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Acceleration of phospholipid flip-flop in the erythrocyte membrane by detergents differing in polar head group and alkyl chain length

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Abstract

The detergents, alkyltrimethylammonium bromide, *N*-alkyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (zwittergent), alkane sulfonate, alkylsulfate, alkyl-β-D-glucopyranoside, alkyl-β-D-maltoside, dodecanoyl-*N*-methylglucamide, polyethylene glycol monoalkyl ether and Triton X-100, all produce a concentration-dependent acceleration of the slow passive transbilayer movement of NBD-labeled phosphatidylcholine in the human erythrocyte membrane. Above a threshold concentration, which was well below the CMC and characteristic for each detergent, the flip rate increases exponentially upon an increase of the detergent concentration in the medium. The detergent-induced flip correlates with reported membrane-expanding effects of the detergents at antihemolytic concentrations. From the dependence of the detergent concentration required for a defined flip acceleration on the estimated membrane volume, membrane/water partition coefficients for the detergents could be determined and effective detergent concentrations in the membrane calculated. The effective membrane concentrations are similar for most types of detergents but are 10-fold lower for octaethylene glycol monoalkyl ether and Triton X-100. The effectiveness of a given type of detergent is rather independent of its alkyl chain length. Since detergents do not reduce the high temperature dependence of the flip process the detergent-induced flip is proposed to be due to an enhanced probability of formation of transient hydrophobic structural defects in the membrane barrier which may result from perturbation of the interfacial region of the bilayer by inserted detergent molecules.

Keywords: Erythrocyte membrane; Detergent; Partition coefficient; NBD-phospholipid; Flip-flop

1. Introduction

Detergents are soluble amphiphiles that are able to distribute into cell membranes and thereby, at low concentrations, produce mixed detergent/lipid bilayers. Upon an increase of the detergent concentra-

Abbreviations: C8-, C10-, C12-, C14TAB, octyl, decyl, dodecyl and tetradecyl trimethylammonium bromide; C6, C10S, hexane and decane sulfonate; C8-, C10-, C12SU, octyl, decyl and dodecylsulfate; C8-, C10-, C12-, C14Z, *N*-octyl, decyl, dodecyl and tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate; C6-, C8-, C10-, C12GP, hexyl, octyl, decyl and dodecyl-β-D-glucopyranoside; C10-, C12MP, decyl and dodecyl-β-D-maltoside; C10-, C12EO8, octaethylene glycol mono-decyl and -dodecyl ether; C12EO3, triethylene glycol mono-dodecyl ether; C12MEGA, dodecanoyl-*N*-methylglucamide; C12OOH, dodecanoic acid; NBD-PC, 1-oleoyl-2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyl]-*sn*-glycero-3-phosphocholine; DIDS, 4,4′-diisothiocyanostilbene-2,2′disulfonic acid

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tion, a critical ratio of detergent to membrane lipid is reached at which saturation of the bilayer with detergent occurs [1]. For phospholipid bilayers, detergent to phospholipid ratios at saturation vary from 0.3 to 2.5 dependent on the type of detergent [2–4]. Above this concentration, bilayers and lipid/detergent micelles coexist which results in solubilization of membrane lipid and release of mixed micelles into the aqueous phase [2–4]. Solubilization of phospholipid bilayers by various detergents will be completed at detergent to phospholipid ratios between 1 and 4 [3,5]. Prior to solubilization of the cell membrane by detergents at concentrations well below their CMC, detergents have been shown to expand the membrane surface area of erythrocytes as detectable by their antihemolytic effects [6]. At these lower concentrations, detergents perturb membrane structure [3,5,7–9] and thereby increase leak permeability [2,3,6,9] that results in colloid-osmotic hemolysis of erythrocytes [6].

Previously, leak permeability induced by insertion of local anesthetics, antibiotics, bacterial toxins and alcohols into the erythrocyte membrane has been found to go along with acceleration of the transbilayer movement of phospholipid probes [10–14]. Acceleration of this movement by simple 1-alkanols with 2–8 C-atoms has been found to be comparable at the same membrane concentration [13].

In the present study, neutral, zwitterionic, anionic and cationic detergents with different alkyl chain length (C6–C14), at antihemolytic concentrations, are shown to accelerate the slow non-mediated flip of NBD-labeled phosphatidylcholine (PC) from the outer to the inner membrane leaflet of red blood cells. After determination of membrane/water partition coefficients, membrane concentrations of detergents were calculated and effectiveness of various detergents was then compared.

2. Materials and methods

2.1. Materials

Erythrocyte concentrates were obtained from the local blood bank and used within 10 days. Erythrocytes were isolated by centrifugation and washed three times with isotonic saline. The incubation medium (pH 7.4) contained (mmol l^{-1}): KCl (90), NaCl (45), phosphate (12.5) and sucrose (44) (= KNPS) or Dextran 4 (M_r = 4000–6000) (= KNPD). The latter additives protected the cells against colloid-osmotic hemolysis by detergents.

The detergents, octyl, decyl, dodecyl and tetradecyl trimethylammonium bromide (C8-, C10-, C12-, C14TAB), hexane and decane sulfonate (C6-, C10S) as well as decylsulfate (C10SU) were purchased from Fluka (Deisenhofen, Germany). Dodecylsulfate (C12SU) was from Serva Electrophoresis (Heidelberg, Germany). *N*-Alkyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonates (C8-, C10-, C12-, C14Z), alkyl-β-D-glucopyranosides (C6-, C8-, C10-, C12GP), alkyl-β-D-maltosides (C10-, C12MP), triethylene glycol monododecyl ether (C12EO3), octaethylene glycol monoalkyl ethers (C10-, C12EO8), dodecanoyl-*N*-methylglucamide (C12-MEGA), octylsulfate (C8SU), 1-octanol and dodecanoic acid were obtained from Sigma (Deisenhofen, Germany). Triton X-100 was from Roche Molecular Biochemicals (Mannheim, Germany). Detergents were of highest purity available, meaning at least 98–99% pure or in the case of C8SU 95% pure.

1-Oleoyl-2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-aminohexanoyl]-*sn*-glycero-3-phosphocholine (18:1,6-NBD-PC) was obtained from Avanti Polar Lipids (Alabaster, AL) and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) from Calbiochem (Bad Soden, Germany).

2.2. Methods

2.2.1. Measurement of the inward translocation (flip) of NBD-phospholipids

After insertion of trace amounts of fluorescent NBD-phospholipid (8 nmol/ml packed cells) into the outer membrane leaflet of erythrocytes (7 min, 0°C), cells were washed once, resuspended in KNPS containing detergent (hematocrit 0.6–23%) and incubated at 37°C. The time-dependent flip of the NBD-phospholipid to the inner membrane leaflet was measured by following the increase of fluorescence in the inner membrane leaflet, F_i, using the albumin extraction procedure [13] or the dithionite method [15]. At various time intervals, 55–950-μl samples of the suspension were taken and in the event of low hematocrit cells were isolated by centri-

fugation. In the first method, these samples were mixed with medium containing albumin (final concentration 1.3–2 g dl^{-1}) and incubated (2 min, 21°C). After centrifugation, the albumin extraction was repeated once and cells washed with KNPS. In the second method, samples of cell suspension or isolated cells were incubated (2 min, 0°C) with 700 μl of reducing solution containing 80 mmol l^{-1} dithionite, 100 μmol l^{-1} DIDS and 80 mmol l^{-1} Tris (pH 10). After centrifugation, cells were washed twice with medium (0°C).

Cells were then hemolyzed with 100 μl of water and lipids extracted by addition of 800 μl of isopropanol. Following centrifugation of the extract, fluorescence of the supernatant (F_i) was measured [13,16]. The fluorescence in the inner membrane leaflet was related to the total isopropanol-extractable fluorescence, F_{tot}, in the same amount of hemolyzed cells. An exponential function, $q\bullet[1-\exp(-k_1\bullet t/q)]$, was fitted to the kinetic data of the non-extractable fractions (F_i/F_{tot}), where q represents the fraction of probe in the inner leaflet under stationary conditions and k_1 the rate constant for the unidirectional flip [16].

2.2.2. Determination of membrane/water partition coefficients

Membrane/water partition coefficients, K_p (mol l^{-1} membrane phase/mol l^{-1} aqueous phase), were determined according to the method of Lissi et al. [17]. To this end, the concentration dependence of flip acceleration by a given detergent was measured at various hematocrits (0.6–23%). The rate constants derived from the flip kinetics were plotted against detergent concentrations initially present in the medium and the concentrations required to enhance the flip rate constant from about 0.0003 min^{-1} in the absence of detergent [16] to 0.0020 min^{-1} were determined. Plots of total moles of the detergents (n_d) in the cell suspensions, at these concentrations, against the corresponding membrane volumes (V_m), with n_d and V_m calculated for 1 l of aqueous phase (V_{water}), gave linear relationships. From the slopes and intercepts of the straight lines fitted to the data points, the membrane concentrations (C_m) and the membrane/water partition coefficients (K_p) of the detergent were calculated using the formula [17]: $n_d = C_m\bullet V_{water}/K_p + C_m\bullet V_m$.

The volume of aqueous phase was taken as the sum of volumes of medium and intracellular aqueous space. The latter was assumed to be 65% of cell volume. The fraction of membrane volume per cell was assumed to be 0.8%. Taking a volume for the human erythrocyte of about 90 μm^3 and a cell surface area of 135–140 μm^2, a membrane fraction of 0.8% assumes an effective thickness of the membrane of about 5 nm for the distribution of detergents.

3. Results

The slow flip of the phospholipid probe NBD-PC (k_1 about 0.0003 min^{-1}, [16]) from the outer to the inner membrane leaflet of human erythrocytes is accelerated in the presence of all detergents investigated. This acceleration could be measured in the absence of significant extent of colloid-osmotic hemolysis by protecting cells with sucrose or Dextran 4 (see Section 2.2). As typical examples, flip kinetics of NBD-PC in the presence of increasing concentrations of C10TAB and C10GP are shown in Fig. 1. In the cases of alkyl-TAB, alkyl-Z, alkyl-MP, C12EO3, alkyl-EO8, and Triton X-100, flip kinetics were measured using the albumin extraction technique. In the cases of alkyl-GP, C12MEGA, alkyl-S and alkyl-SU, kinetics had to be measured with the dithionite reduction method, since the albumin technique produced flip kinetics with an initial (<5 min) steep increase of the non-extractable probe followed by a slower continuous increase of non-extractable probe (data not shown). This initial fraction of non-extractable probe was observed (data not shown) for the echinocyte-forming detergent C12-SU but also for C12-GP that produces stomatocytes following a very short period of echinocytosis (<1 min at 37°C). The extent of initial non-extractability increased upon an increase of detergent concentration and correlated with the formation of spherostomatocytes upon albumin addition (data not shown) which probably preferentially extracts detergent from the outer membrane leaflet. It seems therefore possible that a limited access of albumin to the deep membrane invaginations of spherostomatocytes prevents extraction of the probe from this membrane area and causes the initial steep increase of non-extractability. For flip in presence of detergents routinely measured

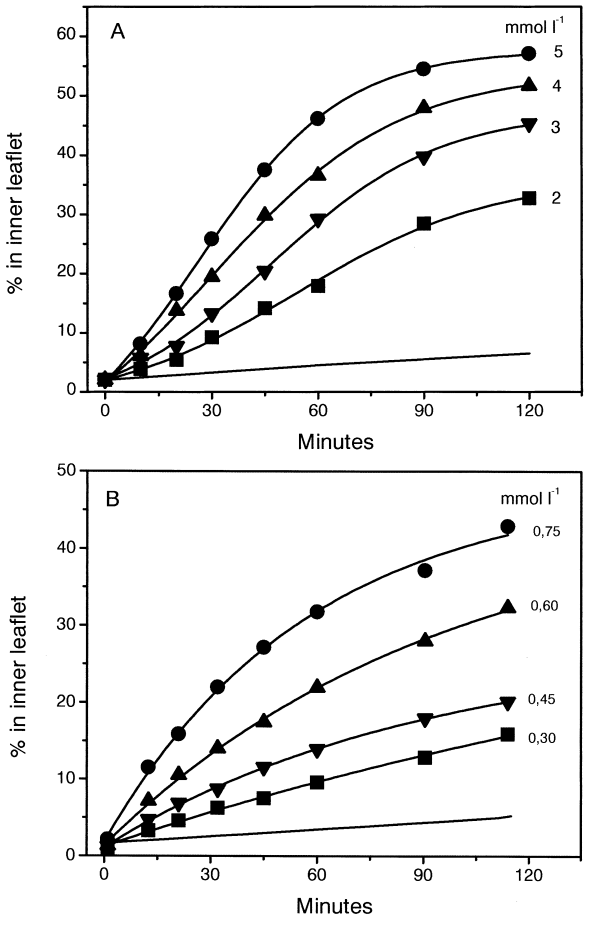

Fig. 1. Enhancement of translocation of NBD-PC from the outer to the inner membrane leaflet of human erythrocytes at various concentrations of C10TAB (A) and C10GP (B). After insertion of NBD-PC into the outer membrane leaflet, its time-dependent flip to the inner leaflet was followed by measuring the decrease of membrane fluorescence remaining after albumin extraction (A) or dithionite treatment (B) (see Section 2.2). (A) C10TAB: 2 (■), 3 (▼), 4 (▲) and 5 (●) mmol l^{-1}. (B) C10GP: 0.3 (■), 0.45 (▼), 0.6 (▲) and 0.75 (●) mmol l^{-1}. Flip kinetics without detergent: no symbol.

with the albumin technique and flip in presence of lower concentrations of alkyl-SU and alkyl-GP, both methods of flip measurement gave similar kinetics (data not shown). Only with TAB, the dithionite method gave somewhat flatter flip kinetics. From these observations, significant changes in the distribution of NBD-PC between the inner and outer membrane leaflet during extraction of detergents from the membrane by albumin seem to be unlikely.

Maximal flip acceleration before lysis occurs differs for the various detergents. It is about 15-fold for alkyl-S, alkyl-SU and alkyl-Z, somewhat more for alkyl-MP and alkyl-GP, about 50-fold for alkyl-TAB and 100-fold or more for Triton X-100 and alkyl-EO8 (k_1 about 0.0300 min^{-1}). Erythrocytes from freshly drawn blood and erythrocytes stored for up to 10 days gave comparable concentration-dependent flip accelerations by a given detergent (data not shown).

For the flip kinetics, rate constants were calculated (see Section 2.2) and plotted against the concentration of detergent in the medium before addition of cells. The effectiveness of the detergents increases with the length of their alkyl chain (Fig. 2). An in-

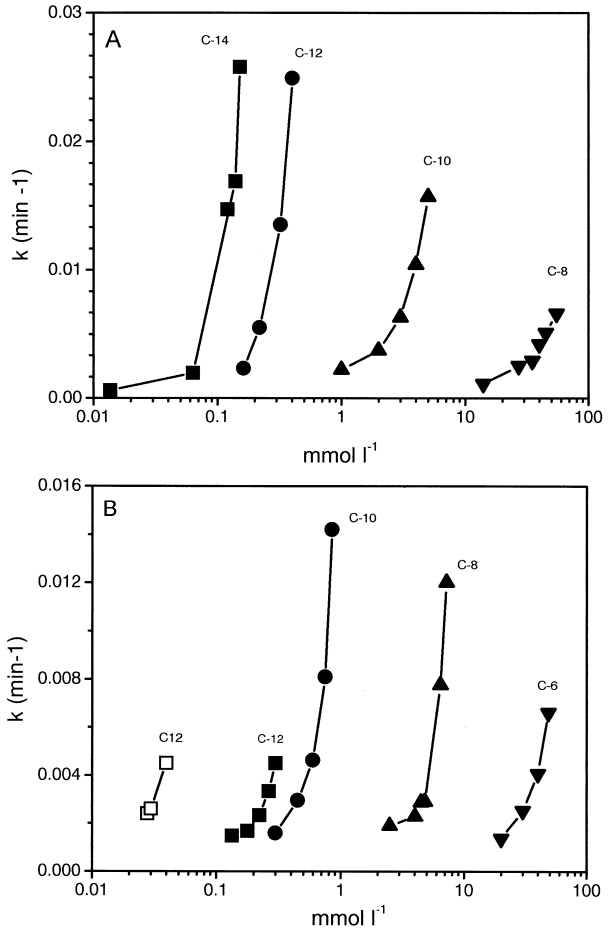

Fig. 2. Dose–response curves for the acceleration of flip rate of NBD-PC by C10TAB (A) and C10GP (B). From the flip kinetics shown in Fig. 1, rate constants were determined and plotted against the detergent concentration present in the medium prior to addition of cells. (A) TAB: C14 (■), C12 (●), C10 (▲), C8 (▼) at 12% hematocrit. (B) GP: C12 (■), C10 (●), C8 (▲), C6 (▼) at 12% hematocrit and C12 (□) at 1.3% hematocrit.

crease of the alkyl chain length by two C-atoms, shifts the dose–response curve for a given type of detergent at 12% hematocrit to about 10-fold lower concentrations. This shift becomes smaller when going from C12 to C14TAB and from C10 to C12GP. However, shifts to 10-fold lower concentrations are achieved upon a decrease of the membrane content of the suspension by decreasing the hematocrit from 12% to 1.3% as shown in Fig. 2B for C12GP. The smaller shift of the dose–response curve at 12% hematocrit is explained by the strong decrease of concentrations of C14TAB and C12GP in the medium upon addition of cells as a result of the high extent of partitioning of these detergents into the membrane according to their membrane/water partition coefficients (see below). In line with this idea, dose–response curves for detergents with short alkyl chains (C6–C8) and thus smaller membrane/water partition coefficients are not shifted by a decrease of hematocrit (data not shown).

When the detergent concentrations in the medium required to increase the flip rate constant, k_1, from about 0.0003 min^{-1} in absence to 0.0020 min^{-1} in presence of detergent at 1.3–2% hematocrit are plotted against the number of C-atoms of the detergents, linear relationships are obtained (Fig. 3). From the slopes of the lines, it can be derived that the detergent effectiveness increases 10- to 14-fold upon an increase of the alkyl chain by two C-atoms.

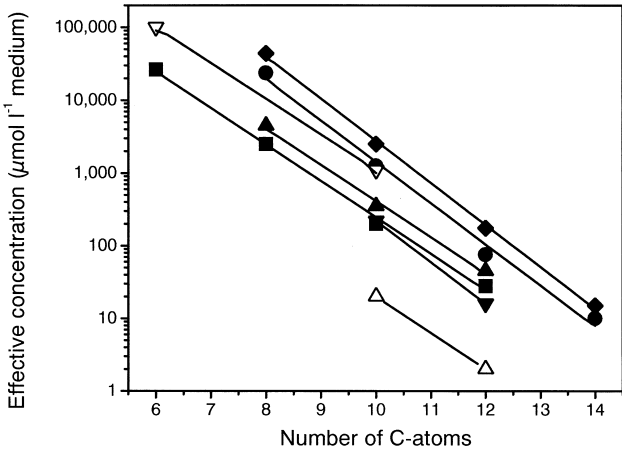

Fig. 3. Dependence of the detergent concentrations required to produce flip acceleration on the number of C-atoms of the detergent hydrocarbon chain. Concentrations of various detergents in the medium, prior to addition of cells at 1.3% or 2% hematocrit, represent those required to obtain flip rate constants of 0.0020 min^{-1}. GP (■), MP (▼), SU (▲), TAB (●), Z (♦), EO8 (△), S (▽).

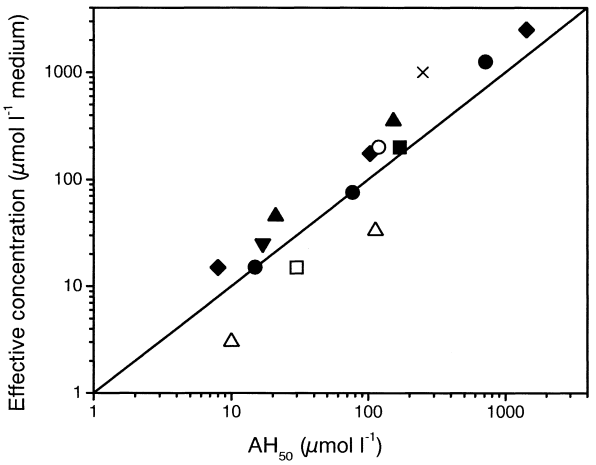

Fig. 4. Correlation between concentrations of various detergents required for flip acceleration and those reported to suppress hypotonic hemolysis. Concentrations of detergents required to increase the flip rate constant to 0.0020 min^{-1} are plotted against concentrations reported [18–20] to produce 50% of the maximum suppression of hypotonic hemolysis at about 1% hematocrit. C12 GP (■), C12MP (▼), SU (▲), TAB (●), Z (♦), EO8 (△), Triton X-100 (□). For reasons of comparison data [21,22] for octanol (×) and dodecanoic acid (○) are shown.

A plot of the detergent concentrations which yield $k_1 = 0.0020$ min^{-1} against the corresponding detergent concentrations reported [18–20] to produce 50% of the maximum suppression of hemolysis in hypotonic medium (AH_{50}) gives a linear relationship (Fig. 4). Since the antihemolytic effect is assumed to be due to membrane expansion [21], flip acceleration correlates with membrane expansion. Such a correlation between membrane expansion estimated by AH_{50} [21,22] and flip acceleration is also found for simple hydrocarbon derivatives such as 1-octanol and dodecanoic acid (Fig. 4). In the case of EO8, flip acceleration is obtained at a considerably smaller membrane expansion than for other detergents.

In order to compare flip accelerations by various detergents, their effective membrane concentrations and membrane/water partition coefficients were determined according to the method of Lissi et al. [17]. To this end, the detergent concentrations in the medium, prior to addition of cells, which yield $k_1 = 0.0020$ min^{-1} at various hematocrits (membrane volumes) were determined (cf. C12GP in Fig. 2B). From these concentrations, moles of detergent per

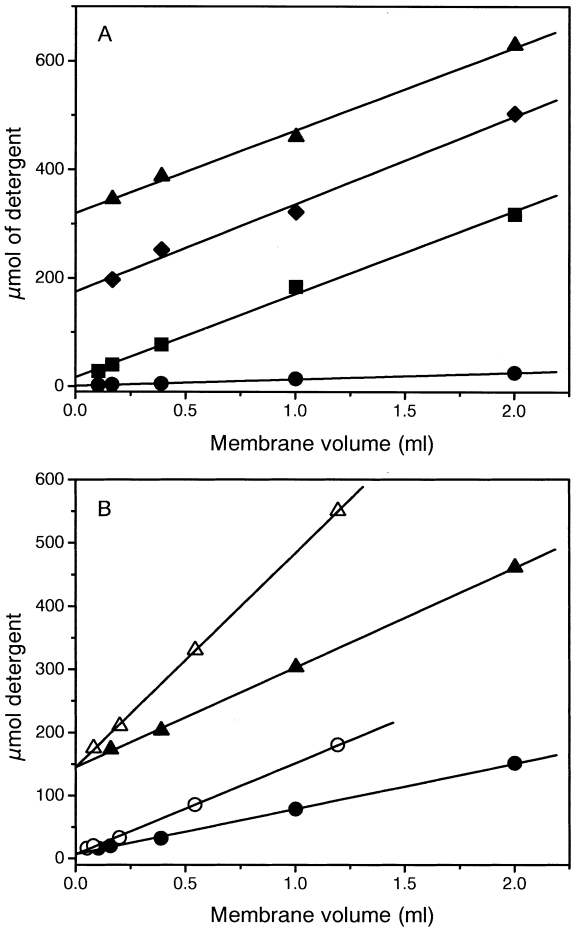

Fig. 5. Dependence of the amounts of various detergents which yield $k_1 = 0.0020$ min^{-1} on the corresponding membrane volumes. (A) The amounts of C10SU (▲), C10GP (♦), C12GP (■) and C12EO8 (●) and the corresponding membrane volumes (outer and inner leaflet) calculated per liter of medium and aqueous intracellular space. (B) The amounts of C12MP (circles) and C12Z (triangles) and the corresponding membrane volumes calculated for the outer and inner membrane leaflet per liter of medium and aqueous intracellular space (closed symbols) or the outer membrane leaflet per liter of medium (open symbols). See Section 2.2 for details.

liter of aqueous phase were calculated and plotted against the corresponding membrane volumes (see Section 2.2). The data points could be fitted by straight lines (Fig. 5). The slopes of the lines represent detergent concentrations in the membrane and the *y*-intercepts represent detergent concentrations in the aqueous phase at infinite small membrane volume. From the ratios of slopes and *y*-intercepts, membrane/water partition coefficients for various detergents were calculated (see Section 2.2). In Table 1, detergent concentrations in the membrane which yield $k_1 = 0.0020$ min^{-1} and membrane/water partition coefficients for various detergents are listed. Partition coefficients vary from about 300 for C10EO8 and C10MP to almost 20 000 for C12EO8 and C14Z. In the case of C14Z, the coefficient has a very high S.D. which results from the low concentration of the detergent in the aqueous phase remaining after a high extent of accumulation of the detergent into the membrane even at very low membrane concentrations. Partition coefficients for C12Z and C12TAB (1090 and 1294) are considerably smaller than those for the other C12 detergents (4785–16 040). This is probably due to the positive charges adjacent to the alkyl chain of Z and TAB which results in a less deep penetration into the hydrophobic domain of the alkyl chains of these detergents than those of the other detergents. From Table 1, it becomes evident that an increase of the alkyl chain length by two C-atoms increases the partition coefficient 10- to 50-fold.

Comparison of detergent concentrations in the membrane which yield $k_1 = 0.0020$ min^{-1} (Table 1) shows that SU, C12Z and GP have the lowest effectiveness while the non-ionic detergents EO8 and Triton X-100, with respectively 8 and 10 ethylene glycol residues, have the highest effectiveness, which means that about 10-fold lower concentrations of these non-ionic detergents are required than of all other detergents to produce the same extent of flip acceleration. The effectiveness of a given membrane-inserted detergent does not depend on its alkyl chain length.

Since the observed rate of inward net movement of NBD-PC in erythrocyte membranes is the result of a simple inward flip and a floppase-mediated outward translocation [23,24] possible variable effects on floppase activity by different detergents could complicate comparison of their membrane concentrations which yield $k_1 = 0.0020$ min^{-1}. To check this, detergent effects on flip were carried out in the presence and absence of floppase activity. Floppase inhibition by a pretreatment of cells with *N*-ethylmaleimide [23] did not change or only slightly decreased or increased ($< 10\%$) the membrane concentrations. Only in the case of C12MEGA, a considerable decrease of the membrane concentration from 130 mmol l^{-1} before (Table 1) to 90 mmol l^{-1} after suppression of floppase was observed (data not shown).

In calculations of membrane concentrations and

partition coefficients, detergents were assumed to distribute into the inner membrane leaflet and intracellular space during flip measurement. Amounts of detergents were therefore calculated per liter of medium and intracellular aqueous phase and the sum of outer and inner membrane leaflets was considered as distributing membrane volume (see Section 2.2). Such a distribution of detergents into the cell interior was derived from observed (data not shown) and reported [18,19] shape transformations of erythrocytes following insertion of detergent into the outer membrane leaflet which initially transforms biconcave cells into echinocytes according to the bilayer couple hypothesis [25]. In the case of C12GP and C12TAB, echinocytes returned to discocytes (half-times <1 and 30 min repectively, at 37°C) and further transformed into stomatocytes. This shape transformation is explained, according to the bilayer couple hypothesis, by translocation of the detergents from the outer to inner membrane leaflet. Such a rapid translocation of GP is supported by indirect calorimetric data [5]. In the case of C12EO8, a very fast transbilayer movement was derived from the release kinetics of C12EO8 from lipid vesicles [8,26]. In the case of C12SU, a very fast movement across the erythrocyte membrane was derived from direct transfer measurements using ^{14}C-labeled C12SU (half-time <1 min at 37°C; B. Deuticke, personal communication).

On the other hand, in the cases of C12MP and C12Z, the extent of distribution of detergent to the inner membrane leaflet during flip measurements is uncertain. At low concentrations, these detergents only slowly if at all move to the inner membrane leaflet. This assumption is based on the observation that upon addition of these detergents biconcave erythrocytes are transformed into echinocytes which are stable for several hours of incubation [18,19,27].

Table 1
Membrane/water partition coefficients and membrane concentrations which yield $k_1 = 0.0020$ min^{-1} for various detergents

Detergent	Partition coefficients for		Membrane concentrations (mmol l^{-1})
	Erythrocyte membranes from this work	PC vesicles from literature	
C12TAB	1294 ± 103		76 ± 4
C10SU	474 ± 28		152 ± 8
C12SU	10 415 ± 1941	6000[b]	168 ± 3
C12Z	1090 ± 21 (2340)		158 ± 2 (339)
C14Z	18 713 ± 15 878 (42 230)		70 ± 3 (141)
C10GP	920 ± 121	2160[c]	162 ± 11
C12GP	9029 ± 3108		153 ± 6
C10MP	267 ± 2 (697)	270[c]	58 ± 1 (152)
C12MP	10 433 ± 2031 (21 135)	6750–28 000[b,c]	72 ± 1 (144)
C12MEGA	4785 ± 327		130 ± 7
C12EO3	–	67 500–13 5000[c]	101 ± 1
C10EO8	287 ± 16		7 ± 1
C12EO8	16 040 ± 8204	8100–28 000[a,b,c]	12 ± 1
Triton X–100	2386 ± 416	2700–12 000[a,b,c]	17 ± 1
Dodecanoic acid at			
pH 5.6[d]	39 626 ± 32 847		93 ± 4
pH 7.4	1471 ± 83		278 ± 8
pH 8.5	1677 ± 40		289 ± 3

From plots of moles of detergents which yield $k_1 = 0.0020$ min^{-1} against corresponding membrane volumes, membrane concentrations and partition coefficients (± S.D) were derived (see Section 2.2.2). Data were calculated using the outer and inner membrane leaflet and 1 l of medium and aqueous intracellular space as the volumes for detergent distribution (see Fig. 5). In case the detergents MP and Z are assumed not to penetrate into the cell interior, data were also calculated per liter of medium and the outer membrane leaflet as the volumes for detergent distribution (numbers in parentheses).

[a]Data taken from [4].

[b]Data taken from [8].

[c]Data taken from [29].

[d]Data were corrected for cell swelling, at this low pH, which resulted in a lower number of cells in the suspension.

Such a slow movement of C12MP is supported by results on lipid vesicles [8]. However, at the detergent concentrations used to determine partition coefficients, slow transformations into discocytes with respective half-times of 60 min and several hours take place (37°C, data not shown) which indicate enhanced transbilayer redistributions. On the other hand, a 3-fold decrease of the membrane concentrations of the detergents and the concomitant decrease of the rates of transbilayer movements gave similar partition coefficients (data not shown). Therefore, movements of zwittergents and maltosides from the outer to the inner membrane leaflet during flip measurements may be very limited or even absent and their partition coefficients were also calculated from plots of the volume of the outer membrane leaflet against the amount of detergent, both calculated per liter of medium (Fig. 5B). By this method of calculation, partition coefficients and concentrations of detergents in the outer membrane leaflet become about 2-fold higher (Table 1, numbers in parentheses).

For reasons of comparison, the effect of a simple hydrocarbon derivative, dodecanoic acid, on NBD-PC flip was measured. At pH 7.4, the concentration in the membrane which yield $k_1 = 0.0020$ min^{-1} was higher (278 mmol l^{-1}) than that for detergents. At this pH, the fatty acid is expected to be mainly present in the membrane in its electrically charged, deprotonated form even upon consideration of a shift of its pK_a value from about 4.9 in aqueous phase to about 6.9 in the lipid bilayer, as reported for lipid vesicles [28]. To get an estimate of the pK_a value for dodecanoic acid in erythrocyte membranes, the pH dependence of flip acceleration by the fatty acid was measured. Membrane concentrations which yield $k_1 = 0.0020$ min^{-1} were then plotted against pH and a sigmoid function was fitted to the data. From this function, a pK_a value of about 6.3 could be derived (data not shown). This means a pK_a shift of 1.4 upon insertion of the fatty acid into the membrane. Upon an increase of pH to 8.5, which increases the fraction of membrane-bound dissociated fatty acid to above 99%, the partition coefficient and the membrane concentration of dodecanoic acid which yield $k_1 = 0.0020$ min^{-1} were comparable to those at pH 7.4. A decrease of pH to 5.6, which increases the fraction of the protonated form of the fatty acid to 83%, decreases the membrane concentration to 93 mmol l^{-1} (Table 1) which is 77 mmol l^{-1} of non-ionic fatty acid. It can be concluded that the fatty acid in its protonated form is considerably more effective than in its deprotonated form.

Since the ratio of the dissociation constants for the fatty acid in the aqueous medium and the membrane is related to the ratio of the membrane/water partition coefficients of protonated and deprotonated fatty acid [28], the partition coefficient of the protonated fatty acid is expected to be about 25-fold higher than that of the deprotonated fatty acid. From the membrane/water partition coefficient at pH 8.5, which will be very close to that of the deprotonated fatty acid, the partition coefficient of the protonated fatty acid can then calculated to be about 40 000 which supports the value with a high S.D. derived from our measurements at pH 5.6.

4. Discussion

In the present study, the slow flip of NBD-PC has been found to be accelerated by anionic, cationic, zwitterionic and neutral detergents in the absence of significant hemolysis. In order to compare flip accelerations by various detergents, their effective membrane concentrations and membrane/water partition coefficients were determined. This was achieved by measurements of the increasing amounts of detergent required to obtain the same rate constant for the flip at increasing membrane volumes in the erythrocyte suspension according to the method of Lissi et al. [17]. This method was originally described to determine partition coefficients for pure lipid bilayers and now appeared to be applicable to determine partition coefficients for biological membranes in the range of about 300 to 15 000. For detergents with lower partition coefficients, the accumulation of detergent into the membrane will only vary to a considerable extent in the range of hematocrit values above 20%. At these high hematocrit values, flip measurements become hardly feasible and the uncertainty on the extent of accumulation of detergent in the intracellular space highly contributes to the uncertainty of the estimated partition coefficients. On the other hand, for detergents with partition coefficients above 15 000 their accumulation in the mem-

brane will be already high at a rather low hematocrit and will only vary to a considerable extent in the range of hematocrit values below about 1%. In this very low range, flip measurements become inaccurate because small amounts of cells in high volumes of medium have to be analyzed. This is reflected by the high standard deviations for the estimated partition coefficients as shown in Table 1 for C14Z and dodecanoic acid.

The membrane/water partition coefficients obtained for the erythrocyte membrane (Table 1) are of the same order of magnitude than those reported [4,8,29] for phosphatidylcholine bilayer vesicles. It should be considered that the membrane volume, in which detergents distribute, can only be estimated since the effective bilayer thickness for detergent distribution and the contribution of membrane proteins to detergent binding are not known. In our calculations, it is assumed that membrane volume is 0.8% of cellular volume (see Section 2.2).

For the electrically charged detergents such as SU and TAB, the partition coefficients at our detergent concentrations in the medium could be considerably lower than those at infinite low detergent concentrations due to the reduced partitioning of the charged detergents into the membrane as a result of the production of a membrane surface potential by the detergent [30]. On the basis of the saturating concentration dependence reported [20] for the adsorption of TAB to erythrocytes, the membrane/water partition coefficient measured for TAB at infinite low membrane concentrations could be 1.3-fold higher than that measured at our experimental concentrations (about 7×10^7 molecules per cell). In the case of SU, the partition coefficient at very low detergent concentrations may be estimated [30] to be at least 2-fold higher than that measured at our experimental concentrations. For the non-ionic detergents, in particular the polyethylene glycol ether detergents for which partition coefficients could be measured at lower concentrations in the membrane, the coefficient is not expected to be significantly higher at still lower concentrations [6,31].

Comparison of the membrane concentrations of various detergents required to increase the rate constant of NBD-PC flip from about 0.0003 min^{-1} to 0.0020 min^{-1} (Table 1) shows that concentrations for anionic, cationic and zwitterionic detergents as well as neutral detergents of the glycopyranosyl type with various alkyl chain length (C10–C14) are quite similar. Interestingly, these concentrations are also quite similar to those reported for 1-butanol [13] and now measured for 1-octanol (150 mmol l^{-1}, data not shown). It is therefore not important for obtaining detergent-stimulated flip whether a neutral small hydroxyl, a larger sugar residue or an ionic group is linked to the alkyl chain.

On the other hand, membrane concentrations of the non-ionic detergents EO8 and Triton X-100 with respectively eight and ten oxyethylene residues are about 10-fold lower than those of the other detergents. Shortening of the oxyethylene chain to three residues strongly reduces effectiveness. This is evidence for a strong membrane-perturbing effect of the long polyoxyethylene domain of the detergents. At the membrane concentrations listed in Table 1, the molar ratio of detergent to phospholipid ranges from about 0.3 for SU, C12Z and GP ('weak' detergents) to about 0.02 for Triton X-100 and EO8 ('strong' detergents; cf. [29]). All ratios are below those reported for detergent-saturated phospholipid bilayers [2–4].

Interference of flip kinetics by exo- or endo-vesiculation following echinocyte or stomatocyte formation by detergents has to be considered. Exovesiculation following echinocyte formation by Z and MP would decrease the total amount of membrane-bound NBD-PC probe (F_{tot}) during measurements of flip kinetics. This was not observed (data not shown). In the case of endovesiculation following stomatocyte formation by, e.g., Triton X-100 and EO8, the time-dependent loss of extractability from the outer leaflet of phospholipid probes with different flip rates, such as NBD-PC and NBD-sphingomyelin [16], would exhibit a lower specificity (cf. [32]) than in the case of echinocyte formation without exo-vesiculation. This was not observed. The ratio of flip rates of NBD-PC and NBD-sphingomyelin was even higher in presence of these stomatocytogenic detergents (ratio about 20) than in presence of echinocytogenic detergents (ratio 3–10, data not shown).

Insertion of detergent molecules into the membrane lipid bilayer results in expansion of the membrane [18–20]. Concentrations of detergents required for membrane expansion correlate to those required for flip acceleration (see Fig. 4). However, membrane

expansion for EO8, at the membrane concentration which yield $k_1 = 0.0020$ min^{-1}, is considerably smaller than that for other detergents. Therefore, a simple causal relationship between membrane expansion and flip acceleration is unlikely. Moreover, membrane expansions by a selective insertion of C12MP into the outer membrane leaflet [19] and a symmetric insertion of C12GP into both outer and inner leaflet produce the same flip accelerations when the concentration of MP in the outer leaflet and that of GP in both leaflets are the same. It may be concluded therefore that a symmetric insertion of detergent into the membrane lipid bilayer is not required for flip acceleration. The reported membrane expansion [19] by the selective insertion of MP into the outer leaflet [27] requires a secondary expansion of the inner membrane leaflet. Such a phenomenon has been reported previously for the selective insertion of lysophosphatidylcholine into the outer membrane leaflet [33]. The membrane expansions by amphiphiles derived from the observed antihemolytic action [6,20,33] and echinocyte formation [34,35] are smaller than those expected from calculated numbers of membrane-inserted amphiphiles.

In order to discuss possible mechanisms of flip acceleration by detergents, the interactions of the various detergents with membrane phospholipids and their perturbing effects on the lipid bilayer have to be considered. The electrically charged head groups of TAB, SU, and Z are expected to interact with the charges of the polar head groups of the phospholipids which thereby alter their orientation relative to the bilayer surface [36]. The sugar moiety of GP has been discussed [5] to penetrate into the bilayer up to the level of the glycerol residue of the phospholipid without affecting the phosphocholine head group. On the other hand, EO8 has been reported [7] to perturb both the hydrocarbon interior and interfacial region of the bilayer as well as the polar head group region. Moreover, evidence has been obtained [31] that every ethylene oxide unit of EO8 is inserted into a similar non-aqueous environment of the membrane. It may be suggested therefore that the stronger membrane-perturbing effect, as measured by flip acceleration, of EO8 and Triton X-100 than that of the other detergents is due to the interaction of the polyoxyethylene domain of the former type of detergents with a broader interfacial region of the phospholipid bilayer. In line with this idea, shortening of the ethylene glycol chain from 8 to 3 units such as is the case for EO3 reduces the effectiveness of the detergent (Table 1).

A local accumulation of detergent in the membrane lipid bilayer or a preferential interaction of detergent with certain lipid species (cf. [29,32]) may be discussed to play a role in membrane perturbation. However, it seems unlikely that the very diverse amphiphiles with non-charged small (alcohols) and large (MP) head groups or detergents bearing one or more charges (TAB, SU, Z) produce the same extent of domain formation at the same membrane concentrations.

Flip acceleration generated by insertion of various detergents and alcohols between the alkyl chains of the phospholipids does not greatly depend on their alkyl chain length (C10–C14 for detergents, see Table 1; C2–C8 for alcohols [13]), meaning it is rather independent on depth of penetration of the alkyl chain into the hydrophobic bilayer. It is concluded, therefore, that the decrease of packing constraints resulting in enhanced segmental motion of the phospholipid hydrocarbon chains and the decrease of the bilayer thickness are unlikely to play a major role in membrane perturbation that is responsible for flip acceleration. Moreover, the temperature dependence of the detergent-induced flip of NBD-PC is high. For EO8 and GP, apparent activation energies were resp. about 80 and 95 kJ/mol (data not shown) compared to a reported [16] value of 65 kJ/mol in the absence of detergent. The high activation energy is not due to a temperature-dependent change of the effective membrane concentration, because the membrane/water partition coefficient for GP only decreases from 9029 at 37°C (Table 1) to about 7500 at 21°C (data not shown). Correction for the temperature-dependent change of effective membrane concentration lowers the activation energies but not below the value for the unperturbed membrane. The transient formation of hydrated defects in the hydrophobic barrier [37] acting as flip sites for phospholipids is therefore unlikely, since this would reduce the energy barrier [38] for transfer of the polar head group of phospholipids [39].

Previously, barrier defects in the native and perturbed membrane that act as leaky pathways and flip sites have been proposed to result from the forma-

tion of fluctuating hydrophobic pores [40]. From our observation that EO8 and Triton X-100 are most effective in flip acceleration followed by alkane diols [13] and non-dissociated fatty acid as well as the idea that these amphiphiles orient with the polyoxyethylene, hydroxyls and carboxyl residues in the membrane/lipid interfacial region close to the glycerol backbone [31,41,42], it may be suggested that perturbation of this region is the major factor in hydrophobic defect formation.

References

[1] D. Lichtenberg, R.J. Robson, E.A. Dennis, Solubilization of phospholipids by detergents. Structural and kinetic aspects, Biochim. Biophys. Acta 737 (1983) 285–304.

[2] M. Ollivon, O. Eidelman, R. Blumenthal, A. Walter, Micelle-vesicle transition of egg phosphatidylcholine and octyl glycoside, Biochemistry 27 (1988) 1695–1703.

[3] T. Inoue, Interaction of surfactants with phospholipid vesicles, in: M. Rosoff (Ed.), Vesicles, Marcel Dekker, New York, 1996, pp. 151–195.

[4] J. Lasch, Interaction of detergents with lipid vesicles, Biochim. Biophys. Acta 1241 (1995) 269–292.

[5] R. Wenk, T. Alt, A. Seelig, J. Seelig, Octyl-β-Glucopyranoside partitioning into lipid bilayers: Thermodynamics of binding and structural changes of the bilayer, Biophys. J. 72 (1997) 1719–1731.

[6] B. Isomaa, H. Hägerstrand, G. Paatero, A.C. Engblom, Permeability alterations and antihaemolysis induced by amphiphiles in human erythrocytes, Biochim. Biophys. Acta 860 (1986) 510–524.

[7] D. Otten, J. Löbbecke, K. Beyer, Stages of the bilayer-micelle transition in the system phosphatidylcholine-C12E8 as studied by deuterium- and phosphorous-NMR, light scattering, and calorimetry, Biophys. J. 68 (1995) 584–597.

[8] U. Kragh-Hansen, M. le Maire, J.V. Møller, The mechanism of detergent solubilization of liposomes and protein-containing membranes, Biophys. J. 75 (1998) 2932–2946.

[9] M. Ueno, Partition behavior of a nonionic detergent, octyl glycoside, between membrane and water phases, and its effect on membrane permeability, Biochemistry 28 (1989) 5631–5634.

[10] E. Schneider, C.W.M. Haest, G. Plasa, B. Deuticke, Bacterial toxins, amphotericin B and local anesthetics enhance transbilayer mobility of phospholipids in erythrocyte membranes. Consequences for phospholipid asymmetry, Biochim. Biophys. Acta 855 (1986) 325–336.

[11] J. Classen, C.W.M. Haest, H. Tournois, B. Deuticke, Gramicidin-induced enhancement of transbilayer reorientation of lipids in the erythrocyte membrane, Biochemistry 26 (1987) 6604–6612.

[12] J. Rosso, A. Zachowski, F. Devaux, Influence of chlorpromazine on the transverse mobility of phospholipids in the human erythrocyte membrane: relation to shape changes, Biochim. Biophys. Acta 942 (1988) 271–279.

[13] C. Schwichtenhövel, B. Deuticke, C.W.M. Haest, Alcohols produce reversible and irreversible acceleration of phospholipid flip-flop in the human erythrocyte membrane, Biochim. Biophys. Acta 1111 (1992) 35–44.

[14] F. Bassé, J. Sainte-Marie, L. Maurin, A. Bienvenüe, Effect of benzylalcohol on phospholipid transverse mobility in human erythrocyte membrane, Eur. J. Biochem. 205 (1992) 155–162.

[15] J.C. McIntyre, R.G. Sleight, Fluorescence assay for phospholipid membrane asymmetry, Biochemistry 30 (1991) 11819–11827.

[16] C.W.M. Haest, D. Kamp, B. Deuticke, Transbilayer reorientation of phospholipid probes in the human erythrocyte membrane. Lessons from studies on electroporated resealed cells, Biochim. Biophys. Acta 1325 (1997) 17–33.

[17] E. Lissi, M.L. Bianconi, A.T. do Amaral, E. de Paula, L.E.B. Blanch, S. Schreier, Methods for the determination of partition coefficients based on the effect of solutes upon membrane structure, Biochim. Biophys. Acta 1021 (1990) 46–50.

[18] B. Isomaa, H. Hägerstrand, G. Paatero, Shape transformations induced by amphiphiles in erythrocytes, Biochim. Biophys. Acta 899 (1987) 93–103.

[19] H. Hägerstrand, T.H. Holmström, M. Bobrowska-Hägerstrand, J.E. Eirksson, B. Isomaa, Amphiphile-induced phosphatidylserine exposure in human erythrocytes, Mol. Membr. Biol. 15 (1998) 89–95.

[20] B. Isomaa, Interaction of surface-active alkyltrimethylammonium salts with the erythrocyte membrane, Biochem. Pharmacol. 28 (1979) 975–980.

[21] P. Seeman, The membrane interactions of anesthetics and tranquilizers, Pharmacol. Rev. 24 (1972) 583–655.

[22] M. Rybczynska, A. Csordas, Chain length-dependent interaction of free fatty acids with the erythrocyte membrane, Life Sci. 44 (1989) 625–632.

[23] M.V. Serra, D. Kamp, C.W.M. Haest, Pathways for flip-flop of mono- and di-anionic phospholipids in the erythrocyte membrane, Biochim. Biophys. Acta 1282 (1996) 263–273.

[24] D. Kamp, C.W.M. Haest, Evidence for a role of the multidrug resistance protein (MRP) in the outward translocation of NBD-phospholipids in the erythrocyte membrane, Biochim. Biophys. Acta 1372 (1998) 91–101.

[25] M.P. Sheetz, S.J. Singer, Biological membranes as bilayer couples. A molecular mechanism of drug-erythrocyte interactions, Proc. Natl. Acad. Sci. USA 71 (1974) 4457–4461.

[26] M. le Maire, J.V. Møller, P. Champeil, Binding of a nonionic detergent to membranes: Flip-flop rate and location on the bilayer, Biochemistry 26 (1987) 4803–4810.

[27] S. Schwarz, C.W.M. Haest, B. Deuticke, Extensive electroporation abolishes experimentally induced shape transformations of erythrocytes: a consequence of phospholipid symmetrization?, Biochim. Biophys. Acta 1421 (1999) 361–379.

[28] J. Miyazaki, K. Hideg, D. Marsh, Interfacial ionization and

partitioning of membrane-bound local anaesthetics, Biochim. Biophys. Acta 1103 (1992) 62–68.
[29] H. Heerklotz, J. Seelig, Correlation of membrane/water partition coefficients of detergents with the critical micelle concentration, Biophys. J. 78 (2000) 2435–2440.
[30] S. McLaughlin, H. Harary, The hydrophobic adsorption of charged molecules to bilayer membranes: A test of the applicability of the Stern equation, Biochemistry 15 (1976) 1941–1948.
[31] H. Heerklotz, H. Binder, G. Lantzsch, G. Klose, Membrane/water partition of oligo(ethylene oxide) dodecyl ethers and its relevance for solubilization, Biochim. Biophys. Acta 1196 (1994) 114–122.
[32] D. Wüstner, T. Pomorski, A. Herrmann, P. Müller, Release of phospholipids from erythrocyte membranes by taurocholate is determined by their transbilayer orientation and hydrophobic backbone, Biochemistry 37 (1998) 17093–17103.
[33] C.W.M. Haest, G. Plasa, B. Deuticke, Selective removal of lipids from the outer membrane layer of human erythrocytes without hemolysis. Consequences for bilayer stability and cell shape, Biochim. Biophys. Acta 648 (1981) 701–708.
[34] J.R. Ferrell, K.J. Lee, W.H. Huestis, Membrane bilayer balance and erythrocyte shape: A quantitative assessment, Biochemistry 24 (1985) 2849–2857.
[35] D. Allan, C. Hagelberg, K.J. Kallen, C.W.M. Haest, Echinocytosis and microvesiculation of human erythrocytes induced by insertion of merocyanine 540 into the outer membrane leaflet, Biochim. Biophys. Acta 986 (1989) 115–122.
[36] P.G. Scherer, J. Seelig, Electric charge effects on phospholipid headgroups. Phosphatidylcholine in mixtures with cationic and anionic amphiphiles, Biochemistry 28 (1989) 7720–7728.
[37] S. Paula, A.G. Volkov, A.N. Van Hoek, T.H. Haines, D.W. Deamer, Permeation of protons, potassium ions, and small polar molecules through phospholipid bilayers as a function of membrane thickness, Biophys. J. 70 (1996) 339–348.
[38] R. Homan, H.J. Pownall, Transbilayer diffusion of phospholipids: dependence on headgroup structure and acyl chain length, Biochim. Biophys. Acta 938 (1988) 155–166.
[39] A. Parsegian, Energy of an ion crossing a low dielectric membrane: Solutions to four relevant electrostatic problems, Nature 221 (1969) 844–846.
[40] B. Deuticke, S. Klonk, C.W.M. Haest, Erythrocyte membrane barrier function: Role of bilayer polarity and skeletal proteins, Biochem. Soc. Trans. 20 (1992) 769–773.
[41] A. Sanson, M. Ptak, J.L. Rigaud, C.M. Gary-Bobo, An ESR study of the anchoring of spin-labeled stearic acid in lecithin bilayers, Chem. Phys. Lipids 17 (1976) 435–444.
[42] S. Ohki, K. Arnold, Surface dielectric constant, surface hydrophobicity and membrane fusion, J. Membr. Biol. 114 (1990) 195–203.