Influence of Some Lysosomotropic Compounds on Calcium Ion Desorption Process from Liposome Membrane

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The effect of a group of model lysosomotropic compounds on the process of Ca^{2+} ion desorption from lecithin liposome membranes was studied. The compounds studied were: hydrochlorides of fatty acids 2-dimethylaminoethyl esters (DM-n) for n = 9, 11, 13 and 15 carbon atoms in the fatty acid alkyl chain and methochloride of 2-dimethylaminoethyl laurate (DM_S-11).

It was found that all the compounds studied caused increased desorption with increasing concentration of the compound. Most effective was the quaternary ammonium salt, DM_S-11. Moreover, it was found that the process of Ca^{2+} desorption from the membrane depended on pH of the medium. Compound DM-11 was more active at pH 8 than at pH 5. The action of DM-n compounds depended on the alkyl chain length, DM-11 and DM-13 being the most active.

Apparently free amines penetrate the phospholipid membranes and incorporate into its hydrophobic core causing structural deformations. Hydrochlorides of fatty acids and the quaternary ammonium salt induce desorption of calcium ions mostly as a result of competitive electrostatic interactions. By quantum chemistry, PM3 method, and methods of molecular modelling we established the higher hydrophilicity of the polar head of DM-n series with respect to the polar head of the DM_S-n compounds. DM-n compounds possess both acceptor and donor properties for hydrogen bonding while DM_S-n are instrumental as acceptors only.

It should be noted, that the results obtained in this paper for model membranes are in accordance with those for biological ones.

Introduction

The physico-chemical properties of compounds of N,N-dimethylaminoethyl esters of fatty acids (DM) depend on pH of the medium, and their effect on living cells depends on whether they assume ionic or nonionic form. They belong to a vast group of lysosomotropic compounds, i.e. they can pass through biological membranes and can concentrate several hundred times in lysosomes of mammalian cells to which they are added (de Duve *et al.*, 1974; Miller *et al.*, 1983; Boyer *et al.*, 1993). Biological activity of lysosomotropic compounds depends not only on properties of the medium but also on their molecular structure (Dubowchik *et al.*, 1993, Lachowicz *et al.*, 1997).

In medium of low pH such compounds undergo protonation and as cationic detergents they can affect the structure and function of biological membranes. The amphiphilic ammonium compounds exhibit, among others, algicidal, fungicidal and bactericidal properties (Witek *et al.*, 1978; Witek and Grobelny, 1978; Rucka *et al.*, 1980; Deviński *et al.*, 1991). Other compounds of similar properties were studied by us previously. We have found that such compounds significantly affect the structure of phospholipid membranes, as well as transport processes occurring within the membrane (Gabrielska *et al.*, 1979, 1981; Kuczera *et al.*, 1983, 1985, 1989, 1996; Przestalski *et al.*, 1983).

Lachowicz *et al.* (1996) showed that DM compounds among others inhibit the growth of yeast *Saccharomyces cerevisiae* and yeast-like organisms (Bień *et al.*, 1995). It was also found that the compounds influence the process of calcium uptake by yeast (Lachowicz *et al.* 1996).

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Therefore, it seemed useful to study the effect of the lysosomotropic substances DM on the process of calcium ion transport into model phospholipid membranes as dependent on pH of the medium and structural properties of the compounds.

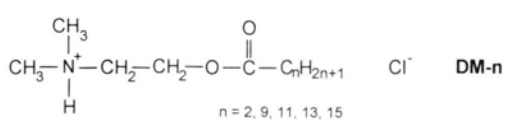
For comparison, the activity of trimethylammonium derivative of DM_S-11 compound was also studied.

Materials and Methods

The compounds studied are presented in Fig. 1. They have been synthesized in our laboratory. The series of compounds DM-n have been synthesized in the form of their hydrochlorides as white crystals, soluble in water and having high purity of about 99% (Bień *et al.*, 1995). A free amine was liberated from DM-11 and by quaternization with methyl bromide transformed into trimethylammonium derivative, DM_S-11.

Egg lecithin was prepared according to the method described by Singleton *et al.* (Singleton *et al.*, 1964). Small unilamellar liposomes (SUV) were prepared from egg yolk lecithin by using sodium cholate in Liposomat (DIANORM) (Weder and Zumbuhl, 1984). The solution used to form vesicles contained a veronal-acetate buffer of pH 7.5 and 0.3 mM radiolabeled $CaCl_2$. During vesicle formation calcium cations were absorbed by liposomes (Kuczera and Żyłka, 1979). The radioactive tracer was removed from the external medium during liposome preparation. The liposome dispersions (6 ml) were diluted in 80 ml buffer of required pH. After 30 min the dispersion was added to the experimental chambers.

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 °C. The inner chamber was filled with the liposome suspension (of relative Ca^{2+} to lecithin concentration equal 1:10), and the outer one with the solution alone. Defined amounts of the compounds studied were added to both compartments to give identical concentrations on both sides of the cellophane wall. The final concentrations ranged between 1.0 and 5.0 mM. Aliquots were taken at chosen time intervals and their radioactivity was measured with a Packard liquid scintillation counter. The experiments were repeated 4–6 times for each compound studied.



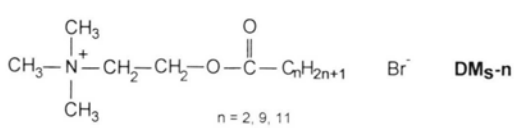


Fig. 1. Compounds studied. DM-n-hydrochlorides of fatty acids 2-dimethylaminoethyl esters, DM_S-n-methochloride of 2-dimethylaminoethyl alkanoates.

The theoretical work-out of the transport and desorption measurements previously described (Mazgis and Kuczera, 1981) was used, with minor modifications. The flux of the ions observed results from the desorption process and permeation through the interior of the liposomes. However the latter flux is negligibly small 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). In order to determine the rate constant of the ion desorption process, the 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).

Solving a system of kinetic equations of balance for the amount of radiotracer present in each compartment, one obtains the following solution for relative radioactivity, U

$$U = (A_\infty - A)/A_\infty = [\beta/(\beta-\alpha)]\, e^{-\alpha t} - [\alpha/(\beta-\alpha)]\, e^{-\beta t} \qquad (1)$$

where: A_∞ – equilibrium radioactivity (in cpm), determined as $A_\infty = [V_0/(V_0+V_i)]\, A + [V_i/(V_0 + V_i)]\, A_i$; A_i and A – radioactivity of samples taken from the inner and outer chamber, respectively; V_i and V_0 – volume of the inner and outer chamber; t – time, α – rate constant of calcium ion desorption process from liposome membrane, β – rate constant of calcium ion transport through cellophane membrane (β was determined in a separate experiment).

Plots of logarithm of relative radioactivity, $\ln U$, against time were constructed from experimental points. Theoretically calculated curves from equa-

tion (1) were fitted to them using a computer programmed Newton iteration method that allows to determine the optimal value of the rate constant α.

Quantum mechanical computations of DM-9 and DM_S-9 structures were performed with Mopac6 section of Cerius2 (Cerius2, 1997) by PM3 (Stewart 1989, 1990) method. Visualizations of electrostatic potential and hydrogen bonding ability mapped on Connolly surfaces for PM3 optimized structures were performed with Sybyl v. 6.3 (Sybyl, 1997). LogP values for DM-2 and DM_S-2 structures were computed with Cerius2 (Cerius2, 1997).

Results

In Fig. 2 an example of the relation between logarithm of relative radioactivity of samples and time is presented. It can be seen that radioactivity of samples in the outer chamber increases with time and is DM-11 concentration dependent.

The final results, presented in Figs. 3, 4 and 5, show the relation between the relative rate constant α/α_0 of calcium ion desorption process and concentration of the compounds studied.

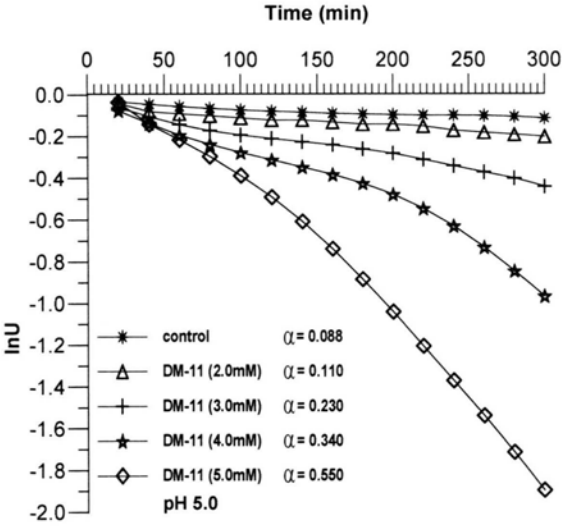


Fig. 2. A representative relationship between logarithm of relative radioactivity, lnU, and time for several concentrations of the DM-11 modifier at the pH 5.0. U = $(A_\infty - A)/A_\infty$, where A – is radioactivity of sample taken from the outer chamber, A_∞ – radioactivity of sample at infinite 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.

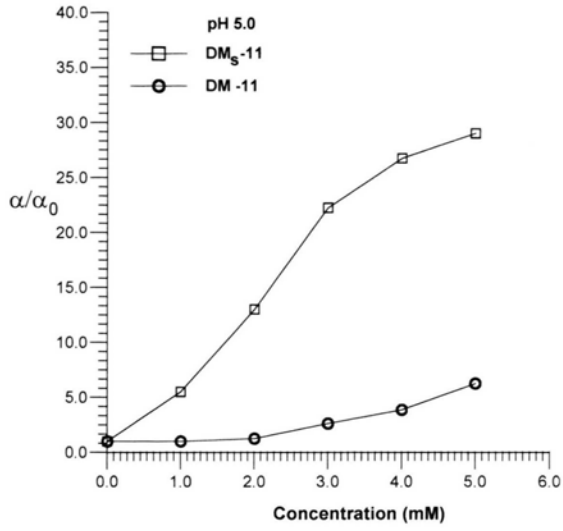


Fig. 3. Relative rate constant, α/α_o, of calcium ion desorption process from liposome membranes against concentration of the DM_S-11 and DM-11 compounds at the pH 5.0. α and α_o are kinetic constants for modified and unmodified membrane, respectively.

It can be seen in Fig. 3 that for the medium of pH 5 in the concentration range studied an increased concentration of DM-11 compound caused only a small increase in the rate constant

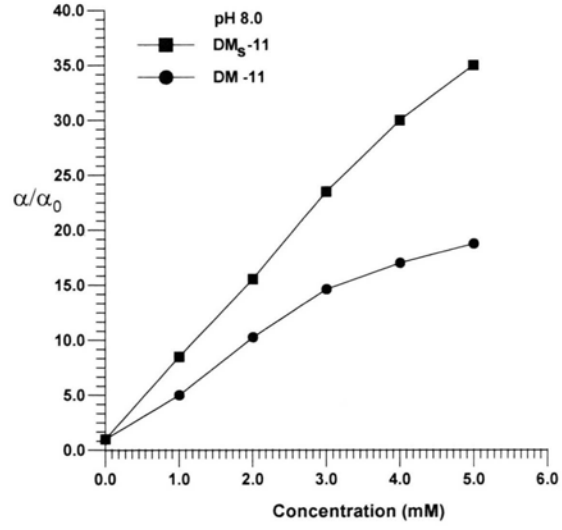


Fig. 4. Relative rate constant, α/α_o, of calcium ion desorption process from liposome membranes against concentration of the DM_S-11 and DM-11 compounds at the pH 8.0. compounds. α and α_o are kinetic constants for modified and unmodified membrane, respectively.

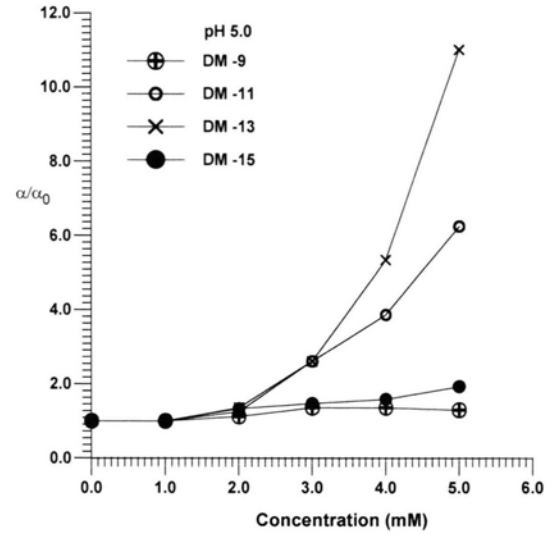


Fig. 5. Relative rate constant, α/α_o, of calcium ion desorption process from liposome membranes against concentration of the DM-9, DM-11, DM-13 and DM-15 compounds at the pH 5.0. α and α_o are kinetic constants for modified and unmodified membrane, respectively.

and a much greater increase for DM_S-11 compound. The results presented in Fig. 4 for pH 8 show also that increased concentration of the compounds studied caused an increase in the rate constants and more effective was also DM_S-11 compound, but the differences are not so pronounced as in pH 5.

Fig. 5 shows the dependence of α/α_0 on concentration of compound DM-n, with n = 9, 11, 13 and 15 carbon atoms in the alkyl chain.

The effect of DM-13 is strongest, a little weaker is that of DM-11; compound DM-15 acts very weakly and DM-9 does not affect the process of calcium ion desorption from liposome membranes.

LogP values for DM-2 and DM_S-2 were determined (Cerius2, 1997) to be -1.220 and -0.520, respectively.

PM3 (Stewart 1989, 1990) quantum computations of the optimized geometries and net atomic charges were performed for DM-9 and DM_S-9. The results of the computations are shown in Fig. 6a for DM-9, in Fig. 6b for DM_S-9 and in Table I. Hydrogen bonding ability was mapped on Connolly surfaces and compared. We observed that DM-9 posseses both acceptor and donor properties while DM_S-9 has only acceptor ability for hydrogen bonding. Electrostatic potential on Connolly surfaces was carefully studied for DM-9 and DM_S-9 and compared. The maximal values of the electrostatic potential are equal to 154.96 e/Å for DM-9 and to 125.45 e/Å for DM_S-9.

Discussion

As it results from the experimental procedure described above, calcium ions are adsorbed at both surfaces of liposomes. The binding sites are localized at the phosphate groups which are the

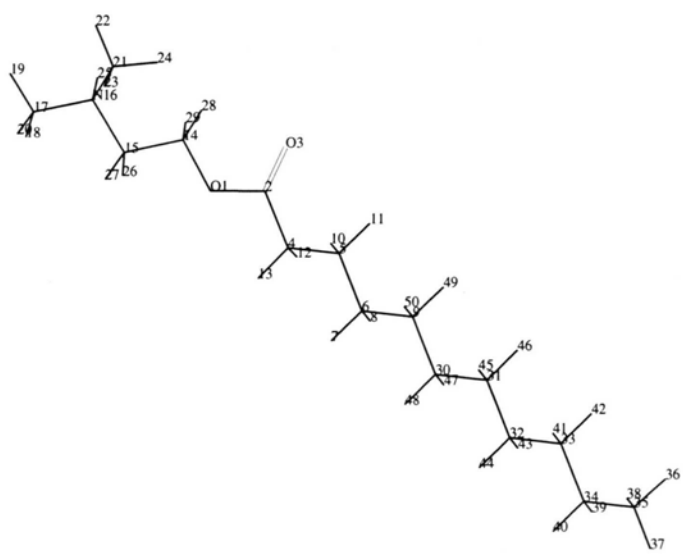


Fig. 6a. Optimized geometry and atomic labels of DM-9 optimized with PM3 method. Labels correspond to Table I.

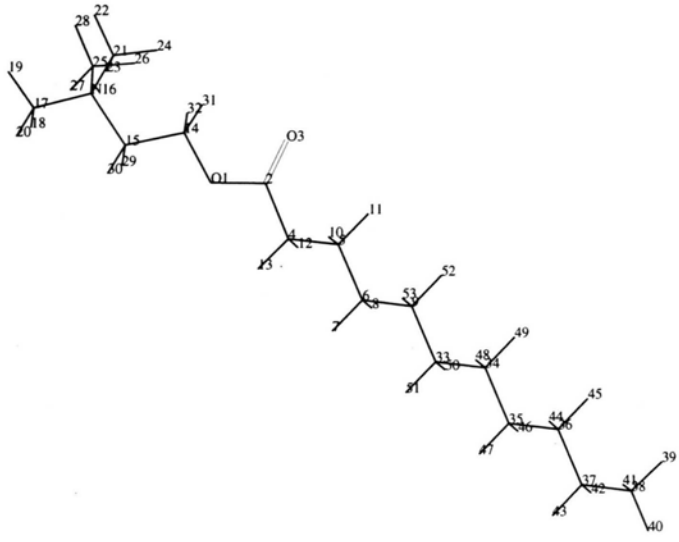


Fig. 6b. Optimized geometry and net atomic labels of DM_S-9 optimized with PM3 method. Labels correspond to Table I.

carriers of the negative part of the delocalized charge of the lecithin polar head groups. Single ions like calcium complex more than one phosphate binding center (Altenbach and Seelig, 1984). The presence of adsorbed positive ions means that the membranes used in our experiments have an initially strong positive charge.

The compound DM_S-11studied now is an ionic surfactant, and in agreement with results obtained previously in our laboratory with different surfactants (Kuczera *et al.,* 1989, 1996; Fogt *et al.,* 1994), and also those reported by other authors (Grupe *et al.,* 1978; Gallova *et al.,* 1990), suggest that adding such compounds to liposome dispersion results in incorporation of the compound into lipid membrane. It can be expected that the hydrophobic part of the compound is localized in the hydrocarbon region of the bilayer, and the hydrophilic part is close to the polar part of the bilayer.

The form the DM compounds studied appear to depend on pH of the solution. In a medium of low pH the ionic form predominates, whereas with increasing pH the number of nonionic free amine molecules increases.

Protonated DM compounds will behave in liposome suspension like the compound DM_S-11, i.e. they locate their hydrophilic parts in the hydrophilic area of the membrane and their chains – along the alkyl chains of lecithin molecules. Free amines, instead, being hydrophobic will locate in the hydrophobic membrane area, or pass into the liposome interior.

DM compounds in either form may induce increased desorption of calcium ions. When in ionic form this can occur as a result of competition between calcium ions and the surfactant cations for negatively charged binding sites localized on the polar moieties of lecithin molecules (Kuczera and Żyłka, 1979; Altenbach and Seeling, 1984), and also as a result of structure disturbance induced both in polar and nonpolar parts of the membrane. In the polar part the disturbance is due to the steric properties of the incorporated polar head, and in the hydrophobic part due to different alkyl chain length of the incorporated molecule and lecithin (Gabrielska *et al.,* 1981; Kuczera *et al.,* 1985). Free amines embedded into membrane will cause structural changes in its hydrophobic part, its loosening, and thus weakening of calcium binding forces.

From Figures 3 and 4 it follows that DM_S-11 compound acts substantially stronger than DM-11, both for pH 5 and pH 8. The two compounds differ in their hydrophilic parts, the size and hydrophobicity of heads. The larger head of compound DM_S-11 brings about greater disturbance in the hydrophilic part of membrane, weakening the binding of calcium with the membrane. This is in accord with our earlier results on other surfactants (Kuczera *et al.,* 1983). The presence of three

Table I: Comparison of net atomic charges of DM-9 and DM_S-9 molecules (atom labels correspond to Fig. 6a and Fig. 6b).

Molecule DM_S-9 labels, atoms types and charges			Molecule DM-9 labels, atom types and charges		
Atom label	Atom type	Charge [a.u.]	Atom label	Atom type	Charge [a.u.]
O1	O	−0.2849	O1	O	−0.2887
2	C	0.3873	2	C	0.3881
O3	O	−0.3500	O3	O	−0.3475
4	C	−0.0918	4	C	−0.0914
5	C	−0.0999	5	C	−0.0997
6	C	−0.1009	6	C	−0.1009
7	H	0.0525	7	H	0.0524
8	H	0.0547	8	H	0.0545
9	C	−0.1013	9	C	−0.1013
10	H	0.0613	10	H	0.0623
11	H	0.0744	11	H	0.0737
12	H	0.0878	12	H	0.0871
13	H	0.0833	13	H	0.0839
14	C	0.0719	14	C	0.0759
15	C	−0.2330	15	C	−0.2582
N16	N	0.6149	N16	N	0.6774
17	C	−0.2371	17	C	−0.2571
18	H	0.1102	18	H	0.1142
19	H	0.1100	19	H	0.1145
20	H	0.1102	20	H	0.1147
21	C	−0.2492	21	C	−0.2688
22	H	0.1124	22	H	0.1159
23	H	0.1107	23	H	0.1151
24	H	0.1191	24	H	0.1226
25	C	−0.2492	25	H	0.0556
26	H	0.1195	26	H	0.1262
27	H	0.1107	27	H	0.1256
28	H	0.1124	28	H	0.0604
29	H	0.1206	29	H	0.0672
30	H	0.1211	30	C	−0.1012
31	H	0.0634	31	C	−0.1017
32	H	0.0632	32	C	−0.1014
33	C	−0.1012	33	C	−0.1030
34	C	−0.1017	34	C	−0.0971
35	C	−0.1014	35	C	−0.1102
36	C	−0.1031	36	H	0.0389
37	C	−0.0971	37	H	0.0405
38	C	−0.1102	38	H	0.0387
39	H	0.0389	39	H	0.0489
40	H	0.0405	40	H	0.0486
41	H	0.0386	41	H	0.0516
42	H	0.0490	42	H	0.0520
43	H	0.0486	43	H	0.0515
44	H	0.0515	44	H	0.0510
45	H	0.0520	45	H	0.0522
46	H	0.0517	46	H	0.0530
47	H	0.0510	47	H	0.0525
48	H	0.0520	48	H	0.0515
49	H	0.0529	49	H	0.0562
50	H	0.0526	50	H	0.0541
51	H	0.0515	–	–	
52	H	0.0561	–	–	
53	H	0.0538	–	–	

methyl groups at the nitrogen atom in DM_S-11 compound instead of two groups and hydrogen in compound DM-11 makes the compound DM_S-11 more hydrophobic than DM-11 with resultant increase in membrane incorporation. The two factors seem to explain the stronger affect of com-

pound DM_S-11 on the process of calcium ion desorption from the lecithin liposome membrane than that of DM-11 compound that occurs in protonated form.

To check this rationale we computed LogP values for DM-2 and DM_S-2 cations. The aim of this computation was to compare the hydrophobicities of the charged head groups of DM-n and DM_S-n structures, by computing the decimal logarithms of their partition coefficients P between water and octanol media for short chain representants of the compouds studied. The LogP value for DM-2 is equal to -1.220 and is much lower than the LogP value for DM_S-2, which is equal to -0.520. This proves, that the head group of DM_S-n is more hydrophobic than the head group of DM-n. The hydrophobicities of the both classes of compounds in obvious way increase with the increase of their chain length, but should differ by the hydrophobic/hydrophillic properties of their head groups making DM-n less hydrophobic than DM_S-n if they are compared for the same chain length. To study this problem in more detail we performed quantum mechanical computations to establish the optimized geometries and net atomic charges for DM-9 and for DM_S-9 cations for comparison. The optimized geometry and net atomic charges for DM-9 are presented in Fig. 6a, for DM_S-9 in Fig. 6b and they are compared in Table I. The geometries of the two compounds do not differ significantly in the hydrophobic tail part, the only changes are seen due to different stoichiometry of the head groups and their topology. The partial net atomic charges of the tail parts of the two compounds are nearly identical, however there are some little changes in net atomic charges on nitrogen, carbon atoms bonded to nitrogen and hydrogens bonded to those carbons. The main difference in the charge decomposition of DM-9 with respect to DM_S-9 can be observed in the greater (0.062 a.u) net charge of nitrogen atom in DM-9.

However, the small changes in net atomic charges of DM-9 with respect to DM_S-9 can result in much greater maximal value of the electrostatic potential of about 29.5 e/Å. The explanation of this fact could have been read from visualization. The closer Connolly surface fits the nitrogen atom the higher are the values of the electric potential. The steric reasons seem to be responsible for the higher maximal value of the electrostatic potential of DM-9 with respect to DM_S-9. The positive net atomic charge of nitrogen results in much greater electrostatic potential on shorter distance and this is the case of the compound with a smaller head group, which is DM-9. The visualisation of hydrogen bonding ability on Connolly surfaces depicts acceptor or donor properties, which can be manifested by the molecules in contact with the surrounding medium. As we established, DM-9 is more hydrophillic due to its nitrogen atom acting as donor for hydrogen bonding. This feature is absent in DM_S-9 structure, whose nitrogen atom does not possess such ability.

In our opinion, the stronger hydrogen bonding molecule can form, the more hydrophilic the molecule is; and also the stronger electric nonbonding interaction with water and other polar molecules or their parts, the higher hydrophilicity of the molecule considered. Thus the nature of the greater hydrophobicity of DM_S-n than the corresponding DM-n molecule can be interpreted both in terms of hydrogen bonding and electric interaction of the molecules with the hydrated region of the phospholipid bilayer.

As mentioned earlier, it follows from figures 3 and 4 that both the compounds DM_S-11 and DM-11 act stronger at pH 8, the difference in the interaction being more substantial for compound DM-11. This feature is characteristic of lysosomotropic compounds (Golstein *et al.,* 1991). The growth-inhibiting activity on the yeast *Saccharomyces cerevisiae* of DM-11 and DM-13 is also pH dependent, increasing with increasing pH (Lachowicz *et al.,* 1996).

In the case of compound DM_S-11, which occurs in ionic form only, its greater activity at pH 5 may be due to changes in membrane charge caused by hydrogen atoms. That supposition may be confirmed by our finding that the kinetic constant for calcium ion desorption, α_0, is two times greater in the absence of the compounds studied at pH 5. Hydrogen ion must be additionally bound by the membrane, increasing its positive charge and weakening Ca^{2+} ion binding. This increased positive membrane potential may cause a decrease in partition coefficient of the surfactants between the membrane and medium.

It seems that the stronger action of compound DM-11 at pH 8 can be explained by accumulation within membrane of a large amount of amine com-

pounds which at high pH are the dominant form. Such substantial amount of molecules incorporated into membrane can induce changes in membrane structure and significant weakening of calcium binding forces resulting in increased desorption of the ions.

The experiments illustrated in Fig. 5 were done at pH 5, i.e. when the ionic form of DM-n dominates. The relationship between the rate constant of the desorption process and alkyl chain length that follows from the figure indicates that a maximum exists in the effectiveness of action of the compound DM-13. Such effect is characteristic for biological activity of many surface-active substances. In a homologous series of compounds the activity increases progressively with increasing chain length up to a critical point, beyond which the compounds cease to be active. This behaviour is called the cut-off effect (Devińsky *et al.*, 1996). As follows from our previous experiments, carried out with homologous series of amphiphilic quaternary ammonium compounds having various intermediate groups between the nitrogen atom and alkyl chain, the maximum in the effectiveness depends on those groups.

In Lachowicz *et al.* (1996) a maximum was also found in the growth-inhibiting activity of *Saccharomyces cerevisiae* and also for DM-13.

The coincidence of the results obtained in this work for compound DM-n with the results for *Saccharomyces cerevisiae*, both with respect to pH dependence and alkyl chain length, confirms the earlier studies with other groups of compounds (Gabrielska *et al.* 1981; Kuczera *et al.*, 1985) that the process of calcium ion desorption from lecithin liposome membranes can be utilized for testing the biological activity of lysosomotropic compounds.

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