A Joint Effect of Cationic and Anionic Amphiphilic Compounds on the Desorption of Calcium Ions from Lecithin Liposome Membranes

Janina Kuczera[a], Teresa E. Kral[a], Marian Podolak[b], Stanisław Przestalski[a]

[a] Department of Physics and Biophysics, Agricultural University, Norwida 25, 50–375 Wrocław, Poland
[b] Institute of Physics, Opole University, Oleska 48, 45–052 Opole, Poland

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The aim of the work was to determine the synergism and antagonism of mixtures of the cationic amphiphilic compounds: N-benzyl-N,N-dimethyl-N-tetradecylammonium chloride (BDTA), N-benzyl-N,N-dimethyl-N-dodecylammonium chloride (BDDA), N-methyl-N-tetradecyl-morpholinium bromide (MTM), N,N,N-trimethyl-N-tetradecylammonium bromide (TMTA) and those of tripropyltin chloride in a mixture with amphiphilic anionic compounds (a homologous series of sodium alkylsulfonates with $n = 2, 4, 6, 8, 10, 12$ and 14 alkyl carbon atoms) in the process of calcium ion desorption from liposome lecithin membranes. Radioactive labels and spin labels were used in the investigations.

All compounds studied caused an increase in the kinetic rate constant of calcium ion desorption from liposome membranes with increasing concentration. In case of mixtures containing cationic and anionic compounds both synergism and antagonism were observed. It was shown that the regulatory effect depended on the properties of both cationic and anionic compounds. A mixture of the most effective cationic compound (BDTA) with each of the anionic compounds decreased the effect. Mixtures of the remaining ammonium compounds with sodium alkylsulfonates produced both synergism and antagonism. Anionic compounds with short chains increased, and with longer chains decreased efficiency, while compounds with comparable chains caused totally blocked desorption. A mixture of an organometallic compound having three short chains with anionic compounds gave a reversed effect: short-chain compounds caused a decreased and those with long-chains – increased efficiency of tripropyltin chloride.

Studies with spin labels supported the conclusion that the most important factor responsible for the regulatory effect is the molecular shape of the complexes formed in a mixture of both groups of compounds – a factor that determines membrane packing density.

Introduction

The authors of the paper have been working for some years to determine the molecular mechanism involved in the action of amphiphilic compounds on biological and model membranes, particularly that of quaternary ammonium salts (Kuczera *et al.,* 1996; Podolak *et al.,* 1996) and also that of organometallic compounds (Gabrielska *et al.,* 1997; Kuczera *et al.,* 1997). Among others, the substances exhibit fungi-, bacteri- and algaecidal properties (Balgavy and Devinsky, 1996). The compounds were found to affect both the structure and function of model phospholipid membranes. We have found that some of the compounds induced marked increase, sometimes by tens of times, in the rate of some processes, depending on the electric charge of the polar head group, its distribution and on the polar head size, as well as on the alkyl chain length and chain multiplicity. We have found a far-reaching correlation between the results of transport experiments and biological tests (Rucka *et al.,* 1983). Some results of the studies have been used to rationally synthesise new compounds of increased biological activity.

Exchanging the chloride or bromide anion in amphiphilic cationic compounds for an amphiphilic anion alters physico-chemical properties of the compounds in aqueous dispersions. In many practical applications mixtures of anionic and cationic amphiphiles exhibit properties much better than those of their individual components (Crove, 1987).

It seemed purposeful to investigate to what extent transport processes in phospholipid model

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Reprint requests to Teresa E. Kral.
Fax: (+048)-71-205172
E-mail: tek@ozi.ar.wroc.pl

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membranes and some of its properties were affected by a mixture of amphiphilic cationic and anionic compounds. Preliminary investigations on the process of calcium ion desorption from the lecithin liposome membrane were conducted by us with one cationic compound, dodecyloxymethylene methylmorpholinium chloride and homologous series of amphiphilic anionic compounds, i.e. sodium *n*-alkylsulfonates (Fogt *et al.,* 1994).

In order to determine more closely the molecular mechanism of action of such mixtures it seemed necessary to verify that effect for a number of cationic compounds that differ in their polar parts and alkyl chain length. By choosing an anionic compound with a proper alkyl chain length, the effectiveness of cationic compound could be altered in a broad range from a several-fold increase to its total alleviation. Such results indicated the possibility to control the effectiveness of amphiphilic cationic compounds.

This paper presents the results of studies on the effectiveness of cationic compounds and anionic amphiphile mixtures on the process of calcium ion desorption from lecithin liposome membranes for various systems, as follows: A system composed of equimolar amounts of sodium alkylsulfonates and cationic detergents that possessed equal number of carbon atoms in the alkyl chain but whose polar parts of cationic amphiphiles differed in their physico-chemical properties; another system was composed of equimolar amounts of sodium alkylsulfonates and cationic compounds differing much in steric properties; a system composed of a selected anionic surfactant and cationic once with different alkyl-chain lengths and a system composed of sodium alkylsulfonates and cationic detergent differing in their concentrations.

The ESR spin probe method was applied to some of the above-mentioned systems to take into consideration the membrane structure density changes when discussing the effect of the compounds studied and the molecular mechanism of their action on the lipid membrane.

Materials and Methods

Amphiphiles used are presented in Table I. Purity of the compounds was not less than 98%.

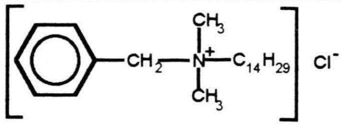
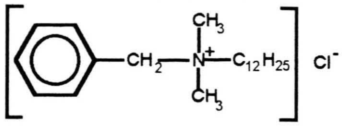
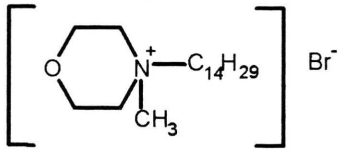
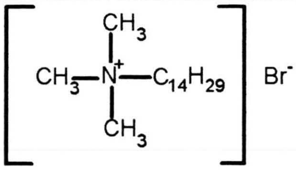
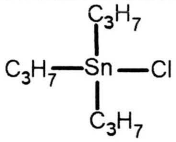
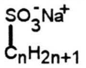
Experiments on calcium ion desorption

Liposomes. Small unilamellar liposomes (SUV) were prepared from egg yolk lecithin (EYL) using sodium cholate in the Liposomat (DIANORM) (Weder and Zumbuhl, 1984). Lecithin was prepared according to Singleton *et al.* (1965). The solution used to form vesicles contained a veronal-acetate buffer, pH 7.5, and 0.3 mmol/dm^3 $CaCl_2$ labelled with the radioactive tracer ^{45}Ca (3×10^8 Bq/mmol). During vesicle formation calcium cations were absorbed at the outer and inner liposome membranes. The radioactive tracers were removed from external medium during liposome preparation.

The measuring set-up was composed of 16 vessels, each containing an outer chamber with a coaxially-mounted inner cylindrical chamber with cellophane side walls. The chambers were kept at 25^{0}C. The inner chamber was filled with a liposome suspension, and the outer one with the solution alone. Defined amounts of the stock solution of amphiphiles studied were added to both compartments to get identical concentrations on both sides of the cellophane wall. The final detergent concentrations of each component ranged between 0.5 and 7.0 mmol/dm^3. Aliquots were taken at chosen time intervals and their radioactivity was measured with a liquid scintillation counter (PACKARD). The experiments were repeated 4–6 times for each concentration of the compounds studied.

The theoretical description of the transport and desorption measurements described previously (Mazgis and Kuczera, 1981) was used with minor modifications. To determine the rate constant α of ion desorption, a three-compartmental analysis was used. Calcium ions released from the liposome membrane (first compartment) are in the inner chamber (second compartment) from there they pass through a cellophane membrane to the outer chamber (third compartment). The flux of the ions observed results from the desorption process from the membrane and permeation from the interior of the liposomes. However, the latter flux is negligible because of the very low concentration of Ca^{2+} in the bulk inner medium and its very low permeability through the lipid bilayer (Kuczera and Żyłka, 1979). The time dependence of the activity of the media in the inner and outer chambers

Table I. Compounds studied.

Code	Chemical structure	Chemical name	Company
BTDA		N-benzyl-N,N-dimethyl-N-tetradecylammonium chloride	FLUKA
BDDA		N-benzyl-N,N-dimethyl-N-dodecylammonium chloride	ALDRICH
MTM		N-methyl-N-tetradecylmorpholinium bromide	*
TMTA	 $[CH_3-N^+(CH_3)_2-C_{14}H_{29}]\,Br^-$	N,N,N-trimethyl-N-tetradecylammonium bromide	SERVA
TPT	 $(C_3H_7)_3Sn-Cl$	tripropyltin chloride	MERCK
ASn	 $SO_3^-Na^+-C_nH_{2n+1}$ n = 2,4,6,8,10,12,14	sodium alkylsulfonates	FLUKA

* Compound synthesised in the Institute of Organic Chemistry and Polymer Technology of the Technical University of Wrocław.

characterizes the resultant desorption process from the liposome membranes and permeation process through the cellophane membrane. The latter was measured in separate experiments. Plots of the logarithm of relative radioactivity, $U = (A_\infty - A)/A_\infty$ (where A – is radioactivity of the medium in the outer chamber and A_∞ is the equilibrium radioactivity after infinite time in any chamber), against time were constructed from the experimental points and the theoretically calculated curves were fitted to them. Figs. 1a and 1b present examples of the fitting of the theoretical curves (solid lines) to the experimental points for various concentrations of MTM compound (Fig. 1a) and for its mixture with AS8 compound (Fig. 1b).

Experimental conditions. To determine the effect of the electrical and steric properties of ion pairs of cationic and anionic amphiphiles on calcium ion desorption process, equimolar mixtures of one of the cationic surfactants BDTA, MTM, TMTA or TPT and a homologue of the series of anionic surfactants ASn were used (see Table I).

To determine the effect of chain length of a cationic surfactant on the process studied, two homologues, BDDA and BTDA with 12 and 14 carbon

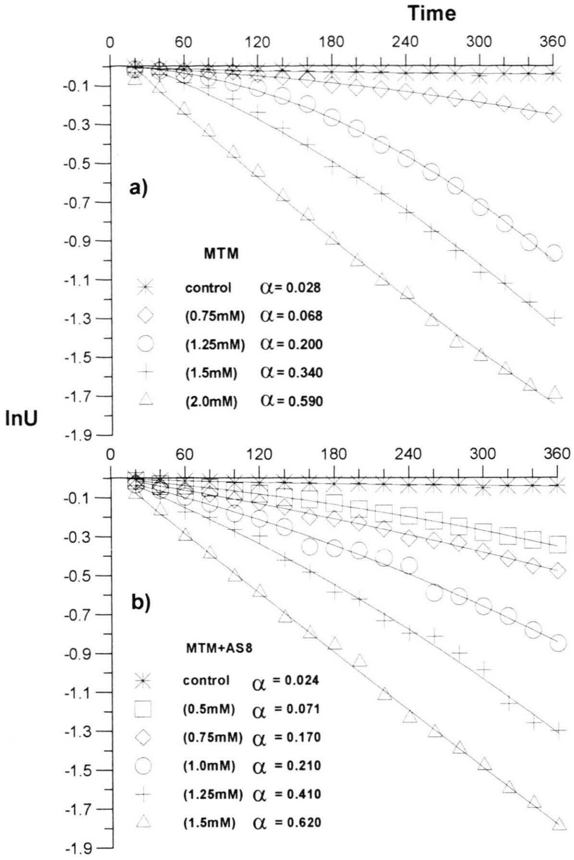


Fig. 1. A representative relationship between logarithm of relative radioactivity, lnU, and time for several concentrations of the MTM modifier **(a)** and of the MTM+AS8 modifier **(b)**. U = $(A\infty - A)/A\infty$, where A – is radioactivity of sample taken from the outer chamber, $A\infty$- radioactivity of sample at infinite time. The theoretical curves (solid lines) have been fitted to experimental points. Values of the kinetic constants α, given in the legend, have been determined from a three-compartment analysis.

atoms in the alkyl chain, respectively, were used in equimolar mixtures with sodium octylsulfonate AS8.

To determine the effect of molar ratio of cationic and anionic surfactants, the MTM compound was used in a mixture with sodium hexyl- (AS6) or octylsulfonate (AS8). The concentration of MTM was constant with 1 mM, and the concentration of the ASn compounds were changed in the range between 0.25 and 2.50 mM.

The ESR measurements

The spin probe method with 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl probe, containing a nitroxyl group attached to the carbon atom localized between the first hydrocarbon group from the end and fourteenth group from the beginning of the fatty acid alcyl chain of the probe molecule, FA (1,14), was used. The probe has been delivered by SYVA (USA). The liposomes were prepared from egg yolk lecithin (EYL) in the process of sonication of phosphoric buffer (pH 7.5) and lecithin composition. EYL concentration in a sample was 0.04 M and the spin probe – (FA [1,14]) to EYL molar ratio was 0.01. MTM and ASn compounds were introduced to the samples after liposome formation and their concentration was varied within the range of 0–50 mol% with respect to EYL (mol ASn/mol EYL × 100%). In the case of MTM and ASn mixtures the concentration of MTM in the samples was constant and equal to 25 mol%, while that for ASn was varied from 0 to 50 mol%. ESR spectra of FA (1,14) dissolved in the samples were taken with SE-X/25 spectrometer (Technical University of Wrocław). From the spectra the parameter τ (Hemminga, 1983; Podolak *et al.,* 1996), the quantity inversely proportional to the rotational velocity of the probe, was determined.

Results and Discussion

The results of kinetic studies on the calcium ion desorption process are presented in Figs. 2, 5 and 6, where the relative rate constants are plotted against concentration of the cationic compounds when studied alone and in a mixture with the anionic homologue series. The relative rate constant α/α_0 is defined as the ratio of the rate constant of calcium ion desorption in the presence of compounds studied to that measured in the absence of modifiers and is a measure of the compounds efficiency. The standard error was below 10%. As follows from the results presented, cationic compounds induce multiple increase in the rate constant compared with unmodified membrane. Effectiveness of the compounds increases with concentration. As it was discussed earlier (Fogt *et al.,* 1994) the observed desorption of Ca^{2+} ions from liposome membrane in the presence of cationic compounds is the result of competition between that cation and cationic compounds for the negatively charged binding sites localised on the polar moieties of the lecithin molecules. As it results from Figs. 2a, 2b, 2c and 2d, the most potent is the BDTA compound, less are MTM and TMTA, and TPT is the least effective. One may suppose that the differences in the effectiveness of action of the compounds BDTA, MTM, TMTA in the calcium desorption process follow from the values of dipole moments of their polar heads, from the values of point charges on some atoms and also from steric properties (Kuczera *et al.*, 1996).

Alkylsulfonates when acting separately did not cause any effect on the rate of calcium ion desorption from the membrane. For a mixture of anionic and cationic compounds an antagonistic or synergistic action is observed depending strongly on alkyl chain length of the ionic surfactants.

From Fig. 2a it follows that the BDTA cation when acting together with anionic amphiphiles brings about decreased effects compared with its single action. The decrease is slight for short-chain anions but gets more pronounced with increasing ASn chain length, and for AS14 the compound BDTA becomes completely inactive. For all the

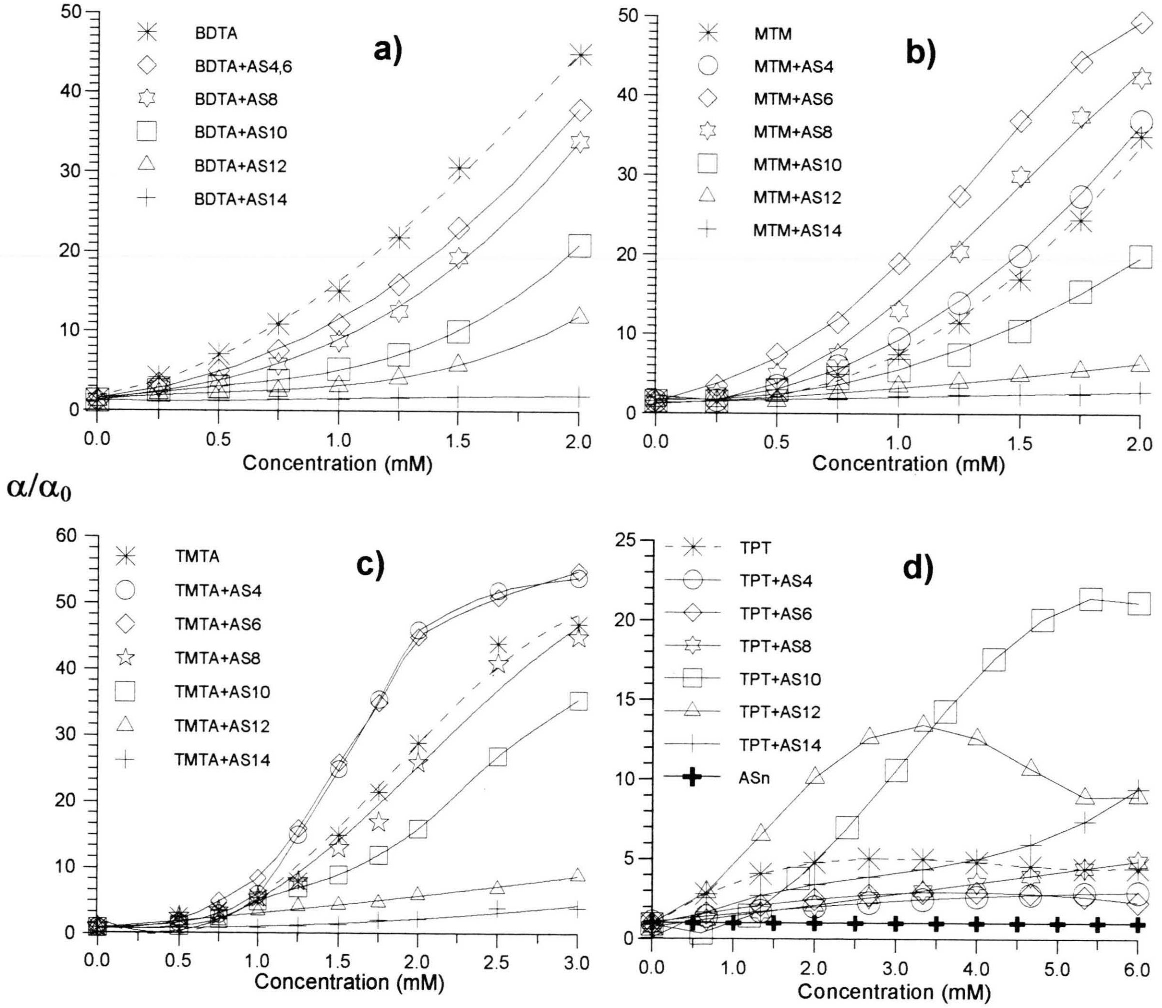


Fig. 2. Relative rate constant, α/α_o, of calcium ion desorption process from liposome membranes against concentration of cationic compounds and its mixtures with sodium alkylsulfonates: **a)** – BDTA and BDTA+ASn, **b)** – MTM and MTM+ASn, **c)** – TMTA and TMTA+ASn and **d)** – TPT and TPT+ASN. α and α_o are kinetic constants for modified and non-modified membrane, respectively.

BDTA+ASn combinations the effectiveness increases with concentration. For the joint action of MTM cation with ASn anions a regulatory effect induced by amphiphilic anionic compounds can be observed in Fig. 2b. Depending on the alkyl chain length, changes are observed beginning from a several-fold increase in effectiveness for the MTM+AS6 system up to total inactivation for the MTM+AS14 system. The compounds AS8 and AS4 caused an increased, AS10 and AS12 – a decreased effectiveness of the MTM cation action. The results for the systems TMTA+ASn are shown in Fig. 2c. We observe increased effectiveness of action for the mixture of compounds TMTA+AS4 and TMTA+AS6 compared with TMTA alone, and decreased effectiveness for all the remaining TMTA+ASn mixtures.

In the case of a mixture of cationic and anionic amphiphiles calcium ion desorption may be the result of competition between calcium ions and cationic species as is the case for cationic surfactants. The basic factor responsible for the lowering in effectiveness of cationic detergents in ion desorption process seems to be charge neutralisation resulting from formation of ion pairs with cationic compounds. The longer the chain of a compound of group ASn the more stable the ion pair it forms with a cationic compound in a membrane, the greater their number in the membrane and stronger the resulting inactivation of cationic compound. This might explain the effect of the BDTA+ASn mixture.

However, the different behaviour of systems containing the compounds MTM+ASn and TMTA+ASn (see also Fig. 4), showing both increased and decreased effectiveness, indicates that other factors must also play an essential role. It seems that an important factor may be the steric properties of the ion pair formed, that follow from the length of the alkyl chain of ASn molecule and also from shape of cationic compounds. The almost total inactivation caused by ion pairs formed of the cations BDTA, MTM and TMTA with the anions AS12 and AS14 may result from cylindrical shape of the pairs and dipolar character of the polar part that makes them similar to the membrane phospholipids. Ionic pairs formed of compounds BDTA, MTM, TMTA and ASn that differ much in their alkyl chain length will form conical structures. The larger the size of the polar part and the greater the difference in alkyl chain length, the greater the disturbance in the membrane structure. An incorporation of any amphiphilic species to liposome membrane must be accompanied by the creation of defects (gaps and kinks) in the hydrophobic part of the membrane which should result in some changes of the structure and packing density of the polar part.

Figs. 3a and 3b exhibit the dependence of the relative value of parameter τ (τ/τ_k) of FA(1,14) spin probe embedded in EYL liposome membranes on concentration of MTM and ASn admixtures (Fig. 3a) and in the EYL liposome membranes on the ASn admixtures concentration at constant concentration of MTM equal to 25% (Fig. 3b). The value of τ/τ_k parameter of the sample containing only MTM admixture (without ASn) of 0.87 was the reference point for τ/τ_k parameter values of other samples (Fig. 3b). From Fig. 3a it follows that an increase in MTM, AS8 and AS10 concentration caused a decrease in the value of τ/τ_k parameter (increase in liposome fluidity). However, admixture of AS14 compound caused a small decrease in membrane fluidity for concentrations smaller than 12 mol% and increased fluidity above this concentration. The compound AS8 shows the largest activity in fluidizing liposomes membranes. From Fig. 3b it follows that at constant MTM concentration an increase in AS8 concentration causes a decrease in τ/τ_k parameter (increase in membrane fluidity) in comparison to the reference value (0.87). The AS14 compound caused an increase in τ/τ_k parameter (decrease in fluidity) for concentrations smaller than 25 mol% and an increase in membrane fluidity above that concentration. The AS10 compound caused a little increase in membrane fluidity for concentrations larger than 20 mol%.

Fig. 2d. shows the dependence of the relative rate constant of the process of calcium ion desorption on concentration of the organometallic compound TPT and mixtures of it with a homologue series of anionic ASn compounds. At variance with mixtures of the compounds considered previously, they act significantly weaker. The organometallic compound TPT, partially dissociated into ions in water solution, differs considerably from BDTA, MTM, TMTA compounds (Kuczera *et al.*, 1997). From its structure one can conclude that the three alkyl chains it has will adhere together due

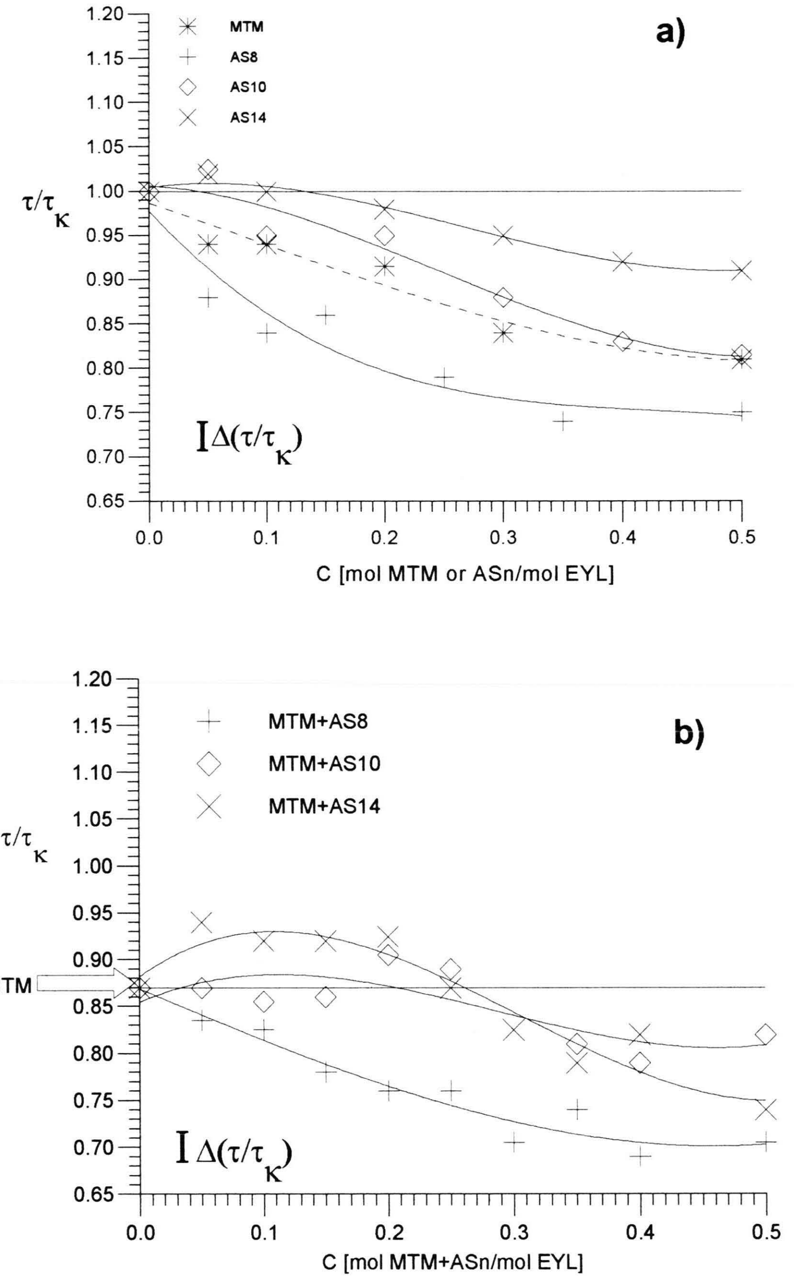


Fig. 3. Dependence of relative value of parameter τ (τ/τ_K) of FA (1,14) spin probe embedded in EYL liposome membranes (**a**) on concentration C of the admixtures MTM, AS8, AS10, AS14 and (**b**) on concentration C of AS8, AS10, AS14 compounds mixed with MTM. In this case the MTM to EYL molar ratio was constant and equal to 0.25. Symbol τ_K denotes τ parameter for control sample (without MTM and ASn compound admixtures). The value of τ_K parameter was constant and equal to 4.2 ns. The horizontal line represents the τ/τ_K parameter for the control sample (without MTM and ASn admixtures) (Fig. 3a) and the sample without admixture of ASn compounds (Fig. 3b). The figure presents the mean error of a measurement series.

to hydrophobic interaction, conferring to the molecule a semispherical shape. The hydrophilic part of TPT molecule will interact with the hydrophilic part of membrane, while the hydrophobic part will locate in the membrane most outer hydrophobic part (Gabrielska *et al.*, 1997). Compound TPT may thus compete with Ca^{2+} ions for adsorption centres, but because of the large cross section of the molecule the interaction will be weaker than of the other cations considered in this work. The inactivation of a cationic compound is not caused by anionic compounds with longest alkyl chains, as has been found for single long-chain cationic compounds, but anionic compounds with short chains, AS4 and AS6 (see also Fig. 4). The effectiveness of TPT is increased the most by AS12 and AS14. It seems that this effect can be explained by weaker effect on the outer layer of membrane of the union of compound TPT with a short-chain anion, because of larger size of the composite molecules and charge neutralisation. Such molecular systems are more hydrophobic and can penetrate deep into the membrane and even across it. In the mixture with long-chain ASn compounds the ion pair formed has a conic shape, with more screened polar part and possibility to anchor in the membrane with the long chain, which may result in a stronger interaction with binding centres in the membrane and increased desorption constant.

The modification of the action of cationic amphiphilic compounds by anionic amphiphilic compounds may also depend on other parameters. Figs. 5 and 6 present preliminary results for two parameters: chain length of cationic compound

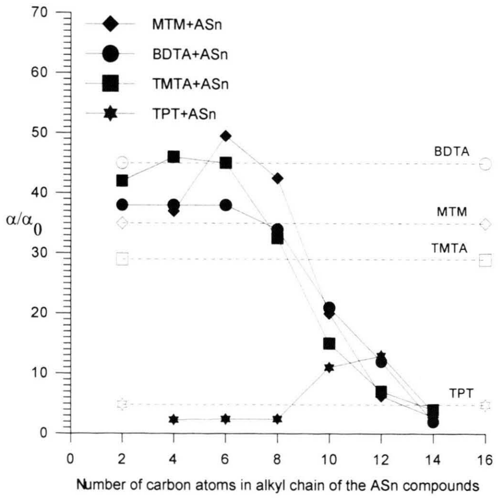


Fig. 4. Relative rate constant, α/α_o, of calcium ion desorption process from liposome membranes against number of carbon atoms in alkyl chain of the ASn compounds for the MTM+ASn, BDTA+ASn, TMTA+ASn and TPT+ASn mixtures at the concentration equal 3 mM. α and α_o are kinetic constants for the modified and non-modified membrane, respectively.

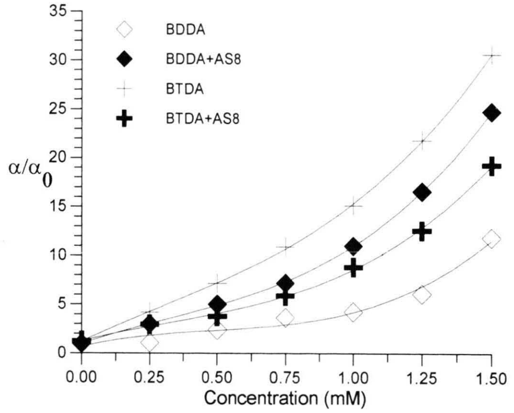


Fig. 5. Relative rate constant, α/α_o, of calcium ion desorption process from liposome membranes against concentration of the BDTA and BDDA compounds and their mixtures with AS8; BDTA+AS8 and BDDA+AS8. α and α_o are kinetic constants for modified and non-modified membrane, respectively.

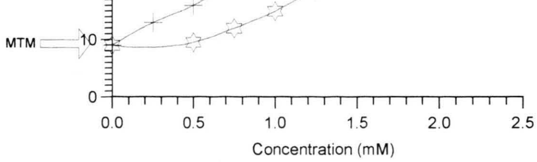


Fig. 6. Relative rate constant, α/α_o, of calcium ion desorption process from liposome membranes against concentration of the AS6 or AS8 compounds in the mixture with MTM compound. The concentration of the MTM compound was kept constant to 1 mM. On the vertical axis was signed value of the relative rate constant, α/α_o, for the MTM compound acting alone in this concentration. α and α_o are kinetic constants for modified and non-modified membrane, respectively.

and molar ratio of amphiphilic cation to amphiphilic anion.

From Fig. 5 follows the important observation that the two homologues, BDTA and BDDA, that differ only in two ethylene groups in the chain, when combined with an identical amphiphilic anion causes a considerable amplification of the action of an amphiphilic cation in the former case and considerable depression in the latter case. Compound BDTA with 14 carbon atoms in the alkyl chain is three times as effective as its analogue with 12 carbons in the chain. This may be caused by a deeper immersion in the membrane of BDTA than BDDA, deeper placement of the hydrophilic part in the membrane and stronger competition with calcium ions. The BDDA+AS8 mixture acts stronger than the BDDA compound separately and somewhat stronger than the mixture BDTA+AS8. It is also worth noting that the regulatory effect of those mixtures is reversed. The AS8 compound enhances the action of BDDA but weakens that of BDTA. The ion pair formation with the amphiphilic AS8 anion increases the hydrophobic properties of the pairs and causes charge neutralization. In the case of the very effective BDTA, as mentioned previously, the charge neutralization effect predominates, whereas in the case of BDDA ion alone, which is less deeply embedded, the increased hydrophobicity of the pair formed may be important; because it enables the molecule to penetrate the membrane deeper, which makes the action of the two pairs, BDTA+AS8 and BDDA+AS8, similar.

Fig. 6 illustrates the effect of cationic (MTM) and anionic (AS6 and AS8) surfactants as dependent on their molar ratio. On the abscissa the anionic compounds concentration is marked. The MTM compound concentration was constant and equal to 1 mM. On the ordinate are marked values of α/α_0 for MTM acting alone at 1 mM concentration. It can be seen that for both the mixtures the process rate increases with increasing concentration of alkylsulfonates. The rate constants level off when ASn concentration is twice as high as MTM concentration. The reason for such a course of the plots may be increasing concentration of ion pairs MTM+AS6 or MTM+AS8 in the membrane with increasing ASn concentration. ASn anions can penetrate the positively charged membrane in excess and thus lower the positive membrane surface potential. At sufficiently high concentration of ASn compounds in the membrane a reduction in Ca^{2+} efflux from the membrane occurs, and also counteraction to further influx of ASn ions to membrane and equilibrium obtains.

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