A Spin Label Study of Perturbation Effects of N-(1-methyldodecyl)-N, N, N-trimethylammonium Bromide and N-(1-methyldodecyl)-N, N-dimethylamine Oxide on Model Membranes Prepared from *Escherichia coli* -Isolated Lipids*

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Abstract. Interaction of bactericidal surfactants N-(1-methyldodecyl)-N, N, N-trimethylammonium bromide (2-ATDBr) and N-(1-methyldodecyl)-N, N-dimethylamine oxide (2-ATDNO) with phospholipid membranes prepared from *Escherichia coli* — isolated lipids was studied by ESR spectroscopy using *m*-doxyl stearic acid (*m*-DSA, $m = 5, 12, 16$) and N-cetyl-N, N-dimethyl-N-tempoylammonium bromide spin labels located in different membrane depths. 2-ATDBr was found to be a more potent membrane perturbant than 2-ATDNO both at equal membrane and sample concentrations; this is in compliance with the respective antimicrobial activities of these agents. Using the statistical model of hydrocarbon chains in lipid bilayers, the probabilities of the formation of gauche conformations and the effective energy differences between the trans and gauche conformations were calculated from *m*-DSA order parameters for two different bilayer regions. Based on these parameters, a molecular model of the location of surfactant molecules in bilayer has been formulated. It has been suggested that at low concentrations the surfactant molecules are located in structural defects between lipid clusters in the bilayer. After filling up these defects, the surfactant molecules penetrate into the clusters between lipid molecules, expand the bilayer laterally and increase the amount of gauche conformations in the hydrocarbon chains in the hydrophobic core of the bilayer.

Key words: Surfactant bactericides — Membranes — Alkylammonium bromides — Alkylamine oxides — Spin label ESR

* Part V. of the series Interaction of Surfactants with Model and Biological Membranes, part XXIII. of the series Amine Oxides and part XXI. of the series Quaternary Ammonium Salts.

Introduction

Organic quaternary ammonium salts as well as amine oxides have antimicrobial properties under certain structural conditions. They affect both the function and the structure of the cytoplasmic membrane. The primary mechanism of the antimicrobial action of these agents has been supposed to be physical pertubation of the lipid bilayer part of the membrane, while changes in metabolism are probably secondary to this perturbation. Studies of structural changes in model phospholipid membranes can characterize more closely the membrane effects of these compounds.

In the present work, the effects on membrane structure of a cationic surfactant, N-(1-methyldodecyl)-N, N, N-trimethylammonium bromide (2-ATDBr), and of a structurally analogous, non-ionic surfactant, N-(1-methyldodecyl)-N, N-dimethylamine oxide (2-ATDNO) were studied. Membranes were prepared from lipids isolated from *Escherichia coli* cells, and the method employed was electron spin resonance (ESR) spectroscopy of spin labels located in different membrane depths.

Materials and Methods

2-ATDBr and 2-ATDNO were prepared according to Lacko et al. (1977) and Devinsky et al. (1985). Total phospholipids were isolated from cells of *Escherichia coli* Ec 377/79 (CNCM, Prague, Czechoslovakia) at the end of the exponential phase of growth. The cells were cultivated on nutrient agar (Imuna, Šarišské Michaľany, Czechoslovakia). Phospholipids were isolated according to Folch et al. (1957). The spin labels, *m*-doxyl stearic acids (*m*-DSA; $m = 5, 12, 16$), were purchased from Syva (Palo Alto, USA), and N-cetyl-N-tempoyl-N, N-dimethylammonium bromide (CAT-16) from Technika (Sofia, Bulgaria). The solvents were redistilled before use.

Samples for ESR measurements were prepared as follows: Ethanolic solutions of the respective spin label, lipids, and 2-ATDBr or 2-ATDNO were weighted into polyethylene test microtubes. After evaporating the solvent in a stream of nitrogen, the samples were evacuated (10^{-2} Pa) at 25° C. Immediately before experiment, redistilled water was added in a weight ratio of lipid: $H_2O = 1 : 50$, and the lipid was dispersed by sonication in Tesla UC 005 AJ1 (Czechoslovakia) bath sonicator for 5 minutes. The final concentration of the spin label was $2.5 \cdot 10^{-4}$ mol . l^{-1}, those of 2-ATDNO or 2-ATDBr were between 10^{-4} and 10^{-1} mol . l^{-1}, and that of the lipid was 20 g . l^{-1}.

ESR spectra were recorded using an ERS-230 X-band spectrometer (ZWG AdW, Berlin, GDR) with a microwave power output of 5mW and a modulation amplitude of $2—5 \cdot 10^{-5}$ T.

To determine the relative membrane perturbing efficiences of 2-ATDBr and 2-ATDNO, the order parameter S was calculated from ESR spectra according to

$$S = f_A(A_{\parallel} - A_{\perp})/[A_{zz} - (A_{xx} + A_{yy})/2] \quad (1)$$

$$f_A = (A_{xx} + A_{yy} + A_{zz})/(A_{\parallel} + 2A_{\perp}) \quad (2)$$

where $A_{\parallel}$ and $A_{\perp}$ are time averaged components of the axially symmetric hyperfine splitting tensor $\bar{A}$, parallel and normal to the magnetic field direction, respectively, obtained from the outer and inner extrema, respectively, of the spin label spectra in the membranes; A_{ii} are components of

diagonalized $\bar{A}$ tensor measured in the crystal; and f_A is the polarity correction factor (see Gaffney (1976) and Hemminga (1983)). Occasionally, the outer splitting could not be measured due to a low signal: noise ratio. In these cases a simplified equation was used to calculate S (Gordon and Sauerheber 1977):

$$S = 0.5\{3[(A_{zz} + A_{xx}) - 2A_{\perp}]/(A_{zz}^{\perp} - A_{xx}) - 1\} \quad (3)$$

The rate of molecular reorientation of the spin label can be described by rotational correlation time τ. In the limit of the fast spin label motion ($5 \cdot 10^{-11}\text{s} < \tau < 3 \cdot 10^{-9}\text{s}$)

$$\tau_B = \Delta H_0 \sqrt{3}\pi 2.8 \cdot 10^6 [8b\Delta\gamma H_0/15]^{-1} \cdot [(I_0 : I_{-1})^{1/2} - (I_0 : I_{+1})^{1/2}] \quad (4)$$

$$\tau_c = \Delta H_0 \sqrt{3}\pi 2.8 \cdot 10^6 [b^2/4]^{-1} \cdot [(I_0 : I_{-1})^{1/2} + (I_0 : I_{+1})^{1/2} - 2] \quad (5)$$

$$-\Delta\gamma = \beta [g_{zz} - 0.5(g_{xx} + g_{yy})] : \hbar \quad (6)$$

$$b = 4\pi [A - A_{\perp}] : 3 \quad (7)$$

where ΔH_0 is the width of the center ($m = 0$) ESR line (in Gauss), H_0 is the magnetic field induction in the position of the center line (in Gauss), I_m are the amplitudes of lines for magnetic quantum number m, β is the Bohr magneton, $\hbar$ is the Planck constant, g_{ii} are the components of the diagonalized g-factor tensor measured in the crystal, and A and $A_{\perp}$ are components of the $\bar{A}$ tensor in frequency units s^{-1} (Schreier et al. 1978; Berliner 1982; Hemminga 1983).

Both the order parameter S and the correlation time are (different) quantitative measures of membrane fluidity.

Results

The dependence of the order parameter on concentrations of 2-ATDBr and 2-ATDNO

Fig. 1 shows typical changes occurring in the order parameter S of the spin labels located in the model membranes, in dependence on the sample concentration c of 2-ATDBr or 2-ATDNO, at a constant temperature (25°C). It can be seen that the order parameters S are lower in samples containing 2-ATDBr in comparison with those containing 2-ATDNO. Furthermore, the order parameters S decrease with the increasing surfactant concentration c above the critical value of $c = 5\ \text{mmol} \cdot \text{l}^{-1}$. As shown in Fig. 1*A* (insert), this decrease can be approximated by a linear function from which the slope $\Delta S/\Delta c$ can be calculated. This parameter characterizes the efficiency of the compounds studied in perturbing the membrane structure at different depths, depending upon the type of the spin label used, at an equal surfactant concentration c in the sample. As clearly seen from Table 1 summarizing the values of $\Delta S/\Delta c$, 2-ATDBr is a more efficient membrane perturbant than 2-ATDNO.

The dependence of the order parameter on temperature and membrane depth

The temperature dependence of the spin label order parameter is linear within

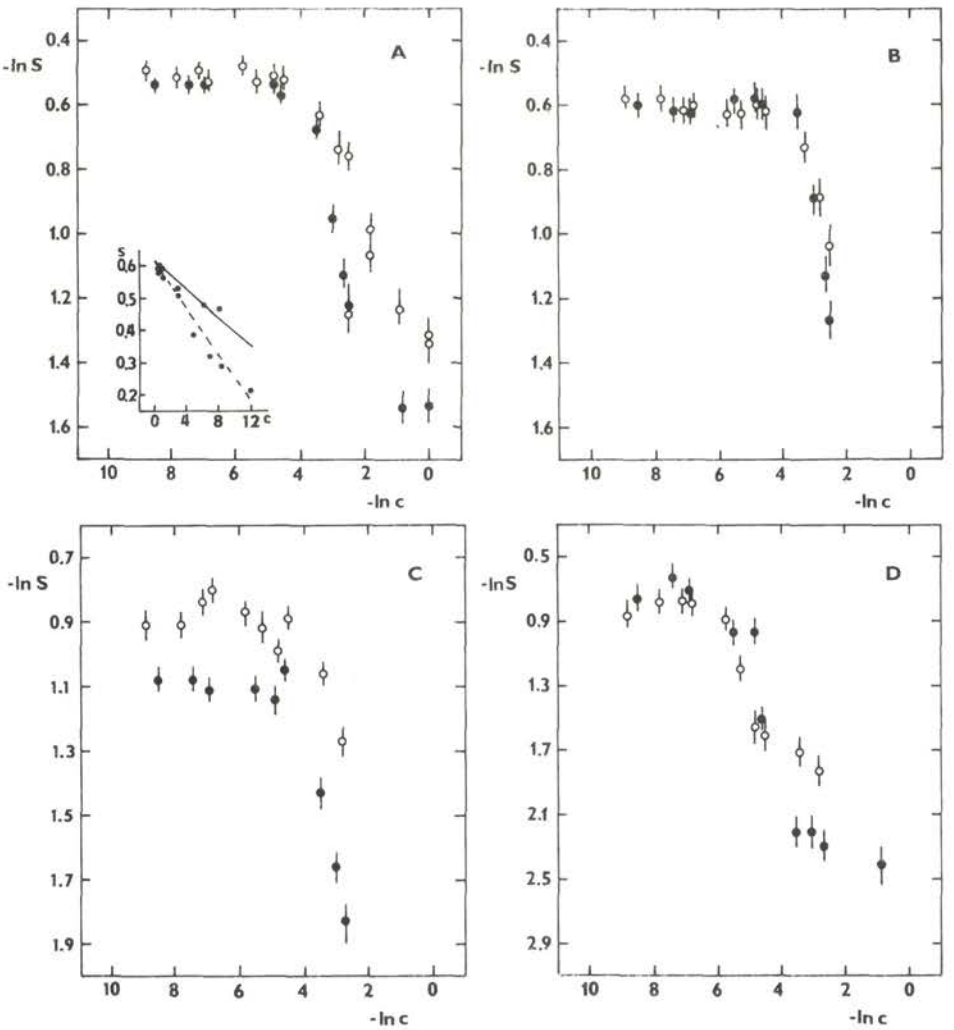

Fig. 1. The relationship of the order parameter S and surfactant concentration c (mol . l^{-1}). *A*: spin label 5-DSA; *B*: spin label 12-DSA; *C*: spin label 16-DSA; *D*: spin label CAT-16. Open symbols: 2-ATDNO; closed symbols: 2-ATDBr. In the insert the values of c are given in 10^{-2} mol . l^{-1} units.

the temperature range studied and for the given experimental error (Fig. 2). To compare the efficiency with which the studied compounds perturb the membrane at different membrane depths, we calculated parameter P (see Ondriaš et al. 1983):

$$P = (\Delta S/\Delta c) : (\Delta S/\Delta T)_0 \qquad (8)$$

Table 1. Parameters characterizing the perturbation effects of 2-ATDBr and 2-ATDNO surfactants on model membranes prepared from *Escherichia coli*-isolated lipids and their antimicrobial effects on *Escherichia coli* cells. The MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) values were taken from the literature (Leitmanová et al. 1989).

Spin label	Parameter	Unit	2-ATDBr	2-ATDNO
CAT-16	$\Delta S/\Delta c$	$mol^{-1}.l$	-6.8 ± 0.7	-4.4 ± 0.5
5-DSA	$\Delta S/\Delta c$	$mol^{-1}.l$	-3.8 ± 0.2	-2.1 ± 0.2
12-DSA	$\Delta S/\Delta c$	$mol^{-1}.l$	-4.2 ± 0.3	-2.3 ± 0.2
16-DSA	$\Delta S/\Delta c$	$mol^{-1}.l$	-2.8 ± 0.2	-2.3 ± 0.3
CAT-16	P	$mol^{-1}.l.K$	827 ± 85	535 ± 61
5-DSA	P	$mol^{-1}.l.K$	704 ± 37	389 ± 37
12-DSA	P	$mol^{-1}.l.K$	816 ± 58	447 ± 39
16-DSA	P	$mol^{-1}.l.K$	553 ± 41	471 ± 61
5-DSA	K_p	—	90 ± 10	360 ± 50
5-DSA	IP	$mol^{-1}.l$	0.13 ± 0.02	0.042 ± 0.007
—	MIC^{-1}	$mmol^{-1}.l$	5.6 ± 0.6	3.6 ± 0.5
—	MBC^{-1}	$mmol^{-1}.l$	0.84 ± 0.07	0.025 ± 0.002

where $(\Delta S/\Delta c)$ is the slope of S vs. c dependence at a constant temperature, and $(\Delta S/\Delta T)_0$ is the slope of S vs. temperature dependence at zero concentration of the surfactant. Parameter P expresses the change in temperature ΔT of the control sample required to obtain a change of order parameter ΔS in the sample identical with that resulting from the effect of an unit concentration of 2-ATDNO (or 2-ATDBr) at a constant temperature. Parameter P thus allows to compare the perturbing effect of a given compound with that of temperature.

The values of parameter P for various membrane depths (Table 1) indicate that sample cooling does not compensate for the perturbing effects of the compounds studied, i.e. that the surfactant induced changes in the membrane structure are anisotropic.

Partition coefficients and intrinsic perturbing effects

A comparison of intrinsic perturbing effects of 2-ATDBr and 2-ATDNO requires the comparison of changes in S induced by identical membrane concentrations of the agents. This in turn requires the knowledge of the lipid/water partition coefficients of the compounds studied for the model system employed. The partition coefficient K_p is defined as

$$K_p = (n_l/V_l) : (n_w/V_w) \qquad (9)$$

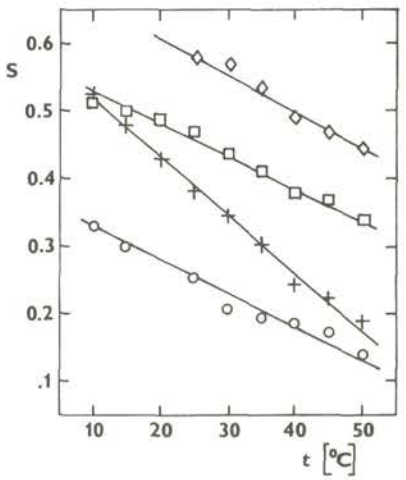

Fig. 2. The relationship of the order parameter S and temperature t in the absence of surfactants. Diamonds: spin label 5-DSA; squares: spin label 12-DSA; crosses: spin label CAT-16; circles: spin label 5-DSA.

where n_l and n_w are the numbers of surfactant molecules in the lipid and water phases, respectively, V_l and V_w are the volumes of lipid and water phases respectively, and for the sake of simplicity, the density of both phases is taken equal to 1 g/ml. Provided that the order parameter S depends linearly on the surfactant concentration in the lipid phase, it is easy to show that the change in S, ΔS, after the addition of water (in volume V_w) containing k surfactant molecules per unit volume, to volume V_l of dry lipid is

$$\Delta S = \alpha k K_p V_w : (K_p V_l + V_w) \tag{10}$$

where α is a constant. ΔS is the change in S:

$$\Delta S = S_0 - S_w \tag{11}$$

where S_0 is the value of S for the control sample without added surfactant, and S_w is the value of S after the addition of volume V_w of water with a constant concentration of surfactant c_w.

Table 1 shows the values of K_p obtained by fitting Eqs. (9) — (11) to experimental data such as those from Fig. 3. The polar part of 2-ATDBr is dissociable, while that of 2-ATDNO is not, i.e. in system containing water 2-ATDBr is more polar than 2-ATDNO. Hence, it is not surprising to obtain higher value of K_p for 2-ATDNO.

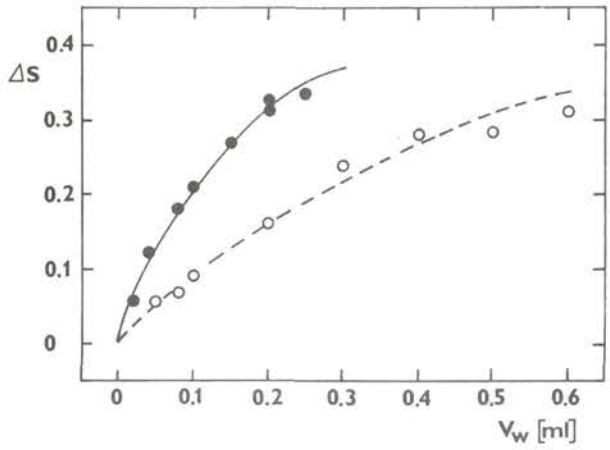

Fig. 3. Change in the order parameter ΔS of the 5-DSA spin label in relation to the amount of water V_w (containing a constant concentration $c_w = 50$ mmol . l^{-1} of surfactants) added to 2 mg of dry *Escherichia coli* lipids. Open symbols: 2-ATDNO; closed symbols: 2-ATDBr.

The values of partition coefficient K_p can now be used to calculate the membrane surfactant concentration, c_m, at a given sample concentration, c:

$$c_m = K_p \,.\, c[1 + V_l/V_w] : [1 + K_p \,.\, V_l/V_w] \tag{12}$$

Since the value of K_p for 2-ATDNO is higher than that for 2-ATDBr, the decrease of the order parameter S in the presence of 2-ATDBr is even more pronounced than that in the presence of the same membrane concentrations of 2-ATDNO (cf. Figs. 1*B* and 4). To quantitatively characterize the intrinsic perturbing effects, parameter IP (= intrinsic perturbation), was introduced, expressing the change in order parameter at unit surfactant concentration in the membrane:

$$\mathrm{IP} = \lim_{V_w \to \infty} \Delta S / K_p c_w \tag{13}$$

where c_w is the (constant) surfactant concentration in the added water volume V_w (see Ondriaš et al. 1983). The values of experimentally obtained IP (Fig. 3) clearly show that 2-ATDBr is more efficient in inducing membrane perturbations than 2-ATDNO both at identical sample concentrations, and at the same membrane concentrations (see Table 1).

Trans-gauche isomerization of phospholipid hydrocarbon chains

The decrease of the order parameter S of *m*-DSA spin labels can be due to an

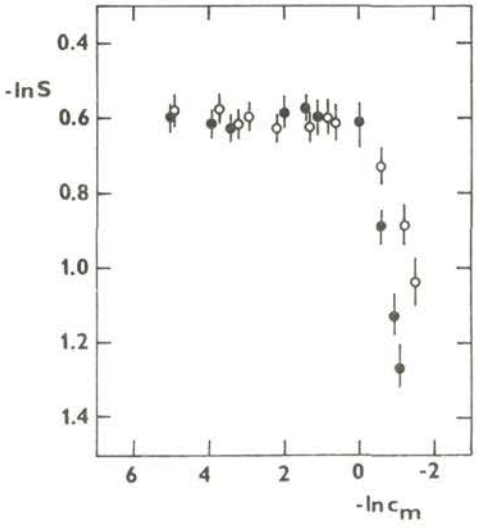

Fig. 4. The relationship of the order parameter S of the 12-DSA spin label and surfactant concentration c_m (mol . l^{-1}) in the membrane. Open symbols: 2-ATDNO; closed symbols: 2-ATDBr.

increased concentration of gauche conformations in the lipid hydrocarbon chains, but also to changes in the motion of the long axes of the lipid molecules as whole. According to Seelig (1970; 1971), the order parameter for rotation around a single C-C bond S_σ can be obtained from value S of *m*-DSA spin label:

$$S = S_\sigma^n . S_o \tag{14}$$

where n is the number of C-C bonds between the doxyl and carboxyl groups of *m*-DSA spin label, and S_o the order parameter for the motion of the long axis of the *m*-DSA spin label.

Supposing that the order parameter S_σ is approximately constant between two different *m*-DSA spin labels, that the rotations around adjacent C-C bonds are mutually dependent, and that the combinations of conformations $g^\mp g^\mp$ and $g^\pm g^\pm$ are disfavoured, Marsh (1974) found for the probability of gauche conformations p_g and for the effective energy difference between trans and gauche conformations E_g at absolute temperature T

$$p_g = [1 - (1 + 8\sigma)^{-1/2}] : 2 \tag{15}$$

$$1 - S_\sigma^2 = 9\sigma : [1 + 8\sigma + (1 + 8\sigma)^{1/2}] \tag{16}$$

$$\sigma = \exp(-E_g/RT) \tag{17}$$

where R is the molar gas constant. Thus the values of S obtained for different

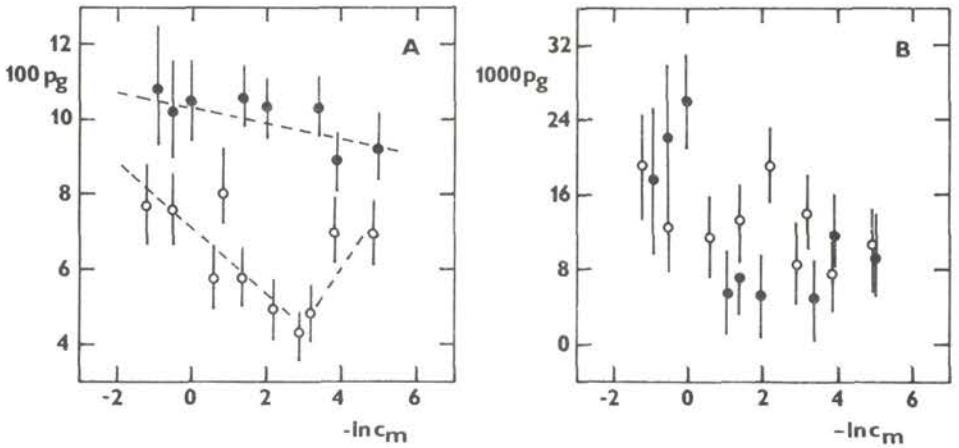

Fig. 5. The relationship of the probability of gauche conformations p_g in the lipid hydrocarbon chains and the membrane surfactant concentration c_m (mol . l^{-1}). *A*: calculated from the order parameters S of 12-DSA and 16-DSA spin labels; *B*: calculated from the order parameters S of 5-DSA and 12-DSAspin labels. Open symbols: 2-ATDNO; closed symbols: 2-ATDBr. The lines were drawn by eye to indicate trends of changes.

m-DSA spin labels can be used to calculate p_g and E_g as a function of 2-ATDBr or 2-ATDNO membrane concentration c_m.

The probability of gauche conformations p_g calculated from S values of 16-DSA and 12-DSA spin labels increases with the increasing membrane concentration c_m of 2-ATDBr, while in the presence of 2-ATDNO p_g initially decreases (up to $c_m = 49$ mmol . l^{-1}) and then increases with the increasing membrane concentration of 2-ATDNO (see Fig. 5*A*). A similar concentration dependence was observed for the effective energy difference between trans and gauche conformations calculated from S data of 16-DSA and 12-DSA spin labels (Fig. 6*A*). The value of E_g initially increases with the increasing 2-ATDNO concentration (up to $c_m = 49$ mmol . l^{-1}), and then decreases, while the values of E_g in samples with 2-ATDBr gradually decrease with the increasing 2-ATDBr concentration. The values of p_g were higher and the values of E_g were lower in the presence of 2-ATDBr, than those observed in the presence of 2-ATDNO, within the entire range of concentrations studied.

The dependences of p_g and E_g on c_m as calculated from S values of 12-DSA and 5-DSA spin labels were quite different. The values of p_g were substantially smaller (and those of E_g larger) than those reported above: this indicates that the ordering of the hydrocarbon chains is higher near the polar region than near the lipid bilayer centre (compare Figs. 5, *A* and *B*, and Figs. 6, *A* and *B*).

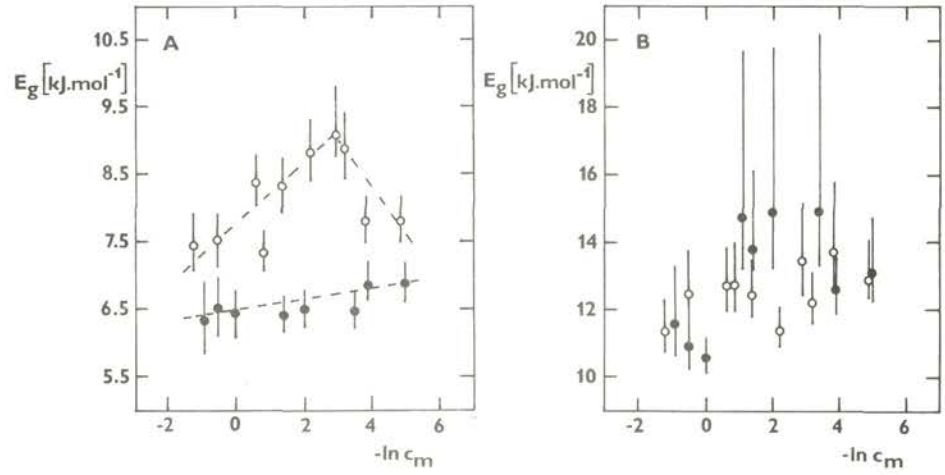

Fig. 6. The relationship of the effective energy difference between trans and gauche conformations E_g in the lipid hydrocarbon chains and the membrane surfactant concentration c_m (mol . l^{-1}). *A*: calculated from the order parameters *S* of 12-DSA and 16-DSA spin labels; *B*: calculated from the order parameters *S* of 5-DSA and 12-DSA spin labels. Open symbols: 2-ATDNO; closed symbols: 2-ATDBr. The lines were drawn by eye to indicate trends of changes.

Furthermore, the value of p_g increases and that of E_g decreases with increasing c_m concentration for both surfactants studied. Within the experimental error, there is no difference in p_g (and E_g) values between the samples containing 2-ATDNO or 2-ATDBr at the same membrane concentration c_m.

Correlation time

The correlation times τ_B and τ_C are measures of rotational motion rates of the spin label in membranes. Only for the 16-DSA label, $\tau_C = \tau_B$. Differences in values of τ_C and τ_B may be due to motion anisotropy, to effects of molecular ordering, and to motion in the slow regime ($\tau \geq 3 . 10^{-9}$ s). In the slow regime, Eqs. (4) — (7) for the calculation of correlation times are no longer valid (Schreier et al. 1978; Berliner 1982; Hemminga 1983). Therefore, the only experimental values τ which can be used for an exact evaluation of the effects of 2-ATDNO and 2-ATDBr on the membrane dynamics are those obtained with 16-DSA and 12-DSA labels (Fig. 7).

It can be seen that at low membrane concentrations c_m of 2-ATDNO, the value of τ for the 16-DSA label increases with the increasing c_m reaching a maximum at $c_m = 49$ mmol . l^{-1}. Thereafter, the value of τ decreases with the increasing c_m. With 2-ATDBr, only a decrease in τ with the increasing c_m was

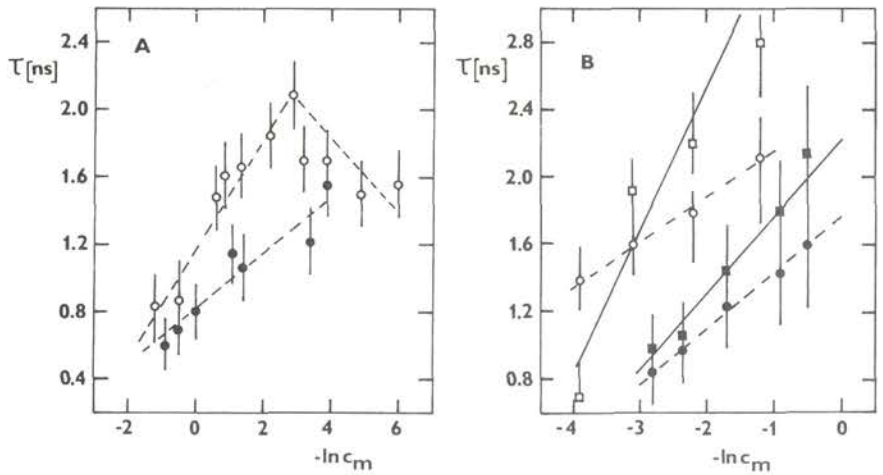

Fig. 7. The relationship of the correlation time τ of the 16-DSA and 12-DSA spin labels and the membrane surfactant concentration c_m (mol.l^{-1}). Open symbols: 2-ATDNO; closed symbols: 2-ATDBr. *A*: spin label 16-DSA, $\tau_B = \tau_C$. *B*: spin label 12-DSA, circles and dotted lines: τ_C; squares and full lines: τ_B. The lines were drawn by eye to indicate trends of changes.

observed. The rotational motion of the 16-DSA spin label is thus affected by 2-ATDNO and 2-ATDBr probably via mechanism(s) such as trans-gauche isomerization of hydrocarbon acyl chains near the lipid bilayer centre (compare Figs. 5*A*, 6*A*, and 7*A*). The motion of the 12-DSA spin label was sufficiently fast only at the highest concentrations of the surfactants. As clearly seen from Fig. 7*B*, these membrane concentrations of both surfactants studied introduce a decrease of τ for the 12-DSA label.

Finally, except at lowest c_m concentrations values of τ for 16-DSA and 12-DSA were lower in samples containing 2-ATDBr as compared to those containing the same amount of 2-ATDNO in the membrane. This can be interpreted using the concept of membrane microviscosity. If the paramagnetic fragment of a *m*-DSA spin label is approximated by a rigid sphere of radius a rotating in a medium with viscosity η, then the Stokes-Einstein relationship yields

$$\tau = (6D)^{-1} \tag{18}$$

$$D = kT/8\pi a^3\eta \tag{19}$$

where D is the isotropic rotational diffusion coefficient, k is the Boltzmann constant, and T is the absolute temperature (Hemminga 1983). Thus Fig. 7 indicates that the microviscosity of the membrane hydrophobic region of the

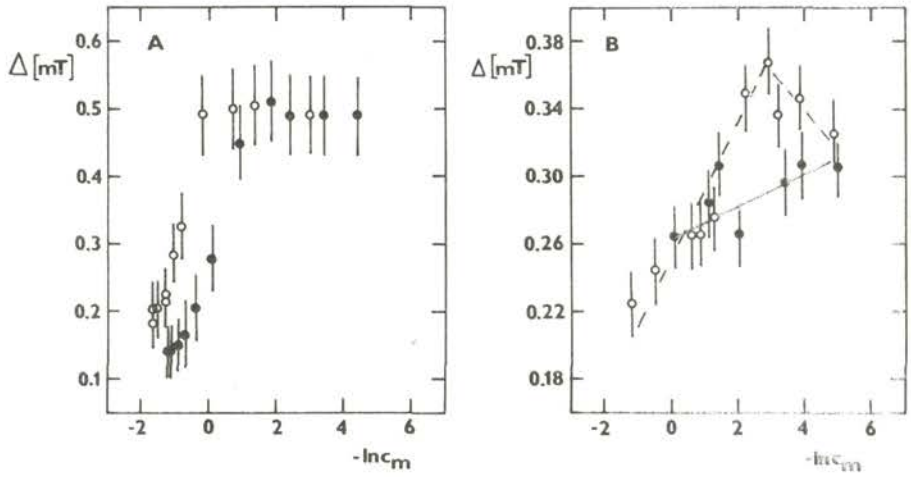

Fig. 8. The relationship of the half-width at half-height of the low field ESR hyperfine extremum Δ and the membrane surfactant concentration c_m (mol . l^{-1}). *A*: CAT-16 spin label; *B*: 5-DSA spin label. Open symbols: 2-ATDNO; closed symbols: 2-ATDBr. The lines were drawn by eye to indicate trends of changes.

membrane at the levels of carbon 16 and 12 is lower in the presence of 2-ATDBr than of 2-ATDNO.

To characterize membrane dynamics in the polar region (spin label CAT-16) or in the hydrophobic region at the level of carbon 5 (spin label 5-DSA) where the spin labels move in slow regime ($\tau \geq 3 . 10^{-9}$ s), the half-width at half-height of the low field ESR hyperfine extremum Δ was employed. Mason and Freed (1974) and Mason et al. (1977) have shown that this index of spin label motion is sensitive to correlation times as long as $\tau \leq 3 . 10^{-6}$ s, i.e. also in the slow motion regime ($3 . 10^{-9}$ s $\leq \tau \leq 3 . 10^{-6}$ s) where the use of Eqs. (4) — (7) is inappropriate. Fig. 8*A* clearly illustrates that the values of Δ decrease (i.e. spin label mobility increases) with the increase in concentration c_m also in the polar region of membrane. At identical membrane concentrations 2-ATDBr is again more efficient than 2-ATDNO in increasing the spin label mobility. For 5-DSA the value of Δ initially increases with increasing concentration c_m of 2-ATDNO, reaches a maximum at approx. $c_m = 49$ mmol . l^{-1} and decreases thereafter (see Fig. 8*B*).

Although the experimental data for samples containing 2-ATDBr are burdened with a significant experimental error it seems that Δ decreases with large concentration of 2-ATDBr and that the values of Δ are lower than those

measured with identical concentrations of 2-ATDNO. The relationship of the motional parameters on the surfactant concentration c_m thus seems similar for both 5-DSA and 16-DSA.

Discussion

The aim of our work was to study how antimicrobials (such as organic quaternary ammonium salts and amine oxides) influence the stucture and dynamics of model phospholipid membranes. Surfactants 2-ATDBr and 2-ATDNO were selected for this purpose as they show strong inhibitory and bactericidal activities on *Escherichia coli* cells in antimicrobial activity tests (Leitmanová et al. 1989, Table 1). ESR spectroscopy of spin labels was employed. The ESR spectra measured in all samples (except those for the 16-DSA label which showed a sufficiently fast motion to be treated as nearly isotropic) were of the anisotropic axially symmetric powder pattern type.

Since the used spin labels have also surface-active properties, distribution of the labels in the systems studied might be quite intricate. The labels can be located in various environments: in the aqueous phase as isolated molecules or aggregated into micelles, in surfactant micelles, or in membrane lipids. The observed changes in the physical parameters of spin labels might thus simply indicate redistribution of labels between these environments. Hence, it must be proved that the observed changes relate to membrane properties.

The motion of isolated label molecules in the aqueous phase is isotropic. The presence of labels both in the membrane and in the aqueous phase would result in the superposition of two ESR signals, one anisotropic ("membrane signal") and the other one isotropic ("aqueous signal"), due to the slow exchange rate of the used labels between membrane lipids and the aqueous phase as compared to the ESR time scale ($\nu_{ex} \leq 10^8\ s^{-1}$). A similar superposition has been observed in model phospholipid membranes at high *m*-DSA: phospholipid molar ratios (Moules et al. 1982) and also in phospholipid membranes where CAT-type spin labels with short alkyl substituents were used (Eriksson and Westman 1981). A superposition of "membrane" and "aqueous" signals is easily recognizable and it was not observed in our systems. Micelle formation by spin labels gives rise to isotropic spectra broadened by dipolar and exchange interaction between the paramagnetic nitroxyl groups (Sackmann 1983). Consequently, if micelle formation by spin labels had occurred in our experiments, the ESR spectra would have been expected to be single very broad lines. Therefore we may conclude that the labels in our experiments were not located in the aqueous phase either as isolated molecules or as micelles. Spin labels located in micelles of the surfactants studied display seemingly isotropic spectra in the fast

motion limit, most probably because of a fast exchange between the micelle interior and the aqueous phase (Šeršeň et al. 1989). If in our experiments the labels were localized in surfactant micelles, the ESR spectra would have consisted either of an isotropic pattern or of a superposition of an isotropic and a "membrane"-type spectrum. It is true that the 16-DSA label displays isotropic spectra so that it could be located in the surfactant micelles. However the 5-DSA label gave clearly "membrane" type spectra so that it is improbable that one positional isomer of stearic acid (5-DSA) is localized in membrane lipids whereas the other one (16-DSA) in the surfactant micelles. Hence it can be suggested that the "isotropic" spectra of the 16-DSA label are in fact "membrane" spectra motionally averaged due to the well known flexibility gradient in the hydrophobic core of the membranes (Seelig 1971; Ivkov and Berestovskij 1982; Houslay and Stanley 1982; Sackmann 1983). Based on the above it can be concluded that the used spin labels were localized in the membranes and that they indeed do report on membrane properties.

It is possible that the spin labels tested form complexes with the studied surfactants rather than with membrane lipids. Owing to this both positively (CAT-16) and negatively (*m*-DSA) charged labels were tested. The CAT-16 label cannot form complexes with molecules of 2-ATDBr because of electrostatic repulsion but the negatively charged *m*-DSA labels can. If strong complexes had formed in our systems (separated from the membrane phospholipid phase), the ESR spectra of *m*-DSA labels would have consisted of two overlapping "membrane" signals, one increasing with increasing 2-ATDBr concentration; the ESR spectra of CAT-16 labels would have displayed just one "membrane" signal at any 2-ATDBr concentration. As only one "membrane"-type spectrum was observed in each sample at all 2-ATDBr concentrations studied with both types of spin labels (CAT-16 and *m*-DSA), it can be concluded that no strong surfactant-label complexes were formed in the membrane and the obtained ESR spectra report on the physical properties of different label binding sites within the membrane between which the label exchange is fast on the ESR time scale due to fast lateral diffusion. Thus the observed and calculated physical parameters are values statistically averaged over different membrane sites within the characteristic time interval of ESR spectroscopy.

The statistical weight of the sites where the label interacts with the surfactant molecules is expected to increase with the increasing concentration of the surfactant. Does this fact have any impact on our conclusions? With the 5-DSA label the partition coefficient, K_p, was higher for 2-ATDNO than for 2-ATDBr. The calculation of K_p involves relative changes in the order parameter S due to changes in volume of the aqueous phase V_w (see Eqs. (9) — (11)). Therefore, the calculated values of K_p cannot be influenced by differences in interactions of the 5-DSA with the studied surfactants. The second principal finding is that both

surfactants studied increase the "fluidity" characterized by different physical parameters. Primary data obtained experimentally are S, τ and Δ. All the measured parameters indicate that 2-ATDBr induces of a more significant membrane perturbation than does 2-ATDNO (despite of a lower partition coefficient K_p). This was observed with two different types of spin labels: CAT-16 which cannot form complexes with 2-ATDBr and *m*-DSA which can. Hence, the principal conclusion concerning 2-ATDBr being a more potent membrane perturbant than 2-ATDNO is valid irrespective of the various label-surfactant interactions. This corresponds also to the inhibitory (MIC) and bactericidal (MBC) activities of both compounds.

We shall now discuss molecular details of membrane perturbations, and will use mainly data obtained with the *m*-DSA labels. The main finding is that p_g, E_g, τ, and Δ show extrema at critical membrane concentrations of 2-ATDNO, while no extrema were seen using 2-ATDBr surfactant. Obviously the absolute values of p_g, E_g, τ and Δ rather than trends could be influenced by the differences in interactions of *m*-DSA labels with 2-ATDBr and/or 2-ATDNO.

We can restrict our discussion to variable p_g (Fig. 5*A*) since discussion concerning other parameters would proceed the same line of reasoning. Let us suppose that the interaction od *m*-DSA labels with 2-ATDBr molecules is stronger than that with 2-ATDNO molecules due to a significant electrostatic contribution of dissociated polar groups of *m*-DSA and 2-ATDBr. In this case *m*-DSA labels would sample the 2-ATDBr neighbourhood more frequently than that of 2-ATDNO. Both surfactants influence the value of p_g. Since the statistical weight of the sites near 2-ATDBr is higher, scaling the data for the same statistical weight as have the sites near 2-ATDNO would decrease the value of p_g for samples with 2-ATDBr. Since the energy of the label-surfactant interaction in the membrane is unknown, the scaling factor cannot be calculated. Consequently, the following discussion will be restricted to qualitative features.

It is well known that surface active organic quaternary ammonium salts influence the gel-liquid crystal phase transition (Arnold et al. 1976; Eliasz et al. 1976; Frischleder and Gleichmann 1977; Grupe et al. 1978a; Sarapuk et al. 1985) and lateral packing (Cirák et al. 1988) in model membranes prepared from synthetic phosphatidylcholines; increase the ionic permeability of black bimolecular membranes, and decrease their stability (Ter Minassian-Saraga and Wietzerbin 1970; Antonov et al. 1976; Grupe et al. 1978b; Sarapuk et al. 1984). These compounds increase ionic efflux as well as efflux of organic matter from lipid vesicles (Grupe et al. 1978b; Sunamoto et al. 1983, 1984), change the surface charge of lipid liposomes (Grupe et al. 1978c; Rydhag and Gabrán 1982; Przestalski et al. 1983; Requena and Haydon 1985) thus influencing the equilibrium distance of lipid bilayers (Hauser 1984; Matsumura et al. 1986), affect the conformation of the polar part (Balgavý et al. 1984; De Haan et al. 1984; Bayer

1986) as well as that of acyl chains (Sarapuk et al. 1985; Cirák et al. 1988; Gallová et al. 1989) in phosphatidylcholine bilayers and, at higher concentrations, destabilize the lipid bilayer inducing the formation of non-bilayer phases in membranes (De Smedt et al. 1976; Rydhag et al. 1982; Balgavý et al. 1984; De Haan et al. 1984). It is possible that all these effects may have a common underlying phenomenon, namely anisotropic perturbations of the membrane structure following the incorporation of the surfactant into the lipid part of the membrane.

The values of parameter P determined in various membrane depths indicate that changes in the membrane structure are anisotropic. In our previous paper (Ondriaš et al. 1984) values of P were normalized for two different local anesthetics to obtain identical numerical values of P for 5-DSA spin label for both anesthetics; then the ratios of normalized P values in different membrane depths were used to estimate the extent of membrane disordering in terms of both its depth and the structure of the anesthetic. Repeating the same for the surfactants studied in the present paper we obtained $P_P:P_5:P_{12}:P_{16} = = 1.18:1.00:1.16:0.79$ for 2-ATDBr and $P_P:P_5:P_{12}:P_{16} = 1.38:1.00:1.15:1.21$ for 2-ATDNO, where P_P, P_5, P_{12}, and P_{16} are the normalized values of parameter P for CAT-16, 5-DSA, 12-DSA, and 16-DSA, respectively. It is obvious from the above that 2-ATDNO, despite of its lower absolute disordering efficiency, perturbes the polar region and the centre of the hydrocarbon core of the membrane (in comparison to the depths of carbons 5 and 12) relatively more than does 2-ATDBr. However it is difficult to comment further on this result, because parameters P were calculated from values of S which include two effects: conformational changes and changes in the motion of the long axes of lipid molecules as whole. Molecular insight into the conformational changes could provide the calculated values of P_g and E_g.

Let us first discuss the results obtained for 2-ATDBr. It is obvious from Figs. 5 and 6 that the probability of gauche conformations in the lipid hydrocarbon chains increases and the effective energy difference between the trans and gauche conformations E_g decreases with increasing concentration of 2-ATDBr. These effects can be explained by a model of the interaction of 2-ATDBr with phospholipids in a lipid bilayer (Fig. 9). Since membrane lipids of *Escherichia coli* contain mainly phosphatidylethanolamine (80%) with a hydrocarbon chain lenght of 16—18 carbons (Lugtenberg and Peters 1976; Jain and Wagner 1980) the structure of 1,2-dipalmitoylphosphatidylethanolamine was chosen for the sake of simplicity (Fig. 9). Quaternary alkylammonium salts charge lipid membranes positively (Requena and Haydon 1985) so that they interact with membrane lipids in dissociated form. Because of a high dissociation of the ammonium group, the positive charge on the 2-ATDBr nitrogen is quite high. Consequently the electrostatic attraction between the 2-ATDBr nitrogen and the

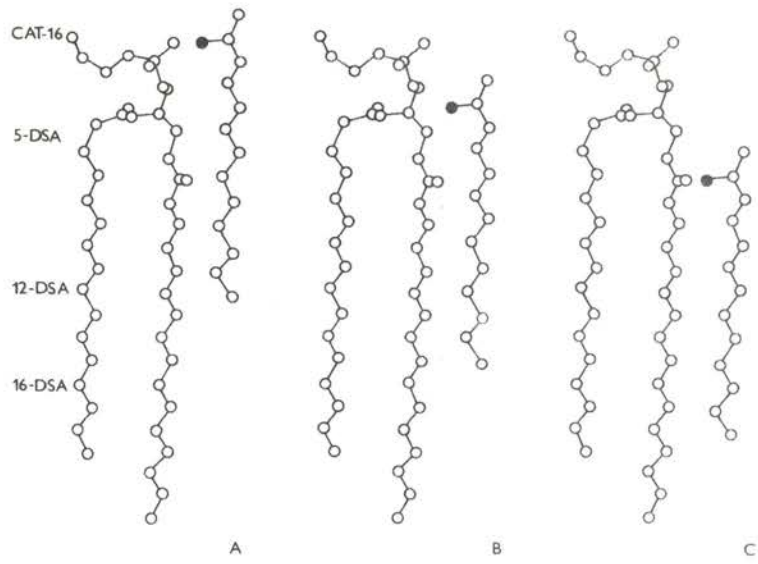

Fig. 9. Proposed localizations of 2-ATDBr (A) and 2-ATDNO (B, C) in the phosphatidylethanolamine bilayer. CAT-16 and 5-DSA, 12-DSA, and 16-DSA denote localizations of paramagnetic group of the spin label in the bilayer as suggested by Castle and Hubbell (1976) and Barratt and Laggner (1974), respectively. The structure of the phosphatidylethanolamine molecule in the bilayer as found by Hitchcock et al. (1975). The full circles denote ammonium and amine oxide groups of 2-ATDBr and 2-ATDNO, respectively.

negatively charged phosphate group of the phospholipid will result in a very high polar interaction. The most probable localization of the positively charged ammonium group of the 2-ATDBr ion thus is at the level of the phospholipid phosphate group. The 1-methyldodecyl chain of 2-ATDBr extends into hydrophobic region of the bilayer parallel to phospholipid hydrocarbon chains ending between the paramagnetic fragments of spin labels 12-DSA and 16-DSA. Since this localization pushes the neighbouring phospholipids apart (lateral expansion of the membrane), their hydrocarbon chains below the end of the 1-methyldodecyl chain of 2-ATDBr have more space and less motional constraint. This should give a higher value of p_g and a lower value of E_g for the hydrocarbon region between labels 12-DSA and 16-DSA and an increased rate of the rotational motion of the 16-DSA spin label. The changes in the region between 5-DSA and 12-DSA are expected to be rather small. Our results are consistent with this hypothesis (see Figs. 5—8). It is important to note that the lateral expansion of the membrane and localization of the ammonium group of

Fig. 10. Interaction of the 2-ATDNO polar head group fragment with the ester group of the phospholipid.

2-ATDBr near the phospholipid phosphate group also results in a less motional constraint in the polar region above the ammonium group of 2-ATDBr. The decrease of the order parameter S of CAT-16 (Fig. 1*D*) and of its motion parameter Δ (Fig. 8*A*) with the increasing concentration of 2-ATDBr is thus consistent with the supposed changes also in the polar region of the membrane.

The decrease of p_g (and increase of E_g) at low membrane concentrations of 2-ATDNO followed by an increase in p_g (and decrease of E_g) at higher membrane concentrations observed for the membrane hydrocarbon region between spin labels 12-DSA and 16-DSA suggest that the predominant localizations of 2-ATDNO in the membrane are different at low and high membrane concentrations. At high concentrations, where p_g increases and E_g decreases, the localization of 2-ATDNO could be the same as that of 2-ATDBr, with the polar group being at the level of the phospholipid phosphate group and with the terminal methyl group of 1-methyldodecyl chain ending between the paramagnetic fragments of 12-DSA and 16-DSA. The consequences of this localization are the same as in the case of 2-ATDBr and as observed experimentally at high 2-ATDNO concentrations. However, 2-ATDNO bears a non-dissociable polar group and the positive charge on its nitrogen is rather small and non-expressive in comparison to 2-ATDBr. Therefore, hydrophobic forces can play a more significant role in the interactions of 2-ATDNO with phospholipids. This is also seen from the increased value of the partition coefficient K_p as compared to that of 2-ATDBr (Table 1). The 2-ATDNO chain can penetrate more deeply into the hydrocarbon core of the bilayer. From the structures of 2-ATDNO and the phospholipid polar parts, and because of the relatively high electron density localized on the phospholipid carbonyl group we infer an interaction between polar parts by a mechanism as illustrated in Fig. 10 (which locates the 2-ATDNO nitrogen on the level of phospholipid carbonyl groups). With this location the terminal group of the 1-methyldodecyl chain of 2-ATDNO is localized below the doxyl group of 16-DSA spin label (see Fig. 9). We postulate that this site is the second type of the binding site of 2-ATDNO in the membrane which is occupied and saturated at low membrane concentrations of 2-ATDNO.

However from the simple model of lipid bilayer (Fig. 9) it is not clear why conformation of lipid chains between 16-DSA and 12-DSA labels should significantly change with this localization of 2-ATDNO as the 1-methyldodecyl chain of 2-ATDNO is rather flexible and can adapt to the packing constrains in the bilayer without significantly influencing the conformation of the lipid chains.

However, the lateral packing of the phospholipid in the bilayer is heterogeneous: as suggested by Ivkov and Berestovskij (1982) and Sackmann (1983) the lipid bilayer consists of clusters of densely packed lipids and of hydrophobic defects between these clusters. The defects are characterized by less densely packed and less ordered lipid chains than in clusters. Therefore the defects can be easily filled in by different "impurity" molecules. After a defect has been filled in, the packing of hydrocarbon chains becomes more dense. We can thus suppose that at low concentrations the binding sites of 2-ATDNO are localized in the hydrophobic defects. Our experimental results support this hypothesis: the filling in of the defects leading to the more dense packing are expected to result in an increase of E_g and a decrease of p_g. After reaching some critical values of E_g and p_g, the packing in defects is probably as dense as in clusters and the molecules of 2-ATDNO can interact with lipids both in clusters and defects, though in a different manner. We believe that this "different manner" is the same as proposed for 2-ATDBr, leading to an increase of p_g and a decrease of E_g.

The cluster model explains qualitatively the mode of 2-ATDNO interaction with lipid bilayers and predicts the trends of changes of physical parameters as observed in experiments. However it remains to explain why the mode of interaction of 2-ATDBr with the membrane is different (or not), why 2-ATDNO is localized deeper in the "empty" defects than in the "filled in" defects or in the clusters, and why the defects are saturated at a particular membrane concentration of 2-ATDNO (approx. $c_m = 49\ \text{mmol}.\text{l}^{-1}$). All the above problems are mutually related and satisfactory answers can be found. First of all, the defects are **hydrophobic**, i.e. the preference for binding with the sites in defects should depend on the hydrophobicity of the particular molecule. White et al. (1981, 1986) and King et al. (1985) studied the interactions of hexane (which is practically water insoluble in comparison to 2-ATDNO) with dioleoylphosphatidylcholine bilayers using neutron diffraction and partition coefficient determination. They observed that hexane dissolved in a bilayer has an approximately zero partial molar volume at low membrane concentrations (up to the critical hexane: lipid molar ration od 1 : 3). As the molar ratio passed this critical value the partial molar volume increased, the hydrocarbon thickness of the bilayer decreased, the bilayer expanded laterally, and the free energy of hexane transfer from pure liquid to the bilayer decreased from $1.26\ \text{kJ}.\text{mol}^{-1}$ to $0.84\ \text{kJ}.\text{mol}^{-1}$. Furthermore, at low concentrations hexane is localized largely in an 1 nm wide

zone at the centre of the bilayer. According to Simon et al. (1977) the free energy of hexane transfer to lipid bilayer consists of a large negative enthalpic contribution offset by a large negative entropic contribution. The entropic contribution is high because hexane is relatively more ordered in the bilayer than in pure liquid. As suggested by White et al. (1981, 1986) and King et al. (1985) hexane fills in "voids" in the bilayer, but the ensuing tighter packing causes a compensating decrease in entropy. Let us suppose that hexane is localized in defects between clusters at low concentrations up to a molar ratio of 1 : 3, but goes into the clusters at higher molar ratios. Then we can infer that the defects are localized deeply in the hydrophobic bilayer centre, i.e. they are highly hydrophobic. At higher molar ratios there should follow a decrease in the free energy of transfer because of hexane insertion between tightly packed chains both in defects and clusters: contribution of entropy should be more important. Indeed, a decrease in free energy has been observed. Furthermore, the thickness of the hydrophobic region decreases at high concentrations. This can occur only due to lateral expansion of the membrane and to the formation of gauche conformers. So we are convinced that the mode of interaction of hexane with lipid bilayers is similar to that of 2-ATDNO. Hexane is much more hydrophobic than 2-ATDNO; the contribution of enthalpy in the case of hexane can be expected to be more important than for 2-ATDNO, i.e. defects should accomodate more hexane than 2-ATDNO. This is again observed experimentally: the critical 2-ATDNO concentration in the bilayer ($c_m = 49\ \text{mmol}.\text{l}^{-1}$) corresponds to a 2-ATDNO: lipid molar ratio of about 1 : 14 to 1 : 17. These figures are substantially smaller than 1 : 3 found for hexane. (The same applies for volume ratios at these critical molar ratios.) Now we can answer all the questions posed in the introduction of Discussion. 2-ATDNO may be localized deeper in the defects than in clusters simply because the defects are hydrophobic and localized deeply in the bilayer, near the bilayer centre. The particular critical membrane concentration at which the binding sites in the defects are saturated probably depends on the "hydrophobicity" of the compound or, more correctly, on the balance of enthalpic and entropic contributions. Both are changing with the changing packing in the bilayer. Consequently, the mode of 2-ATDBr interaction with the lipid bilayer could be indentical with that proposed for 2-ATDNO (and hexane), and the differences could be only quantitative rather than qualitative. 2-ATDBr is more hydrophilic than 2-ATDNO (and much more than hexane), so that critical concentrations for 2-ATDBr may be lower than for 2-ATDNO. Therefore, no increase of E_g and decrease of p_g due to the presence of 2-ATDBr was observed in our experiments; the defects are probably not filled in as extensively with 2-ATDBr as with 2-ATDNO, and the observation of expected smaller changes in p_g and E_g was hampered by a relatively large experimental error.

Evidence for the existence of defects and clusters in lipid bilayers has been provided recently by the small angle diffusion scattering of neutrons (Bezzabotnov et al. 1987) as well as by computer experiments using Monte Carlo simulation of two-dimensional microscopic interaction models involving different conformations of the lipid chains (Mouritsen and Zuckermann 1985). We would like to suggest that the mode of interaction as observed for 2-ATDNO and inferred for 2-ATDBr and hexane is a general phenomenon which is expected also for different types od amphiphilic and hydrophobic compounds, including anionics. In fact, it has been observed (but not interpreted using the cluster model of bilayer) for anesthetics and beta-blockers by various investigators (Rosenberg et al. 1975; Neal et al. 1976; Rosenberg 1980; Rosenberg and Alila 1982; Ondriaš et al. 1987).

The actual size, geometry, probability of formation and dynamics of clusters and defects depend on the actual composition of the bilayer, and especially on the presence of proteins. Therefore, the critical membrane concentration of a particular compound, where the dependence of p_g and E_g changes similar to that in our experiments, will be dependent not only on the chemical structure of the compounds, but also on membrane composition. The thickness of the bilayer hydrophobic region increases (decreases) with the decrease (increase) of p_g and the activity of (trans)membrane enzymes critically depends on this thickness (Houslay and Stanley 1982). Therefore, an observation of biphasic effect of non-specifically interacting membrane-active compound on the activity of (trans)membrane enzymes could be an evidence for a similar mode of interaction also with the complex systems such as investigated in our paper. We would like to note that similar effects were observed in vivo using *Escherichia coli* cells (Leitmanová et al. 1989).

Acknowledgement. One of the authors (P. B.) wishes to thank Drs. J. Ostanevich and V. Gordelij of the Joint Institute for Nuclear Research in Dubna (USSR) for the hospitality and stimulating discussions.

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Final version accepted October 27, 1988