Effect of Non-Lytic Concentrations of Brij Series Detergents on the Metabolism-Independent Ion Permeability Properties of Human Erythrocytes

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ABSTRACT Subcritical micellar concentrations (sub-CMC) of Brij-series detergents alter ion movements between human erythrocytes and their environment when metabolism has been slowed down by incubation at zero degrees centigrade. The effect of nonhemolytic concentrations of detergents on the erythrocyte K^+ and Na^+ movements is described. Results indicate a significant difference in monovalent cation movements, depending on the number of hydrophilic polyoxyethylene units (n). There is an increasing loss of K^+ and gain of Na^+ as n increases from 4 to 20. Where $n \geq 21$, ion movements are not significantly different from those found in erythrocytes not exposed to detergents. The carbon chain length of the detergent fatty acid residue (10–18 carbons) appears to be relatively unimportant, but detergents with unsaturated (oleic acid) hydrophobic regions potentiate K^+ release and Na^+ uptake when compared to the corresponding saturated fatty acid (stearic acid). The erythrocyte stabilizing effect of detergents against hypo-osmotic shock correlates well with the increase of monovalent ion traffic and the mobility of membrane lipids revealed by fluorescence anisotropy measurements.

INTRODUCTION

During the past three decades, a large number of non-ionic detergents have become available for the purification of membrane proteins. Among these the polyoxyethylene-octylphenols (NP-40, Triton X-100, and Triton X-114), the polyoxyethylene-sorbitans (Tween series detergents), the polyoxyethylene-alcohols (Lubrol series), and the polyoxyethylene fatty acid ether (Brij series) detergents are particularly popular (Fig. 1). The hydrophile-lipophile balance (HLB) values of the most frequently used members of these detergent groups fall between 12 and 18. Detergents in the low HLB region (HLB, 12–15) tend to be more efficient in extracting intrinsic membrane proteins, whereas those in the higher HLB range are frequently used for the extraction of external membrane proteins (Gennis, 1989). Because the membrane proteins must be transferred into detergent micelles during isolation, detergent concentrations are maintained at or above the critical micellar concentration (CMC). Owing to their practical application, detergent interactions with the lipid and protein components of plasma membranes have been studied in considerable detail (Jacobs et al., 1966; Jörgensen and Skou, 1971; Umbreit and Strominger, 1973; Kirkpatrick and Gordesky, 1974; Slinde and Flatmark, 1976; Gennis, 1989).

The transfer of plasma membrane constituents into detergent micelles is often associated with the apparently simultaneous collapse of cytoplasmic architecture. Milder detergent action removes lipid components and some of the membrane proteins, but the cytoplasmic architecture is maintained for a relatively long period of time (Schliwa et al., 1981, 1987; Kellermayer et al., 1986). Unexpectedly, plasma membrane fenestration does not result in immediate monovalent ion equilibration between the cell and its environment. All cell types treated with Brij 58 maintained high cytoplasmic K^+ and low Na^+ concentrations for much longer periods of time than would theoretically be required for total equilibration of an aqueous compartment of the size of the cell with its environment (Kellermayer et al., 1984, 1986, 1994; Cameron et al., 1988; Miseta et al., 1991). Ion equilibration appeared to be correlated with the collapse of cytoplasmic architecture and the release of cytoplasmic components, as shown by x-ray microprobe data for nucleated chicken erythrocytes (Cameron et al., 1988). Similar results were obtained when the density distribution of Brij 58-treated L929 cells was studied (Ridsdale and Clegg, 1991).

Despite these earlier observations, the ion equilibration process between detergent-treated cells and their environment remains a complex and largely unexplored phenomenon and depends on the detergent properties and on the blood group characteristics of the erythrocytes (Pazos-Sanou and Mata-Segrada, 1985; Rao et al., 1987; Isomaa et al., 1987; Isomaa and Hagerstrand, 1988). In theory ion equilibration in the presence of detergent can proceed in three ways: i) beyond their CMC values, the most frequently

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$$R - O - (CH_2 - CH_2 - O)_n H$$

FIGURE 1 The chemical structure of Brij series polyoxyethylene adduct non-ionic detergents. R represents a fatty acid residue. n is the number of oxyethylene repeats.

used detergents may cause erythrocyte disintegration, i.e. hemolysis ensues; ii) detergent-mediated opening of the plasma membranes occurs before lysis and presumably leads to the start of accelerated ion traffic between the cell and its environment; and iii) detergent molecules may themselves incorporate into plasma membranes, changing plasma membrane permeability without necessarily removing lipids. This third way is best studied when detergents are applied at nonlytic concentrations (Tragner and Csordás, 1987; Isomaa and Hagerstrand, 1988; Bogner et al., 1989; Bielawski, 1990). In addition, detergents may alter erythrocyte metabolism and/or interfere with membrane proteins involved in ion regulation (Jörgensen and Skou, 1971; Gennis, 1989).

Ways i) and ii) are largely eliminated, if sub-CMC, nonhemolytic concentrations of detergents are used. Although it is known that detergents facilitate K^+ loss and Na^+ uptake in sub-CMC, nonhemolytic concentrations, only a limited number of studies, in part with different aims, have been carried out on the detergent properties, which are involved in this process.

In the present report, we describe the dependency of detergent-mediated ion permeability changes on a) the size of the hydrophilic polyoxyethylene chain, and on b) the nature of the hydrophobic fatty acid residue of Brij series detergents (Fig. 1). Our results indicate that surprisingly small differences in either the hydrophobic or the hydrophilic residue can dramatically change the monovalent cation permeation properties of the erythrocyte. We also demonstrate a significant correlation between erythrocyte membrane permeability changes, the mobility of membrane lipids, and stabilization against osmotic lysis caused by detergent treatment.

MATERIALS AND METHODS

Materials

Heparinized blood was obtained from healthy volunteers, samples being shifted to wet ice immediately after collection.

Polyoxyethylene 2-stearyl ether (Brij 72), polyoxyethylene 4-stearyl ether, polyoxyethylene 10-stearyl ether (Brij 76), polyoxyethylene 20-stearyl ether (Brij 78), polyoxyethylene 21-stearyl ether (Brij 721), polyoxyethylene 100-stearyl ether (Brij 700), and polyoxyethylene 10-oleyl ether (Brij 96), 1,6-diphenyl-1,3,5,-hexatriene (DPH), and trimethylammonium DPH (TMA-DPH) were purchased from Sigma (St. Louis, MO). All other chemicals were purchased from Reanal (Hungary). Detergent stocks (10 mM/L) were prepared in a 50% water, 50% ethanol solution, except for Brij 72, which was dissolved in ethanol.

Measurements of erythrocyte K^+, Na^+, and water levels

Blood samples were centrifuged at 3000 × g for 10 min, and the plasma and buffy coat were removed. The erythrocyte pellets were resuspended in ice-cold 0.15 M NaCl solution and centrifuged as described above, and the supernatant was removed. The washing procedure was repeated twice. For experimental purposes, erythrocytes were resuspended to give a hematocrit (Htc) of 4% in ice-cold TBSR solution (10 mM Tris-HCl, pH 7.4, 148 mM NaCl, and 2 mM RbCl). This solution served as the control. In other cases, TBSR was complemented with one of the above-described detergents. The final concentration was usually 40 μM, a concentration at which none of the above-mentioned detergents proved hemolytic. Ten-milliliter samples were taken immediately after the start of incubation, and at required time points up to 4 h. The samples were centrifuged, as described above, and the supernatants were removed. The erythrocytes were transferred into microcentrifuge tubes of known weights and centrifuged at 16,000 × g for 10 min. The supernatants were removed, and residual pellets were weighed before being freeze-dried in a Savant speed vacuum (SC-110) system. Dry weights were subsequently measured, a 0.5 to 1 ml of 1 M HCl was added to each sample, and the sample was then incubated at room temperature on a rocker table for not less than 24 h.

K^+ and Na^+ levels were measured in a flame photometer (Eppendorf EFOX 5070). Actual ion concentrations were calculated after correction for dilution factors and were based on sample weight data. Each data point represents the mean value of five measurements. Owing to variations in the detergent resistance of different blood samples (Pazos-Sanou and Mata-Segrada, 1985), variations of data increased significantly if experiments were repeated with blood samples obtained from different donors. Nevertheless, we repeated each experiment at least three times. Results of representative experiments are presented in this report.

ATP measurements

Erythrocyte ATP levels were measured with a bioluminescent firefly luciferin/luciferase kit obtained from Boehringer, and a Berthold Biolumat LB-9505, as described by Kőszegi (1988).

Osmotic resistance investigations

Classic osmotic resistance experiments were carried out on the erythrocytes. A 0.15 M (300 mOsm/L) stock solution of NaCl was prepared and diluted serially with distilled water. The osmolarity of the 15 solutions prepared fell between 270 and 88 mOsm/L as measured by a Wescor 5500 vapor pressure osmometer. The total volume of the individual samples was 4.4 ml. Equimolar sub-CMC concentrations of different Brij series detergents were added to each series respectively. Aliquots (150 μl) of isotonic saline-washed blood samples (Htc 50%) were added to each sample and incubated at 4°C for 2 h. After incubation the samples were centrifuged at 4000 × g for 5 min, and 3-ml supernatants were removed for photometric measurements, which was carried out by a Perkin-Elmer Lambda 2 spectrophotometer at 540 nm.

Erythrocyte ghost preparation and fluorescence emission anisotropy measurements

Erythrocyte ghosts were prepared by the method of Dodge et al. (1962). Ghosts were labeled with DPH or with its cationic derivative TMA-DPH in phosphate-buffered saline at 25°C for 30 min. Various detergent molecules were added in equimolar (20–40 μM) concentrations simultaneously, and the ghosts were shifted to ice for at least 1 h. The protein concentration of ghosts was ~100 μg/ml, and the final concentration of DPH or TMA-DPH was 1 μM. The protein concentration of ghost preparations was measured by Lowry's method (Lowry et al., 1951). Measurements were carried out with a Hitachi MPF-4 spectrofluorimeter equipped with polarization ac-

cessories at 4°C. Wavelengths were 360 nm and 425 nm for excitation and detection, respectively. Fluorescence anisotropy was calculated according to the equation: $r = (I_{vv} - GI_{vh})/(I_{vv} + 2GI_{vh})$, where I_{vv} and I_{vh} are the fluorescence intensities measured with a vertical polarizer, and a vertically or horizontally mounted analyzer, respectively ($G = I_{hv}/I_{hh}$) (Donner and Stoltz, 1985). The fluorescent intensities of dyes were always measured in the presence of detergents, but without ghosts. Results obtained were subtracted from those obtained in the presence of labeled ghosts. For each sample, fluorescence was corrected for the scattering effect of unlabeled ghosts.

RESULTS

K^+ release from detergent-treated human erythrocytes

Human erythrocytes were incubated in the presence of 40 μM of each detergent at a Htc of 4% at 0°C for a maximum of 4 h. None of the detergents induced significant hemolysis at these concentrations (<5% by the end of treatment). Erythrocyte ATP concentrations remained remarkably constant and similar to those of the native blood during these treatments (data not shown). Fig. 2 illustrates results from an experiment with polyoxyethylene-(*n*)-stearyl ethers where the number (*n*) of oxyethylene units varied from 4 to 100. The results in Fig. 2, *a* and *b*, indicate that polyoxytheylene-4-stearyl ether had little effect on K^+ release and on Na^+ uptake of erythrocytes, but K^+ release increased with increase of the oxyethylene chain length until $n = 20$. Particularly striking was the finding that polyoxyethylene-4-stearyl ether induced changes in erythrocyte K^+ and Na^+ concentrations that resembled the changes induced by polyoxyethylene-4-stearyl ether or in control (untreated) erythrocytes. Further increase of *n* does not cause a significant difference. Similar results were obtained when polyoxythylene-(*n*)-lauryl ethers were used. Because the experiments were carried out at low temperature, there is no active transport, and therefore rubidium uptake was parallel to Na^+ uptake (data not shown).

Erythrocyte water content remained remarkably constant, being similar to that of normal untreated (control) human erythrocytes (67–69%) after incubation.

In a similar set of experiments, the number of polyoxyethylene residues was kept constant ($n = 10$). Compared with untreated erythrocytes polyoxyethylene-10-stearyl ether treatment caused a modest acceleration of K^+ release and Na^+ uptake compared with untreated erythrocytes (Fig. 3). The results clearly demonstrate that the chain length of saturated fatty acids had little effect on electrolyte movements (Fig. 3). Likewise, incubation of erythrocytes with stearic (C = 18), cetyl (C = 17), or lauric ethers (C = 12) caused effects similar to that of polyoxyethylene-10-stearyl ether. However, a striking difference in monovalent cation movements occurred where polyoxyethylene (10)-oleyl ether (C = 18) was used. The loss of K^+ and the gain in Na^+ ions was dramatically accelerated.

Erythrocyte water contents also remained remarkably constant and close to those of untreated (control) human erythrocytes under these conditions.

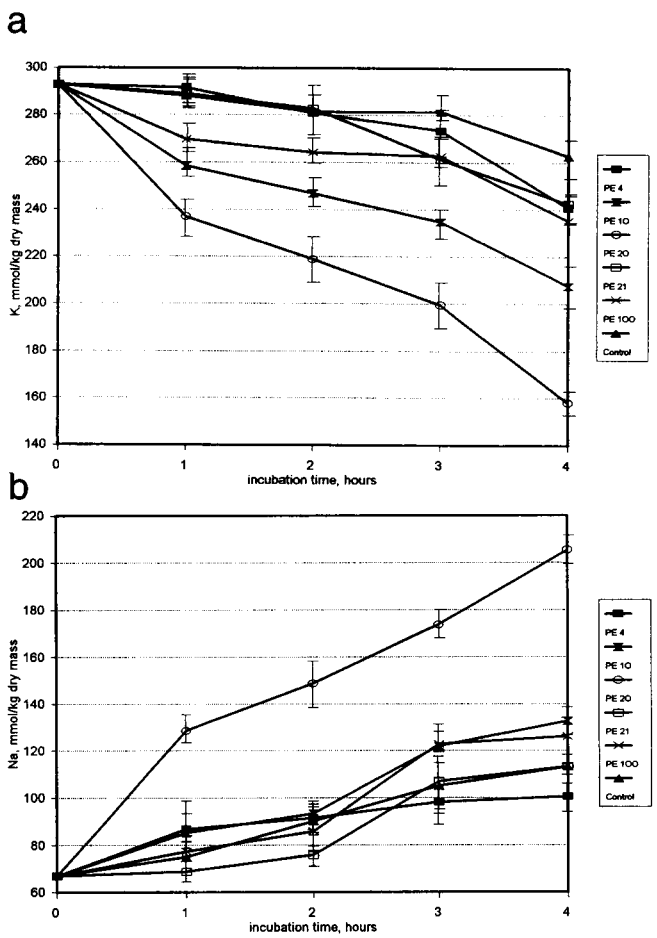

FIGURE 2 Incubation of human erythrocytes in the presence of *n*-stearyl ethers. Variations in the number of polyoxyethylene residues (*n*) in stearic-acid-ether type Brij series detergents alter the release of potassium ions from human erythrocytes incubated on wet ice. Equimolar, 40 μM concentrations of detergents were used at an Htc of 4%. Increasing potassium losses were observed where *n* increased from 4 to 20. A sudden reversal of the effect took place if $n \geq 21$. Complementary changes are seen in erythrocyte Na^+ concentrations (*b*). Each data point represents the mean of five measurements.

The osmotic resistance of detergent-treated erythrocytes

Classic osmotic resistance experiments were carried out with iso-osmotic saline-washed human erythrocytes at 4°C. Solutions (except controls) were complemented with equimolar concentrations of various Brij series detergents. The detergent concentrations were 40 μM. The results (Fig. 4) indicate that the addition of a 4-stearyl ether cause little effect on hemolysis compared to the untreated erythrocytes. The shift of the lysis curve to the right in Fig. 4. suggests that there was protection against osmotic lysis in 10-stearyl ether, and 20-stearyl ether-treated erythrocytes, the latter being the more effective. 21-Stearyl ether also protected erythrocytes against osmotic lysis, but it was a less effective protector against hemolysis than 20- and 10-stearyl ethers, respectively. Although some hemolysis occurred in mildly hypo-osmotic mediums complemented with polyoxyethyl-

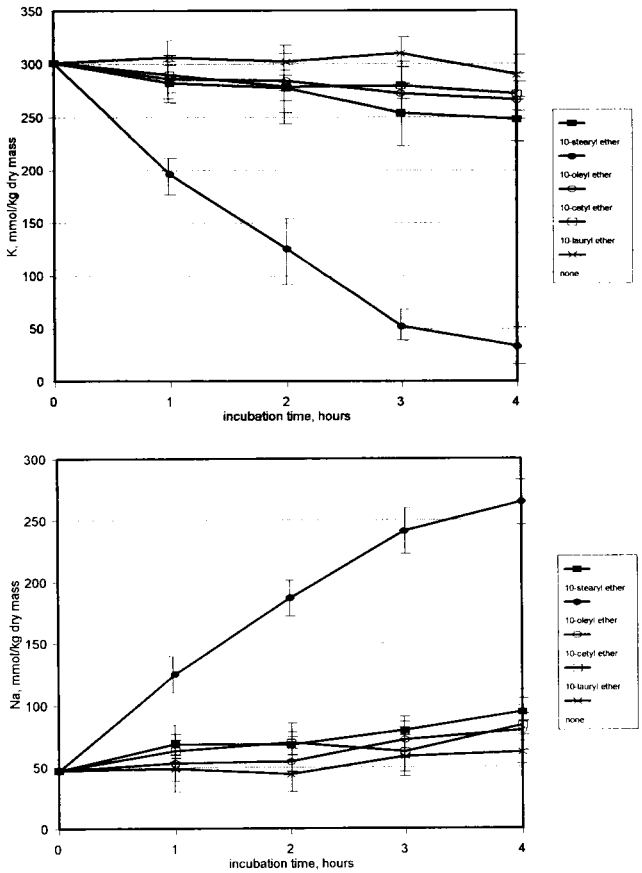

FIGURE 3 Incubation of human erythrocytes in the presence of 10-fatty-acyl ethers. The size of the saturated fatty acid residue did not play a significant role in the loss of potassium ions in various Brij series detergents, if the number of polyoxyethylene repeats was $n = 10$. Equimolar 40 μM concentrations of detergents were used at a Htc of 4%. Nevertheless, the substitution of the saturated stearic acid residue with an unsaturated oleic acid residue resulted in significant acceleration of the equilibration processes. Complementary changes are seen in erythrocyte Na^+ concentrations (*b*). Each data point represents the mean value of five measurements.

ene 10-oleyl ether, it protected the erythrocytes against osmotic lysis better than any of the stearyl ether derivatives. Note especially that the above rank order of polyoxyethylene adduct detergents in protecting erythrocytes against hemolysis is very similar to their effect in accelerating monovalent cation traffic between the erythrocyte and its environment.

Fluorescence anisotropy in detergent-treated erythrocyte ghosts

Erythrocyte ghosts were isolated and labeled with DPH or TMA-DPH as described in Materials and Methods. Changes caused by short-chain ($n = 2$–4) hydrophobic detergents are negligible, but 10- and 20-stearic ethers caused a significant decrease in fluorescent anisotropy ($p < 0.01$), whereas longer stearic acid ethers did not significantly alter results (Table 1). The 10-oleic acid ether-treated

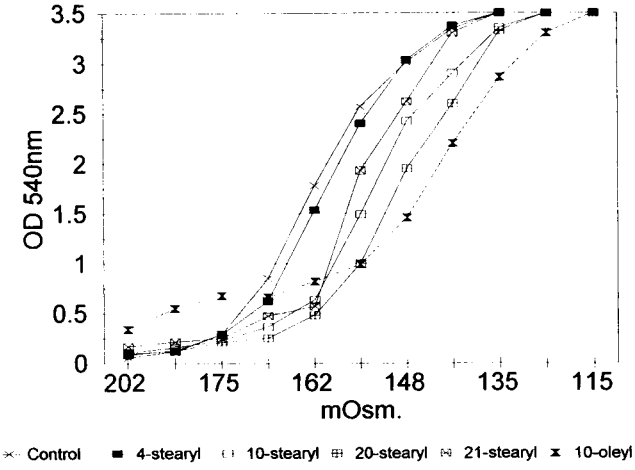

FIGURE 4 Osmotic lysis of human erythrocytes in serial dilutions of 0.89% NaCl solutions at 0°C. The shift of the lysis curves toward the right indicates increased osmotic resistance. All detergents used cause increased resistance. The rank order agrees well with the detergent potential to increase erythrocyte leakiness.

erythrocyte ghosts displayed the largest decrease in fluorescence anisotropy.

Note that the rank order of changes agrees with those seen in case of detergent-mediated increase in ion traffic and in protection against osmotic lysis.

DISCUSSION

The action of detergents on plasma membrane ion permeability properties is surprisingly complex where detergents are applied beyond their CMC values.

Monomer detergent molecules interact exclusively with plasma membranes when detergents are applied in sub-CMC concentrations. Here the complexities in ion equilibration seen above CMC values are largely eliminated as there is little or no lysis of cells, and the effects of membrane-incorporated detergent molecules can be more precisely studied. In previous studies a number of chemically unrelated amphiphiles have been shown to protect erythrocytes against hypotonic lysis (Seeman, 1972; Csordás and Schauenstein, 1984; Isomaa et al., 1986; Isomaa and Hagerstrand, 1988). It was generally assumed that the protecting effect depended upon the concentration of amphi-

TABLE 1 Fluorescence anisotropy in normal and in detergent-treated erythrocyte ghosts

Probe: Detergent	*n*	⟨*r*⟩ DPH (mean ± SD)	⟨*r*⟩ TMA-DPH (mean ± SD)
None	6	0.241 ± 0.001	0.296 ± 0.002
2-Stearyl ether	6	0.243 ± 0.002	0.290 ± 0.004
4-Stearyl ether	6	0.244 ± 0.001	0.289 ± 0.005
10-Stearyl ether	6	0.231 ± 0.007	0.263 ± 0.009
20-Stearyl ether	6	0.229 ± 0.004	0.257 ± 0.006
21-Stearyl ether	6	0.241 ± 0.003	0.281 ± 0.005
10-Oleyl ether	6	0.201 ± 0.003	0.247 ± 0.012

philes incorporated into the plasma membrane. Therefore a direct expansion of the plasma membranes was postulated to be the reason behind increased osmotic resistance of amphiphile-treated erythrocytes. In their excellent studies, Isomaa and Hagerstrand (1988) and Tragner and Csordas (1987) tested several detergents at 37°C but did not elucidate a relationship between increased osmotic resistance and increased monovalent cation permeabilities.

To eliminate complexities arising from possible alterations of erythrocyte metabolism and ion transport properties, we studied the effects of sub-CMC, nonhemolytic concentrations of Brij series detergents at 0°C. Polyoxyethylene fatty-acid ethers (Brij series detergents) added to normal human erythrocytes in nonhemolytic concentrations facilitate the loss of K^+ and the uptake of Na^+ and at 0°C (Fig. 2). The number of polyoxyethylene units (n) seems to be critical in the equilibration process. Small detergent molecules ($n = 2–4$) with low HLB values (HLB $\cong$ 5.4–10) did not significantly facilitate K^+ loss and Na^+ uptake. Increasing oxyethylene unit numbers are associated with increased ion permeability, but there is a sharp reversal of the effect of $n \geq 21$. This same effect had been observed in previous (unreported) studies with Brij 58 ($n = 20$) and Brij 35 ($n = 23$) detergents.

The length of the fatty acid residue appears to be relatively unimportant in ion movements in the size range investigated ($12 \leq C \leq 18$). This is the size range for the alkyl chain of most abundant lipids in the cell membranes (Gennis, 1989). We found that 10-stearic acid ether behaves in a manner similar to that of 10-lauric acid ether in facilitating ion release (Fig. 3). This observation was somewhat surprising because the hydrophobic tail region of detergents is known to be intercalated between membrane lipids (McIntosh et al., 1980; Gennis, 1989; Bressauer et al., 1985). Isomaa and Hagerstrand (1988) studied the effects of a variety of octa-ethyleneglycol-alkylethers (C_{10}-C_{16}) on the ion-exchange properties of human erythrocytes. They found that these derivatives potentiated K^+ release in the rank order $C_{14} > C_{12} > C_{10} > C_{16}$. However, they also noted "that passive fluxes of potassium were only slightly increased below CAH_{75}, but markedly increased at CAH_{max}." (CAH_x is a percentage expression of the antihemolytic concentrations of detergents.) Consequently they compared different concentrations of detergents, based on the assumption that at equiprotecting concentrations the number of detergent molecules in the lipid phase is similar, because of the different oil/water partitioning of detergents. However, the HLBs of 10-stearyl and 10-oleyl ethers are virtually identical, yet these two derivatives show a marked difference in their abilities to potentiate monovalent cation exchanges (Fig. 3).

A further observation is that equimolar concentrations of detergents cause protection against hemolysis in a rank order, which agrees well with detergent capabilities in facilitating ion exchange between the cell and its environment (Fig. 4). This observation supports the idea that an accelerated equilibration between the detergent-treated erythrocytes and their environment could be important in protection against hypotonic hemolysis. Fast compensatory movements of hydrated ions and small molecules presumably work against swelling induced by low osmolarity solutions, where fast net water uptake can itself cause disintegration of the plasma membrane and lysis of the cell. A direct expansion of the plasma membranes is unlikely to play a role in this phenomenon, because the oil/water partition of detergents follows an inverse order. Consequently, hydrophobic (low HLB) detergent molecules are more abundant in the plasma membranes than hydrophilic (high HLB) ones, yet the former offer little if any protection against hypotonic lysis (Fig. 4).

Unfortunately, a more direct comparison between the findings of others (Tragner and Csordás, 1987; Isomaa and Hagerstrand, 1988) and our data is not possible because their experiments were carried out at higher temperatures, and their detergents, although similar, were not identical to ours.

Further correlations were explored, when we used two fluorescent probes to follow alterations of membrane anisotropy caused by non-ionic detergents. DPH is assumed to be located in the hydrocarbon region of membranes, and there is evidence that its cationic derivative TMA-DPH is located near the phospholipid polar headgroups (Donner and Stoltz, 1985). Nevertheless, DPH and TMA-DPH signals changed similarly within our experimental setup (Table 1).

Results indicate that hydrophobic ($n = 2–4$) stearic acid ethers cause little alteration in fluorescent anisotropy, but 10- and 20-stearic acid ethers cause decreased fluorescent anisotropy in the case of detergent-treated red blood cell ghosts. Although the equilibrium distribution ratio of 10-oleyl ether must be similar to its 10-stearyl sibling, the former caused a more pronounced decrease in fluorescent anisotropy than the latter, indicating that the physicochemical nature of the interacting hydrophobic tail group may be more important than considered previously by many membrane researchers.

In conclusion, by using cold incubation and non-lytic concentrations of Brij series detergents we explored a relationship between detergent properties and accelerated ion traffic. A significant correlation was demonstrated between the increased osmotic resistance of detergent-treated erythrocytes, the detergent-mediated acceleration of ion traffic, and the detergent-dependent increased mobility of membrane lipids.

Although the mechanism of action of various detergents is considered in the light of their physicochemical properties, the fact that minor changes in detergent properties may cause dramatic alterations of ion traffic supports the idea that specific detergent-lipid and detergent-protein interactions should also be considered (Kirkpatrick et al., 1974; Gennis, 1989; Sun et al., 1968; Moore et al., 1989; Broring et al., 1989).

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