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ERYTHROCYTE WATER PERMEABILITY

THE EFFECTS OF ANESTHETIC ALCOHOLS AND ALTERATIONS IN THE LEVEL OF MEMBRANE CHOLESTEROL

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Summary

1. Treatment of human erythrocytes with anesthetic *n*-alkanols (pentanol, hexanol and heptanol) results in a decrease in the osmotic water permeability of the red cell membrane.

2. The alcohol-induced changes in osmotic water permeability are proportional to the alcohol concentration and roughly parallel the changes in the rotational mobility of the lipid-soluble membrane probe diphenylhexatriene that are induced by the alcohols.

3. Enrichment of the red cell membrane in cholesterol also results in a decrease in the osmotic water permeability.

4. Moderate depletion (9% or 40%) of membrane cholesterol is without effect on the osmotic water permeability, even though this treatment results in a significant increase in the rotational mobility of diphenylhexatriene in the membrane lipids.

The water permeability of human erythrocytes has previously been estimated from the kinetics of uptake of ^{3}H-labeled water [1—3], from the kinetics of osmotically induced red cell volume changes [4—6] and from the exchange of water measured by NMR [7—10]. The prevailing opinion is that when experiments are properly performed and analyzed, the same value of the water permeability is obtained from diffusion and osmosis experiments [5,11]. The water permeability of human red cells can be inhibited about 90% by the sulfhydryl reagents *p*-chloromercuribenzoate, *p*-chloromercuribenzenesulfonate and dithiodinitrobenzoate [12,13]. This has been interpreted to mean that the

largest fraction of red cell water flux is protein-mediated. Under certain conditions that minimize nonspecific labeling, only the band III polypeptides of the red cell membrane are labeled with dithiodinitrobenzoate [14]. This suggests that band III may be involved in mediating water transport.

Information on the effects on water permeability of altering the physical state of membrane lipids is relatively scarce. It was reported that addition of cholesterol to planar lipid bilayers [15] or liposomes [16,17] resulted in a decrease in water permeability. Addition of hexanol, an anesthetic alcohol, to vesicles formed by sonication of egg lecithin [18] or to unsonicated egg liposomes [17] caused an increased permeability to water. The information available suggests that, in pure lipid membranes, increasing the fluidity of the acyl chains of the membrane lipids increases water permeability. In red blood cells it has been reported that anesthetics (chlorpromazine, ethanol, pentanol, hexanol and octanol) also cause an increase in the osmotic water permeability [19].

In this paper we report our studies of the effects of anesthetic alcohols, cholesterol enrichment and cholesterol depletion (all treatments which are known to alter the mobility of membrane phospholipids) on the osmotic water permeability of human red cells.

Materials and Methods

Materials

Blood was obtained from normal human volunteers by venipuncture. The blood was mixed with acid/citrate/dextrose solution (1 vol. solution to 7 vol. whole blood) and stored at 4° C for no more than 2 days before use. *n*-Pentanol, *n*-hexanol and *n*-heptanol were obtained from Fisher Chemical Co., Sigma Chemical Corp. and Eastman Organic Chemicals, respectively. Cholesterol was purchased from Steraloids, Inc. (Wilton, NH), batch No. 1726. Dipalmitoyl phosphatidylcholine was obtained from Gibco, lot No. R582553. 1,6-Diphenyl-2,3,5-hexatriene was the product of the Aldrich Chemical Co., Puriss grade. Penicillin-streptomycin was purchased from Microbiological Associates. All other chemicals were reagent grade or better.

Measurement of the osmotic water permeability of human erythrocytes

The stop-flow apparatus described by Blum and Forster [6] was used to follow the time course of shrinking after red cells were mixed with hypertonic solutions. The transmittance of the red cell suspension was followed at 583 nm. Shrinking the cells caused a decrease in light transmission which was proportional to the magnitude of the volume change [6]. The red cells were washed three times in isotonic saline, pelleting them each time at 2000 rev./min for 10 min in the Omni rotor of the Sorvall RC 2B centrifuge. The washed cells were suspended at 3—4% hematocrit in isotonic saline or in phosphate-buffered saline at pH 7.4. The phosphate-buffered saline consisted of 150 mM NaCl and 5 mM sodium phosphate. The stop-flow apparatus mixed equal volumes of the red cell suspension and a hypertonic solution (usually 0.462 M NaCl). The output of the phototube amplifier was recorded on a storage oscilloscope at a sweep-speed of 50—200 ms/cm and Polaroid photographs of the stored traces

were taken. The kinetics of the shrinking of the red cells were obtained by analyzing the photographs.

Calculation of the osmotic water permeability, L_p

The decrease of light transmission as the cells shrank was found to follow first-order kinetics. That is to say, a plot of the logarithm of 1 minus the extent of completion of the change in light transmission was linear in time. From the slope of this semi-logarithmic plot, the apparent first-order rate constant (k) for the shrinking process was obtained. The half-time for shrinking was computed as $\ln 2/k$. The osmotic water permeability was then calculated from Eqn. 5 of Forster [11], which can be written as

$$tL_p = \frac{V_{w\infty}}{C_0 A V_{w0}} \left[V - V_0 - V_{w\infty} \ln \frac{V_\infty - V}{V_\infty - V_0} \right] \tag{1}$$

where t is time; V_{wo} and V_{wo} are the osmotically active cell water volumes at times zero and infinity, respectively; V, V_0 and V_∞ are the total cell volumes at times t, 0 and ∞; A is the red cell area; and C_0 is the initial internal osmolarity of the cellular contents. Defining the extent of completion of the volume change, n, as $(V - V_0)/(V_0 - V_\infty)$, we obtain

$$tL_p = \frac{V_0 V_{w\infty}}{C_0 A V_{w0}} \left[\frac{V_0 - V_\infty}{V_0} n - \frac{V_{w\infty}}{V_0} \ln(1 - n) \right] \tag{2}$$

When $t = t_{1/2}$, both n and $1 - n$ are 1/2, giving

$$t_{1/2} L_p = \frac{V_0 V_{w\infty}}{C_0 A V_{w0}} \left[0.5 \frac{V_0 - V_\infty}{V_0} + 0.6931 \frac{V_{w\infty}}{V_0} \right] \tag{3}$$

Eqn. 3 is then used to compute L_p.

It is known that the n alkanols, like other anesthetics, cause an expansion of the membrane area of model and natural membranes. From the data of Seeman et al. [20,21] on the effect of n-alkanols on the membrane area of human red cells, we calculated that the maximum expansion of membrane area caused by the highest concentrations of pentanol, hexanol and heptanol which we used is about 6%. We did not take this increase in membrane area into account in computing L_p.

Determination of the effects of anesthetic alcohols on L_p

Pentanol, hexanol or heptanol was added to phosphate-buffered saline in an amount calculated to give the desired concentration. The mixture was shaken repeatedly in a 50 ml tube (with Teflon-lined screw cap) until the alcohol completely dissolved. Aliquots of packed red cells were added to the alcohol-containing buffer to produce hematocrits of between 3 and 4%. Measurements were then made with the stop-flow apparatus as described above. When measurements were made soon after mixing cells with alcohol the results were similar to those obtained as when the cells sat in the alcohol-containing buffer for 2—3 h.

Effect of anesthetic alcohols on the steady-state osmotic behavior of human red cells

The osmotically active water volumes of the red cell at times zero and 'infinity' enter into the computation of L_p (Eqn. 3). It was thus necessary to determine the influence of the alcohols on the osmotically active water volume. Red cells were equilibrated with the desired concentration of alcohol in buffer. To 6 ml of 30% (approx.) hematocrit suspension were added repeated 100-μl aliquots of 1.54 M NaCl. After each addition the suspension was mixed and triplicate samles were taken for hematocrit determination in microhematocrit tubes. From the change in hematocrit the change in volume (ΔV) of the red cells caused by the addition of salt was computed. The osmotically active water volume at isotonicity (V_{wo}) as a fraction of total red cell water volume (V_0) was determined by plotting $\Delta V/V_0$ vs. the reciprocal of tonicity according to the equation of Ponder [22]

$$\frac{\Delta V}{V_0} = \frac{V_{w0}}{V_0} - \frac{V_{w0}}{V_0} \cdot \frac{1}{T} \tag{4}$$

According to this equation, a straight line would be obtained with both slope and y-intercept equal to V_{w0}/V_0.

Altering the cholesterol content of the red cell membrane

We used methods described by Cooper and his associates [23] to change the cholesterol composition of the red cell membrane. Phosphatidylcholine/cholesterol vesicles were used to enrich or deplete the red cell membrane cholesterol. 40 mg dipalmitoyl phosphatidylcholine and either 2, 23 or 80 mg cholesterol were put in the bottom of a fluted stainless steel sonication vessel. 10 ml Hanks' balanced salts solution (without Ca^{2+} or Mg^{2+}) were added and the mixture subjected to ultrasonic bombardment by a Heat Systems Ultrasonics sonicator (Model W-375) using the 0.5 inch diameter horn. The temperature of the mixture was kept at about 45°C during sonication by means of a water-jacketed vessel perfused by a refrigerated circulator. Sonication times ranged from 30 min to 75 min; the higher cholesterol mixtures required longer to disperse. The sonicated mixtures were centrifuged at 16 000 rev./min in the JA-20 rotor of the Beckman J-21 centrifuge and the pellets were discarded. 3 ml heat-inactivated autologous serum were added to the lipid vesicle suspension and 10 000 units of both penicillin and streptomycin were added. Thrice-washed red cells (10 ml 10% hematocrit) were added to 10 ml of the lipid vesicle-serum mixture in a 250 ml Erlenmeyer flask. The flask was flushed thoroughly with N_2 and capped and then incubated at 37°C on an incubator-shaker for 12—18 h. After the incubation the mixture was chilled in ice-cold isotonic saline and the cells separated from the vesicles by washing four times in ice-cold isotonic saline, centrifuging each time at 2000 rev./min in the Omni rotor of the Sorvall RC-2B centrifuge.

Estimation of the cholesterol/phospholipid molar ratio and membrane fluidity of the vesicle-treated red cells

Lipids were extracted from a sample of the vesicle-treated erythrocytes with 10 : 6 (v/v) isopropanol/chloroform [24]. Cholesterol was determined by the

$FeCl_3$ method [25] and total lipid phosphorus was estimated by the Method of Bartlett [26]. For the purposes of computing the cholesterol/phospholipid molar ratio, it was assumed that the average phospholipid had a molecular weight of 750.

Membrane fluidity was estimated by means of the hydrophobic probe 1,6-diphenyl-2,3,5-hexatriene as previously described [23,27]. Red cell ghosts were prepared from the vesicle-treated cells by hypotonic hemolysis [28]. Ghosts were added to isotonic saline containing 2 μM diphenylhexatriene and incubated at 37°C for 20—30 min to allow equilibration of the probe with the membrane lipids. The molar ratio of diphenylhexatriene/lipid was always less than 1 : 100 based on phospholipid determinations on the ghosts by the method of Bartlett [26]. The fluorescence polarization of diphenylhexatriene in the ghost membrane was determined with an Elscint MV-1 Microviscometer. This instrument excites the sample with light from a mercury lamp which is polarized by passage through a Glan-Thompson polarizer. The emission intensities parallel ($I_/$) and perpendicular ($I_\perp$) to the polarization of the exciting light are simultaneously measured by balanced phototubes and the polarization (p) computed as $p = (I_/ - I_\perp)/(I_/ + I_\perp)$.

Results and Discussion

The osmotic water permeability of normal human erythrocytes

When normal red cells were mixed with hypertonic saline, so that the initial extracellular osmolarity was twice isotonic, we found the water permeability to range from 0.107 to 0.465 cm^4/osmol per s. The average value of 43 determinations was 0.261 and the standard deviation was 0.072. Using cells from a particular donor the standard deviation was no more than 5% of the mean value, so we conclude that the variation we saw in L_p represents real variation among individuals. Our mean value of L_p is consistent with the values reported by others [5,6].

Effects of anesthetic alcohols on the osmotic water permeability of human erythrocytes

We found that the addition of pentanol, hexanol, or heptanol led to decreased osmotic water permeability (Fig. 1). The largest number of experiments was done with hexanol (15—18 measurements at each hexanol concentration), so that more confidence can be put on the hexanol results than on those with pentanol (4—12 measurements at each concentration) and heptanol (4—8 measurements at each concentration). In terms of aqueous concentration, heptanol is more potent than hexanol, which is more potent than pentanol. The relative potencies are in rough quantitative agreement with the reported partition coefficients for these alcohols between the red cell membrane and an aqueous phase [21].

Our results are in conflict with those of Seeman et al. [19], who reported that ethanol, pentanol, hexanol and octanol all increased the osmotic water permeability of human red cells. The discrepancy between our results and theirs may be due to differences in methodology and instrumentation. The permeability values they obtained for untreated red cells are similar to our

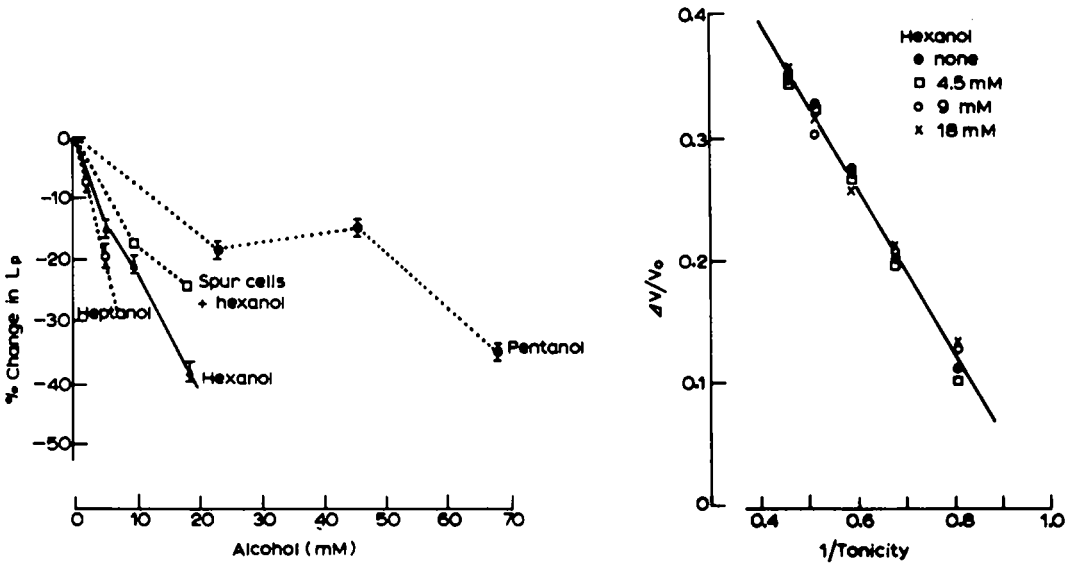


Fig. 1. The effects of alcohols on the osmotic water permeability (L_p) of human erythrocytes. The percent change in L_p (± S.E.) relative to the L_p of the same cells in the absence of alcohol is plotted vs. the aqueous concentration of alcohol. Shown are data for normal red cells in the presence of pentanol (●), hexanol (▲) and heptanol (○). Also shown are data from the red cells of one patient with spur cell anemia in the presence of hexanol (□).

Fig. 2. The effects of hexanol on the steady-state osmotic behavior of normal human erythrocytes. The red cells were mixed with hypertonic NaCl solutions of five different tonicities. The change in volume of the red cells (ΔV) as a fraction of the volume in isotonic saline (V_0) was determined as described in the text. $\Delta V/V_0$ is plotted vs the reciprocal of the tonicity according to the equation of Ponder [22] (Eqn. 4 in the text).

values, however. Our data are more numerous and more internally consistent than those of Seeman et al.; in none of our experiments did the addition of alcohol lead to increased water permeability.

Effects of anesthetic alcohols on the steady-state osmotic behavior of human red cells

As can be seen from Eqn. 3, it is possible that the alcohols might affect the calculated value of L_p via an effect on the fraction of intracellular water that is osmotically active. Fig. 2 shows the 'Ponder plots' for human red cells in the presence of various concentrations of hexanol. In three separate experiments (that shown in Fig. 2 and two additional experiments) hexanol was without significant effect on the slope or intercept of the 'Ponder plot'. We conclude that hexanol, at concentrations up to 18 mM, does not significantly alter the fraction of red cell water that is osmotically active.

Effect of altering the level of membrane cholesterol on osmotic water permeability

In two experiments red cells were enriched or depleted of cholesterol by incubation with phosphatidylcholine/cholesterol vesicles. As shown in Table I, in both experiments depletion of membrane cholesterol lead to no significant

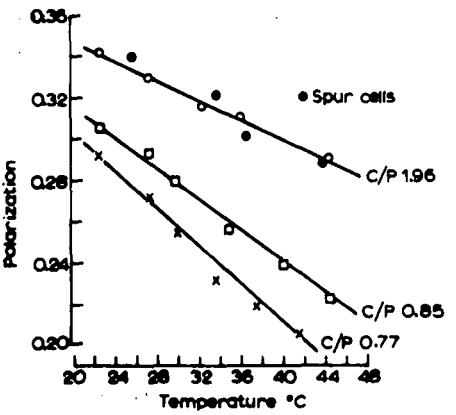
TABLE I

THE EFFECTS OF CHOLESTEROL ENRICHMENT AND DEPLETION ON THE OSMOTIC WATER PERMEABILITY (L_p) OF NORMAL HUMAN RED CELLS

The data for two separate experiments are shown.

Treatment	Percentage change in cholesterol/phospholipid ratio	Percentage change in L_p
Cholesterol depletion	−9	+2.2
	−39.6	+4.4
Cholesterol enrichment	+131	−36.7
	+165	−25.8

change in L_p, but cholesterol enrichment caused a significant decrease in L_p. In one experiment hexanol was added to the cholesterol-enriched cells. This resulted in a further decrease of L_p. The effects of cholesterol enrichment and depletion on the 'fluidity' of membrane lipids was estimated by means of the hydrophobic membrane probe, diphenylhexatriene. In agreement with our previous results [29], we found that cholesterol depletion led to a decrease in the polarization of diphenylhexatriene fluorescence, while cholesterol enrichment had the opposite effect (Fig. 3). Thus cholesterol removal had a 'fluidizing' effect on membrane lipids, while cholesterol enrichment had a 'rigidifying' effect. It should be stressed that the fluidity sensed by any probe molecule represents a weighted average of the molecular mobility of the probe in different regions of the membrane. Diphenylhexatriene partitions selectively



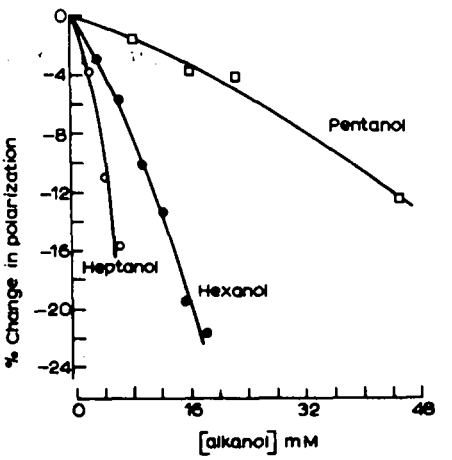


Fig. 3. The effect of cholesterol enrichment or depletion on the fluorescence depolarization of diphenylhexatriene in the membranes (ghosts) of normal human erythrocytes that were incubated overnight with high cholesterol liposomes (○), 'normal cholesterol' liposomes (□), and low cholesterol liposomes (X). The final cholesterol to phospholipid molar ratio (*C/P*) attained is shown near each curve. Data for the cholesterol-enriched cells of a patient with spur cell anemia are shown for comparison (●).

Fig. 4. The effects of alcohols on the polarization of the fluorescence of diphenylhexatriene in the membranes of ghosts of normal human erythrocytes. The percent change in fluorescence polarization is plotted versus the aqueous alcohol concentration for pentanol (□), hexanol (●) and heptanol (○).

into the hydrophobic core of the membrane and avoids the polar head groups, but apparently partitions equally between areas of liquid and condensed lipids [30]. The polarization of diphenylhexatriene fluorescence, then, should reflect something approaching the average mobility of diphenylhexatriene throughout the hydrophobic core of the membrane without much bias in favor of any one region.

Effect of anesthetic alcohols on the 'fluidity' of red cell membrane lipids

As judged from their effects on the polarization of diphenylhexatriene fluorescence, the alcohols have a 'fluidizing' influence on the lipids of the red cell membrane (Fig. 4). The relative potencies of pentanol, hexanol and heptanol in decreasing the polarization of diphenylhexatriene fluorescence are similar to their relative potencies in decreasing the L_p. This is consistent with, but certainly does not prove, the effect of the alcohols on L_p being due to their effect on the membrane lipids.

Erythrocytes from a patient with spur cell anemia

Erythrocytes were obtained from a 47-year-old female with spur cell anemia associated with liver disease. The osmotic water permeability of these cells was 0.269 cm^4/osmole per s, which is very close to the average for normal red cells (0.261). The spur cells were enriched in cholesterol, as expected, having a molar ratio of cholesterol/phospholipid that was more than twice that of normal cells. The polarization of diphenylhexatriene in the spur cell membrane was increased to an extent that is consistent with the increase in membrane cholesterol (Fig. 3). The addition of 9 or 18 mM hexanol to the spur cells caused a decrease in the osmotic water permeability (Fig. 1). The data suggest that the L_p of the spur cells may be less sensitive to hexanol than that of normal cells. This may be the result of the increased membrane cholesterol, but it is risky to generalize on the basis of only one sample of spur cells. Since the L_p of normal cells varies widely (0.261 ± 0.072) (S.D.) cm^4/osmol per s), the significance of the finding that the spur cells had a normal L_p is not clear.

Interpretations

Effects of cholesterol and n-alkanols on water permeability in red cells

One of the purposes of this study was to evaluate the possible influence of membrane fluidity on osmotic water transport in red blood cells. As described above increasing membrane cholesterol decreased the osmotic water permeability, as did the addition of anesthetic *n*-alkanols (C_5—C_7). Addition of cholesterol to the membranes resulted in decreased membrane fluidity, while addition of the alcohols increased membrane fluidity. The simplest interpretation of these results is that fluidity is not the relevant variable in the effect of these treatments on water permeability. We thus want to consider other possible explanations for the similarity of the effects of cholesterol and *n*-alkanols on osmotic water permeability in red cells.

Effects on membrane area

Anesthetic alcohols have been found to expand the area of biological mem-

branes [20,31]. The alcohol molecules intercalate between phospholipid molecules, forcing them apart. The degree of membrane expansion far exceeds the volume occupied by the alcohol molecules [20]. Cholesterol, on the other hand, causes phospholipids to pack closer together in certain membrane systems [32]. However, when human erythrocytes are enriched with cholesterol, there is an expansion of the area of the red cell membrane [23], so that in this respect the alcohols and cholesterol have similar effects on the red cells membrane.

Effects on fluidity at various depths within the lipid bilayer

Adding cholesterol to phospholipid model membranes above their phase transition has either no effect [33] or slightly increases [34] the mobility of the polar heads of the phospholipids. Cholesterol dramatically decreases the mobility of the acyl chains of the phospholipids [35,36], but there is little effect on the mobility of the terminal metheylene groups [33,37]. Anesthetic alcohols have been found to increase the mobility of spin labels located in the region of the phospholipid polar head groups [38,39] and to enhance acyl chain mobility. At high concentrations of longer chain *n*-alkanols (above C_{10}) show an ordering effect on membrane lipids [38,40], suggesting that those carbons of the acyl chain that are in close proximity to the alkanol may experience a decrease in mobility.

Location of cholesterol and n-alkanols in the membrane

It appears that cholesterol is inserted among the phospholipids such that the 3β-hydroxyl group is hydrogen-bonded to carbonyl oxygen of a fatty acyl group, while the α and β surfaces of the steroid nucleus interact in specific ways with the acyl chains of typical natural phospholipids [41,42]. The hydroxyl group of *n*-alkanols would be likely also to reside in the glycerol backbone or the polar head group region of the bilayer, with the methylene chain parallel to the fatty acyl chains of the phospholipids. Thus there are apparently important similarities in the membrane locations of cholesterol and *n*-alkanols.

Effects of cholesterol and n-alkanols on phase transition behavior

Jain and Wu [40] showed that different classes of small molecules have different effects on the phase transition behavior of dipalmitoyl phosphatidylcholine liposomes. The localization of the molecule in the bilayer appears to be the prime determinant of the nature of its effect on the shape of the transition and the temperature at which it occurs. Jain and Wu distinguished four distinct responses of the phase transition, depending on whether the molecule was located in the phosphorylcholine region, the area of the glycerol backbone, the C_1—C_8 methylene region, or the C_9—C_{16} region of the bilayer. Both cholesterol [43] and the *n*-alkanols from pentanol to decanol [40] have the effect of lowering the temperature of the phase transition (both the temperature of onset and the midpoint) and of broadening the phase transition. This suggests that the interaction of cholesterol and the *n*-alkanols (C_5—C_{10}) is similar in that both lower the temperature at which the acyl chains of dipalmitoyl phosphatidylcholine can begin to melt and both lower the size of the cooperative unit in the phase transition.

Thus, both the localization of cholesterol and *n*-alkanols in the membrane and their effects on the phase transition behavior of membrane lipids are similar. Though the mechanisms by which cholesterol and *n*-alkanols decrease water permeability in red cells remains obscure, these similarities in the membrane localization and action of cholesterol and the *n*-alkanols may help us to explain how they both depress water permeability.

Evidence for protein-mediation of water transport in red blood cells

Water transport in red blood cells can be inhibited as much as 90% by the sulfhydryl inhibitors *p*-chloromercuribenzoate [12], *p*-chloromercuribenzenesulfonate [12,13] and dithiodinitrobenzoate [13]. This suggests that water transport is mediated by membrane proteins and is consistent with the observation that red cells have about 10 times greater water permeability than lipid bilayer model membranes.

Addition of cholesterol to model membrane systems has been found to decrease water permeability [15,16] while addition of *n*-alkanols increases water permeability [17]. In natural membranes, anesthetics in general, and *n*-alkanols in particular, have been found to inhibit all facilitated transport systems thus far reported [44], but anesthetics enhance simple diffusion in model and natural membranes. Thus, our finding that *n*-alkanols inhibit osmotic water flux in red cells is consistent with the idea that water transport is protein-mediated.

Acknowledgements

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