introduction of reactants ensured the best mixing. Therefore, the mixing chamber used in the present experiments has this geometry because mixing must be completed in <3 ms, before the first sampling of filtrate. The close packing of cells during the preparation procedure might be expected to cause adhesion of cells, which could prevent or at least delay mixing. Experiments with [51]CrEDTA, however, indicate that this source of error can be excluded. CrEDTA does not permeate the red cell membrane and remains in the extracellular medium. The radioactivity of [51]CrEDTA in
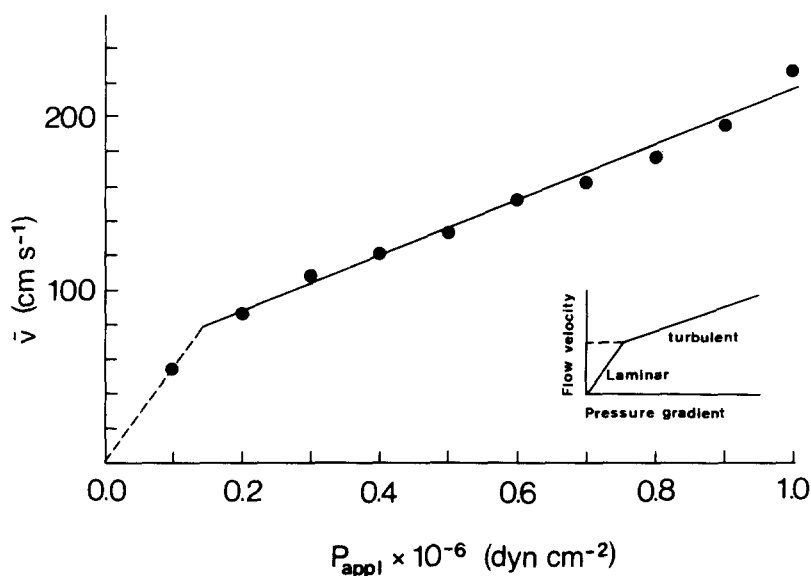

FIGURE 1.   Pressure-flow diagram of control experiments with the flow tube. The velocity of flow ($\bar{v}$, cm s^{-1}) is depicted vs. the applied pressure (P_{appl}, dyn cm^{-2}), driving the cell suspension through the tube. The inset illustrates how the slope of the curve changes in this depiction when laminar flow becomes turbulent (after Richardson and Neergaard, 1972). The application of P_{appl} is discussed in the text. The fully drawn part of the curve is described by the equation $P_{appl} = 70.5 \times \bar{v}^{1.79}$, which yields an exponent close to the empirically found value of 1.75 at turbulent flows (cf. Eq. 5). It appears that flow becomes turbulent at linear velocities of 80–100 cm s^{-1}.

the sample of packed cells in the syringe is therefore due solely to radioactive-labeled extracellular medium trapped between the cells during the packing procedure. The radioactivity in the filtrates obtained during an experiment is thus a measure of how rapidly and perfectly the trapped medium between the cells is mixed with the suspending medium. Fig. 2 shows the results from experiments performed at linear flow velocities between 160 and 230 cm s^{-1} (Fig. 2A) and between 275 and 440 cm s^{-1} (Fig. 2B). The radioactivity in the filtrates is depicted as the fraction of the radioactivity in the suspension collected at the outlet of the flow tube. Note the difference in time scale of the
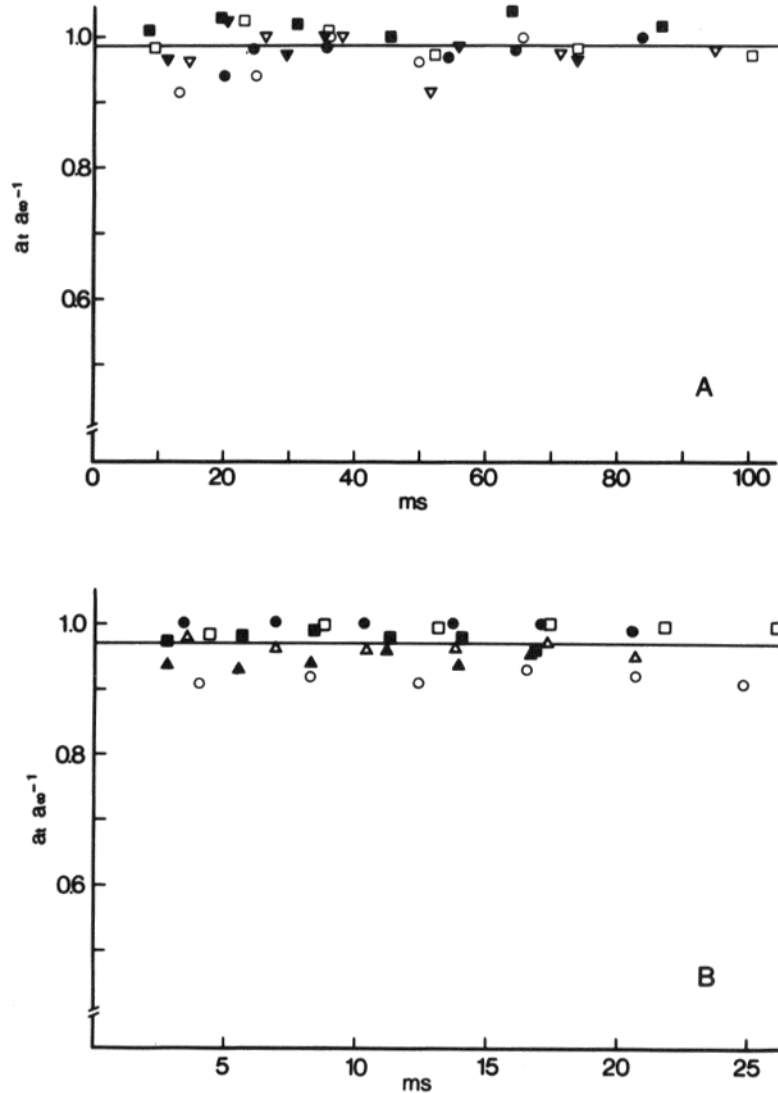
FIGURE 2.   The efficiency of the mixing chamber of the flow tube determined by means of $^{51}CrEDTA$. The 7–8% (wt/wt) extracellular medium trapped between the cells during the packing procedure was labeled with $^{51}CrEDTA$, which cannot permeate the red cell membrane. The radioactivity of $^{51}CrEDTA$ in the filtrates of the subsequent experiments, expressed as a fraction of the "equilibrium" sample collected at the outlet of the tube ($a_t a_\infty^{-1}$, ordinate) indicates that mixing is efficient and accomplished before the cell suspension passes the first filtering unit, which at the high flow velocities (Fig. B) was in < 3 ms.

two abscissas. In each experiment the first filtrate had attained the average radioactivity, even at the largest flow velocities at which filtrates were obtained 2.5–3 ms after mixing was initiated (Fig. 2B). Furthermore, the curves show that the ratio between radioactivity in the filtrates and the "equilibrium" sample was close to 1. In Fig. 2A, $a_t a_\infty^{-1}$ was 0.99 (SD 0.03, $n = 34$); the ratio of the high flow-velocity experiments was 0.97 (SD 0.04, $n = 36$). Satisfactory mixing is accomplished before the cell suspension passes the first filtering unit.

UNSTIRRED LAYERS The results with ^{51}CrEDTA indicate rapid and efficient mixing, but it cannot be excluded that the rate of transport across the red cell membrane may be limited by diffusion through stagnant layers of medium adjacent to the cell membrane. The analysis of this problem is facilitated by assuming that the unstirred layers can be regarded as an equivalent membrane in series with the red cell membrane. The steady state permeability of the unstirred layers is defined as (House, 1974):

$$P_u = D_u \Delta x^{-1} \qquad (\text{cm s}^{-1}), \tag{6}$$

where $D_u (\text{cm}^2 \text{s}^{-1})$ is the aqueous diffusion coefficient of the solute and $\Delta x (\text{cm})$ is the operational thickness of the unstirred layers. P_u contributes to the measured permeability coefficient, P_t, as

$$P_t^{-1} = P_m^{-1} + P_u^{-1} \qquad (\text{s cm}^{-1}), \tag{7}$$

where P_m is the "true" membrane permeability coefficient. If P_m is much larger than P_t, i.e., if the movement of the solute is limited by diffusion through unstirred layers, Eqs. 6 and 7 lead to:

$$\Delta x \simeq D_u P_t^{-1} \qquad (\text{cm}). \tag{8}$$

Holz and Finkelstein (1970) and Poznansky et al. (1976) took advantage of the large n-butanol permeability of artificial lipid membranes to determine the thickness of unstirred layers in their model systems. Therefore, butanol was obviously chosen for determination of unstirred layer thickness in the dilute cell suspension moving through the flow tube.

Butanol efflux experiments were performed with 1 mM butanol at flow rates from 350 to 450 cm s^{-1}. The radioactivity in the filtrates (a_t) attained a steady state value of 0.93 (SD 0.03, $n = 21$) of the equilibrium sample within 6 ms (Fig. 3A). When the butanol concentration was increased to 100 and 250 mM (Fig. 3B), steady state radioactivity (mean 0.96, SD 0.04, $n = 42$) was found even in the first filtrate for each run. This indicates that the total diffusion barrier consisting of the serial diffusion barriers of the membrane and the adjacent unstirred layers was reduced because of the raised butanol concentration. High butanol concentrations fluidize the red cell membrane and nonspecifically increase the permeability to cations (for a review, see Seeman, 1972), to anions, and to a large nonelectrolyte such as sucrose (Wieth et al., 1974). It is thus most likely that the increased butanol permeability at >100 mM is caused by a decrease of the diffusion barrier of the membrane. Assuming that the efflux of labeled butanol follows first-order kinetics in all experiments, radioactive equilibrium is attained within 5–6 half-times. $T_{1/2}$ is
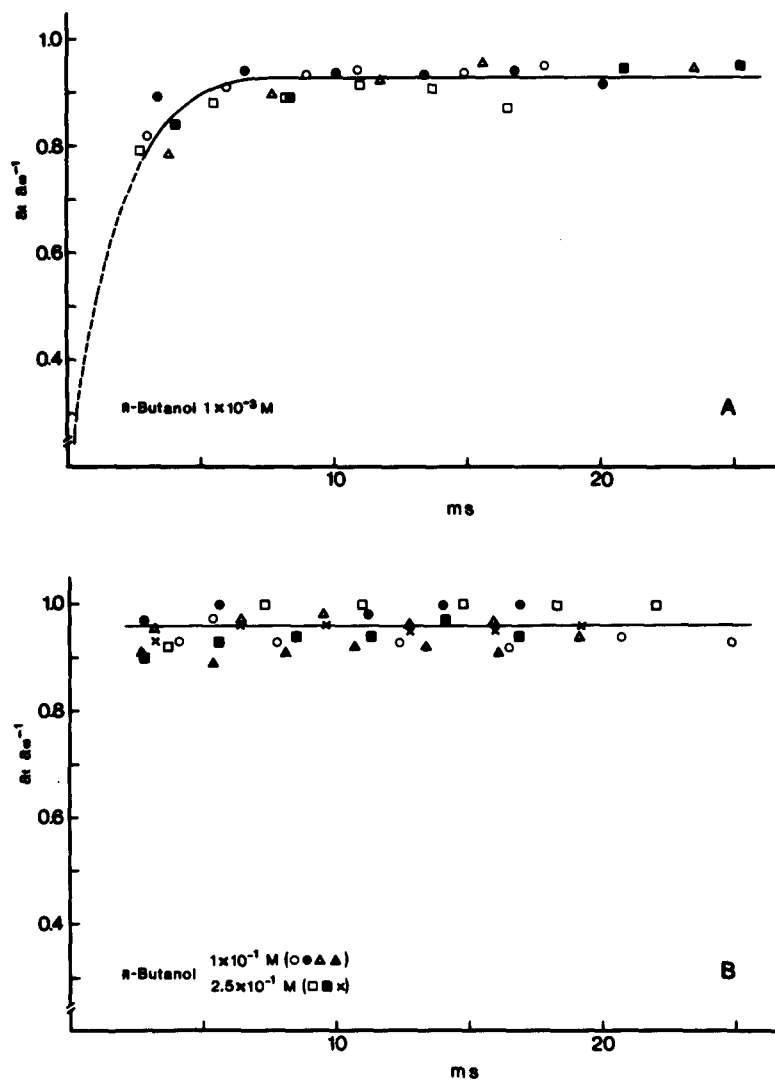
FIGURE 3. Unstirred layer thickness of a dilute red cell suspension (<0.5%) moving through the flow tube, determined by measuring the rate of [^{14}C]butanol efflux from labeled cells. A depicts results of five differentially symbolized experiments at 1 mM butanol, and B shows the results of four experiments at 100 mM butanol and three experiments at 250 mM butanol. The rate of butanol efflux was increased by raising the butanol concentration from 1 to 100 and 250 mM, because the membraneous diffusion barrier diminished at high butanol concentrations. The experiments were carried out within similar ranges of flow rates. As discussed in the text, the unstirred layer thickness can be estimated to be < 2 × 10^{-4} cm at flow velocities above 350 cm s^{-1}.

thus ~1 ms at 1 mM butanol and $\leq$0.5 ms at the higher butanol concentrations. According to Eq. 2, the respective equivalent permeability is 3×10^{-2} and $\geq 6 \times 10^{-2}$ cm s^{-1}. The increase of permeability in the high-butanol experiments is probably caused by an increase of the membrane permeability to a very large value, i.e., $P_m >> P_u \simeq P_t$. According to Eq. 6, and assuming a diffusion coefficient for butanol in water of 1×10^{-5} cm^2 s^{-1} (Lyons and Sandquist, 1953; Gary-Bobo and Weber, 1969), one finds that the permeability coefficient of the unstirred layers, P_u, could reflect diffusion of butanol through an equivalent aqueous layer with a thickness of $\leq 1.7 \times 10^{-4}$ cm. Both series of experiments were performed at the same flow rates, and one can thus calculate a P_m of butanol at 1 mM of 6×10^{-2} cm s^{-1}. The results of calculations summarized in Table I therefore suggest that the movement of butanol at 1 mM (25°C) is evenly restricted by the membrane and the adjacent unstirred layers. Furthermore, the table shows that the unstirred layer thickness increases to 1.5×10^{-3} cm when the linear flow velocity is reduced below 200 cm s^{-1}.

Sha'afi et al. (1967) determined unstirred layers to be ~6×10^{-4} cm in their stopped-flow apparatus, in which flow is stopped abruptly before the

TABLE I
ESTIMATE OF UNSTIRRED LAYERS

n-Butanol	Velocity of flow	$T_{1/2}$	P_t	P_m	Δx
mM	$cm\ s^{-1}$	ms	$cm\ s^{-1} \times 10^3$		$cm \times 10^4$
100, 250	350–450	$\leq$0.5	$\geq$61(= P_u)	∞	1.7
1	350–450	~1	30	61	1.7
1	150–200	~5	6	61	15

isolation of the test sample of blood in the observation chamber. Flow velocities before stopping were similar to the velocities used in the present study. It is therefore not surprising that the unstirred layers in their set-up were somewhat larger, because in the continuous flow apparatus the continued stirring down the observation tube implies both a delay and a lesser increase of thickness of unstirred layers (Rice, 1980).

In a recent hydrodynamic study of turbulent flow of red cell suspensions in a continuous-flow system, Gad-el-Hak et al. (1977) concluded that previous findings suggesting that the red cell membrane is rate limiting for oxygen uptake in dilute red cell suspensions can be explained by the existence of an operational unstirred layer adjacent to the cell membrane of 1.4×10^{-4} cm. From theoretical considerations of hydrodynamics and diffusion processes in continuous flow systems, Rice (1980) concluded that, depending on the design of the apparatus, the mixing time at best may be ~1 ms or a little less, and that the unstirred layers will be $1-2 \times 10^{-4}$ cm thick. The hydrodynamic estimations of unstirred layers in the two studies thus agree with the present estimate of $\leq 1.7 \times 10^{-4}$ cm at optimal experimental conditions. It must be noted that even at optimal conditions of mixing, the intracellular solvent

volume remains unstirred. Thus, the operational unstirred layers of $\leqq 1.7 \times 10^{-4}$ cm in part reflects an intracellular diffusion barrier of half the red cell thickness of 0.5–1.2×10^{-4} cm.

The butanol experiments illustrate that the movement of solute across the red cell membrane may be partially or totally rate controlled by diffusion through unstirred regions adjacent to the two sides of the membrane. An operational thickness of $\sim 2 \times 10^{-4}$ cm constitutes a diffusion barrier with an equivalent permeability of 5×10^{-2} cm s^{-1}, assuming a diffusion coefficient for the solute of 1×10^{-5} cm^2 s^{-1}. A measured permeability coefficient of 5×10^{-3} cm s^{-1} will be $\sim 10\%$ less than the corresponding membrane permeability coefficient. The measured permeability coefficients can be corrected for unstirred layers, but the flow tube is still unsuitable for determinations of permeability coefficients above 10^{-2} cm s^{-1}, because a reliable determination of the rate of tracer efflux cannot be obtained.

The Permeability of Red Cells to Aliphatic Alcohols

The permeability coefficients of human red cells to the straight-chained aliphatic alcohols were determined at steady state conditions (cf. Methods). Fig. 4 shows tracer washout curves for the different alcohols. The permeability coefficients for the different alcohols are proportional to the slopes of the curves, which vary with the chain length. The variations in permeability coefficient, however, are not a monotonous function of the chain length, as shown in Fig. 5. The minimum permeability coefficient, 2.1×10^{-3} cm s^{-1}, was obtained with ethanol. The permeability coefficient increased when the chain length decreased to methanol (P^{meth} 3.7×10^{-3} cm s^{-1}), and when the chain length increased to n-propanol (P^{prop} 6.5×10^{-3} cm s^{-1}) and n-butanol ($P^{but} \geqq 60 \times 10^{-3}$ cm s^{-1}; cf. THE CONTINUOUS FLOW-TUBE METHOD). The increase of permeability did not proceed beyond this point, however, as the permeability for n-hexanol was 8.7×10^{-3} cm s^{-1}. These permeabilities were all obtained at an extracellular alcohol concentration of 1 mM. The permeability coefficients for methanol, ethanol, and propanol were concentration independent between 1 and 500 mM (Figs. 4 and 5). (Results at 100 mM are not shown.) The concentration-independent permeability coefficients indicate that transport of these alcohols proceeds by simple diffusion. A different picture was seen with the higher alcohols. When the butanol concentration was increased to 100 and 250 mM, or the hexanol concentration was increased to 25 mM, the rate of self-exchange increased to values above the upper detection limit of the continuous flow tube. The permeability increase suggests that the erythrocyte membrane can be modified by raising the alcohol concentrations, possibly by a fluidizing effect of butanol and hexanol on the membrane lipids (see Seeman, 1972), which may reduce the diffusion barrier of the membrane to these alcohols.

The fully drawn part of the curve of Fig. 5 is qualitatively similar to the permeability pattern described for the homologous series of amides, from formamide through valeramide, in red blood cells both from human and dog (Sha'afi et al., 1971; Galey et al., 1973; Naccache and Sha'afi, 1973), and in

planar lipid bilayer membranes and phospholipid vesicles (Cohen, 1975; Finkelstein, 1976; Poznansky et al., 1976). The permeability pattern for the larger amides has not been determined. But results similar to those presented here have been obtained for alcohol permeability of dog red cells (Garrick et al., 1980).[1] The permeability patterns for the amides were interpreted (Sha'afi et al., 1971; Sha'afi and Gary-Bobo, 1973) to indicate that the red cell membrane contains both a hydrophilic and a lipophilic pathway, in accordance with the suggestion by Collander and Bärlund (1933) for plasma
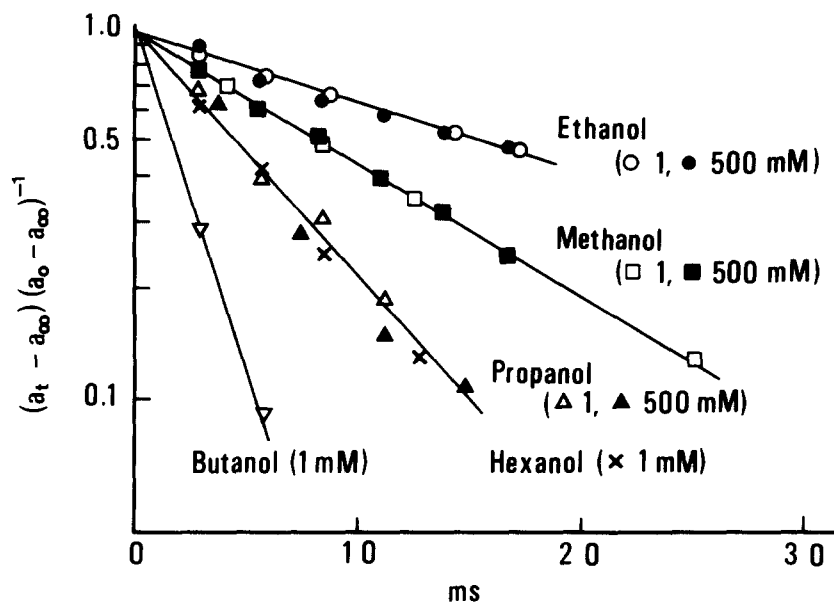

FIGURE 4. The rate of self-exchange of the homologous aliphatic alcohols in human red cells at pH 7.2 and 25°C. The logarithmic ordinate expresses the fraction of tracer that remains in the cells at a given time. The slope of the curves is equal to the rate coefficient of tracer washout (k, s^{-1}) and directly proportional to the permeability coefficient $(P = kVA^{-1}, cm\ s^{-1}, Eq.\ 2)$. The permeability to methanol, ethanol, and n-propanol did not change in the concentration range 1–500 mM (for clarity, experiments performed at 100 mM are not shown). The permeability to butanol and hexanol exceeded the upper limit of determination when the alcohol concentrations were 100 and 25 mM, respectively. The implications are discussed in the text.

membranes. The permeability for the hydrophilic molecules should be determined primarily by molecular size, whereas the permeability for lipophilic molecules should be determined primarily by their lipid solubility. The present

[1] In human red cells Naccache and Sha'afi (1973) determined a gradual decrease of permeability to methanol through butanol, by means of the hemolysis method. The prolonged hemolysis time found with 0.3 M butanol was erroneously interpreted as a decreased permeability with increasing chain length. Butanol nonspecifically increases the membrane permeability to ions, and the prolonged hemolysis time is thus in part due to the increased potassium leak by 0.3 M butanol.

results show clearly that the alcohol permeability is not simply a function of the solute lipophilicity (Table II), as would be predicted from Overton's rule. The permeability coefficient for methanol was more than four times larger than expected from an extrapolation of the pattern seen with ethanol through butanol. The hexanol permeability coefficient, on the other hand, decreased
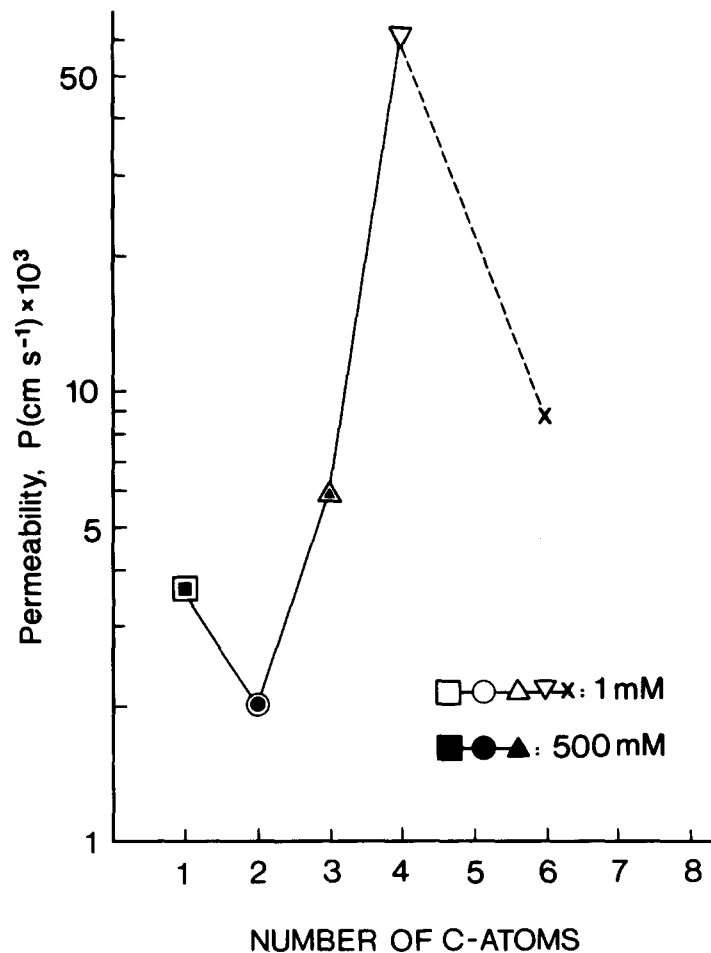

FIGURE 5.   The membrane permeability of human red cells to the homologous series of aliphatic alcohols is depicted as a logarithmic function of the number of carbon atoms in the alcohol molecule. The butanol permeability coefficient plotted in the figure represents the lower limit for butanol permeability.

and was much lower than predicted from the data obtained with the lower alcohols. These results raise three questions: (a) are the permeability coefficients reported here indeed correct?; (b) is the permeability pattern seen here a consequence of the interplay between two (or more) permeability pathways?; and (c) what is the basis for the concentration-dependent permeability coefficients seen with butanol and hexanol?

The methanol permeability has been determined in human red cells by Savitz and Solomon (1971), using a continuous flow method, to be 3.2×10^{-3} cm s^{-1} without correction for unstirred layer effects, and in dog red cells to be 4.7×10^{-3} cm s^{-1} by Garrick et al. (1980) using the entirely different method of linear diffusion through a column of packed cells. The available results are thus in excellent agreement. This does not exclude systematic errors, but this becomes a less likely possibility.

The relative larger permeability of human red cells to methanol raises the question: is a large fraction of methanol transported by a hydrophilic path assumed to transport the hydrophilic formamide, acetamide, and urea (Sha'afi et al., 1971), or is the larger methanol permeability caused by a smaller steric hindrance on diffusion of the small molecule through the lipid bilayer leaflet of the membrane, in analogy with the interpretation of the amide permeability pattern in nonporous artificial lipid membranes (Poznansky et al., 1976).

Although permeation through the hydrophilic and the lipophilic pathways

TABLE II

PERMEABILITY COEFFICIENTS OF ALIPHATIC ALCOHOLS
THROUGH THE RED CELL MEMBRANE AT 25°C, pH 7.2

	P_m	$\alpha \times 10^2$	D_{H_2O}	$P_m\,\alpha^{-1} \propto D_m$
	cm s$^{-1} \times 10^3$		cm^2 s$^{-1} \times 10^5$	
Methanol	3.7*	1.0‡	1.70§	3.70
Ethanol	2.1	3.5	1.26	0.60
n-Propanol	6.5	15.5	1.15	0.42
n-Butanol	≧61	63	1.00	≧0.97
n-Hexanol	8.7	750	0.83‖	0.012

* Corrected for diffusion through unstirred layers.

‡ Olive oil/water partition coefficients; from Lindenberg (1951).

§ Diffusion coefficients in water at room temperature; from Gary-Bobo and Weber (1969).

‖ Calculated from $D^{hexanol} = D^{butanol} \sqrt{\text{mol wt}^{butanol}\,(\text{mol wt}^{hexanol})^{-1}}$.

are not mutually exclusive, Sha'afi et al. (1971) suggested that nonelectrolytes could be classified according to the arbitrarily chosen ether/water partition coefficients for the nonelectrolytes relative to the partition coefficient for water ($\alpha^{H_2O}_{ether}$ 0.003). It should be interposed that Finkelstein (1976) emphasized that one type of hydrophobic solvent in preference to the other as a model for "membrane solubility" appears not to be of substantial importance, because the ratio of nonelectrolyte solubility changes but little for different solutes. If the relative accessibility of hydrophilic and lipophilic pathways to nonelectrolytes can be related to the nonelectrolyte partition coefficients (for a discussion, see Macey, 1979), the fraction of methanol transport through a hydrophilic path must be small. The ether/water distribution coefficient for methanol is 0.14, two orders of magnitude larger than the coefficient for the hydrophilic formamide of 0.0014 (Collander, 1949), and is as large as the distribution coefficient for valeramide (Sha'afi et al., 1971), whose permeation predominantly through a lipophilic path is generally accepted.

Inhibitors of transport of hydrophilic nonelectroytes or water did not inhibit methanol permeability (Table II), which supports the conclusion that methanol predominantly permeates the lipid core of the membrane. In human red cells *p*-chloromercuribenzene sulfonate (PCMBS) inhibits osmotic and diffusional water permeability and permeability to formamide, acetamide, and urea, whereas phloretin inhibits the permeability to formamide, acetamide, and urea, but not to water (Macey and Farmer, 1970; Macey et al., 1972; Owen and Solomon, 1972; Kaplan et al., 1974; Naccache and Sha'afi, 1974; Owen et al., 1974; Wieth et al., 1974; Kaplan et al., 1975; Brahm, 1982, 1983). However, neither compound inhibits methanol permeability.

Urea and thiourea are transported by facilitated diffusion and compete for the same transport system (Wieth et al., 1974; Brahm, 1983) that apparently also transports formamide and acetamide (Solomon and Chasan, 1980). In accordance with the lack of inhibition by PCMBS and phloretin, the inhibitors of urea and thiourea transport, methanol permeability was not reduced significantly by 500 mM urea, which turns off the saturable component of thiourea transport completely (Wieth et al., 1974). Methanol permeability was reduced 40% by 500 mM thiourea, which is far above the concentration of 13 mM thiourea that causes half-inhibition of urea transport (Brahm, 1983). The reduction of methanol permeability by high thiourea concentrations might be due to thiourea forming hydrogen bonds with membrane lipids. According to Stein (1967), urea has the same hydrogen bonding ability as thiourea. The different effects of urea and thiourea might suggest that the thiourea concentration in the membrane phase is >10 times larger than the urea concentration at equal aqueous concentations, as the olive oil/water distribution coefficient for thiourea is >10 times higher than the distribution coefficient for urea (Collander, 1949).

The present results thus suggest that methanol predominantly permeates via the lipid core of the human red cell membrane. Indirect support for this conclusion was found in methanol experiments with chicken erythrocytes. In the chicken red cell membrane, the equally low osmotic and diffusional water permeability and the urea permeability (Farmer and Macey, 1970; Brahm and Wieth, 1977) are as low as the water and urea permeability coefficients of nonporous, cholesterol-containing bilayer membranes. The methanol permeability of chick red cells was, however, even larger (4.4×10^{-3} cm s^{-1} at 25°C; J. Brahm, unpublished result) than the methanol permeability of human red cells, probably because of the different lipid composition of the erythrocyte membrane from the two species.

Fig. 5 shows that hexanol permeability decreased steeply though the lipophilicity was increased further (cf. Table II). The permeability pattern of Fig. 5 illustrates that the assumption that the relative permeability coefficients for lipophilic solutes are determined by the relative distribution coefficients between the membrane phase and the medium is an over-simplification of the permeability concept originally suggested by Overton (1895). The concept implies that the ratio between the diffusion coefficients in the membrane and in aqueous solutions is equal for all nonelectrolytes considered (Orbach and

Finkelstein, 1980). The permeability data of Table II disagree with this assumption. The phenomenological permeability coefficient obviously may also be determined by other factors than the partition coefficient. Lieb and Stein (1969) suggested that nonelectrolyte permeability in biological membranes could be explained in terms used to describe diffusion across synthetic polymeric sheets, where diffusion can be related to the relative molecular weight of the diffusant. The importance of the molecular weight for the permeability coefficient has been questioned (Sha'afi and Gary-Bobo, 1973). Isomeric amides with identical molecular weight and very similar partition coefficients have a lower permeability coefficient if the molecule is branched, which suggests that steric factors may determine the relative permeability. It should be noted, however, that the concept of Lieb and Stein (1969) is not an alternative to, but rather an extension of, the simple lipid solubility-permeability concept (for a discussion, see Andersen, 1978), taking into account that diffusion coefficients in the membrane cannot necessarily be deduced from the value obtained in aqueous solutions. The observation by Sha'afi and Gary-Bobo (1973) confirms the importance of physicochemical properties of the molecule and the membrane components for the diffusion process through the lipid phase of the membrane.

By a rise of hexanol concentration to 25 mM the hexanol permeability coefficient increased above the upper detection limit (results not shown), as was the butanol permeability coefficient at butanol concentrations of 100 and 250 mM (Fig. 3B). It is not likely that a concentration-dependent increase of distribution coefficients for the alcohols would account for the increase of permeability coefficients. The fluidizing effect of the larger aliphatic alcohols on the membrane lipids (Seeman, 1972) suggests that the increased butanol and hexanol permeability coefficients are caused by a decreased diffusion resistance of the lipid phase. Accordingly, hexanol also increased the permeability to the model nonelectroyte 2-(3'-thioglyceryl)-N-ethylmaleimide (TG-MalNEt) by a factor of two (Wilbers et al., 1979) and to a lesser extent it increased methanol permeability (Table III). A similar increase of methanol permeability was found with 1 mM octanol, whereas even 250 mM butanol, which increased butanol's own permeability, showed no effect (Table III). The lack of effect by butanol cannot be due simply to the lower distribution coefficient for butanol (Table III), because aqueous solutions of 250 mM butanol and 25 mM hexanol result in similar concentrations in the membrane phase. The different effect by butanol and the more lipophilic alcohols on methanol permeability is surprising and may reflect that the membrane disorder created by butanol differs from the disorder produced by the more lipophilic solutes (for discussion, see Finean, 1973), such as hexanol and octanol.

It has previously been shown that phloretin doubles the permeability coefficients of hydrophilic amides in nonporous lipid bilayer membranes (Andersen et al., 1976; Poznansky et al., 1976), and the permeability coefficients of 2,3-butanediol (Owen and Solomon, 1972) and TG-MalNEt (Wilbers et al., 1979) in human red cells. Phloretin also increased propanol permeability

above the upper detection limit (results not shown), whereas the effect on methanol permeability was small (Table II). It thus appears that the diffusion coefficient in the lipid phase of the red cell membrane for methanol, for unknown reasons, shows less susceptibility to the fluidification of membrane lipids by phloretin than the diffusion coefficients both for the more hydrophilic and more lipophilic nonelectrolytes.

The present results of the alcohol permeability of human red cells and the similar permeability pattern in dog red cells (Garrick et al., 1980) show that nonelectrolyte permeation through the lipid phase of the cell membrane is determined both by the lipid solubility and by the diffusion coefficient in the membrane for the nonelectrolyte, the relative importance of the two factors varying by the extension of the carbon chain length.

TABLE III

METHANOL PERMEABILITY OF HUMAN RED CELLS AT 25°C AND pH 7.2

$(C_0^{meth} = 1 \text{ mM})$

Compound	Concentration	$P^{meth} \times 10^3$	Range	$P^{meth}_{relative}$
	mM	*cm s^{-1}*		
Control		3.7	3.1–4.2	1.00
			(SD 0.4, $n = 11$)	
PCMBS	1	3.6	3.5–3.7	0.97
Thiourea	15	3.6	3.3–3.8	0.97
—	100	2.8	2.8	0.74
—	500	2.3	2.1–2.7	0.62
Urea	100	3.5	3.5	0.94
—	500	3.2	2.9–3.5	0.86
Salicylate	10	3.0	3.0–3.1	0.80
Phloretin	0.25	4.3	4.2–4.4	1.15
—	0.50	4.5	4.5	1.21
n-Butanol	100, 250	3.6	3.4–4.1	0.97
n-Hexanol	25	5.3	5.0–5.7	1.41
n-Octanol	1	5.6	4.9–5.8	1.50

The methanol permeability coefficient in the presence of the different compounds was determined in two or more efflux experiments.

The permeability coefficients are corrected for diffusion through unstirred layers.

TEMPERATURE DEPENDENCE OF ALCOHOL PERMEABILITY It is a general finding that transport of nonelectrolytes and water by simple diffusion through the lipid phase of biological membranes and bilayer lipid membranes show high activation energies (Wartiovaara, 1949; Price and Thomson, 1969; Redwood and Haydon, 1969; Macey et al., 1972; Galey et al., 1973; Cohen, 1975; Bindslev and Wright, 1976; Poznansky et al., 1976; Brahm and Wieth, 1977; Brahm, 1982, 1983). This was also the case for transport of aliphatic alcohols in human red cells. The permeability to methanol, propanol, and hexanol was determined at 1 mM in the temperature range 5–25°C. The results depicted in the Arrhenius diagram of Fig. 6 show that the apparent activation energy of permeation was equally high, 50–63 kJ mol^{-1}, for all three alcohols. This finding stresses that the three alcohols, whose relative

lipophilicity is 1:16:750 (Table II), most likely are transported through the same pathway. Furthermore, the curves of Fig. 6 suggest that one apparent activation energy of the transport processes determines the rate of transport in the whole temperature range. In contrast, in a recent temperature study of lipophilic solute permeation in dog red cells, Garrick et al. (1982) found that the activation energy of hexanol permeability changed dramatically from 11 kJ mol^{-1} between 37 and 20°C (their Table III) to 133 kJ mol^{-1} between 20
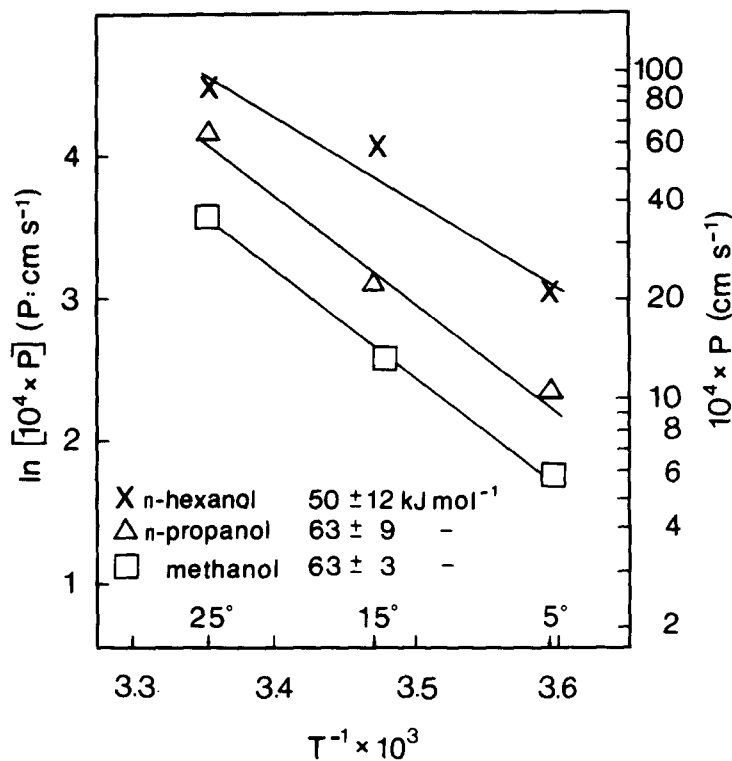

FIGURE 6. Temperature dependence of methanol, n-propanol, and n-hexanol permeability in human red cells at 5–25°C, pH 7.2. The Arrhenius diagram depicts the logarithm of the permeability vs. the reciprocal of absolute temperature. The activation energies were calculated by linear regression analysis of the relation: $\ln P = E_A(RT)^{-1} + \text{constant}$. E_A is the activation energy (J mol^{-1}), R is the gas constant (8.31 J [mol K]$^{-1}$), and T is the absolute temperature (K). The same temperature dependence was found for all three alcohols, although the relative lipophilicity is 1:15:750 by the increase of chain length.

and 15°C (calculated from their Table II). It should be noted that their study included determinations of diffusional water permeability whose activation energy also changed, from 22 kJ mol^{-1} between 37 and 15°C to 54 kJ mol^{-1} between 15 and 10°C. Both sets of results differ from the activation energy of hexanol permeability (Fig. 6) and the constant activation energy of diffusional

water permeability (Brahm, 1982) in human red cells. Hence, the different temperature dependence of hexanol and water permeability in dog and in human red cells may be due to different properties of the two red cell species or may have its origin in the different methods used.

RATE-LIMITING STEP FOR ALCOHOL PERMEATION   At present no evidence is available establishing where the rate-limiting step in alcohol transport through the membrane lipids is located. In its simplest form, permeation through the membrane bilayer leaflet can be separated into three steps: (*a*) adsorption of the solute at the interfacial region between the aqueous solution and the membrane lipids; (*b*) diffusion through the different regions of the hydrocarbon chains; and (*c*) desorption of the solute on the other side of the membrane. As pointed out by Andersen (1978), for permeation through a homogenous membrane, "Permeability coefficients will be determined by whatever part of a membrane represents the largest barrier for solute movement. This barrier may be due to a low concentration, to a low mobility, or to both." The outer regions of the membrane hydrocarbons are generally believed to be less fluid than the membrane interior and may therefore exert a sieving effect on the permeation of lipophilic solutes. According to this interpretation, the small methanol molecule passes the same rate-limiting barrier as the larger hexanol molecule, which agrees with the observation that the apparent activation energy of the transport processes were similar (Fig. 6). If the outer hydrocarbon regions represent a steric hindrance to the permeation of lipophilic solutes, the relatively larger methanol permeability and the relatively lower hexanol permeability reflect the ability of the barriers to discriminate between lipophilic permeants according to their molecular size. Accordingly, 25 mM hexanol, presumably because of its fluidizing effect on these regions of the membrane lipids, reduces the discriminating property of the regions, so that methanol permeability increased somewhat and hexanol permeability increased above the upper detection limit.

Träuble (1971) has suggested that thermal fluctuations in the hydrocarbon chains of the membrane lipids, "kinks," may accomodate the transport of small molecules. It is, however, unlikely that Träuble's model can account for the relative large methanol permeability. The kink mechanism implies an ordered structure of the hydrocarbon phase (Träuble, 1971). This is highly questionable for the interior of a bilayer, which appears to have a much less ordered structure than previously anticipated (Andersen, 1978; Fettiplace and Haydon, 1980). The fact that methanol permeability was increased by 25 mM hexanol and 1 mM octanol, which increase the degree of disorder of the membrane lipids further, makes it unlikely that kink formations of the hydrocarbon chains mediate transport of low molecular weight solutes.

The present study shows that the permeability of a homologous series of lipophilic nonelectrolytes is not a simple function of the relative lipophilicity of the permeants. The observed deviations from Overton's solubility rule stress that the alcohol permeability coefficients are determined both by the lipophilicity and by the mobility in the membrane, the relative importance of the two factors varying with the increase of the molecular chain length.

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