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Alcohols produce reversible and irreversible acceleration of phospholipid flip-flop in the human erythrocyte membrane

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The slow, non-mediated transmembrane movement of the lipid probes lysophosphatidylcholine, NBD-phosphatidylcholine and NBD-phosphatidylserine in human erythrocytes becomes highly enhanced in the presence of 1-alkanols (C_2–C_8) and 1,2-alkane diols (C_4–C_8). Above a threshold concentration characteristic for each alcohol, flip rates increase exponentially with the alcohol concentration. The equieffective concentrations of the alcohols decrease about 3-fold per methylene added. All 1-alkanols studied are equieffective at comparable calculated membrane concentrations. This is also observed or the 1,2-alkane diols, albeit at a 5-fold lower membrane concentration. At low alcohol concentrations, flip enhancement is reversible to a major extent upon removal of the alcohol. In contrast, a residual irreversible flip acceleration is observed following removal of the alcohol after a treatment at higher concentrations. The threshold concentrations to produce irreversible flip acceleration by 1-alkanols and 1,2-alkane diols are 1.5- and 3-fold higher than those for flip acceleration in the presence of the corresponding alcohols. A causal role in reversible flip-acceleration of a global increase of membrane fluidity or membrane polarity seems to be unlikely. Alcohols may act by increasing the probability of formation of transient structural defects in the hydrophobic barrier that already occur in the native membrane. Membrane defects responsible for irreversible flip-acceleration may result from alterations of membrane skeletal proteins by alcohols.

Introduction

The slow non-mediated transbilayer reorientation of phospholipids and related analogues in the erythrocyte membrane becomes highly enhanced after oxidative modification of membrane proteins [1–3], following heat denaturation of the membrane skeletal protein spectrin [4,5] upon an increase of the intracellular Ca^{2+} concentration [6], in the presence of local anesthetics [7] or after insertion of channel-forming antibiotics [7,8] and bacterial toxins [7] into the membrane, as well as following electrophoration of the membrane permeability barrier [9]. Flip acceleration was postulated to result from a perturbation of the membrane lipid bilayer at the lipid/protein interface or in the bulk lipid domain [10,11]. Flip accelerations are usually paralleled by an increase of leak permeabilities to ions and small hydrophilic non-electrolytes [2,3,7,10–12], taken to indicate the formation of aqueous leaks in the hydrophobic barrier. The mechanistic relationship between the two phenomena is presently not clear. The aqueous leaks might be envisaged as the product of a local or general decrease of membrane hydrophobicity on top of fluctuating defect structures existing in the native permeability barrier. Leak permeabilities and colloid-osmotic lysis were also observed upon incubation of erythrocytes with aliphatic alcohols [13–17]. Recently, an alcohol-induced increase of erythrocyte membranes permeability to large hydrophobic ions could be described in terms of an alcohol-induced increase of the dielectric constant of the hydrophobic membrane core ('membrane polarity'), using the Born-Parsegian equation [18]. Such a decrease of membrane polarity will lower the energy barrier which has to be overcome by ions penetrating the lipid bilayer, and, thus, probably also by the polar head-group of a phospholipid in the course of flip-flop. Since ion permeability is exponentially related to the height of this energy barrier, even a small decrease of the barrier will enhance permeability and flip rates to a great extent. This concept has been questioned on the basis of experiments on pure lipid membranes [19], showing that alkanes, which are not expected to change the membrane dielectric constant, increase proton leak-

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Abbreviations: NBD-PC, 1-oleoyl, 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyl]-*sn*-glycerol-3-phosphocholine; PS, phosphatidylserine; PEG, polyethylene glycol).

permeability like 1-alkanols. There is presently no consensus concerning the mechanism of alcohol-induced enhancement of the permeability of lipid membranes to charged compounds in contrast to the permeability to non-electrolytes, for which stimulating effects of alcohols are usually assigned to an enhancement of membrane 'fluidity' [14,18].

The present study aimed to investigate the effect of insertion of 1-alkanols and 1,2-alkane diols into the erythrocyte membrane on the translocation of phospholipid probes between the two leaflets of the membrane bilayer.

Materials and Methods

Materials

Human blood anticoagulated by citrate was obtained from the local blood bank and used with 7 days. After centrifugation, the supernatant and the buffy coat were carefully removed and erythrocytes washed three times with isotonic saline. 1-[^{14}C]Palmitoyl-L-lyso-3-phosphatidylcholine (spec. act. 56 mCi/mmol) was purchased from Amersham. The alcohols were of the highest purity available. Fatty-acid-free bovine serum albumin was from Paesel (Frankfurt) and Dextran 4 (M_r 4000–60000) from Serva (Heidelberg). Bee venom phospholipase A_2 was from Boehringer-Mannheim. Phospholipase D (*Streptomyces* species) and poly(ethylene glycol) (PEG) 6000 were from Sigma. 1-oleoyl-2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino-hexanoyl]-*sn*-glycero-3-phosphocholine (NBD-PC) and *N*-dansyl-phosphatidylethanolamine were obtained from Avanti Polar Lipids (Birmingham, USA). NBD-phosphatidylserine (PS) was prepared from NBD-PC by transphosphatidylation using phospholipase D according to Ref. 20 in a biphasic system of ethylacetate and water containing Mes buffer (pH 5.6), 3.4 mol l^{-1} serine and 80 mmol l^{-1} Ca^{2+} (60 min, 30°C). After addition of EGTA and lipid extraction, NBD-PS was purified by thin-layer chromatography.

Incubation medium A used in experiments with PEG contained (mmol l^{-1}): KCl (90), NaCl (45) and Na_2HPO_4/ NaH_2PO_4 (12.5) (pH 7.4). Incubation medium D used in experiments with alcohols was Medium A supplemented with 40 mmol l^{-1} Dextran 4 (M_r 4000–6000) (final osmolarity 380 mmol l^{-1}). Dextran provides protection against colloid-osmotic lysis by alcohols. Incubation medium C contained (mmol l^{-1}): KCl (140), Hepes (20) and $MgCl_2$ (0.5).

Methods

Measurement of flip-flop. The movement of [^{14}C]lysophosphatidylcholine to the inner membrane leaflet after its prior insertion into the outer leaflet was measured by the time-dependent decrease of extractability of the lipid probe from the cells by albumin as described before [1]. Briefly, 10 μl of radioactively-labeled palmitoyllysophosphatidylcholine in ethanol (2 nmol) was transferred to a 1-ml tube and evaporated to dryness. Subsequently, 80 μl erythrocyte suspension in medium A or D (hematocrit 50%) were added and the vial shaken for 2 min at room temperature. After addition of 1 ml of medium, the cells were sedimented by centrifugation, the supernatant removed and 360 μl of medium added. This suspension was then transferred to a new tube and incubated at 37°C. After different incubation periods, 20-μl samples were mixed with 400 μl of medium at 0°C, the cells isolated by centrifugation and the supernatant removed. To quantify the total membrane-bound ^{14}C-labeled lysophosphatidylcholine, cells were lysed with 200 μl water. To quantify the labeled lipid in the inner leaflet, the cells were extracted twice (1–2 min, 0°C) with 400 μl medium containing 1.5% albumin, washed once with 1 ml medium and lysed with 200 μl water. Lysates were added to 3 ml scintillation fluid and radioactivity was counted. The albumin-inextractable (inner leaflet) fraction of [^{14}C]lysophosphatidylcholine was calculated from the ratio of radioactivities in the two lysates. Flip rates were derived from the time-dependent increase of this inner leaflet fraction during the initial phase.

To measure the flip of NBD-phospholipids, 5 μl of a stock solution of NBD-PC or NBD-PS in ethanol (0.1 mmol l^{-1}) were mixed (22°C) with 1 ml of an erythrocyte suspension (hematocrit 10%). After 5 min cells were washed with buffer and flip rates measured using the albumin extraction method described above. Cells from 100-μl samples were isolated by centrifugation and hemolysed either directly (total fluorescence) or following albumin extraction (inner leaflet fluorescence) by mixing with 100 μl water. Subsequently, lipids were extracted with 800 μl isopropanol [21]. After centrifugation, the supernatants were transferred to a cuvette and fluorescence was measured at 523 nm (excitation at 472 nm; spectrofluorimeter Jobin-Yvon JY3).

Flip measurements in the presence of alcohol. After insertion of the lipid probe into the membrane, the cells were resuspended in medium D (hematocrit 10%), containing the alcohols and the flip rates were measured as described above. Alcohol concentrations given refer to the amount of the alcohol per liter of final erythrocyte suspension.

Flip measurements after pretreatment with alcohol. Erythrocytes were suspended in medium D (hematocrit 10%) containing alcohol and incubated for 15 min at 37°C, unless indicated otherwise. To remove the alcohol cells were isolated by centrifugation, resuspended in medium D and incubated (5 min, 22°C). This washing procedure was repeated twice. Subsequently, cells were isolated by centrifugation, the lipid probe was inserted into the membrane and flip rates measured as described above.

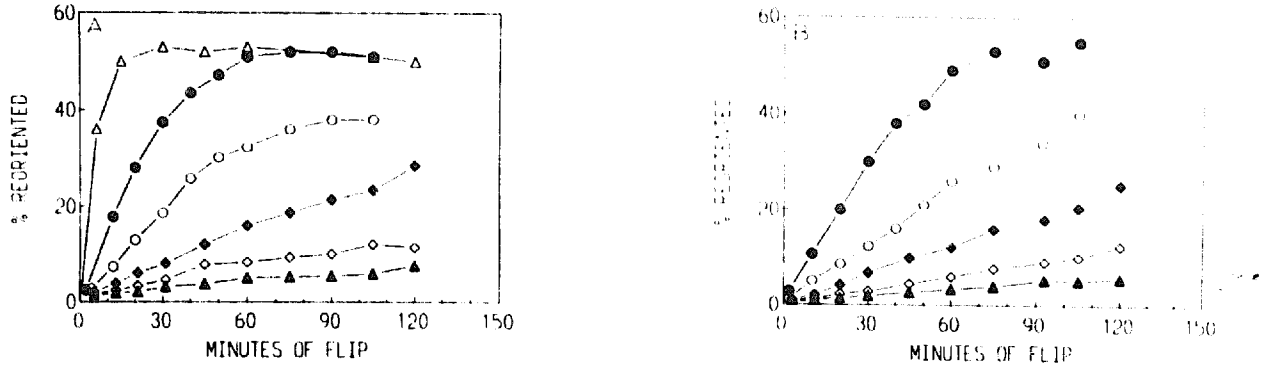

Fig. 1. Concentration-dependent enhancement of the flip rate of lysophosphatidylcholine in the presence of 1-butanol (A) and 1,2-butanediol (B). Transbilayer reorientation (flip) of lysophosphatidylcholine was defined by the time dependent increase of inextractability of the lipid probe by albumin (see Materials and Methods). (A) 1-Butanol (mmol l^{-1}): 0 (▲), 113 (◇), 135 (◆), 158 (○), 180 (●) and 225 (△). (B) 1,2-Butanediol (mmol l^{-1}): 0 (▼), 540 (◇), 720 (◆), 810 (○) and 1080 (●).

Flip measurements in presence of poly(ethylene glycol) (PEG) 6000. Erythrocytes preloaded with lipid probe were suspended (hematocrit 10%) in medium A containing 12, 15 or 17% PEG (w/w). In order to have the same number of cells in every sample taken for flip measurements despite the progressive aggregation of cells in the presence of PEG, the suspension was immediately divided into 50-μl aliquots which were then incubated at 37°C. After increasing time intervals, one of these aliquots was mixed with 400 μl of ice-cold medium D and the albumin-inextractable fraction of the lipid probe determined as described above.

Measurement of the surface dielectric constant. Surface dielectric constants of the membrane were estimated using the method described for lipid vesicles by Ohki and Arnold [22]. Since the *N*-dansyl-phosphatidylethanolamine originally used in this method does not spontaneously insert into erythrocyte membranes we used its lyso-derivative. This was prepared by enzymatic cleavage of *N*-dansyl-phosphatidylethanolamine in the presence of 0.1 mg Triton X-100 in 1 ml of a solution containing 5 mmol l^{-1} Hepes and 1 mmol l^{-1} $CaCl_2$ with 20 IU phospholipase A_2 (37°C). After 15 min, the enzymic activity was blocked by EDTA (3 mmol l^{-1} final concentration). *N*-dansyl-lysophosphatidylethanolamine (1% of membrane phospholipid) was then inserted into the membrane of resealed ghosts (10% suspension) prepared according to Ref. 23. After centrifugation the ghosts were resuspended into medium C (1% suspension) and the fluorescence spectrum scanned with a spectrofluorimeter (Jobin-Yvon JY3) using an excitation wavelength of 340 nm. Shifts by alcohols of the emission maximum were assumed to report polarity changes of the environment of the polar head-groups of membrane phospholipids.

By comparison of these shifts with shifts of the emission maximum of *N*-dansyl-lysophosphatidylethanolamine in various organic solvents with known dielectric constants an apparent membrane surface dielectric constant could be derived. For the unmodified membrane, this was about 27.

Results

Acceleration of flip in the presence of alcohols

1. 1-Alkanols. The presence of 1-alkanols in the flip medium results in acceleration of the slow inward translocation of ^{14}C-labeled lysophosphatidylcholine. As a typical example, flip acceleration by increasing concentrations of 1-butanol is shown in Fig. 1A. In order to obtain a more quantitative comparison of the effectiveness of different alcohols, initial flip rates were derived from the time-course of translocation of the lipid probe. Values obtained in the presence of alcohol (k) were normalized to those in its absence (k_0) and plotted vs. the logarithm of the alcohol concentration (Fig. 2). The slope of the curves is independent of the alcohol used. When the data are plotted on a double logarithmic scale, they can be fitted by straight lines. From the slopes of these lines, an apparent order of

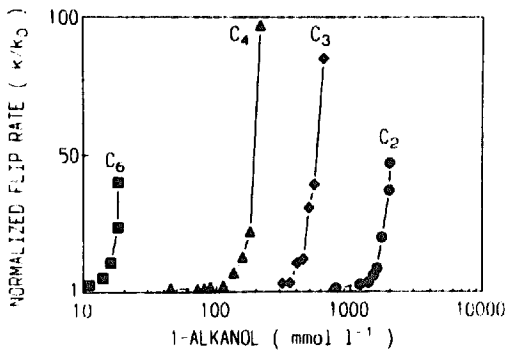

Fig. 2. Dose-response curves for the enhancement of the flip rate of lysophosphatidylcholine by various 1-alkanols. The normalized initial flip-rates (k/k_0) for hexanol (■), butanol (▲), propanol (◆) and ethanol (●) derived from the time-dependent increase of transbilayer reorientation (cf., Fig. 1) are plotted against the logarithm of the alcohol concentration. k, flip rate in presence of alcohol; k_0, flip rate in the absence of alcohol (0.028 ± 0.008 h^{-1}, $n = 47$).

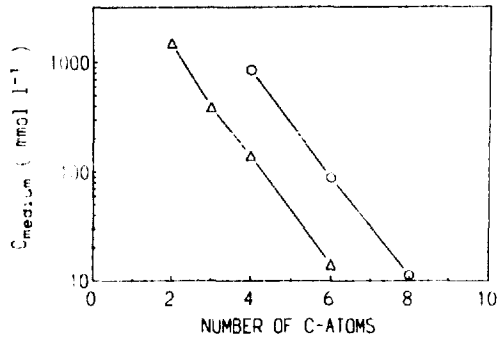

Fig. 3. Semilogarithmic plot of medium concentrations of various 1-alkanols (△) and 1,2-alkane diols (○) required to obtain a 6-fold acceleration of lysophosphatidylcholine flip vs. the number of C-atoms of the alcohol.

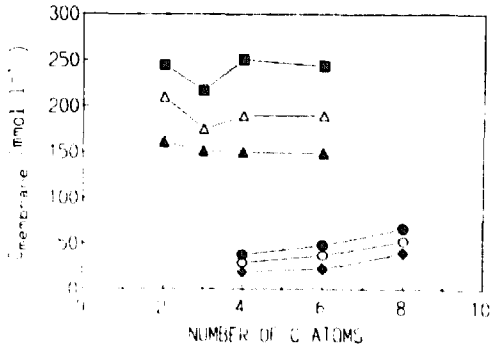

Fig. 4. Effect of increasing chain-lengths of 1-alkanols and 1,2-alkane diols on membrane concentrations required for equal extents of flip acceleration. For concentrations of 1-alkanols (3 upper curves) and 1,2-alkane diols (3 lower curves) in the medium required to accelerate flip 2- (▲, ◆), 6- (△, ○) and 20-fold (■, ●), membrane concentrations were calculated using established erythrocyte membrane/water partition coefficients of 0.14 (ethanol), 0.43 (1-propanol), 1.36 (1-butanol), 13.5 (1-hexanol), 0.034 (1,2-butanediol), 0.42 (1,2-hexanediol) and 5.16 (1,2-octanediol). The equieffective membrane concentrations were then plotted against the alcohol chain-length.

about 5 was derived for the concentration dependence of alcohol-induced flip. Such a high order was also observed for alcohol-induced hemolysis [16,24] and can be described in terms of a cooperative membrane effect of the alcohols. The effectivity of the alcohols increases with the length of their hydrocarbon chain. The threshold concentration is about 1000 mmol l^{-1} for ethanol and decreases 10-fold for every increase of the chain length by 2 C-atoms, i.e., to about 100 mmol l^{-1} for butanol and to about 10 mmol l^{-1} for hexanol (Fig. 2).

A plot of the logarithm of alcohol concentrations in the medium required to produce a 6-fold increase of flip rate vs. the alcohol chain-length gives a straight line (Fig. 3). On average, each additional methylene increases the alcohol potency 2.9-fold. Since the various alcohols have different membrane solubilities, a comparison of their effectivity is traditionally based on membrane concentrations. Equieffective alcohol concentrations in the aqueous medium were converted into equieffective membrane concentrations using membrane/water partition coefficients. Directly measured partition coefficients for the erythrocyte membrane are only available for pentanol, hexanol, heptanol and octanol from the work of Seeman [25]. Since the membrane/water partition coefficients of various drugs were regularly found to be 5-times smaller than the corresponding octanol/water partition coefficients, he calculated membrane/water partition coefficients for ethanol, propanol and butanol from their octanol/water partition coefficients. We plotted Seeman's membrane/water partition coefficients against the alcohol chain-length on a semilogarithmic scale and fitted the data by a linear regression. Using membrane/water partition coefficients derived from this regression line (see the legend to Fig. 4), membrane concentrations of various alcohols producing 2-, 6- and 20-fold increases of flip rates were determined and replotted against the number of C-atoms of the alcohols. From Fig. 4, it becomes clear that the alcohols are approximately equieffective at the same membrane concentrations, independent of their chain length.

Flip acceleration by butanol was also observed for a fluorescently labeled diacylphosphatidylcholine (Fig. 5). Starting at about 70 mmol l^{-1}, the slow non-mediated flip of NBD-phosphatidylcholine (k approx. 0.02 h^{-1}) became significantly accelerated. The threshold concentration of butanol in the medium was somewhat lower in the case of this probe than in the case of the monoacylphosphatidylcholine probe (Fig. 5, dotted line). As observed for the flip of lysophosphatidylcholine, the alcohol potency increases with increasing

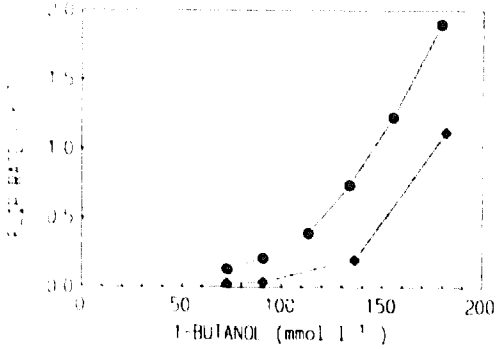

Fig. 5. Dose-response for the enhancement of the flip rate of NBD-labeled phospholipids by 1-butanol. In order to measure the non-mediated flip of NBD-phosphatidylserine (◆), the flippase activity was blocked by ATP-depletion of the cells [26]. This was obtained by a treatment of cells with iodoacetate (5 mmol l^{-1}, 15 min, 37°C) followed by an incubation (60 min, 37°C) in medium containing 5 mmol l^{-1} inosine. This treatment decreases cellular ATP to < 2% (< 20 μmol l^{-1} cells) of the original level (see Ref. 65). The flip of NBD-phosphatidylcholine (●) was measured in freshly washed (high ATP) cells. For reasons of comparison, flip rates of lysophosphatidylcholine in the presence of 1-butanol (dotted line) are also shown).

chain length (Fig. 6). By conversion of alcohol concentrations to membrane concentrations using membrane/water partition coefficients (see the legend to Fig. 4, for octanol a value of 135 was used), similar accelerations of NBD-phosphatidylcholine flip are obtained at comparable membrane concentrations of 1-alkanols with 2 to 8 C-atoms (data not shown).

Moreover, 1-butanol also accelerated the non-mediated flip of NBD-phosphatidylserine (Fig. 5) as determined after prior blockage, by ATP-depletion of cells, of the aminophospholipid flippase which normally catalyzes its transbilayer reorientation [26]. ATP-depletion suppressed the flip rate by > 99%, whereas no measurable effect on the slow non-mediated flip of NBD-PC was observed (data not shown). The flip of NBD-PS remaining after ATP-depletion (k approx. 0.01 h^{-1}) is slower than that of the non-mediated flip of NBD-PC (k approx. 0.02 h^{-1}). This apparent effect of the structure of the polar head-group of the phospholipid on the flip probably reflects the intrinsic properties of the flip process and may be responsible for the shift of the dose-response curve of NBD-PS to higher butanol concentrations compared to that of NBD-PC.

Interestingly, the presence of butanol at concentrations below that required to enhance non-mediated flip of NBD-phosphatidylserine in ATP-depleted cells (10–50 mmol l^{-1} butanol) also stimulated NBD-phosphatidylserine translocation by the aminophospholipid flippase in ATP-rich cells (data not shown). A similar effect was very recently observed for benzylalcohol [27].

2. 1,2-Alkane diols. The equieffectivity of 1-alkanols (C_2–C_8) at the same membrane concentrations suggests that the introduction of OH-groups into the membrane is the determinating factor in the stimulating effect of 1-alkanols on flip acceleration. If this is true, alkane diols should be more effective than 1-alkanols. To test this assumption, we investigated the effect of a second hydroxyl-group at the 2-position of 1-alkanols on the flip accelerating effect of alcohols. Like the 1-alkanols, the 1,2-alkane diols produced a

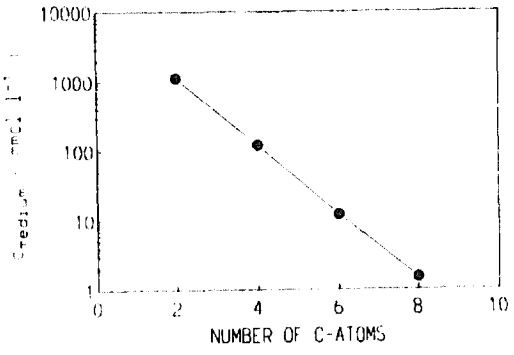

Fig. 6. Semilogarithmic diagram of medium concentrations of various 1-alkanols required to obtain flip rates for NBD-phosphatidylcholine of 0.5 h^{-1}, plotted against the number of C-atoms of the alcohol.

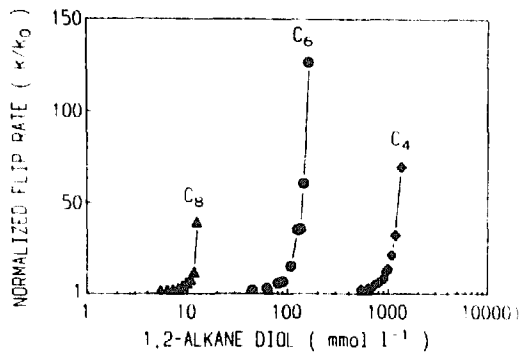

Fig. 7. Dose-response curves for the enhancement of the flip rate of lysophosphatidylcholine by various 1,2-alkane diols. The normalized initial flip rates (k/k_0) for 1,2-butanediol (♦), 1,2-hexanediol (●) and 1,2-octanediol (▲) are plotted against the logarithm of the alcohol concentration.

concentration-dependent increase of the flip of lysophosphatidylcholine, albeit at higher concentrations in the incubation medium. As a typical example, flip acceleration by 1,2-butanediol is shown in Fig. 1B. The threshold concentration of 1,2-butanediol in the medium required for flip acceleration is about 5-fold higher than that of 1-butanol. In Fig. 7, the normalized initial flip rates (k/k_0) obtained for various diols are plotted against the logarithm of their concentrations. As expected, the threshold concentrations in the medium required to obtain flip acceleration decrease with increasing chain length.

From a semilogarithmic plot of the equieffective concentrations of diols in the medium against their chain length (Fig. 3) it becomes clear that each additional methylene increases the potency of the diol 3-fold. The equieffective concentrations of diols in the medium were converted into membrane concentrations. Since no measured partition coefficients for the erythrocyte membrane are available, numbers were indirectly derived using (i), available octanol/water partition coefficients for 1,2-ethanediol [28] and 1,2-butanediol [19] and (ii), the presumption that the linear relationship between the logarithm of the octanol/water partition coefficients of 1,2-alkane diols and their chain length has the same slope as that for alkanols and alkanoic acids [29]. This is a reasonable assumption since the slope reflects the $V\delta G$ for partition per methylene group. Octanol/water partition coefficients were converted into membrane/water partition coefficients using a ratio between the two of 5 [25]. A plot of the membrane concentrations of alkane diols required to obtain 2-, 6- and 20-fold increases of flip rates against the chain length of the diols gives lines with slightly positive slopes (Fig. 4). Considering the uncertainties in the estimation of the partition coefficients of alkane diols, it may be concluded that the potency to accelerate flip is essentially independent of the chain length.

Comparison of the membrane concentrations of 1,2-alkane diols required to induce flip acceleration with those for 1-alkanols indicates that the introduction of an additional OH-group into the C-2 position of an alkanol increases the effectiveness of the alcohol about 5-fold, i.e., a diol would be intrinsically more effective than a mono-alkanol.

In addition to enhancement of flip rates, alcohols also affect the stationary distribution of the phospholipid probes between the two membrane leaflets. The preference of lysophosphatidylcholine for the outer membrane leaflet of untreated cells [30] was shifted to an essentially equal distribution in the presence of alcohols (cf., Fig. 1A and B). Symmetry of distribution in the presence of alcohols was also observed for NBD-phosphatidylcholine (data not shown). Moreover, at high flip rates in the presence of butanol (200 mmol l^{-1}) the flippase activity is overruled and NBD-phosphatidylserine distributes more symmetrically, 65% in the inner leaflet as compared to 85% in the case of the absence of butanol (data not shown).

Flip acceleration by a pretreatment of erythrocytes with alcohols

In further experiments, we studied the reversibility of the alcohol-induced flip acceleration. To this end, erythrocytes were treated with various alkanols for 15 min at 37°C and the alcohol was subsequently removed by washing. In the case of 1-alkanols, such a pretreatment does not affect the flip rates up to concentrations 1.5-fold of those required to produce the first detectable flip acceleration in the presence of the alcohol. In contrast, at higher concentrations the alcohol-enhanced flip rates are only partly reversible. Both, 1-alkanols (Fig. 8A) and 1,2-alkane diols (Fig. 8B) induce a partly irreversible flip-enhancement. The dose-response curves for these irreversible effects run parallel to those obtained in the presence of alcohols (Figs. 2, 7 and 8), but are shifted to higher concentrations. Related to membrane concentrations, these irreversible effects are independent of the alkanol chain-length like the reversible effect.

The irreversible flip-acceleration increases with increasing time of exposure of cells to the alcohols (data not shown). Moreover, its extent also increases with the temperature of the alcohol pretreatment (data not shown). From the temperature dependence for 1-butanol pretreatment an apparent activation energy of 88 kJ/mol could be derived for this process. Since partitioning of butanol from water into the membrane has been shown [31–33] to increase with temperature, the observed temperature-dependence is partly due to a temperature-dependent increase of the membrane concentration of butanol. A correction of the measured apparent activation energy for this contribution of partitioning is hampered by the uncertainty, regarding the extent of temperature-dependence of membrane partitioning of butanol (see below) and by the very steep dependence of the flip acceleration on the membrane concentration of the alcohol (see Fig. 2).

Temperature-dependence of the alcohol-induced flip

In order to further characterize the alcohol-enhanced flip, its temperature-dependence was investigated. To this end, flip rates of lysophosphatidylcholine were measured at different temperatures in the presence of various alcohols. The apparent activation energies calculated from the temperature dependence of accelerated flip rates are 136 ± 7 kJ/mol for 1-hexanol (18 mmol l^{-1}), 138 ± 7 kJ/mol for 1-butanol (225 mmol l^{-1}) and 119 ± 9 kJ/mol for 1,2-hexanediol (108 mmol l^{-1}) and, thus, somewhat higher than the value for the flip in the absence of alcohol (107 ± 7 kJ/mol) (n = 3–4, ± S.D.). As outlined above, these apparent activation energies have to be corrected for temperature-dependent changes of membrane parti-

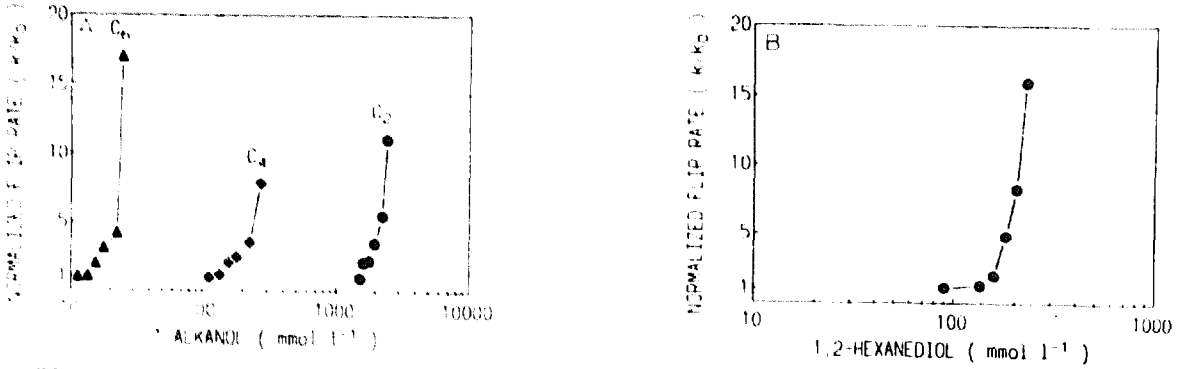

Fig. 8. Irreversible enhancement of flip rates of lysophosphatidylcholine by 1-alkanols and 1,2-alkane diols. (A) 1-Alkanols. Erythrocytes were pretreated (15 min, 37°C) with ethanol (●), 1-butanol (◆) or 1-hexanol (▲) at varying concentrations. (B) 1,2-Hexanediol. Erythrocytes were pretreated (15 min, 37°C) with 1,2-hexane diol (●) at varying concentrations. For reasons of comparison, the dose-response curve for the enhancement of flip rate by the presence of 1,2-hexanediol is shown (dotted line). Following the removal of the alcohols by washing of the cells' lysophosphatidylcholine flip was measured at 37°C. The normalized initial flip rates (k/k_0) are plotted against the logarithm of the alcohol concentration.

tioning of alcohols. Published data for artificial lipid membranes suggest that the membrane partition coefficient of 1-butanol increases between 1.07- and 1.48-fold [31–33] upon an increase of temperature from 30 to 40°C. Although this increase appears small, it will account for a considerable correction of the extent of flip acceleration due to the steep dose-response curves (Fig. 2) for the alcohol-induced flip. In view of the uncertainties in absolute values and the temperature-dependence of the partition coefficients, any correction of flip rates would be subject to marked ambiguity. We, therefore, decided not to attempt such corrections.

For cells pretreated with hexanol (18 mmol l^{-1}, 15 min, 37°C), an apparent activation energy of 88 kJ/mol was derived from the temperature-dependence of lysophosphatidylcholine flip. In this case, no correction for partitioning is required. Although this activation energy is still high, it is somewhat lower than that for flip in native cells (107 kJ/mol).

Effect of PEG on flip rate and changes of surface dielectric constant

One of the tentative explanations for effects of aliphatic alcohols on membranes claims changes of interfacial polarity, i.e., the dielectric constant at the membrane surface. Such changes have in fact been reported for artificial membranes exposed to poly(ethylene glycol) [22]. In order to test whether an altered interfacial polarity might account for the flip enhancement by 1-alkanols and 1,2-alkane diols, two types of experiments were carried out: (a), Flip rates were measured in the presence of PEG. In these experiments, concentrations of PEG 6000 of up to 12% (w/w) did not result in changes of flip rates. At concentrations of 15 and 17%, PEG accelerates flip 2–3-fold (data not shown). These accelerations are small compared to those produced by alcohols. Higher concentrations of PEG could not be tested as a result of irreversible aggregation of erythrocytes by PEG which interfered with the extraction of the lipid probe by albumin required for flip quantification. (b), In a further set of experiments, we determined surface dielectric constants in the presence of alcohols. Membrane surface dielectric constants as measured by the membrane probe *N*-dansyl-lysophosphatidylethanolamine decreased from about 27 (see Materials and Methods) in the absence of PEG to 24 at 15% PEG and to values below 20 at 20% PEG. This lowering, by PEG, of the apparent surface polarity of erythrocytes is in line with results obtained with lipid vesicles [22]. In contrast, alcohols at concentrations shown to accelerate flip rates up to 20-fold did not affect the apparent surface polarity. Higher alcohol concentrations even increased the apparent surface polarity. For ethanol at 1.9 mol l^{-1} and hexanol at 25 mmol l^{-1}, it was 30. Moreover, even high concentrations of 1,2-butanediol (up to 2 mol l^{-1}) did not affect the apparent membrane-surface polarity.

Discussion

In this study, the exposure of erythrocytes to 1-alkanols and 1,2-alkane diols has been shown to result in a concentration-dependent acceleration of the inward translocation of lipid probes such as ^{14}C-labeled monoacylphosphatidylcholine and fluorescent NBD-diacylphospholipids. Large increases of flip rates by alcohols could be measured without interfering colloid-osmotic hemolysis by protecting the cells with Dextran 4 (see Materials and Methods). The 1-alkanols with 2 to 8 C-atoms are equieffective at similar membrane concentrations calculated on the basis of membrane/water partition coefficients. The same is true for 1,2-alkane diols. Surprisingly, the introduction of an additional OH-group into an 1-alkanol at the C-2-position increases the effectivity about 5-fold. In line with this finding, 1,2-alkane diols were reported earlier to be more effective than 1-alkanols in increasing the cation permeability of lipid vesicles [19]. The same is true for stimulation of K^+-leakage, non-electrolyte permeability and permeability to the hydrophobic cation methyltriphenylphosphonium in erythrocytes (Deuticke, B., unpublished data). In an earlier study [34], stimulation, by an alcohol analogue, of flip-flop in erythrocytes was reported. Reorientation of diacylphosphatidylcholine was shown to be enhanced by low concentrations (1.2 mmol l^{-1}) of hexylglycerol. Assuming hexylglycerol to be structurally related to 1,2-decanediol, the extrapolation of dose-response curves for the 1,2-alkane diols (Fig. 5) to 1,2-decanediol would predict flip acceleration above a threshold concentration of about 1 mmol l^{-1}, which is in the effective range [34]. In the present study, we found that a strong enhancement of flip rates by alcohols goes along with a more symmetric steady-state of distribution of both the outer leaflet phospholipid analogues, NBD-lysophosphatidylcholine and NBD-phosphatidylcholine, and of the inner leaflet phospholipid analogue phosphatidylserine. In line with this observation, benzylalcohol was recently shown to abolish the asymmetric distribution of spin-labeled phosphatidylcholine between the two membrane leaflets [27].

At the threshold concentrations for flip acceleration by 1-alkanols and 1,2 alkane diols, the molar ratios of alcohol to membrane and to phospholipid are about 1:4 and 1:20. Flip accelerations at low membrane concentrations of alcohols are reversible upon removal of the alcohol by washing. Such a reversibility of flip enhancement was previously found for the local anesthetic tetracaine [7]. Pretreatment of erythrocytes with higher alcohol concentrations reveals an irreversible

component of flip acceleration becoming evident after the removal of the alcohol.

The hydrophobic membrane core can be regarded as the rate-limiting barrier for the transbilayer movement of the polar head group of phospholipids (see, e.g., Ref. 35). Therefore, it would be desirable for the discussion of the present results to know the concentrations and concentration profiles of the various alcohols in the membrane. While the absolute values for membrane concentrations of alcohols are a matter of considerable uncertainty for reasons already outlined in the Results section, there is general agreement concerning ratios between partition coefficients and, thus, membrane concentrations for homologous alcohols [25,29,36]. Studies on alcohol effects can thus be evaluated rather reliably in comparative terms, while conclusions based on absolute values have to be regarded with caution.

A second uncertainty concerns the intramembrane concentration profiles of alcohols. Lipid and biological membranes are highly anisotropic with respect to polarity and 'fluidity' and the alcohols can therefore not be expected to be distributed homogeneously. On the one hand, it has been proposed that short chain 1-alkanols up to three C-atoms prefer the hydrophilic polar head-group domain of the phospholipids [37–40] while medium chain 1-alkanols with 4–10 C-atoms distribute into the outer hydrophobic segments of the acyl-domain [37,40]. Others have suggested that alcohols with 1–4 C-atoms distribute into the hydrophobic core of the membrane [38,40]. In any case, the long chain 1-alkanols (> C-10) align between the fatty-acid chains of the membrane lipids [37,40]. No information is available on the localization of short-chain 1,2-alkane diols. On the basis of their greater hydrophilic moiety they will probably prefer the hydrophilic interfacial region of the membrane.

Different concentration profiles for alcohols of different chain length are somewhat difficult to reconcile with the very regular and monotonous variation of the effects of homologous alcohols with their partition coefficients, which means that normalized to their membrane concentrations the effects are independent of chain length. This chain-length independence concerns effects on functions and properties of the membrane located in both, the protein- and the lipid-domain, such as the anesthetic potency [41], inhibition of transport of sulfate [42], choline [43] and glucose [44] in erythrocytes, transformation of biconcave erythrocytes into echinocytes [16], increase of cation permeability in erythrocytes and resulting hemolysis [16] and flip acceleration in erythrocytes (Fig. 4).

Several models for the action of alcohols but also of other compounds with anesthetic properties on membranes have to be considered [45]. Two of these seem to be very attractive in view of the amphiphilic nature of the alcohols and the higher potency of 1,2-alkane diols than 1-alkanols to accelerate flip rates (Fig. 3) and to increase leak fluxes (Ref. 19, and Deuticke, B., unpublished data). First, one might envisage an increase of the polarity of the hydrophobic membrane barrier following the introduction of alcohols. Second, alcohols might perturb the membrane/water interface. Moreover, an increase of membrane fluidity by the alcohols may contribute to flip acceleration, while the gradual irreversible flip-acceleration at higher alcohol concentrations suggests a causal role for a perturbation of membrane proteins.

Insertion of alcohols into the membrane has been found to go along with a decrease of membrane-lipid order-parameters [39,46]. However, a decrease of lipid order (increase of membrane 'fluidity') is unlikely to be the major mechanism of flip acceleration by alcohols for a number of reasons. One, alterations of membrane-lipid order by variation of the membrane-cholesterol level or of temperature [46,47], comparable in their extent to alterations of order produced by alcohols [46,48], result only in minor changes of flip rates (4–5-fold [30]) compared to those produced by alcohols (100-fold). Two, lipid-order decreases linearly with increasing alcohol concentration [49,50], whereas flip acceleration by alcohols increases exponentially. Three, the alcohol effects on the flip of phospholipids (this study) and on transport of the hydrophobic ions [18] are strong compared to alcohol effects on the non-mediated transport of nonelectrolytes, e.g., erythritol (Deuticke, B., unpublished data). One would not expect such differences in the case of an effect based on changes of membrane lipid-order. Moreover, changes of membrane order by an extreme variation of the membrane cholesterol level have only small effects on both, diffusion of non-electrolytes through the lipid phase [51] and phospholipid flip [30].

The anesthetic potency of 1-alkanols correlates with their capacity to break hydrogen bonds at the lipid/water interface [52]. Binding of alkanols was, therefore, claimed to cause dehydration and increased hydrophobicity of the membrane/water interface as well as to increase the fluidity of the membrane core. Hydrogen bonds between water and lipid, between adjacent lipids and between lipid and protein may be involved [53]. Poly(ethylene glycols) (PEGs) are established modifiers of the membrane/water interface [22], ultimately inducing cell fusion [54]. According to our results, low concentrations of PEG produce only a minor increase of flip rates in erythrocytes (2–3-fold, see also Ref. 54) in spite of a considerable decrease of apparent surface polarity. In contrast, 1-alkanols and 1,2-alkane diols do not affect the apparent surface polarity in spite of a considerable flip acceleration. At high concentrations, 1-alkanols even seem to shift the polarity in the opposite direction. These results do not support

the view that a modified hydration of the phospholipid headgroup region contributes to flip acceleration by alcohols.

Instead of perturbing the membrane/water interface, alcohols could exert their major effect on the hydrophobic domain. The hydrophobic membrane core with its low dielectric constant is likely to constitute the main energy barrier for the transfer of the polar head group of phospholipids to the opposite side of the membrane (see e.g., Ref. 35). Transfer of ions through the membrane will become highly enhanced by even very small increases of the membrane dielectric constant [55] as may be derived from the Born-Parsegian relationship [56,57];

$$\delta W_B = q^2/2r(1/\epsilon_m - 1/\epsilon_w) \quad (1)$$

This equation relates the dielectric constant of the membrane core (ϵ_m) to the height of the energy barrier (δW_B) that has to be overcome by an ion of radius r and charge q when passing the hydrophobic core. ϵ_w is the dielectric constant of the aqueous phase. δW_B is related to the ion-permeability by:

$$P = P_0 \exp - \delta W_B \quad (2)$$

P_0 is the permeability of an identical, but uncharged analogue. Alcohol-induced increases of ion-permeability of artificial and natural membranes have been assigned to such changes of the dielectric constant [58] and have even been modelled quantitatively [18]. A comparable quantitative prediction of the expected effects of alcohols is not possible for the flip of phospholipids with zwitterionic head-groups as used in our study. However, assuming a relationship similar to Eqn. 1 to be valid, one can compare effects on flip rates of predicted changes of membrane polarity. Rough estimates of membrane dielectric constants produced by various alcohols at equieffective membrane concentrations may be obtained using the Clausius-Mosotti equation for the calculation of dielectric constants of mixtures of solvents (cf., Ref. 18). For a membrane concentration of an 1-alkanol of 200 mmol l^{-1}, which produces a considerable increase of flip rates (6-fold in case of lysophosphatidylcholine), one obtains an increase of the dielectric constant of the membrane core from a normal value of 2.10 (taken from Ref. 18) to 2.14 (ethanol), 2.17 (butanol), 2.17 (hexanol), 2.19 (octanol) and 2.11 (1,2-butanediol). Thus, the calculated increases of the membrane dielectric constant vary with the chain lengths of the alcohol and the increase by butanediol is very small compared to changes by 1-alkanols. This is not readily compatible with the equal extent of flip enhancement. Moreover, calculated increases of the membrane dielectric constant for increasing alcohol concentrations do not correlate (after conversion into predicted changes of flip rates using Eqns. 1 and 2) with the increases of flip rates actually observed under these conditions. These inconsistencies may indicate the inapplicability of Eqn. 1 to zwitterions or of the Clausius-Mosotti equation to anisotropic liquids. Alternatively, the flip acceleration by alcohols could be due to a local instead of a global increase of membrane polarity. This possibility will be discussed next.

The erythrocyte membrane is a less effective permeability barrier than a phospholipid bilayer, which is in turn a lower and certainly more complex barrier than a pure hydrocarbon sheet [19,59]. Moreover, rates of phospholipid flip are higher in erythrocytes than in pure lipid systems [60]. To explain this difference transient defects in the heterogeneous biomembranes acting as leak pathways [59] and flip sites (see e.g., Ref. 7) have been discussed. On the basis of this concept, it may be postulated that short-chain alcohols (C_2–C_8) increase the probability of the formation of such transient defects and/or alter their barrier properties. The 1,2-alkane diols would be particularly effective. The alterations might result from a perturbation of the lipid annulus surrounding intrinsic membrane proteins, either by displacement of lipid from the annulus [61] or secondary to a direct modification of intrinsic proteins. Modifications of intrinsic proteins by alcohols, either directly or secondary to a perturbation of the lipid domain, are indicated by the inhibitory effects of alcohols on carrier-mediated transports [62]. The butanol concentration required for 50% inhibition of anion [42] and choline transport [43] is about 60 mmol l^{-1}. At these low concentrations of butanol, the aminophospholipid flippase is stimulated (data not shown, cf., also Ref. 27). Thus, intrinsic proteins are already modified at alcohol concentrations lower than those required to produce a threshold flip-acceleration (about 100 mmol l^{-1}).

In addition to their effects on intrinsic proteins, alcohols have been found to perturb membrane skeletal proteins as indicated by irreversible changes of the cell shape (Ref. 63 and Deuticke, B., unpublished data), increase of the membrane skeletal volume [64] and increase of the membrane shear modulus [17] by alcohols. Perturbation of the membrane skeleton could induce formation of small clusters of intrinsic proteins that increase the probability of formation of transient defect structures in the hydrophobic domain acting as flip sites. Such a perturbation could be responsible for reversible flip-acceleration. Perturbation of skeletal proteins lasting after removal of the alcohol might be involved in the irreversible flip acceleration by alcohol pretreatments (Fig. 8) analogous to the causal role of oxidative modification of the membrane skeleton in flip acceleration [1] and leak permeability [10,23]. A contribution to the irreversible flip-acceleration (up to

15-fold, Fig. 8) of a modified bilayer resulting from an alcohol-induced decrease of the asymmetric distribution of endogenous phospholipids, as anticipated by the phospholipid analogues, can not be excluded.

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References

1 Bergmann, W.L., Dressler, V., Haest, C.W.M. and Deuticke, B. (1984) Biochim. Biophys. Acta 769, 390–398.
2 Deuticke, B., Heller, K.B. and Haest, C.W.M. (1987) Biochim. Biophys. Acta 899, 113–124.
3 Deuticke, B., Henseleit, U., Haest, C.W.M., Heller, K.B. and Dubbelman, T.M.A.R. (1989) Biochim. Biophys. Acta 982, 53–61.
4 Franck, P.F.H., Op den Kamp, J.A.F., Lubin, B., Berendsen, W., Joosten, P., Briët, E., Van Deenen, L.L.M. and Roelofsen, B. (1985) Biochim. Biophys. Acta 815, 259–267.
5 Classen, J., Deuticke, B. and Haest, C.W.M. (1989) J. Membr. Biol. 111, 169–178.
6 Henseleit, U., Plasa, G. and Haest, C.W.M. (1990) Biochim. Biophys. Acta 1029, 127–135.
7 Schneider, E., Haest, C.W.M., Plasa, G. and Deuticke, B. (196) Biochim. Biophys. Acta 855, 325–336.
8 Classen, J., Haest, C.W.M., Tournois, H. and Deuticke, B. (1987) Biochemistry 26, 6604–6612.
9 Dressler, V., Schwister, K., Haest, C.W.M. and Deuticke, B. (1983) Biochim. Biophys. Acta 732, 304–307.
10 Deuticke, B. Poser, B., Lütkemeier and Haest, C.W.M. (1983) Biochim. Biophys. Acta 731, 196–210.
11 Haest, C.W.M., Heller, K., Kunze, I., Dressler, V. and Deuticke, B. (1983) Biomed. Biochim. Acta 42, 127–129.
12 Schwister, K. and Deuticke, B. (1985) Biochim. Biophys. Acta 816, 332–348.
13 Burt, D.H. and Green, J.W. (1971) Biochim. Biophys. Acta 225, 46–55.
14 Deuticke, B. (1977) Rev. Physiol. Biochem. Pharmacol. 78, 1–97.
15 Wilbers, K.H., Haest, C.W.M., Von Bentheim, M. and Deuticke, B. (1979) Biochim. Biophys. Acta 554, 388–399.
16 Chi, L.-M., Wu, W., Sung, K.-L.P. and Chien, S. (1990) Biochim. Biophys. Acta 1027, 163–171.
17 Chi, L.-M. and Wu, W. (1991) Biochim. Biophys. Acta 1062, 46–50.
18 Orme, F.W., Moronne, M.M. and Macey, R.I. (1988) J. Membr. Biol. 104, 57–68.
19 Barchfeld, G.L. and Deamer, D.W. (1988) Biochim. Biophys. Acta 944, 40–48.
20 Juneja, L.R., Kazuoka, T., Yamane, T. and Shimizu, S. (1988) Biochim. Biophys. Acta 960, 334–341.
21 Peuchant, E., Wolff, R., Salles, C. and Jensen, R. (1989) Anal. Biochem. 181, 341–344.
22 Ohki, S. and Arnold, K. (1990) J. Membr. Biol. 114, 195–203.
23 Klonk, S. and Deuticke, B. (1992) Biochim. Biophys. Acta 1106, 143–150.
24 Williams, R.J., Bross, J., Takahashi, T. and Meryman, H.T. (1984) Cryo-letters 5, 269–276.
25 Seeman, P. (1972) Pharmacol. Rev. 24, 583–655.
26 Colleau, M., Hervé, P., Fellman, P. and Devaux, P.F. (1991) Chem. Phys. Lip. 57, 29–37.
27 Bassé, F., Saint-Marie, J., Maurin, L. and Bienvenüe, A. (1992) Eur. J. Biochem. 205, 155–162.
28 Leo, A., Hansch, C. and Elkins, D. (1971) Chem. Rev. 71, 525–616.
29 Stein, W.D. (1986) in Transport and diffusion across cell membranes, pp. 87–107, Academic Press, Orlando.
30 Bergmann, W.L., Dressler, V., Haest, C.W.M. and Deuticke, B. (1984) Biochim. Biophys. Acta 772, 328–336.
31 Katz, Y. and Diamond, J.M. (1974) J. Membr. Biol. 17, 101–120.
32 Bradley, D.J. and Richards, C.D. (1984) Br. J. Pharmacol. 81, 161–167.
33 Johnson, S.M. and Bangham, A.D. (1969) Biochim. Biophys. Acta 193, 92–104.
34 Jain, M.K., Jahagirdar, D.V., Van Linde, M., Roelofsen, B. and Eibl, H. (1985) Biochim. Biophys. Acta 818, 356–364.
35 Pownall, H.J. and Smith, L.C. (1989) Chem. Phys. Lip. 50, 191–211.
36 Jain, M.K., Gleeson, J., Upreti, A. and Upreti, G.C. (1978) Biochim. Biophys. Acta 509, 1–8.
37 Brasseur, R., Chatelain, P., Goormaghtigh, E. and Ruysschaert, J.-M. (1985) Biochim. Biophys. Acta 814, 227–236.
38 Hitzemann, R.J., Schueler, H.E., Graham-Brittain, C. and Kreishman, G.P. (1986) Biochim. Biophys. Acta 859, 189–197.
39 Hitzemann, R.J. (1989) Biochim. Biophys. Acta 983, 205–211.
40 Jain, M.K. and Wu, N.M. (1977) J. Membr. Biol. 34, 157–201.
41 Pringle, M.J., Brown, K.B. and Miller, K.W. (1981) Mol. Pharmacol. 19, 49–55.
42 Forman, S.A., Verkman, A.S., Dix, J.A. and Solomon, A.K. (1985) Biochemistry 24, 4859–4866.
43 Devés, R. and Krupka, R.M. (1990) Biochim. Biophys. Acta 1030, 32–40.
44 Lacko, L., Wittke, B. and Geck, P. (1973) J. Cell. Physiol. 83, 267–274.
45 Albrecht, R.F. and Miletich, D.J. (1988) Gen. Pharmacol. 19, 339–346.
46 Kutchai, H., Cooper, R.A. and Forster, R.E. (1980) Biochim. Biophys. Acta 600, 542–552.
47 Cooper, R.A., Leslie, M.H., Fischkoff, S., Shinitzky, M. and Shattil, S.J. (1978) Biochemistry 17, 327–331.
48 Pang, K.-Y.Y. and Miller, K.W. (1978) Biochim. Biophys. Acta 511, 1–9.
49 Pang, K.-Y.Y., Braswel, L.M., Chang, L., Sommer, T.J. and Miller, K.W. (1980) Mol. Pharmacol. 18, 84–90.
50 Miller, K.W., Firestone, L.L., Alifimoff, J.K. and Streicher, P. (1990) Proc. Natl. Acad. Sci. USA 86, 1084–1087.
51 Grunze, M., Forst, B. and Deuticke, B. (1980) Biochim. Biophys. Acta 600, 860–869.
52 Chiou, J.-S., Ma, S.-M., Kamaya, H. and Ueda, I. (1990) Science 248, 583–585.
53 Uedo, I. (1991) in Drug and anesthetic effects on membrane structure and function (Aloia, R.A., Curtain, C.C. and Gordon, L.M., eds.), pp. 15–33, Wiley, New York.
54 Huang, S.K. and Hui, S.W. (1990) Biophys. J. 58, 1109–1117.
55 Dilger, J.P., McLaughlin, S.G.A., McIntosh, T.J. and Simon, S.A. (1979) Science 206, 1196–1198.
56 Parsegian, A. (1969) Nature 221, 844–846.
57 Flewelling, R.F. and Hubbell, W.L. (1986) Biophys. J. 541–552.
58 Reyes, J. and Latorre, R. (1979) Biophys. J. 28, 259–280.
59 Deamer, D.W. and Nichols, J.W. (1989) J. Membr. Biol. 107, 91–103.
60 Zachowski, A. and Devaux, P. (1990) Experientia 46, 644–656.
61 Lopes, C.M.B. and Louro, S.R.W. (1991) Biochim. Biophys. Acta 1070, 467–473.
62 Deuticke, B., Grebe, R. and Haest, C.W.M. (1990) in Blood cell biochemistry (Harris, J.R., ed.), Vol. 1, pp. 475–529, Plenum Press, New York.
63 McLawhon, R.W., Marikovsky, Y., Thomas, N.J. and Weinstein, R.S. (1987) J. Membr. Biol. 99, 73–78.
64 Vertessy, B.G. and Steck, T.L. (1989) Biophys. J. 55, 255–262.
65 Bröring, K., Haest, C.W.M. and Deuticke, B. (1989) Biochim. Biophys. Acta 986, 321–331.