Effects of Some Cyclic Elements Containing Amphiphilic Compounds on Stability and Transport Properties of Model Lecithin Membranes

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Abstract. The paper presents results of studies on changes in model membrane properties induced by some single-chain amphiphilic quaternary ammonium salts with fungicidal activity, N-dodecyl-N-methylmorpholinium chloride (IVC), N-dodecyloxymethylene-N-methylmorpholinium chloride (IVD), N--dodecyl-N-methylpiperidinium chloride (VIC), N-dodecyloxymethylene-N--methylpiperidinium chloride (VID) and N-dodecyl-N,N-dimethyl-N-benzylammonium chloride (IB). Two different lipid systems were used, namely, unilamellar liposomes and black planar membranes (BLM). All the compounds studied interfered with sulphate ion transport across liposomal membranes as well as with calcium ion desorption from the membranes. The compounds were found also to change the stability of black lecithin membranes. Generally the sequence of effectiveness of the salts studied on both models used was: IB > VID > IVD > VIC > IVC, although there were some differences for particular processes. The results obtained are discussed in view of a possible mechanism of the interaction between the salts studied and the lipid model including the role the salt head group structure and charge distribution can play in this interaction.

Key words: Lecithin membranes — Amphiphiles — Transport — Stability

Introduction

It was found earlier that some amphiphilic ammonium salts (AAS) have strong biological activities. Some of them have fungicidal properties, particularly when applied to *Alternaria tenuis* and *Botritis cinerea* (Witek et al. 1978a, b), others act against algae *Oscillatoria* sp. or bacteria *Sphaerotilus natans* (Rucka et al. 1983).

However, the molecular mechanism of this action is still not exactly known. It can be assumed that initially the cell membrane is attacked, and thus any change in the cell membrane may alter the transport of the substance across the membrane. To explain the molecular mechanism of the effects of the amphiphilic ammonium salts, we performed some studies on model membranes, in particular on phospholipid membranes. Studies of processes occurring in phospholipid membranes in the presence of amphiphilic compounds may supply information on both the effects of these compounds on the processes studied and the membrane structure. Our earlier studies on the structure of phospholipid membranes and ion transport processes across lecithin membranes modified by amphiphilic nitrovinylbenzylammonium chlorides and their analogues (Gabrielska et al. 1979; Kuczera et al. 1983), by a homologous series of alkoxymethylene-trimethylammonium chlorides (Gabrielska et al. 1981; Sarapuk et al. 1981), and by some derivatives of glycine esters (Kuczera et al. 1985; Sarapuk et al. 1984, 1985), allowed us to conclude that the activity of all these modifiers strongly depends on the properties of both the hydrophilic and the hydrophobic part of the molecules as well as on the presence of some special groups in the molecule. Cyclic elements are very often present in biologically active substances, and their role may be of great importance. It seemed reasonable to study compounds containing cyclic elements of different structure and with different charge distribution, in their interaction with phospholipid membranes, both liposomes and planar membranes and to compare the actions on three processes: desorption of calcium ions, permeability of sulphate ions, and membrane stability.

Materials and Methods

The amphiphilic ammonium salts (AAS) studied are shown in Fig. 1. They were synthesized in our laboratory; elemental analysis and spectral data confirmed the identity of the compound structures shown in Fig. 1. The purity of the compounds was not less than 98%. Egg yolk lecithin prepared according to the technique described by Singleton et al. (1965) was used in both liposome and planar membranes experiments. The solution used to form vesicles and then for elution contained a veronal-acetate buffer, pH 7.5; 0.2% sodium azide and 0.3 mmol/l Na_2SO_4 labelled with S-35 for sulphate ion transport studies, or 0.3 mmol/l $CaCl_2$ labelled with Ca-45 for experiments with transport of calcium ions. The respective solution was added to nitrogen dried lecithin and, after washing it from the walls of the flask, the suspension was mechanically shaken to give multilamellar liposomes. In order to obtain unilamellar vesicles, the "cholate" method described by Brunner et al. (1976) was employed: sodium cholate was added to disintegrate the multilamellar structures. After further mechanical shaking the suspension was filtered through a Sephadex G-50 column using buffer solution without radioactive tracers at 4 °C. By this procedure unilamellar liposome suspension containing radioactive ions in the liposomes is separated from the cholate micelles as well as from the undispersed lecithin structures and radioactive ions in the medium. 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 in a water bath and the solutions were well stirred. The inner chamber was filled with liposome suspension, and the outer one with the solution alone. Defined amounts of the AAS compounds studied were added to both solutions to give identical concentrations in both chambers. The salt concentrations ranged between (0.5—5) mmol/l and (1—10) mmol/l for sulphate and calcium ion experiments, respectively. The solutions were sampled at chosen time intervals and their radioactivity was measured with Intertechnique liquid scintillation counter. The experiments were repeated 3—5 times for each concentration and each compound studied.

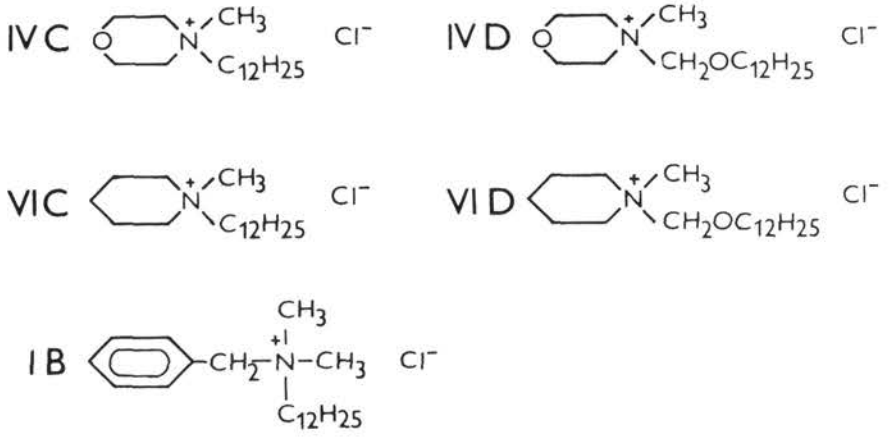

Fig. 1. Structural formulae of the amphiphilic ammonium salts studied. Abbreviations: IVC: N-dodecyl-N-methylmorpholinium chloride; IVD: N-dodecyloxymethylene-N-methylmorpholinium chloride; VIC: N-dodecyl-N-methylpiperidinium chloride; VIC: N-dodecyloxymethylene-N-methylpiperidinium chloride; IB: N-dodecyl-N,N-dimethyl-N-benzylammonium chloride.

Planar bilayer lecithin membranes (BLM) were formed from a stock solution of 1.5% (w/v) egg lecithin in *n*-decane (International Enzymes Ltd, Windsor, England). All data concerning egg yolk lecithin extraction, its purification as well as details of the measurement set-up were described earlier (Sarapuk et al. 1981). All solvents and chemicals were used without further purification. Measurements were performed at room temperature and physiological salt was used as the bath solution. The AAS studied were added to the bath solution shortly after BLM formation. The pipetting of the salt was continued until the membranes broke and no new ones could be formed.

Results

BLM

Black lecithin membranes, once they reached their steady-state level of conductance, were satisfactorily stable for several hours. Typical values of specific BLM resistances were $2 \times 10^6\ \Omega.\mathrm{cm}^2$ to $7 \times 10^6\ \Omega.\mathrm{cm}^2$, depending on the sample.

However, resistance of membranes formed from the same sample differed less than 20% which is a reasonable reproducibility. Both parameters mentioned, i.e., stability and resistance of BLM, were changing as soon as the respective salt was being added to the bath solution (no differences were detected whether when AAS were acting on one side of the membrane nor when they were acting on both sides). The life-time of BLM was shortened with increasing salt concentrations until the membrane could be kept for no more than 3—5 minutes. This means that new membranes could not assume bimolecular structure before they broke, as the "blackening" process took about 10 minutes. Such salt concentrations are called critical (CC); they are shown in Table 1; the experimental error was below 5%.

Table 1. Values of critical concentrations (CC) of the salts studied.

AAS	IB	VID	IVD	VIC	IVC
$\frac{\text{mol}}{\text{dm}^3}$	1.6×10^{-5}	4.1×10^{-5}	4.1×10^{-5}	1×10^{-4}	2.1×10^{-4}

Liposomes

As shown earlier by Kuczera and Żyłka (1979) who used the radiotracer method, calcium ions are bound in the lecithin membrane, with approx. 90% of all calcium ions contained in the liposome. The kinetic constant discussed in the present work concerns mainly the fluxes of labelled calcium ions which are desorbed from the lecithin membrane. On the contrary, sulphate ions are not bound in the lecithin membrane, and the rate constant thus concerns the ion permeation flux through the membrane.

The ions studied, Ca^{2+} and SO_4^{2-}, pass through the liposome membrane and then the cellophane membrane to enter the outer chamber. The time dependence of the activities of samples taken from the chambers characterizes the resultant permeation process across both membranes. In order to determine the rate constant for ion permeation across the liposome membrane, a three-compartment analysis was used, as described by Mazgis and Kuczera (1981). According to this method, plots of time dependences of $\ln \frac{A_\infty - A}{A_\infty}$, are constructed, where: A are the radioactivities of samples taken from the outer chamber, and A_∞ are the activities of the samples after infinite time. To find the rate constants, theoretically calculated curves are drawn and compared with plots of experimental results.

Results obtained by this approach are shown in Figs 2 and 3 where the relative rate constant is plotted against ammonium salt concentration for calcium and sulphate ions, respectively. The standard error was below 5%. The relative rate constant α/α_0 has been defined as the ratio of the rate constant of the ion transport process in the presence of the compound studied (α) to that measured in the absence of AAS (α_0). It is obvious that increasing AAS concentrations in the liposome suspension result in an increase in the kinetic constants. The extent of changes is dependent on the AAS used. For calcium ion desorption, the greatest change in α/α_0 was observed with IB followed by VID, IVD, VIC and IVC. For sulphate ion permeation the sequence was somewhat different: again, IB was the most effective one, but there was no difference between the effectivity of VIC and VID, and between IVD and IVC, the latter two having the weakest effect on the relative rate constants.

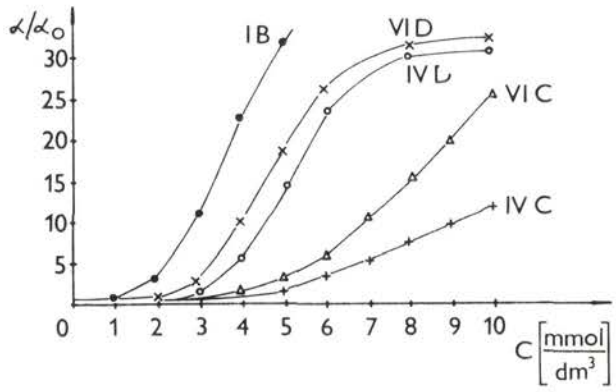

Fig. 2. Relationships between the relative rate constant for calcium ion desorption from the liposome membrane and concentrations of compounds studied in the medium.

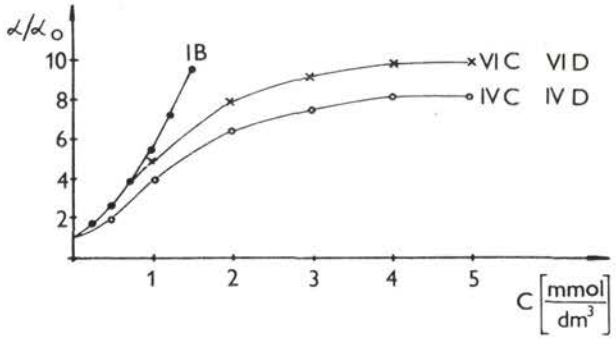

Fig. 3. Relationships between the relative rate constant for sulphate ion transport across the liposome membrane and the concentrations of the compounds studied in the medium.

Discussion

Our results, in agreement with those obtained previously in our laboratory with similar amphiphilic, single-chain ammonium salts (Gabrielska et al. 1979, 1981; Kuczera et al. 1983, 1985; Sarapuk et al. 1981, 1984, 1985) as well as those related by other authors (Bangham and Lea 1978; Grupe et al. 1977; Klose and Hollerbuhl 1981) suggest the existence of an interaction mechanism based on the incorporation of such compounds into the 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. As we concluded earlier on the basis of both transport (Gabrielska et al. 1979, 1981; Kuczera et al. 1983, 1985) and stability studies (Sarapuk et al. 1981, 1984, 1985), the incorporation of single-chain molecules into a lipid membrane may produce deformations in form of gaps in the polar membrane layer, and decrease the packing density of alkyl chains within the membrane, resulting in increased fluidity. This could be confirmed by microcalorimetric, EPR and NMR methods (Frischleder et al. 1984; Przestalski et al. 1983; Sarapuk et al. 1985). When sulphate ion transport processes are considered, all the above deformations may have the same effect as pores for ion transport, and as a result, the rate constant increases. In addition, the membrane becomes positively charged as a result of the incorporation of positive ammonium ions. This in turn may result in accumulation of anions near the membrane surface and their increased permeation across the membrane. Also, the participation of a flip-flop mechanism involving the incorporated AAS compounds in transporting the companion anions cannot be ruled out either. The greater the amphiphile concentration the more molecules penetrate into the bilayer, and the stronger these factors will influence the transport process; this will result in a greater increase in the transport rate constant. The results shown in Fig. 3 support this assumption. The saturation segment of the curves corresponds to the adsorption curve of the ionic surfactants onto nonpolar adsorbents (Rosen 1978). The increased calcium ion desorption rate constant, due to the incorporation of AAS compounds, may be a result of both structural changes at the polar head region and competition between calcium ions and the incorporated ammonium ions. Positively charged fragments of the AAS compounds interacting with the liposome membrane weaken the strength of the bond between the adsorbed calcium ions and the polar heads of the lecithin molecules, thus facilitating the release of calcium ions from the membrane, or exchange of these ions with the medium.

In comparing Figs 2 and 3 it should be noted that, contrary to the sulphate ion transport no change in the relative rate constant for the calcium ion desorption process was observed below certain values of AAS concentrations.

The reason for such a behavior seems to be mainly the positive surface membrane charge in the presence of calcium ions, so that several AAS ions are needed to break the calcium ion bonds with the membrane, and thus to trigger the desorption process, while at concentrations low enough, each AAS molecule contributes to the increase observed with increasing AAS concentration in the sulphate ion permeation process. The triggering concentration of AAS is greater the weaker the action of particular AAS compounds on the membrane.

Changes in BLM stability may be due to structural disturbances of the membrane caused by the AAS studied at sufficiently high concentrations. It seems that under these conditions AAS may first form domains; then, hydrophilic pores may be formed due to increasing surface fluctuations (Petrov 1981), and pore expansion can result in irreversible membrane breakdown (Dimitrov and Jain 1984).

The results obtained with both liposome membranes and planar BLM show that IB is the most active compound for both membrane types. Different activities on the processes analyzed may be observed with other compounds. Owing to the different chemical structure of the compounds studied molecular interactions between the lecithin membrane and the compounds may also be different. This may result in differences in both, partition coefficients for various compounds and structural changes of the membrane. Comparing the chemical structure of the amphiphilic compounds studied (Fig. 1) it is obvious that the only difference between them is the type of the ring and the presence of the oxymethylene group at the beginning of the alkyl chain. Based on studies by Frischleder et al. (1984) the differences in the partition coefficients for the compounds studied in this paper can be expected to be negligibly small. The rings differ from each other mainly in their shape and in the electron distribution.

The benzene ring present in IB has delocalized π-electrons, while the morpholinium ring of IVC and IVD has only a residual charge at the oxygen atom; the piperidinium ring of VIC and VID has no polar element. Our results suggest that the delocalized π-electroncs of the flat benzene ring strongly enhance the interaction of the amphiphilic molecule with the polar head groups of the membrane. The interaction may be strong enough to dehydrate the polar part of the IB molecule and to cause a deep intercalation into the membrane, thus inducing significant deformations in that region weakening calcium ion binding, facilitating sulphate ion permeability and enhancing membrane disturbances at sufficient high concentrations. The weaker activity of the compounds of group IV as compared with the compounds of group VI seems to be due to the absence of a polar element in the piperidinium ring, and to the weak residual charge in the morpholinium ring. Moreover, both rings are not flat and from the steric point of view, they may cause smaller deformations in the membrane. The

compounds of group VI, as follows from the results obtained with transport processes, act stronger than those of group IV. This may be explained by assuming that the small residual charge present in the morpholinium ring prevent a deeper penetration of the molecules into the membrane. The weak interaction of the head of group IV compounds with the membrane is not sufficient to dehydrate the polar part of the ring to allow a deeper penetration; this does not apply for the piperidinium ring since it is non-polar. It may be concluded that a strong polarity of the head of an amphiphilic molecule may greatly enhance its activity, whereas a weak one may even have an opposite effect. On comparing the effects of the AAS salts containing oxymethylene group between the nitrogen atom and the alkyl chain (IVD and VID) with those without this group (IVC and VIC), there are differences in calcium ion desorption but almost no differences in sulphate ion permeability. The nonpolar oxymethylene group elongates the alkyl chain to a similar extent as do two methylene groups. In our earlier studies (Gabrielska et al. 1981) we observed that elongation of the alkyl chain from 12 to 14 carbon atoms (exactly in this range of alkyl chain length) was associated with an 8-fold increase in the rate constant of calcium ion desorption, but only with a slight increase in sulphate ion permeation. Substantial changes in membrane structure are only observed when neighbouring phospholipid molecules are separated from each other due to a prolongation of the chain. Most probably this phenomenon occurs with calcium ion desorption as a result of a change in the number of carbon atoms in the chain from 12 to 14. It can be noted that results obtained in stability study with planar membranes are more similar to those obtained for calcium ion desorption than to those for sulphate ion permeation. In both cases IB enhanced the activity approximately two times as compared to IVD and VID. IVD and VID were about twofold more effective than IVC and VIC. Structure changes in the membrane polar layer seems to be of great importance for both membrane stability and calcium ion desorption. The only difference is that critical concentrations for salts IVD and VID are the same, while they are different for calcium ion desorption. The introduction of the oxymethylene group, resulting in a prolongation of the alkyl chain, seems to have a greater impact on membrane structure changes than the removal of polarity in the piperidinium ring. Similarly as in a previous study, the compounds in the present study were also tested for their fungicidal activity (personal communication). The fungicidal activity correlated very closely with the effects of the compounds on model phospholipid membranes. In almost all cases the piperidinium compounds showed a stronger fungicidal activity than those with the morpholinium ring.

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