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Modification of the Erythrocyte Membrane Dielectric Constant by Alcohols

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Summary. Aliphatic alcohols are found to stimulate the transmembrane fluxes of a hydrophobic cation (tetraphenylarsonium, TPA) and anion (AN-12) 5–20 times in red blood cells. The results are analyzed using the Born-Parsegian equation (Parsegian, A., 1969, *Nature (London)* **221**:844–846), together with the Clausius-Mossotti equation to calculate membrane dielectric energy barriers. Using established literature values of membrane thickness, native membrane dielectric constant, TPA ionic radius, and alcohol properties (partition coefficient, molar volume, dielectric constant), the TPA permeability data is predicted remarkably well by theory. If the radius of AN-12 is taken as 1.9 Å, its permeability in the presence of butanol is also described by our analysis. Further, the theory quantitatively accounts for the data of Gutknecht and Tosteson (Gutknecht, J., Tosteson, D.C., 1970, *J. Gen. Physiol.* **55**:359–374) covering alcohol-induced conductivity changes of 3 orders of magnitude in artificial bilayers. Other explanations including perturbations of membrane fluidity, surface charge, membrane thickness, and dipole potential are discussed. However, the large magnitude of the stimulation, the more pronounced effect on smaller ions, and the acceleration of both anions and cations suggest membrane dielectric constant change as the primary basis of alcohol effects.

Key Words red blood cell · dielectric constant · permeability · hydrophobic ions · alcohols · bilayer · Born energy

Introduction

Lipid bilayers are virtually impermeable to most ions because the electrostatic energy of the ion is much lower in a water medium with high dielectric constant (~80) than in a typical bilayer with low dielectric constant (~2). Only one ion class, the hydrophobic ions, readily permeates lipid bilayers. These ions are large organic structures, which would have little affinity toward water were they not charged. In phospholipid bilayers their permeabilities are 10^{-9} to 10^{-6} cm/sec (cations) or 10^{-3} to 10^{1} cm/sec (anions) in contrast to values around 10^{-12} cm/sec for common inorganic ions (Flewelling, 1984). The measurements and analysis of Ketterer, Neumcke and Läuger (1971) have provided a successful model for hydrophobic ion transport in terms of a single energy barrier within the membrane. More recently Flewelling and Hubbell (Flewelling, 1984; Flewelling & Hubbell, 1986; Honig et al., 1986) have extended these ideas. Current models generally attempt to account for the following forces:

1. *Surface potentials* produced by fixed charges at the membrane boundary (McLaughlin et al., 1970; Theuvenet & Borst-Pauwels, 1976);
2. The *dielectric energy* difference for an ion between water and membrane phases (Born, 1920; Neumcke & Läuger, 1969; Parsegian, 1969; Dilger et al., 1979; Flewelling, 1984);
3. *Dipole potentials* caused by oriented molecular dipoles near the surface of the bilayer (Szabo, 1974; Andersen et al., 1976);
4. *Hydrophobic interactions*.

In addition to these forces, membrane fluidity (Shinitzky, 1984) and thickness (Benz & Läuger, 1977; Dilger, Fisher & Haydon, 1982) may also play a role.

In previous investigations we have shown that the hydrophobic cation, tetraphenylarsonium (TPA), penetrates the erythrocyte membrane by simple diffusion, and that it may be used as a probe of the electrostatic properties of the red cell bilayer. In addition, the transport kinetics of TPA in red cells can be easily accounted for in terms of a single energy barrier, but not by a model based on the Goldman equations (Macey & Orme, 1980; Hunziker, Orme & Macey, 1985). In this paper we examine the effects of higher alcohols on the permeability properties of red cells to hydrophobic cation and anion permeants. Butanol stimulation of red cell K^+ permeability has been appreciated for many years (Ponder, 1948; Jacobs, 1951), and we find a corresponding stimulation of hydrophobic ion permeation amounting to some five- to 20-fold increase above the normal untreated membrane.

Our analysis of these results was prompted by papers of Reyes and Latorre (1979), and Dilger et al. (1979). These authors used the Born-Parsegian equation (Parsegian, 1969) to explain solvent-induced permeability changes in planar lipid membranes on the basis of alterations in the membrane dielectric constant. Lacking a method for measuring changes in dielectric constant, we estimated it by using the Clausius-Mossotti equation together with published estimates of alcohol partitioning into red cell membranes. Using these estimates together with the Born-Parsegian equation, allows us to predict the dependence of hydrophobic ion permeability on alcohol concentration. The prediction agrees remarkably well with our experimental measurements. In addition, these same considerations are able to quantitatively account for the very large alcohol-induced conductance increases in sheep erythrocyte lipid bilayers reported by Gutknecht and Tosteson (1970). Some of this material has been presented in a preliminary report (Macey & Orme, 1984).

Theory

Our object is to calculate the alcohol-induced change in permeability of a hydrophobic ion. We begin by relating the aqueous concentration of alcohol, C, to the volume it occupies within the membrane. Let C_m denote the concentration of alcohol within the membrane, and let K denote the membrane/water partition coefficient, then $C_m = KC$. If v_a is the molar volume of the alcohol, the membrane volume fraction $f(C)$ will be given by

$$f(C) = v_a C_m = v_a KC. \tag{1}$$

From $f(C)$, the membrane dielectric constant $D_m(C)$ can be calculated using the Clausius-Mossotti equation for liquid mixtures (Benedek & Villars, 1979) as follows:

$$D_m(C) = \frac{(2Q + 1)}{(1 - Q)} \tag{2}$$

where:

$$Q = f(C)\frac{(D_a - 1)}{(D_a + 2)} + (1 - f(C))\frac{(D_m(0) - 1)}{(D_m(0) + 2)}.$$

D_a and $D_m(0)$ are the dielectric constants of the alcohol and native membrane, respectively.

The value of $D_m(C)$ can then be used to estimate the work $E(C)$ of moving an ion of radius r from an aqueous solution to the center of the membrane with thickness x_m. Denoting the dielectric constant of water by D_w, the Born-Parsegian formulation (Parsegian, 1969) gives:

$$E(C) = \frac{N(ze)^2}{2r}\left[\frac{1}{D_m(C)} - \frac{1}{D_w}\right] - \frac{N(ze)^2}{x_m D_m(C)} \ln \frac{2D_w}{(D_m(C) + D_w)} \tag{3}$$

where N is Avogadro's number, z is the ionic valence, and e is the electronic charge. [For further refinements *see* Neumcke and Läuger (1969), and Flewelling and Hubbell (1986).]

Finally, we utilize this last result by assuming that transport is limited by a single sharp energy barrier at the center of the membrane. The permeability will be exponentially related to the height of the electrical barrier by

$$P(C) = P_o \exp\left[\frac{-E(C)}{RT}\right] \tag{4}$$

where $E(C)$ is the dielectric energy calculated from Eq. (3), and P_o is equivalent to the membrane permeability for an identical but uncharged form of the permeant. To compare the permeability ratio in the presence or absence of alcohol, $P(C)/P(0)$, we assume that P_o is independent of alcohol and obtain:

$$\frac{P(C)}{P(0)} = \exp\left[\frac{-(E(C) - E(0))}{RT}\right]. \tag{5}$$

If the values of K, v_a, D_w, D_a, $D_m(0)$, and x_m are known (e.g. *see* the Table), then Eq. (5) predicts the relative change of permeability as a function of alcohol concentration. From substitution of the Parsegian term for the dielectric energy, it is apparent that the permeability ratio will be a function of the change in dielectric constant, the radius of the permeant ion, and changes in membrane thickness. (The assumption that P_o is independent of alcohol in the membrane is reviewed in the Discussion.)

Materials and Methods

Tetraphenylarsonium (TPA) chloride was obtained from I.C.N. Pharmaceuticals, Plainview, N.J. The spin probe, AN-12 (*see below*), was a generous gift of Rolf Melhorn of the University of California, Berkeley. All other chemicals were of reagent grade or better.

Fresh blood was obtained from human donors and washed three times by centrifugation in isotonic saline. For TPA efflux experiments, 20 to 30 μM TPA was added to a 30% cell suspension. After a 30-min incubation at room temperature, the cells were packed by high-speed centrifugation and the supernatant aspirated.

Table. Parameters used to calculate ionic permeability ratios from the Born-Parsegian equation

Parameter	Value at 22°	Value at 30°	Reference
Membrane dielectric constant	2.0–2.2	2.0–2.2	Requena & Haydon (1975)
Membrane thickness, Å	30	30	Cornell & Separovic (1983)
Ionic radius of TPA, Å	4.2	4.2	Marcus (1985)
Ionic radius of K, Å	1.33	1.33	Marcus (1985)
Dielectric constant of water	79.4	76.5	Malmberg & Maryott (1956)
Dielectric constant:			
Butanol	18.0	17.1	D'Aprano et al. (1979)
Hexanol	13.5	12.8	
Octanol	9.7	9.0	
Molar volume, cc/mole:			
Butanol	91.8	92.4	Small (1986)
Hexanol	124.9	125.8	
Octanol	158.0	159.1	
Membrane/water partition coefficient:			
Butanol	1.5	1.5	Seeman (1972)
Hexanol	13.0	13.0	
Octanol	151.8	151.8	
$\frac{N(ze)^2}{RT}$	283.1	275.6	

Erythrocyte ghosts were made by a slight modification of the technique of Funder and Wieth (1976). Hemolysis was achieved by mixing one volume of cells (at 50% hematocrit) with nine volumes of 4 mM $MgSO_4$ + acetic acid. The acetic acid concentration was adjusted to give a pH of 5.9 to 6.2 after mixing (Schwoch & Passow, 1973). For fresh cells addition of 4 mM acetic acid was required, while 1 to 2 mM was adequate for blood bank blood. Instead of adding concentrated salt to raise the ionic strength just prior to resealing (Funder & Wieth, 1976), the cells were spun at 48,000 × *g* for 4 min and the supernatant was removed. Salt solutions just above the isotonic level buffered at pH 7.4 were then added. All operations to this point were done at 0°C. The cells were then sealed in the usual way by incubating 1 hr at 37°C. This procedure had the advantage that it gave a more complete wash-out of intracellular material.

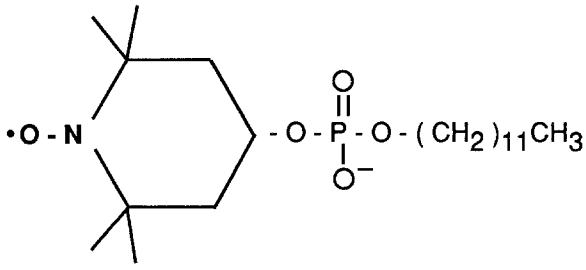
Equilibrium membrane potentials were calculated from the external and internal pH as described by Macey, Adorante and Orme (1978). The TPA and AN-12 experiments were done under conditions where little change in membrane potential would be expected, and monitoring of extracellular pH (Macey et al., 1978) showed that this was the case.

TPA-selective electrodes were made, as described previously (Hunziker et al., 1985), by casting a polyvinyl chloride membrane across the tip of a disposable pipette tip. Electrodes contained 2 mM of the tetraphenylboron salt of TPA dissolved in the membrane plasticizer (dibutylphthalate). They consistently had slopes above 55 mV for a 10-fold concentration change in range of 0.1 μM to 1 mM TPA.

The membrane volume fraction of alcohol was calculated using Eq. (2). Partition coefficients for red cell ghosts were taken from Seeman, Roth and Schneider (1971). The butanol partition coefficient was 1.5 (*see* Discussion). For butanol the molar volumes are 91.8 and 92.4 cc/mole at 22 and 30°C, respectively (Small, 1986). The highest butanol volumes used in this study, corresponding to an aqueous concentration of 320 mM, were 4.4% in the membrane. Temperature corrections for the dielectric constants of the alcohols were made from the data of D'Aprano, Donato and Caponetti (1979) and Lawrence, McDonald and Stevens (1969). For butanol dielectric constants of 18.0 and 17.1 at 22 and 30°C, respectively, were calculated by extrapolation of the literature data. For purposes of initial calculation the native membrane dielectric constant was assumed to be 2.1. From the data of Cornell and Separovic (1983) an estimate of 30 Å for the red cell hydrocarbon thickness was obtained. These parameters along with data for hexanol and octanol are summarized in the Table.

TPA efflux was measured from previously loaded cells at 30°C, using ion-selective electrodes. Time constants were determined by a least-squares procedure and the permeabilities calculated as previously described (Macey & Orme, 1980; Hunziker et al., 1985). Bound TPA was calculated from the difference between free measured concentrations in cells, and the total added concentration. All of our experiments were done in the range where TPA binding is a linear function of concentration (Macey & Orme, 1980; Hunziker et al., 1985).

Electron spin resonance experiments were performed using the hydrophobic anion nitroxide spin probe designated AN-12 (drawing below). The negative charge is furnished by the phosphate group separating the alkyl chain from the nitroxide ring. The spin probe was synthesized by Rolf Melhorn (Lin, Macey & Melhorn, 1983). Probe concentration was kept at 50 μM or less. Concentrations greater than 200 μM were found to cause some hemolysis.



Measurements were made on an IBM EPR spectrometer, model #ER 200D-SRC. Microwave power was 9.9 mW, klystron

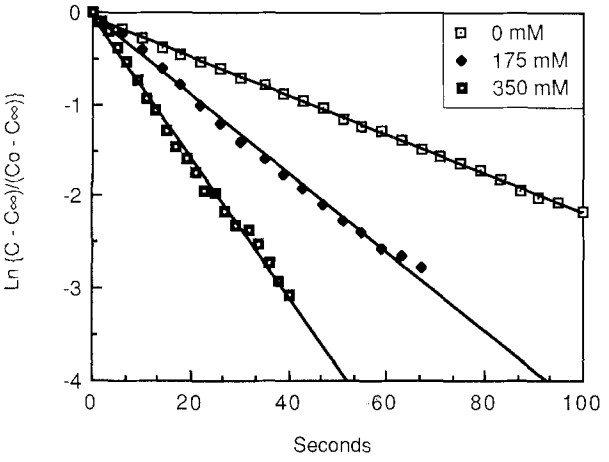


Fig. 1. Logarithmic plot of TPA efflux from red blood cells at different butanol concentrations (*see* legend). Efflux measurements were made at 30°C with ion selective electrodes, as outlined in the text. Symbols: C = external concentration of TPA; C_o = initial external concentration; C_{oo} = final external concentration

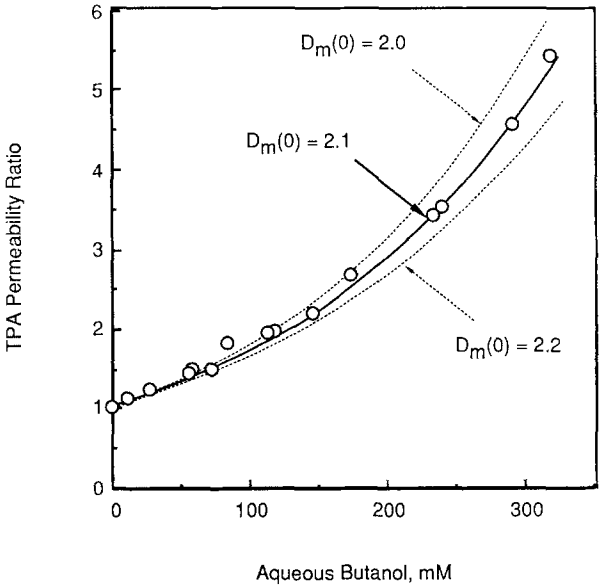


Fig. 2. The Born-Parsegian theory fits TPA permeability ratio data best if the initial membrane dielectric constant is taken to be 2.1 (middle curve). If the initial dielectric constant is assumed to be 2.0 (upper curve) or 2.2 (lower curve) there is a clear divergence from the experimental points. Membrane thickness is assumed to be 30 Å for all three curves

frequency was 9.78 GHz and the spectra were recorded at 3480 ± 50 gauss. Kinetic data was obtained using either one of two simple stop-flow devices. In some cases hand operated coupled syringes were used. Cells in one syringe were challenged with isosmotic solutions containing AN-12 with or without butanol. In other cases a solenoid controlled vacuum aspiration system capable of resolving time constants as fast as 0.2 sec was used. In each case approximately 1 ml of cells (15 to 20% hct.) and challenge solution were mixed, passed through a 1-mm diameter glass capillary in the EPR cavity, and the flow stopped. Fluid velocity was typically 5 meters/sec. AN-12 fluxes were determined from the rate of disappearance of the high field line which is very strongly quenched when the probe is bound to the red cell bilayer (*see* Results).

Most experiments were done with red cell ghosts since whole cells contain nitroxide reducing agents (probably glutathione) which destroy the probe with a time constant of 35 to 70 min. In addition the large intracellular pool of hemoglobin binds significant amounts of AN-12 complicating the kinetic measurements.

Results

Butanol Potentiation of TPA Permeability

TPA flux from preloaded red blood cells follows a simple exponential process which allows calculation of permeability coefficients from time constants (Macey & Orme, 1980; Hunziker et al., 1985). Time constants in the present experiments (at 15% hematocrit) varied from 45 to 93 sec in blood bank blood and from 104 to 150 sec in fresh blood. Average permeabilities calculated from these results are 9.7×10^{-7} cm/sec in blood bank blood and 4.5×10^{-7} in fresh blood. The TPA linear binding constant k_b averaged 3.0 in both fresh and blood bank blood.

Figure 1 shows the dramatic increase in TPA efflux for cells treated with butanol. Permeability increases of about 5 times are attained at an aqueous alcohol concentration of 300 mM (Fig. 3). At this alcohol level the process is exponential and there is little hemolysis. As butanol is raised to near 400 mM permeability increases up to 10 times are observed, but hemolysis makes a significant contribution to ion release.

Our primary result, illustrated in Fig. 2, compares the butanol-TPA data with theoretical curves calculated from the dielectric model, Eqs. (1)–(5). All parameters used for this calculation are obtained from the independent measurements listed in the Table. It can be seen that the agreement between theory and experiment is remarkably good for reasonable values of the native dielectric constant (i.e. for $2.0 < D_m(0) < 2.2$) with the best fit falling into the mid-range, $D_m(0) = 2.1$. This result is especially significant because it is obtained without any adjustment of parameters.

If this interpretation is correct, similar results should be attainable with other alcohols. Anticipated increases in TPA permeability are found when hexanol and octanol are added to the cells, but at considerably lower concentrations (Fig. 3).

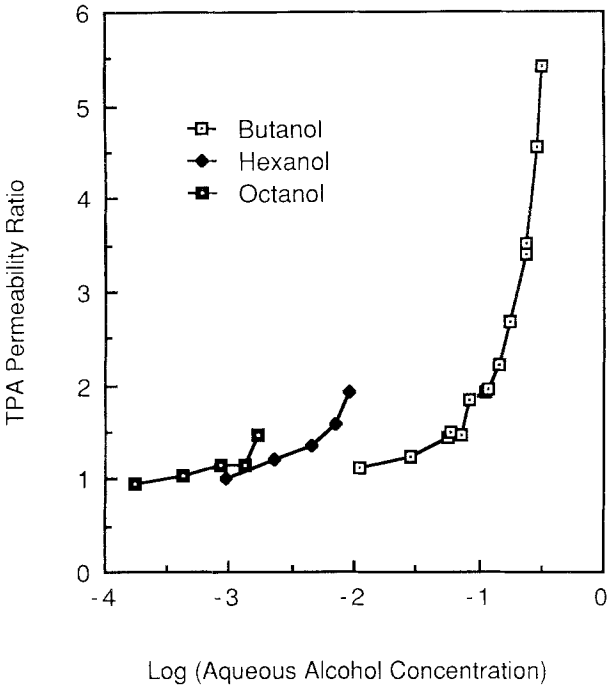


Fig. 3. Alcohols increase TPA permeability of red blood cells. In terms of aqueous concentration octanol is the most effective, followed by hexanol and butanol. The permeability ratio is the permeability at the indicated alcohol concentration divided by the permeability in the absence of alcohol

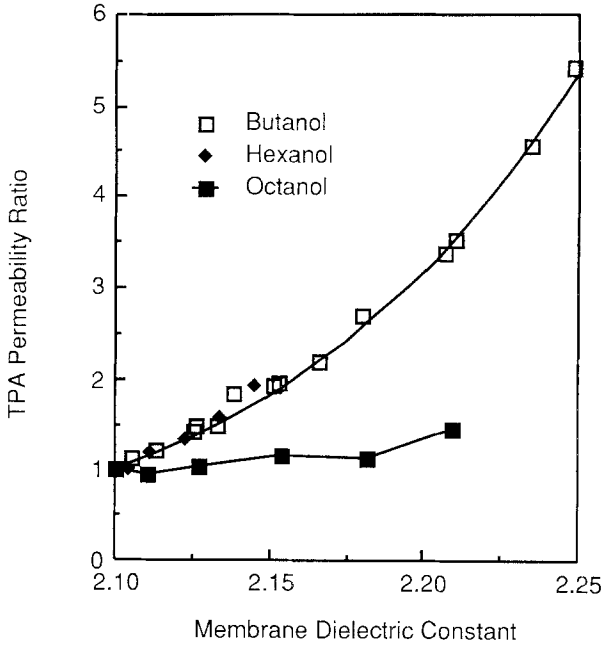


Fig. 4. The permeability ratio data plotted against the membrane dielectric constant calculated from the Clausius-Mossotti equation (*see* text). The data for hexanol and butanol fall on the same theoretical curve (smooth upper curve), while that for octanol (lower curve) deviates from theory. Parameters used to calculated the theoretical curve: membrane thickness = 30 Å; initial membrane dielectric constant = 2.1; TPA radius = 4.2 Å

(Other single experiments with propanol, pentanol, heptanol and nonanol also showed stimulation of the TPA permeability, but were not pursued). The aqueous alcohol concentrations giving a 50% increase in permeability are approximately 2, 7 and 70 mM for octanol, hexanol and butanol, respectively. Using the partition coefficients from the Table to estimate the membrane alcohol levels, hexanol and butanol are equally effective. Octanol, the most effective in terms of aqueous concentration, is the least effective in terms of membrane concentration. Our conjecture is that effects of alcohols on bilayer ion permeability are mediated largely through changes in dielectric constant. If this is true, then the discrepancy seen for the different alcohols in Fig. 3 should disappear if the permeability ratio is plotted as a function of membrane dielectric constant. This result is shown in Fig. 4 where permeability ratios are plotted against the membrane dielectric constant obtained from the Clausius-Mossotti expression (Eq. 2), again using data from the Table. The butanol and hexanol data agree quite well with the dielectric model (solid line), but the octanol data do not. The apparent ineffectiveness of octanol could be the result of using an inaccurate partition coefficient, or because the alcohol localizes its OH group at the surface of the bilayer, resulting in little change to the membrane dielectric constant. In support of the latter idea, Pope, Walker and Dubro (1984) showed by NMR that the octanol OH anchors to the head group region in dimyristoyl phosphatidylcholine vesicles. The latter possibility is of particular interest owing to the well known "cutoff" effect observed for the anesthetic potency of the higher alkanols (Haydon & Elliot, 1986).

Our interpretation would be strengthened even further if it could be shown that butanol effects on electrostatic potentials (dipole and surface potentials) are comparatively small. This can be assessed by examining effects of butanol on anions.

Butanol Stimulation of Organic Anion Permeability

EPR measurements of AN-12 influx in ghosts employs a method similar to the one used by Cafisio and Hubbel (1978) to study cation spin probe fluxes in liposomes. Permeation is observed by monitoring the time-dependent decrease of the aqueous AN-12 high-field peak. Figure 5 compares the AN-12 free signal spectra with the same number of spins in the presence of a packed ghost membrane preparation

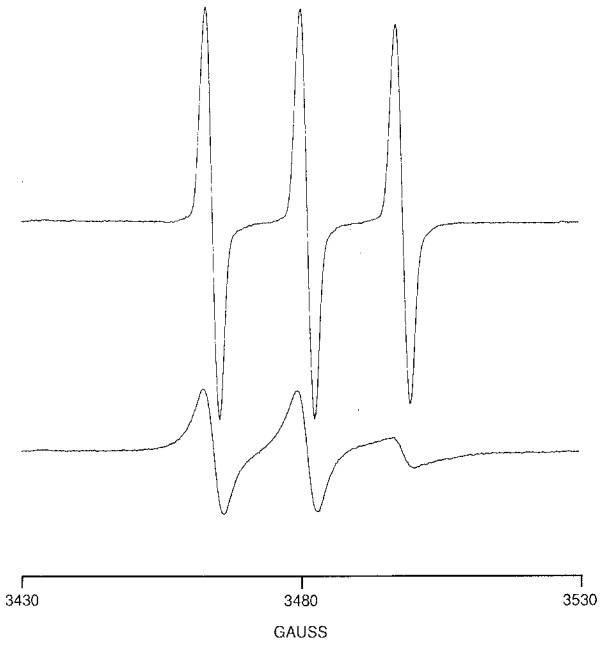


Fig. 5. EPR spectrum of AN-12 in buffer alone (upper trace) and in packed RBC ghosts (lower trace). Spectra are shown at the same gain. Total spin concentration is 50 μM in both cases. Sweep rate equals 1 gauss per second

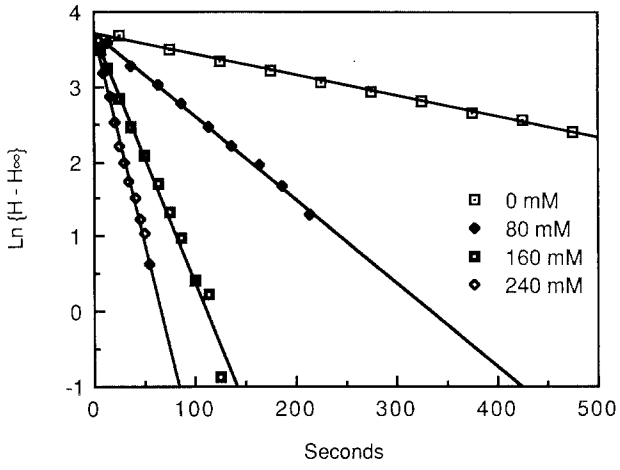


Fig. 6. Logarithmic plot of the ESR high-field peak height H of the AN-12 spin probe as it permeates the red blood cell in the presence of different butanol concentrations (*see* legend). The peak height can be related to changes in external concentration (*see* text) and is thus a measure of ion flux. Influx experiments were done at 22°C

in which all the probe is membrane bound. It can be seen that the membrane high-field signal is reduced by 95%, indicative of extreme anisotropic tumbling in the membrane. Since the membrane signal is so strongly quenched, the high-field line can be used to measure the aqueous free signal. When cells are mixed in the stop-flow AN-12 first binds to the outer surface at a rate faster than we can resolve (<200 msec). Subsequently, it diffuses through the red cell bilayer, binds to the inner surface, and equilibrates with the internal space. This results in further signal quenching which can be readily followed. Decline of the high-field peak occurs exponentially with a time constant of about 250 sec at 22°C and a hematocrit of 10% (Fig. 6). The permeability coefficient of AN-12, in the absence of alcohol, averaged 4.5×10^{-6} cm/sec.

As in the case of TPA, butanol dramatically lowers the time constants for penetration. Figure 6 compares the permeation rates of AN-12 in 0, 80, 160 and 240 mM added butanol. Permeability ratios greater than 20 to 1 are observed at the highest concentration of butanol with no detectable cell lysis. Analysis by Born-Parsegian theory assuming a normal membrane dielectric constant of 2.1, showed that the data fit quite well when the ion is assigned an effective radius of 1.9 Å (*see* Fig. 7). The latter value is reasonable considering the small contribution to charge delocalization expected from the substituent groups on the phosphate.

To rule out the possibility that the AN-12 is being transported via the red cell anion exchanger, experiments were carried out in the presence of 100 μM DIDS, a concentration sufficient to inhibit exchange by 99% and electrogenic flux by 70% (Knauf et al., 1977). DIDS had essentially no effect on the AN-12 flux. In addition, flux measurements at pH 6.0 and 8.0 were compared to eliminate the possibility that AN-12 permeated as a neutral protonated species. A decrease by a factor of 100 would be expected going from the lower pH to the higher one. Experiments showed only a factor of 2 decrease. This is simply accounted for by titration of internal hemoglobin, giving a more negative Donnan potential at the higher pH.

To confirm that AN-12 permeates as an ion, its responsiveness to membrane potential was tested by treating AN-12 equilibrated whole cells with 5 μM valinomycin in the presence of a 10 to 1 (in/out) potassium gradient. Measurement of free external AN-12 showed that hyperpolarizing the cells induced the expected efflux of probe.

Discussion

The stimulation of hydrophobic ion permeability by alcohols is striking and demonstrates effects in a biological membrane previously observed in artifi-

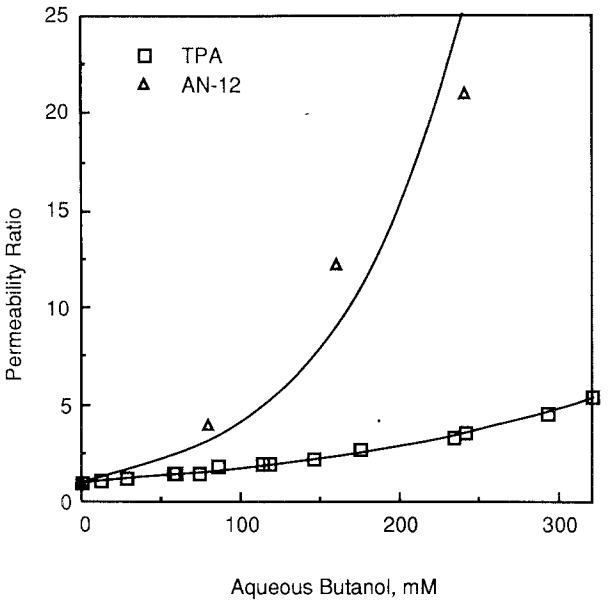


Fig. 7. Butanol increases the permeability of the AN-12 spin probe. The theoretical curve through the AN-12 data points is for an ion with a 1.9 Å radius. The TPA data are shown for comparison

cial systems (Dilger et al., 1979; Reyes & LaTorre, 1979; Gutknecht & Tosteson, 1970). We have shown that these effects can be explained by a theory involving alteration of the membrane dielectric constant. However, it is conceivable that alcohols influence directly or indirectly all of the other factors controlling ion permeability including surface and dipole potentials, hydrophobic interactions, membrane fluidity and thickness.

For a theory based upon the Born-Parsegian analysis to be correct the following conditions must be met in the case of cell membranes:

1) Alcohols must partition into the phospholipid bilayer in sufficient quantities to produce the dielectric changes required by the theory to explain our results.
2) Within the bilayer the alcohol must mix with the hydrocarbon to lower the dielectric energy barrier at the center of the membrane, rather than adsorbing to the surface, concentrating in the polar region or forming clusters of pure alcohol.
3) Permeabilities of both anions and cations must be increased and be strongly dependent on ion size, thereby excluding membrane dipole or surface potential changes.
4) Fluidity changes must be quantitatively insufficient to explain the observed effects.

Partition of Alcohol into the Bilayer

The theoretical curve in Fig. 4 clearly fits the entire experimental range for butanol stimulation of the TPA flux, and was calculated using a partition coefficient of 1.5 obtained from the literature (Seeman, 1972). To increase TPA permeability fivefold the membrane dielectric constant must be raised from 2.1 to 2.247 according to Born-Parsegian analysis. Membrane butanol must rise to 460 mM (a volume fraction of 0.042) to produce this change, and correspond to an aqueous concentration of 306 mM.

Seeman's partition coefficient for butanol is based upon an extrapolation of the empirical relationship between red cell ghost and octanol/water partition coefficients for higher alcohols. Since the experimentally measured ghost partition coefficients were 1/5 those found for octanol, he assumed the relationship also held for the lower alcohols, which he did not measure. This is a reasonable assumption because linear free energy relationships between partition coefficients in different media are well established (Collander, 1951; Leo, Hansch & Elkins, 1971). The use of this value is further supported by the results of Kamaya, Kaneshina and Ueda (1981) who reported a butanol partition coefficient of 1.45 in liposomes made of dipalmitoylphsophatidylcholine. However, Hill (1975) and Katz and Diamond (1974) found coefficients close to 3 for other phospholipids. None of the phospholipid preparations exactly matches those of the red blood cell. However, if we fit the data in Fig. 4 with the assumption that the butanol partition coefficient equals three, then the native membrane dielectric constant would have to be raised to 3.2, which seems very unlikely. Consequently, we believe Seeman's value is reasonably accurate for the red cell.

Location of Alcohols within the Lipid Bilayer

The lipid bilayer is not a homogenous phase. It consists of a polar region of 6 to 10 Å thickness (Finean, 1969; Hauser et al., 1981) and a hydrocarbon region containing cholesterol and the acyl chains of phospholipids in a somewhat disordered array (Finean, 1969). These regions can be further subdivided with respect to properties (Jain & Wu, 1977; Ashcroft et al., 1983). If alcohols are to lower the dielectric barrier they must penetrate into the hydrocarbon region. As indicated earlier, octanol appears to be much less effective in the membrane at increasing the TPA permeability than either butanol or hex-

anol. This may be the result of the alcohols distributing differently in the membrane.

At present there is some disagreement over the distribution of alcohols in membranes. Theoretical calculations, based upon molecular interactions between phospholipids and alcohols, suggest that a uniform alcohol distribution pattern is unlikely. Brasseur et al. (1985) calculate that aliphatic alcohols with chains up to 3 carbons in length have configurations favoring hydrophilic interactions and will concentrate at the membrane surface. Alcohols with 4 to 8 carbons will concentrate in the outer hydrocarbon layer, but will have a tendency to cluster together, while those with 9 or more carbons will interact well with the acyl chains and mix in the hydrocarbon interior.

The organic-phase/water partition coefficients for alkanols into hydrocarbons such as hexadecane (Aveyard & Mitchell, 1969) are about 10 to 20 times less than those measured in phospholipid dispersions. Since the alkanes are often used as models of the hydrocarbon interior of the membrane (Stein, 1986), the difference might be taken as an indication of alcohol partitioning into the more polar regions of bilayers. NMR spectroscopy of the N-methyl groups of phosphatidylcholine in the presence of benzyl alcohol derivatives (Colley & Metcalfe, 1972), and octanol (Pope et al., 1984) led these workers to place the alcohol hydroxyl groups at the membrane surface. Similarly, Kamaya et al. (1981) found that benzyl alcohol lowered the phase transition temperature of phosphatidylcholine vesicles by the same amount regardless of phospholipid chain length. The insensitivity to chain length caused them to assign benzyl alcohol a position in the head group region. However, phenol, a close analog of benzyl alcohol, which also lowers the phase transition temperature, is sensitive to lipid chain length and pH indicating that it is mainly in the hydrocarbon region (Van Dael & Ceutrickx, 1984). Conductance and capacitance measurements indicate that benzyl alcohol changes both the dipole potential and dielectric constant of the solvent-free planar bilayer (Reyes & Latorre, 1979); it must partition into the hydrocarbons as well as the polar region to do this.

Fluorescent probe studies designed to give depth information show that the ability of alcohols to disorder or fluidize membranes depends on alkanol chain length (Zavoico, Chandler & Kutchai, 1985). In particular, pentanol disorders membranes at all levels, but especially so in the center of a bilayer. Tetradecanol on the other hand has little or no effect on ordering, whereas decanol has intermediate effects. The comprehensive differential scanning calorimetry studies of Jain and Wu (1977) show four main types of phase transition profiles when membranes are treated with organic reagents. Alcohols with 1 to 4 carbons have profiles similar to those of low molecular weight hydrocarbons such as benzene, toluene and carbon tetrachloride indicating that they partition chiefly into the hydrocarbon interior. Alcohols with 5 to 10 carbons display a spectrum typical of molecules which partition into the outer hydrocarbon layer.

A factor which may also be important in determining alcohol localization is the regional fluidity of the bilayer. Alcohols are "frozen out" of bilayers below the transition temperature (Colley & Metcalfe, 1972), and it is well known that the outer hydrocarbon region (carbons 1 to 9) is much more rigid than the interior (Seelig, 1977). This would favor partition of small molecules into the center of the membrane.

Although we cannot unambiguously assign a distribution profile for the alcohols used in our study, the fluorescence data of Zavoico et al. (1985), and the calorimetry measurements of Jain and Wu, (1977), give us reasonable confidence in assuming that both butanol and hexanol partition into the red cell membrane interior. Octanol on the other hand may well favor the more ordered region of the membrane hydrocarbon with its OH group anchored to the surface.

Increased Permeabilities for Anions and Cations, Ion Size, and Membrane Thickness

The Born-Parsegian equation predicts that when the membrane dielectric constant is raised, the permeabilities of small ions will increase significantly more than those of large ions. As shown in Fig. 7, 240 mM of aqueous butanol increases the permeability of TPA 3.5 times, and that of AN-12 21 times. The Born-Parsegian interpretation is further supported by the results of Gutknecht and Tosteson (1970), who found that aliphatic alcohols (2 to 10 carbons) increased the KCl conductance of planar bilayers made from sheep erythrocyte lipids by several orders of magnitude. They also showed from transference measurements that the current carried by the smaller potassium ion (r = 1.33 Å; Marcus, 1985) was preferentially stimulated with increasing alcohol concentrations. Figure 8 shows the Gutknecht and Tosteson conductance data for ethanol, pentanol, and heptanol averaged and replotted with theoretical curves based on our model. The theoretical curves are calculated using the potassium crystal radius, and partition coefficients of 0.21, 5.4, and 59.4 for the respective alcohols. These partition coefficients are in the same ratio as those reported by Seeman (1972) for RBC ghosts,

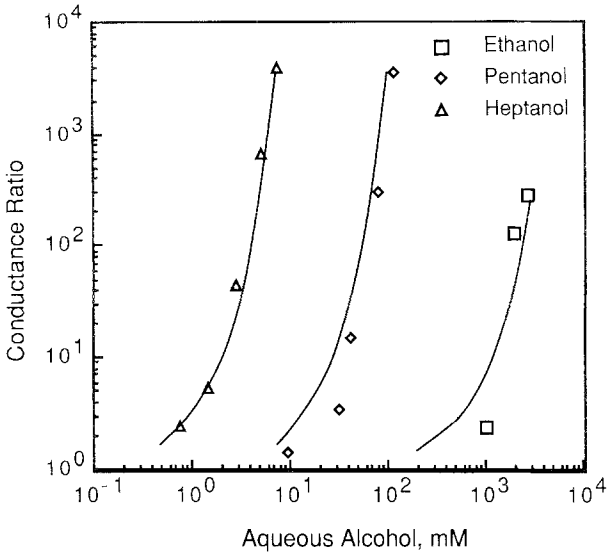


Fig. 8. Alkanol-induced conductance increases in sheep erythrocyte lipid bilayers. Data taken from Gutknecht and Tosteson (1970). Theoretical lines were calculated for ion radius of 1.33 Å. Partition coefficients were increased by 1.5 times over the values given by Seeman (1972)

but are 1.5 times larger. The fits are quite reasonable considering the uncertainties in the data, and span a conductance range better than three orders of magnitude. Similar effects on potassium permeability have been observed in an egg phosphatidylcholine suspension (Bangham, Standish & Miller, 1965) and in intact red blood cells (Ponder, 1948; Jacobs, 1951). In addition, Dilger et al. (1979) found that planar phosphatidylcholine membranes made using chlorodecane (dielectric constant = 4.5) had thiocyanate and perchlorate conductance three orders of magnitude larger than for membranes using decane. They attributed these results to an increase in dielectric constant which was supported by an approximate doubling of the specific capacitance.

Higher alcohols produce monolayer potentials as high as 400 mV, a figure about 10% lower than those of phosphatidylcholine (Adam, 1941). They are known to penetrate monolayers of phospholipids (Haydon & Elliot, 1986), and to lower their potentials through either surface dilution or by orienting to oppose the native dipole potential. An effect on either the membrane surface charges or the dipole potential would affect cation and anion permeabilities in opposing directions. While we cannot exclude small surface effects of alcohols, the large stimulation of both cation and anion permeabilities rules out major surface effects and lends further support to localization of the alcohol in the hydrocarbon region of the membrane. In addition, surface and dipole effects should be independent of ion size contrary to our findings and the other literature cited.

The Born-Parsegian expression predicts that decreasing membrane hydrocarbon thickness will dramatically increase membrane permeability. For example, TPA permeability would be expected to rise almost 10 times were a membrane to shrink from 50 to to 30 Å thickness. Benz and Lauger (1977) found dipicrylamine permeability to increase 20 times when bilayer membrane thickness was varied by incorporating different hydrocarbon solvents. It is hardly likely that alcohols cause thinning of the red cell membrane which is probably only 30 Å thick in the hydrocarbon region. In addition, the theory predicts that thinning the membrane will increase all ionic permeabilities by the same factor. This is contrary to the sixfold greater stimulation of AN-12 versus TPA (butanol = 240 mM) and supports membrane dielectric changes as the main effect produced by the alcohols.

Membrane Fluidity Changes

Aliphatic alcohols, up to 10 carbons, promote membrane fluidity by lowering phase transition temperatures (Jain & Wu, 1977; Kamaya et al., 1981) and by disrupting membrane structure (Colley & Metcalfe, 1972; Paterson et al., 1972; Jacobson & Wobschall, 1974; Van Dael & Ceuterickx, 1984). Nevertheless, it is unlikely that fluidity changes have much to do with any of the alcohol-induced permeability increases discussed.

Fluidity changes produced by alcohol addition to membranes are relatively small. For example, Pang, Chang and Miller (1979) found only a 5% decrease in the order parameter of a viscosity probe incorporated into alcohol-treated phospholipid vesicles, but in parallel experiments the same treatment produced a 3.6-fold increase in valinomycin-K^+ permeability. In addition, a simple viscosity decrease requires permeabilities of all ions to increase in the same proportion. However, our data give a much greater enhancement of the AN-12 permeability compared to TPA. Also, it is hardly likely that fluidity increases could account for the 1000-fold stimulation of the planar membrane conductances discussed earlier.

Lieb and Stein (1971) point out that diffusion through membranes may be more like diffusion through polymers than through liquids. Biological membranes, like artificial polymers, show a steep size dependence to diffusion, large molecules moving much more slowly through them than small ones. The permeabilities of polymer membranes are strongly dependent upon the amounts of organic

solvents or plasticizers incorporated into them. It is quite conceivable that small amounts of alcohol could dramatically raise the permeabilities of such membranes. But one would expect alcohols to stimulate larger ions more than smaller ones in a polymer network, and this is just the opposite of what we have seen in red cell membranes. However, without further experiments we cannot fully exclude this possibility.

Nonelectrolyte permeability, which should be enhanced by increased fluidity, is relatively unaffected by alcohol in the membrane. Erythrocyte methanol permeability is the same in the presence and absence of 250 mM butanol (Brahm, 1983). However, at high concentrations butanol increases its own permeability somewhat. Reyes and Latorre (1979) report benzyl alcohol-induced increases of 40% in the permeability of acetamide. Wilbers et al. (1979), reported a twofold increase for the nonelectrolyte 2-(3'-thioglyceryl)-N-ethylmaleimide in cells treated with hexanol. Although we cannot account for the latter results with any confidence, specific solvation effects through hydrogen bonding by the alcohols along with some fluidity increases could account for the increased permeabilities.

Finally, fluidity increases are clearly dissociated from permeability increases in peroxidized membranes. Peroxidation increases many types of ion permeability (Maridonneau, Braquet & Garay, 1983; Deuticke, Heller & Haest, 1986; Misso, Jones & Paleg, 1986); but oxidation invariably reduces membrane fluidity (Dobretsov et al., 1977; Rice-Evans & Hochstein, 1981; Gut et al., 1985). One can speculate that peroxidation might introduce polar groups into the bilayer lipids, raising the dielectric constant and enhancing ion partition into them. This would explain the general observation that old cells are more permeable to ions than young ones. Preliminary experiments measuring AN-12 permeability in red cells oxidized by treatment with tert-butyl hydroperoxide for 1 hr gave a twofold increase in permeability.

Summation and Some Physiological Consequences

It is clear from the foregoing discussion that there are some uncertainties in the parameters used in our analysis. Yet it is equally clear that these experiments and previous work by others strongly suggest that alcohols increase hydrophobic ion permeability primarily by raising the dielectric constant. The large magnitude of alcohol-induced permeability changes rules out a simple membrane thickness mechanism and casts doubt upon increased fluidity as the mode of action. Differential effects on ions of different sizes rules out a thickness mechanism based upon the Parsegian correction term. Stimulation of both cation and anion permeability eliminates surface and dipole potentials from consideration. Finally, the preferential stimulation of small ion permeabilities rules out most diffusional mechanisms as well as dipole effects. Modification of the membrane dielectric constant comes through these tests as a plausible basis of alcohol interaction. The pattern that emerges is one characteristic of hydrophobic ion permeability, dominated by membrane partitioning rather than by diffusional considerations.

The low dielectric constant of the phospholipid bilayer virtually prevents ions, especially if small and/or multivalent, from crossing the membrane. The cell then has the option of controlling their fluxes through protein channels and pumps. Given the importance of the phospholipid barrier, one might ask how the cell maintains it. Double bonds are particularly susceptible to the introduction of polar groups through peroxidation and the human red cell phospholipids have an average of 1.4 double bonds per acyl chain (Van Deenen & de Gier, 1974). If all of these were to become oxidized, the bilayer concentration of oxygen radicals would be of the order of 2500 mM (assuming the hydrocarbon portion of the bilayer is 50% phospholipid chains), about 5 times higher than the highest alcohol concentrations used in our experiments. Oxidation of 5 to 10% of such groups would produce ion leaks that might threaten the existence of the cell.

Peroxidation may be one cause of the increased TPA permeability we have observed in aged (blood bank) cells. A cell confronted with a major ion leak has two immediate options: it may tolerate the altered intracellular ion concentrations, or it may pump harder with an expenditure of valuable energy. Neither option seems desirable and there are limits to both. Alternatively the cell may have mechanisms for repairing membrane damage and maintaining dielectric constant homeostasis.

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