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INTERACTION OF SOME CHARGED AMPHIPHILIC DRUGS WITH PHOSPHATIDYLCHOLINE VESICLES. A SPIN LABEL STUDY OF SURFACE POTENTIAL EFFECTS

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Adsorptions of amphiphilic drugs (propranolol, alprenolol, metoprolol and tetracaine) to phosphatidylcholine vesicles in media of different pH and NaCl concentrations have been studied. A positively charged spin label amphiphile, N,N-dimethyl-N-nonyl-N-tempoylammoniumbromide, was used to follow the variation in the surface potential by ESR. Competition experiments between the probe molecule and the drugs were carried out. A spin-labeled analogue of propranolol was also employed. We have analysed the results in terms of the theory for the diffuse double layer (Gouy-Chapman) and treated various equilibrium models. A weak, specific adsorption of chloride ions was introduced. For the charged forms of the drugs simulations of the experiments by numerical solution of the system of equations in a satisfactory way furnished intrinsic binding constants, independent of surface potential effects. The common electrostatic surface potential is mainly ruling the competition, and not the number of surface vacancies.

1. Introduction

Several biological processes which are sensitive to drugs take place in membrane systems. Certain drugs are known or supposed to interact specifically with receptors whereas others may instead operate via altered physical properties of the membraneous environment. Much work has been devoted to attempts to understand the action of local anesthetics in nervous systems but many questions still remain to be answered. The catabolism of various drugs involves enzymes in the endoplasmic reticulum of hepatocytes. The amphiphilic character of the drugs is of importance for their adsorption to the membrane surface, and hence also for the kinetics of their metabolism by the membrane bound enzymes. In case of drugs, which are charged at physiological pH like some local anesthetics, adrenergic receptor blockers, and antidepressants, the molecules adsorbed at the membrane surface should affect the electrical potential. The effect of the surface potential has been considered by McLaughlin [1,2] and Lee [3,4] in model studies with phospholipids and drugs. McLaughlin and Harary [5] have tested the applicability of the so-called Stern equation to the adsorption of the amphiphilic ion 2,6-toluidinylnaphthalene sulfonate. They found this equation to well account for the electrostatic phenomena. Recently the importance of the surface potential for the muscular end plate response to drugs has been discussed [6].

We have previously analysed the binding of di- and trivalent metal ions as well as monovalent anions to zwitterionic phosphatidylcholine vesicles in terms of the theory for the diffuse double layer [7,8]. This work is now extended to some ionic drugs like β-adrenergic receptor blockers and a local anesthetic (fig. 1) in order to test whether the theoretical treatment we used earlier will properly apply to the present system.

There are several ways in which the adsorption of a drug may be monitored, directly or indirectly. In this study we are primarily interested in the charged forms of the drugs. We like to follow drug interaction with vesicles by observing the variation in the electrostatic properties. By measuring the ζ-potential for a particle the surface potential may be estimated [2]. Fluorescence spectroscopy offers high sensitivity but may be

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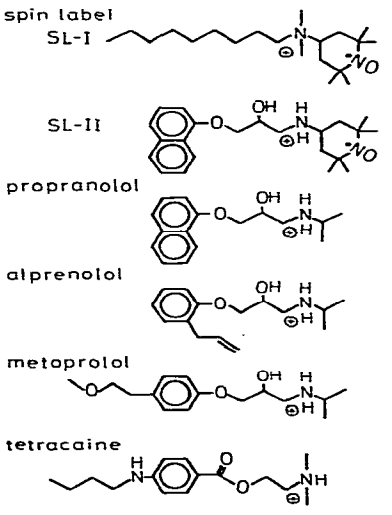

Fig. 1. Formula for the cationic amphiphilic molecules studied.

complicated by inherent artefacts if the quantum yield is influenced in an unpredictable way. Amphiphilic spin labels carrying a net charge can probe the variation in the surface potential [9–11] as observed by electron spin resonance (ESR) spectroscopy. Such a spin label is partitioning between the aqueous and surface phases. We are now observing how the equilibrium for the probe is influenced by the presence of amphiphilic drugs. The amphiphilic character of the spin label may be adjusted to match those of the drugs studied. The spin label molecule mainly employed here was introduced by Castle and Hubbell [9] and has the advantage of being permanently (+) charged and with a convenient hydrophobicity (fig. 1, SL-1). We have also studied the binding of a spin-labeled analogue of propranolol (fig. 1, SL-II) which has been used for receptor studies [12,13]. The application of the spin label technique was examined and the experimental results analyzed in the light of the theory for the diffuse double layer (Gouy-Chapman).

2. Experimental

2.1. Materials

Egg yolk phosphatidylcholine (Grade I) was purchased from Lipid Products (South Nutfield, Surrey, U.K.) and used without further purification. Tetracaine and dl-propranolol were from Sigma Chemical Company (St. Louis, USA). Alprenolol and metoprolol were gifts from AB Hässle (Mölndal, Sweden).

The SL-I amphiphile, N,N-dimethyl-N-nonyl-N-tempoylammoniumbromide (fig. 1) was synthesized essentially according to Castle and Hubbell [9,10]. However, after the first step the by-product of 4-dimethylamino-1,2,2,6,6-pentamethylpiperidine (white crystals; b.p. 83–84°C/3 mm Hg) was removed by distillation in a spinning band column. The spin-labeled analogue of propranolol [12,13], 4-[[2-hydroxy-3-(1-naphthaleneyloxyl)propyl]amino]-2,2,6,6-tetramethylpiperidinyloxyl (fig. 1, SL-II) was purchased from Molecular Probes Inc. (Plano, USA; also SL-I is now available from this source).

2.2. Methods

Vesicles were prepared by sonication during about 1 h of the lipid dispersion under nitrogen with a Heat System Model 350 A Sonifier using a microtip at a low output control (setting 4) and 50% duty cycle. The lipid concentration (about 60 mM) was determined by phosphorus analysis [14]. About 65% of the lecithin molecules were located at the outer bilayer surface as determined by ^{31}P NMR for the average vesicle size [8]. By having 10 mM acetate buffer present the pH value of the samples was generally kept at about 4.7. Aqueous solutions (0.5 M) were prepared from the drugs (fig. 1) by adjusting the pH to about 4 by hydrochloric acid and gentle heating.

ESR measurements were carried out on Varian E-9 and V-4502 spectrometers (extensively reconstructed at this institute) with 4–10 mW microwave power and 0.5 mT field modulation (100 kHz). Digital spectra handling was performed with a Varian 620-i computer. Samples were prepared by evaporating 10 μl of a 2 mM ethanolic stock solution of SL. 100 μl of the vesicle solution was added and vortexed 15 s. Capillaries of 1 mm i.d. were employed. ESR measurement was then immediately started at 25.0 ± 0.5°C. ^{31}P NMR

spectra were recorded as described before [7].

Appropriate equations were solved on a DEC 10 computer. An approximate solution was first obtained and was then used as the input parameters for the full solution. This was calculated using a Harwell Subroutine NSO1A (15) which can solve a system of non-linear equations, provided good enough approximate roots are available.

3. Theory

3.1. Equilibria at a surface

In the following we briefly introduce some of the equations we earlier have used [7,8] to describe the phenomena of cation binding at the zwitterionic surface, and which we are going to test for the adsorption of amphiphiles. Partition of the ionic (or neutral) species X between the interface (I) and surface(s) may be treated as a simple Henry's law distribution,

$$K_X^I = [X]_s/[X]_I \quad [1/Å^2] \tag{1}$$

where $[X]_s$ has the dimension mol/Å^2 and $[X]_I$ mol/l. Castle and Hubbell [9] also applied this type of distribution for the spin label SL-I but used volume concentration for the surface phase by introducing a fictitious surface volume.

Eq. (1) accounts for a distribution according to

$$X(I) \rightleftharpoons X(s) .$$

A specific adsorption to phospholipid sites (P)

$$X(s) + P(s) \rightleftharpoons XP(s)$$

may be evaluated by

$$K_{XP}^s = [XP]_s/[X]_s[P]_s \quad [mol/Å^2] . \tag{2}$$

In case of the amphiphiles we assume this type of adsorption to be dominated by a 1:1 complex. By combining eqs. (1) and (2) we get a Langmuir isotherm relation

$$K_{XP} = K_{XP}^s K_X^I = [XP]_s/[X]_I[P]_s . \tag{3}$$

3.2. Electrostatic conditions. Gouy-Chapman theory

We have for a species with charge z the Boltzmann distribution

$$[X]_I = [X]_b \exp(-zF\psi/RT) \tag{4}$$

which relates the bulk (b) and interfacial (I) concentrations through the surface potential ψ. In the theory for the diffuse double layer the surface charge density σ (e/Å^2) is given by [2,7,8]

$$\sigma^2 = \text{const} \sum_i [X]_{i,b} \{\exp(-z_i F\psi/RT) - 1\} , \tag{5}$$

where the const = 1.34×10^{-5} and $RT/F = 25.7$ mV at 25°C. Here the summation, besides X, should also include concentrations of all the other ions present in the bulk phase. For a zwitterionic membrane σ is governed by the ions adsorbed. Hence

$$\sigma = \sum_i N z_i [X]_{i,s} + \sum_j N z_j [XP]_{j,s} + z_0 , \tag{6}$$

where we have separated contributions according to eqs. (1) and (2), respectively. z_0 corresponds to a conceivable initial charge density. The combination of a Langmuir isotherm with the diffuse double layer theory is sometimes referred to as the Stern equation [2,5].

3.3. Concentration conditions

With the eqs. (1)–(6) we are compelled to apply concentrations, instead of activities. For the phospholipids the surface and volume concentrations are connected by

$$S \cdot [P]_{os} = V \cdot [P]_o . \tag{7}$$

Here S is the total surface exposed (Å^2) and V is the total bulk volume. $[P]_{os}$ is the surface concentration of phospholipids. Assuming a molecular area of 70 Å^2 for egg phosphatidylcholine one gets $[P]_{os} = 1/70 \times N$ mol/Å^2. $[P]_o$ is the volume concentration (mol/l) of the lipids exposed, i.e. the outer layer of the vesicle. Ion concentrations in the bulk phase are always corrected for the amount of ions bound. By means of eq. (7) one gets

$$[X]_b = [X]_o - [X]_s[P]_o/[P]_{os} \tag{8}$$

where $[X]_o$ is the total volume concentration of ions added. On the other hand we have not accounted for the displacement of charged amphiphiles out of the diffuse double layer into the bulk phase, which will give a somewhat too low value for $[X]_b$. Based on our results we have calculated that this may lead to an underestimation of $[X]_b$ at a maximum about 3% when there is a high surface potential.

4. Results

4.1. ESR spectra from the spin labels

In order to be able to correctly apply the spin label method in the study of drug adsorption we first study the probe molecules themselves. Hence, in this section we predominantly scrutinize and extend the technique introduced by Castle and Hubbell [9]. The SL-I amphiphile (fig. 1) senses the surface potential on the outer vesicle area since the transverse diffusion of the probe was shown to be slow [9]. The ESR spectrum (fig. 2) in the presence of vesicles has a component from free (f) probe molecules, in the aqueous phase

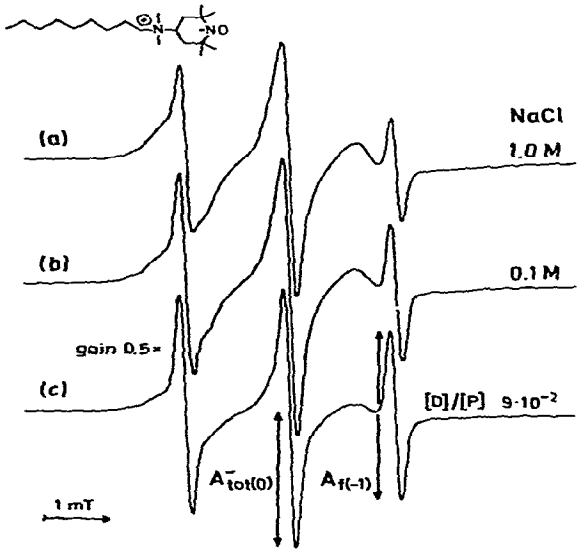

Fig. 2. ESR spectra for the spin label SL-I partitioning between phosphatidylcholine vesicles and aqueous media of different salt concentration, without and with drug present. Spectra from free and bound molecules are overlapping. The number of free spins is related to the amplitude $A_{f(-1)}$, whereas the total number of spins is represented by the amplitude $A^{-}_{tot(0)}$. Concentration of the SL-I was 0.2 mM and for the total lipids about 60 mM. The pH value was kept at 4.7 by 10 mM acetate buffer. a) 1.0 M NaCl; b) 0.1 M NaCl; c) 0.1 M NaCl containing propranolol at a drug to total phosphorus molar ratio of 9×10^{-2}. The gain has been reduced due to the higher concentration of free spin label in the presence of drug. The amplitude ratio $A_{f(-1)}/A^{-}_{tot(0)}$ has increased. Temperature 25°C.

and a broader component from molecules bound to the surface (s). With phosphatidylcholine the sharp lines are present even with high lipid and NaCl concentrations (figs. 2a,b). The linewidths for the free SL-I signal was not influenced by 60 mM phosphatidylcholine present. In order to measure the spectral features of the bound component, different ways to estimate the free signal have been tested. Negatively charged vesicles, containing 8 mol% phosphatidic acid, required low salt concentration in order to minimize the aqueous signal. However, in order to completely quench this component we added $Fe(CN)_6^{3-}$ in about a ten-fold excess of the total SL-I concentration. This may result in a different distribution of the amphiphile due to the altered ionic strength. The remaining background signal, coming from adsorbed molecules, was found to become slightly broadened compared to the original spectrum. In order to obtain a more correct spectrum from bound probe molecules the free component was instead digitally subtracted from the two-component spectrum. The spectrum from SL-I bound to vesicles does not directly demonstrate any preferential orientation of the nitroxide moiety. However, experiments with oriented multilamellar phosphatidylcholine show anisotropic spectra, indicating a bent configuration. The nitroxide x-axis is predominantly deviating from the director (G. Eriksson et al., unpublished work).

A quantitative determination of the probe partitioning can be carried out by integration of the spectral components. This procedure is very demanding and also requires extreme field-frequency control to be able to eliminate the free component completely. Hence, we favour the amplitude ratio procedure evaluated earlier [9]. We followed the ratio $A_{f(-1)}/A^{-}_{tot(0)}$, where $A_{f(-1)}$ is the peak-to-peak amplitude value for the high-field line of the free component, and $A^{-}_{tot(0)}$ is the negative amplitude of the two overlapping centre lines. With no observable g-value shift $A^{-}_{tot(0)} = A^{-}_{f(0)} + A^{-}_{s(0)}$ (fig. 2c). By normalizing the double integrals for the spectra of the free (f) and bound (s) components it was possible to determine the value of $\beta = [A^{-}_{s(0)}/A^{-}_{f(0)}] \times n_f/n_s$, where n is the number of spins. The value of $\beta = 0.16$ for our system is within the range of values reported for SL-I [9].

The fraction of the total number of spin label molecules in the free form can be expressed as

$$n_f/(n_f + n_s) = \beta/(2A^{-}_{tot(0)}/A_{f(0)} + \beta - 1)\,. \qquad (9)$$

For the aqueous spectrum $A^{-}_{f(0)} = A^{-}_{tot(0)} = 0.56\,A_{f(-1)}$. By means of the amplitude ratio procedure we can then estimate the mol fraction from eq. (9).

The ESR spectra for SL-II resembled those for SL-I. However, the signals from the bound components were not identical. The spectral anisotropy with an oriented sample was smaller for SL-II. With SL-II $\beta = 0.12$ was measured.

4.2. Binding studies of spin labels and anions

We first determined the intrinsic binding constant of the SL-I as well as SL-II molecule to phosphatidylcholine vesicles by following the amplitude ratio. The absolute value of this ratio was noticed to be influenced slightly by the reproducibility in the vesicle preparation. Experiments with different electrolyte concentration (NaCl) were carried out. It was observed that the salt concentration slightly shifted the binding curves. The tightest binding occurred at the highest salt concentration (figs. 2a,b). The experiment was done by titrating a dilute SL solution with a more concentrated vesicle solution (fig. 3). Obviously SL-II is binding much tighter than SL-I. In fig. 3 the percentage free molecules are indicated. It is seen that with e.g. 200 times in excess of total lipid molecules about 17% of SL-I is still free, compared to only a few per cent for SL-II.

We also made the reversed experiment, i.e. titrating vesicle solutions (60 mM total lipid) with SL-I in the presence of different salt concentrations (fig. 4). In this case $A_{f(-1)}/A^{-}_{tot(0)}$ increased linearly with the SL-I addition. The charge density is assumed to grow continuously from the original value, extrapolated at zero SL-I concentration. However, the inherent errors, in particular at the low initial probe concentrations, make the absolute value of the intercepts somewhat uncertain. Nevertheless, the intercepts at zero SL concentration responded to the salt concentration, indicating a surface potential difference due to the presence of NaCl already in the absence of amphiphile. The potential difference between the vesicles in 0.1 and 1.0 M NaCl may be estimated from $\Delta\psi = (RT/F)\ln p(0.1)/p(1.0)$, where $p = A^{-}_{f(0)}/A^{-}_{s(0)}$. This should correspond to about 8 mV lower surface potential at the high salt concentration. The cause of the surface charge on the zwitterionic membrane may be contaminations from degradation products. However, the apparent pK value of any traces of fatty acids present should be much higher than 4.7, the pH value in the bulk phase [16]. Moreover, a constant negative surface charge should give intercepts, which respond to the salt concentra-

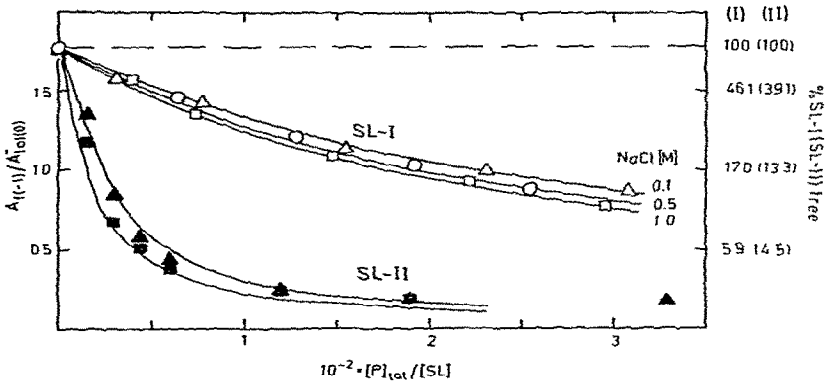

Fig. 3. Titration of the spin labels with phosphatidylcholine vesicles in solutions of different NaCl concentrations. The ESR amplitude ratio $A_{f(-1)}/A^{-}_{tot(0)}$ is followed versus the molar ratio of total phosphorus to spin label. The concentrations of the lipids were about 60 mM. SL-I and SL-II were 0.2 mM. Titrations were done in 0.1 M (△, ▲), 0.5 M (○) and 1.0 M NaCl (□, ■). The full lines are simulated with eqs. (1) and (4)–(8) and the parameters in table 1.

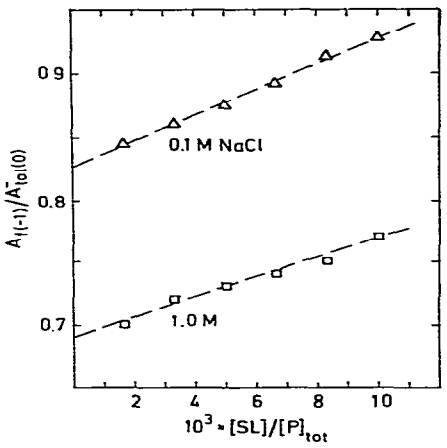

Fig. 4. Titration of phosphatidylcholine vesicles with the spin label SL-I at 0.1 and 1.0 M NaCl. The ESR amplitude ratio $A_{f(-1)}/A_{tot(0)}$ is given versus the molar ratio of spin label to the total phosphorus. The lipid concentration was about 59 mM. pH value adjusted to 4.7.

tion in the reversed order. The same should hold if the activity coefficient for SL-I responded to the salt concentration. In the limit of no surface charge density (fig. 4) the Gouy-Chapman theory alone does not predict any salt effect. We have recently [7,8] discussed the related problem with calcium and lanthanide ion adsorption to phosphatidylcholine, where we introduced a weak, specific anion binding to the membrane by an isotherm. Castle and Hubbell [9] did not consider any anion effect in their experiments. In their experiments with phosphatidylcholine no salt was used.

4.3. Simulations of the binding curves for the spin labels

For simplicity we now assume the anion binding ($X = Cl^-$) to be specific and ruled by eq. (3) with K_{ClP}. Moreover, in all our approaches we anticipate that the anions and the amphiphiles do not compete. As long as the amount of SL molecules added is small $[P]_s \sim [P]_{os}$. It will therefore become difficult to distinguish if the SL molecules ($X = SL$) merely become adsorbed according to eq. (1) or follow an isotherm (eq. (3)). We first tested the Henry's law model for the SL-I adsorption. It is practical to modify eq. (1) by introducing the binding constant $K^*_X = K^1_X/[P]_{os}$ l/mol. Combinations of binding constants for Cl^- (K_{ClP}) and SL(K^*_{SL}), which satisfied the experiments in fig. 3, were estimated by simulations. There is no unique solution and for SL-I we have selected $K^*_{SL} = 150$ l/mol and $K_{ClP} = 0.15$ l/mol as a compromise since these values also satisfy the experiment in fig. 6 (sect. 4.4). It is not possible to get a combination of parameters which also satisfactorily accounted for the slopes of the lines in fig. 4. Problems with simulations of the initial slopes we also met in the experiments with lanthanide ion binding [8]. The values of K_{ClP} reported [8] are of the same order as assumed here. Castle and Hubbell [9] in another type of experiment estimated their binding constant for SL-I to be 500–700 (dimensionless). However, since a hypothetical vesicle volume concentration was introduced by them we have to multiply their values by a conversion factor of 0.42 l/mol, making their constant equal to 200–294, in order to compare with our value. Moreover, a comparison must be done for $K_{ClP} = 0$, where we get $K^*_{SL} = 175$ l/mol for 0.1 M NaCl.

The propranolol analogue spin label SL-II was binding much stronger to vesicles then SL-I. Hence, the experiments in fig. 3 were simulated with $K^*_{SL} = 3000$ l/mol and $K_{ClP} = 0.15$ l/mol, where the binding of SL-II was assumed to follow eq. (1). With no anion effect the binding constant had to be 3625 l/mol at 0.1 M NaCl.

When instead using a Langmuir isotherm (eq. (3)) for SL-I as well as SL-II practically no difference was observed compared to simulations with the Henry's law distribution (not demonstrated in fig. 3). With the type of experiment outlined in fig. 3 it should be difficult to discriminate between the two different models. Although gradually more spin labels become adsorbed upon addition of vesicles there is a concomitant increase in the exposed surface, which will counteract the raise of the surface potential. Since the total spin label concentration remains small no saturation effect is attained.

Once the intrinsic binding constant K^*_{SL} of a charged spin label is known the surface potential may be evaluated if the absolute surface concentration of the probe, $[SL]_s$, can be measured. From eqs. (1), (4) and (8) one gets

$$\psi = \frac{RT}{F} \ln \left\{ K^*_{SL} \left(\frac{[SL]_o}{[SL]_s} [P]_{os} - [P]_o \right) \right\}. \qquad (10)$$

When determining the relative change in the surface potential, $\Delta\psi$, only the corresponding two amplitude ratios have to be known (cf. sect. 4.2).

4.4. Competition experiments with drugs and spin label (SL-I). pH and salt effects

Addition of the charged drugs to vesicles in the presence of SL-I results in an increasing value of $A_{f(-1)}/A^{-}_{tot(0)}$ since a drug adsorption displaces the probe from the surface (cf. figs. 2b and c). In order to be able to apply the amplitude ratio method it is necessary that the line shape of the bound component is not influenced by the presence of drug. This was tested for SL-I by controlling that the β-value remained constant at a drug to phospholipid ratio $[D]/[P]_{tot} = 0.25$ in 0.1 M NaCl for both propranolol and tetracaine (cf. fig. 6). Moreover, propranolol is not influencing the SL-I order with an oriented sample.

Competition between SL and drug (D) molecules at a vesicle surface may be governed by pure electrostatic and/or spatial conditions. However, the packing may also be influenced by the surface charge density. For an amine drug surface potential effects exist for pH-values, at the interface (cf. eq. (4)), below the pK-value of the amine. In fig. 5 vesicles were pH-titrated at a fixed ratio of SL to drug. When raising the pH above the pK-value more SL-I molecules become adsorbed. Without drug present pH variation had no effect on SL-I adsorption, for pH < 10.5. However, with vesicles at pH > 10.5 an enhanced SL-I binding was noticed. The reason for this is not clear, but it seems as the surface potential is becoming negative at extreme pH. Hauser et al. [16] have recently reported that stearic acid in a phosphatidylcholine membrane is fully ionized only at pH > 11. Above this value a negative ζ potential was measured. It is therefore very likely that the SL adsorption reflects traces of fatty acids produced upon sonication [7]. An indication of a gradual alkaline hydrolysis of the phospholipid could not be seen by chromatography. By working at about pH 4.7 any problems with the contamination are normally avoided. For tetracaine we get the apparent pK of about 8.3 and for propranolol 9 to 10. Both values were obtained in the presence of phosphatidylcholine vesicles. We measured (glass electrode) the pH in the bulk phase. By following the pH effect upon the ^{1}H NMR chemical shift of the N-methyl signal of tetracaine in the presence of vesicles a very similar apparent pK value was measured (A. Ehrenberg et al., unpublished work). The pK values for the drugs alone have been reported to be 8.24 [17] and 9.45 [18] for tetracaine and propranolol, respectively.

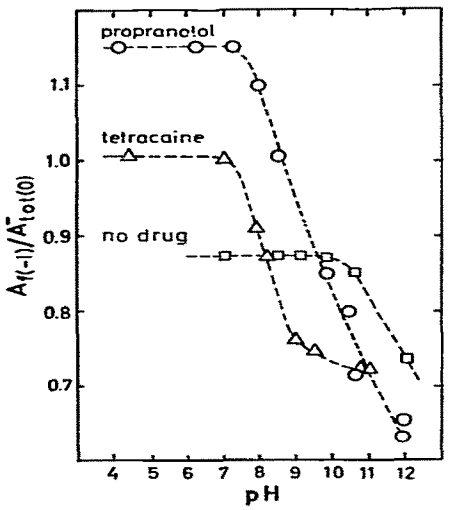

Fig. 5. Distribution of the spin label SL-I as a function of the pH value in solutions with vesicles and drugs. The samples contained either no (□) or 5 mM drug (○, △). 0.1 M NaCl. The SL-I concentration was 0.2 mM. Lipid concentration was 60.5 mM (propranolol) and 64.8 mM (tetracaine).

We observe that the neutral form of the drug is not competing with the SL-I amphiphile. Contrary it seems as the presence of the neutral drug, even at the low concentration used in fig. 5, enhanced the binding of the charged SL-I since the value of the amplitude ratio drops below that measured in the absence of drug. We have no evidence that this effect would be apparent and depend on a gradual change in the spectral shapes, making the amplitude ratio method too primitive. The accessibility of the probe molecules to the membrane is not just influenced by the charge neutralisation but also by other membrane properties. A disordering effect of tetracaine on brain lipids with increasing pH has been reported [19]. This effect should facilitate the intercalation of the probe molecules and may also result in an exposure of the inner surface of the vesicle.

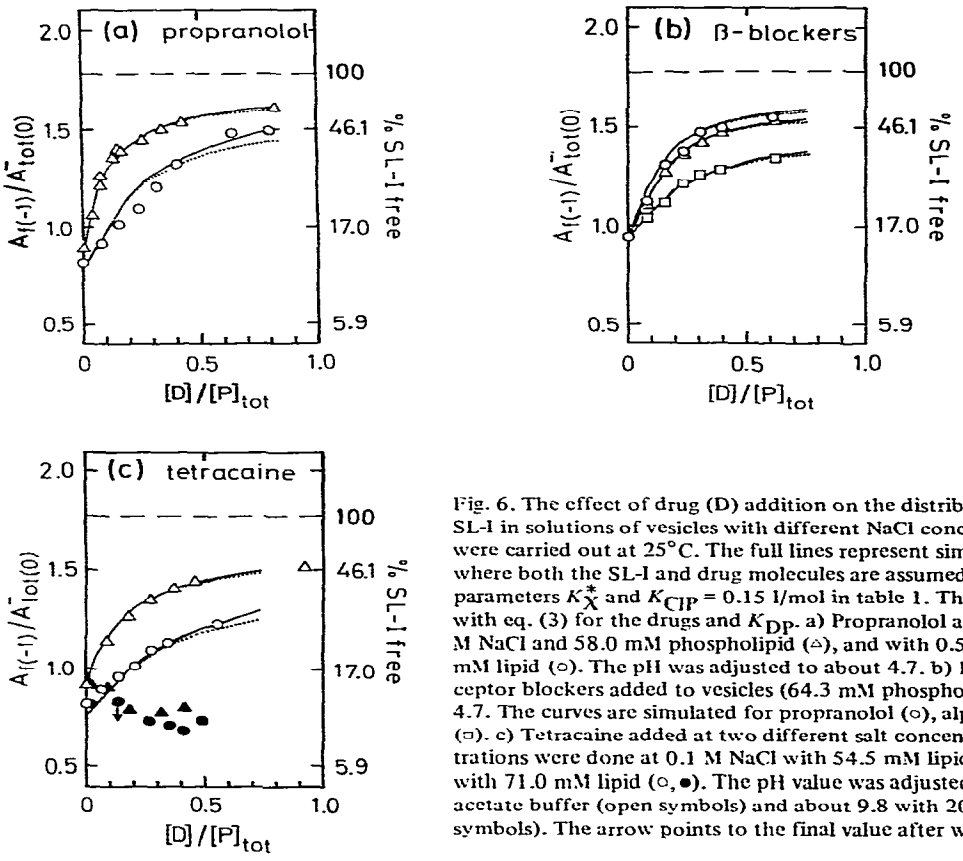

Fig. 6. The effect of drug (D) addition on the distribution of the spin label SL-I in solutions of vesicles with different NaCl concentrations. The titrations were carried out at 25°C. The full lines represent simulated competition curves where both the SL-I and drug molecules are assumed to follow eq. (1) with the parameters K^*_X and K_{ClP} = 0.15 l/mol in table 1. The dotted lines are simulated with eq. (3) for the drugs and K_{DP}. a) Propranolol added. Titrations with 0.1 M NaCl and 58.0 mM phospholipid (△), and with 0.5 M NaCl containing 62.2 mM lipid (○). The pH was adjusted to about 4.7. b) Different β-adrenergic receptor blockers added to vesicles (64.3 mM phosphorus) at 0.1 M NaCl and pH 4.7. The curves are simulated for propranolol (○), alprenolol (△), and metoprolol (□). c) Tetracaine added at two different salt concentrations and pH values. Titrations were done at 0.1 M NaCl with 54.5 mM lipid (△, ▲), and at 0.5 M NaCl with 71.0 mM lipid (○, ●). The pH value was adjusted to about 4.7 with 10 mM acetate buffer (open symbols) and about 9.8 with 20 mM glycine buffer (filled symbols). The arrow points to the final value after waiting for about one hour.

We next titrated vesicles (about 60 mM lipid) in the presence of 0.2 mM SL-I with the drugs (D) propranolol, alprenolol, metoprolol, as well as tetracaine (figs. 6a–c). Different NaCl concentrations were used for propranolol as well as tetracaine. The pH in the bulk was kept well below the pK of the drug by having 10 mM acetate buffer (pH 4.7) present. At this concentration level no significant effects of the buffer on the amphiphile distributions in 0.1 M NaCl were observed. Initially a small difference in SL-I distribution exists depending on the salt concentration (0.1–1.0 M NaCl). Upon drug addition the salt dependence is becoming more pronounced when the SL-I molecules are displaced from the surface. In order to completely expel SL-I, the drug to phospholipid concentration ratio, $[D]/[P]_{tot}$, has to be prohibitively high, even at 0.1 M NaCl. We estimate the increase in surface potential ($\Delta\psi$) to be about + 55 mV when $[D]/[P]_{tot}$ = 0.6 in 0.1 M NaCl for propranolol. About half of the SL-I is then still bound. With higher salt concentration it was even more difficult to displace the SL-I molecules. At 1 M NaCl it was not possible to replace a substantial part of the surface SL-I molecules by propranolol before the drug had aggregated the vesicles, as observed by a gradual increase in turbidity. At 0.1 M NaCl ^{31}P NMR indicated a vesicular form of the membrane. However, for high concentration ratios (> 0.6) propranolol caused a decrease in the resonance linewidth. This may indicate a disorganizing effect on the lipids of the vesicles by the drug.

Of the three β-blockers studied propranolol showed the highest affinity (fig. 6b). Alprenolol was binding slightly weaker whereas metoprolol had only weak bind-

ing to phosphatidylcholine vesicles. This is the same order the drugs expel lanthanide ions from the surface as observed by ^{31}P NMR and centrifugation studies (J. Westman et al., unpublished work). The charged form of tetracaine behaved in the same way as the β-blockers and the SL-I displacement responded to the salt concentration (fig. 6c). At a pH value of 9.8 in the bulk phase tetracaine was not able to compete with SL-I. With low drug concentration the amplitude ratio remained constant immediately after mixing. However, after standing the value dropped (arrow) below the starting value, indicating a tighter binding of SL-I. With higher tetracaine concentrations this happened at once, as also noticed in fig. 5. As the pH > pK practically no salt dependence was observed.

4.5. Simulations of the competition experiments with drugs and SL-I

In the analysis of the experiments in figs. 6a–c we have to account for the bindings of the anions (Cl^-), spin label (SL-I) and the drug (D). The Cl^- adsorption was in all cases treated as a specific binding according to eq. (3). The isotherm for the anions was assumed to be independent of the other adsorbing species. The value of K_{ClP} was selected to give the best possible initial separation, at [D] = 0. Different models have been tested in the simulations. The SL-I and drug bindings may be described by either eqs. (1) or (3). There is no evidence that the two molecules involved would compete for the same sites. The pH experiments indicate that the mutual effect is predominantly electrostatic, governed by eq. (5). We describe the SL-I adsorption with a Henry's law distribution with the binding constant K^*_{SL} = 150 l/mol as derived separately (fig. 3). For the drug distribution we have tested the same distribution as well as the Langmuir isotherm, because of the higher surface concentration obtained for the drug compared to the probe. In the numerical calculations all ions were accounted for. However, the small concentration of acetate ions present (about 5 mM at pH = pK) was neglected. It is not straightforward to arrive at a unique set of parameters for the simulations. There is no dramatic difference in agreement comparing the model where both the SL and the drug are independently obeying Henry's law and the one where the drug instead is following the isotherm. At 0.5 M NaCl concentration, when the membrane surface is more occupied by drug compared to 0.1 M salt, the difference between the models becomes more pronounced. Treating the binding of both amphiphiles by means of a Langmuir isotherm gave less good agreement. The best fit parameters to the experiments in figs. 3 and 6 are found in table 1. For comparison the corresponding values derived at 0.1 M NaCl with the anion binding excluded are also given.

Table 1
Intrinsic binding constants applied to the simulations of the binding of the charged amphiphiles to vesicles of egg yolk phosphatidylcholine at 25°C and a bulk pK of about 4.7. Simulated parameters from a Henry's law type of distribution (K^*_X) and a Langmuir isotherm (K_{XP}) are included. For comparison values derived with no chloride ion binding (K_{ClP} = 0) are also included. The amphiphiles are denoted by X, where X = SL for the spin labels and X = D for the drugs.

amphiphile (X) (cf. fig. 1)	K_{ClP} l/mol	K^*_X l/mol	K_{XP} l/mol
spin label SL-I	0.15	150	do.
	0	175	do.
spin label SL-II	0.15	3000	do.
	0	3625	do.
propranolol	0.15	120	150
	0	80	100
alprenolol	0.15	63	80
	0	48	55
metoprolol	0.15	18	20
	0	15	17
tetracaine	0.15	35	40
	0	27	32

4.6. Competition experiments with propranolol and the spin-labeled analogue (SL-II)

When using the propranolol spin label SL-II in a competition experiment it was necessary to use lower phospholipid concentration (6 mM) than with SL-I, due to the stronger binding of the former. If dl-propranolol and SL-II were binding equally well one should expect the SL-II partitioning to remain constant in the presence of propranolol as long as the total concentration of SL-II plus propranolol was kept constant. As seen in fig. 7 this is obviously not the case. By varying the SL-II concentration from 0 to 0.4 mM, and at the same time the propranolol concentration from 0.4 to 0 mM, a linear dependence for the amplitude ratio was obtained for the SL-II partitioning. This responded

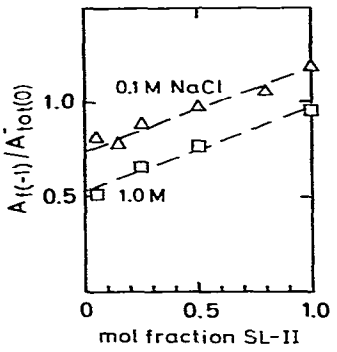

Fig. 7. Distribution of the spin-labeled analogue of propranolol SL-II in the presence of vesicles and in mixtures with different proportions of propranolol. The total concentration of SL-II plus dl-propranolol was kept constant at 0.4 mM. Hence, the mol fraction of SL-II is [SL-II]/0.4. The experimental graphs (broken lines) are for 0.1 M NaCl with 6.2 mM lipid (△), and 1.0 M NaCl with 5.9 mM lipid (□). pH was kept at about 4.7.

to the salt concentration by merely shifting the graph in a parallel manner. By the parameters in table 1 it was not possible by the present simple models to quite satisfactory simulate the slope of the graphs in fig. 7 at both salt concentrations.

5. Discussion

In this study we have investigated a comparatively complex model system with lipids and two additional amphiphiles. The experiments reported here were fitted to the theory for the diffuse double layer with appropriate equilibrium conditions. Simulation of an experiment at a single electrolyte concentration is usually no major problem. As a test of the theory it is more challenging to cover a wide range of salt concentration. It is complicated to distinguish any problems originating from the inherent assumptions behind the treatment of the electrostatics (Gouy-Chapman) and/or those from the thermodynamics of the phase distributions. Nevertheless, we conclude that the present description satisfactorily accounts for the adsorption of the amphiphiles and the electrolyte effect. The problem with the simulations of the experiments in fig. 4 may be related to the results with lanthanide ions at low concentrations [7,8]. We have earlier proposed [8] that a discrete-charge model is more relevant at low surface charge density. Some evidence for a discrete-charge distribution has recently been reported with a polypeptide-lipid interaction [20]. The intrinsic binding constants in table 1 for the two different absorption models (i.e. K_X^* and K_{XP}) are not directly comparable. However, for a given amphiphile the two models predict about the same degree of binding as long as the surface density, $[X]_s/[P]_{os}$, is small.

We have reasons to believe that a change in the properties of the phosphatidylcholine vesicles is at least partly causing the inferior fit seen at the higher salt concentrations, ($\geq$ 0.5 M NaCl) where the drug loading may become appreciable. Nevertheless, in all our simulations it was assumed that only the outer layer of the vesicles was exposed to the adsorbing molecules. Hence in the calculations $[P]_o = 0.65\ [P]_{tot}$ was employed. If the vesicles become permeable a higher lipid concentration should be used. At the moment we have no direct evidence for such permeability of the charged molecules.

In this study we have corroborated our earlier [7,8] findings about anion effects. The specificity among the univalent anions is described by a simple equilibrium model (eq. (3)). A better overall agreement with the present experiments is thus obtained by accounting for the chloride ion effect by means of a specific binding. By ignoring this contribution it is not possible to fit the results over a wide range of salt concentration in a satisfactory way. When the specific chloride ion binding is abolished it is still possible to reasonably well simulate the amphiphile adsorption at a single salt concentration. As seen in table 1 this is only influencing the various binding constants to a small extent with the low value of K_{ClP} derived here. If the anion effect is due to a distinct site binding (as assumed here), or a territorial binding may be complicated to distinguish. The anion effect seems to exist even for the zwitterionic surface (fig. 4). An anion ion condensation phenomenon, in the sense of polyelectrolyte theory, is not expected until a large part of the surface is loaded with the positively charged amphiphile. It is interesting to notice that McLaughlin et al. [21] have substantiated anion effects in their recent study of divalent cation absorption by microelectrophoresis. Measurement by an electrokinetic method [2,22] may facilitate our simulations of amphiphile adsorption. When a unique and relevant solution has been derived

it should approximately agree with the ζ-potential.

Intrinsic binding constants of various drugs with multilamellar dipalmitoyl phosphatidylcholine membranes in 0.1 M NaCl have been reported by Lee [4]. We noticed that the lipid concentration was lower than the one generally used by us with vesicles (about 60 mM). Relatively high drug concentrations were employed. The binding of the drugs was followed indirectly with fluorescence by observing the drug-induced depression in the thermal transition of the lipid. The drug was claimed to be immiscible in the gel phase. Any contribution to the depression by the surface charge was not considered. Using a Langmuir isotherm, without considering the anion effect, 1250 and 1000 l/mol was reported for the binding constants of both the cation and neutral forms of propranolol and tetracaine, respectively [4]. The pK shift was found to be negligible upon binding to the surface. However, due account must then be given of the proton distribution according to eq. (4). The intrinsic binding constants by Lee are substantially larger than ours for the charged drugs with egg yolk phosphatidyl choline. Moreover, independent equilibrium studies (sedimentation and NMR) using multilamellar liposomes with this lipid material indicate that the intrinsic binding constant of the neutral form of tetracaine is much stronger than that of the charged one (J. Westman et al., in preparation).

The experiments with the surface potential probe SL-I are only reflecting the variation of the surface concentration of the charged form of the drug. If the total concentration of this form does not remain constant the situation becomes complex.

The surprisingly strong binding by SL-II might be due to the further hydrophobic contribution to the adsorption by the tempo moiety. This effect must then be stronger than that of the isopropyl part of propranolol itself. For comparison a Langmuir isotherm was applied to the simulations for both SL-II and dl-propranolol. A significantly higher binding constant was obtained for the spin-labeled analogue (table 1). That SL-II is binding substantially stronger than propranolol was also observed by equilibrium dialysis experiments. It is interesting to notice that SL-II has been reported [13] to have a dissociation constant to a β-adrenergic receptor which is 30 times larger than for dl-propranolol. With the analogue SL-II one expects a higher proportion of the non-specific binding to the erythrocyte membranes, making the available total concentration at the receptor site lower.

In the present work we have studied a neutral zwitterionic material. However, there is evidence that the intrinsic (potential independent) binding constant for the adsorption of a charged drug is fairly insensitive to the surface charge density of a membrane containing charged phospholipids (J. Westman et al., in preparation). This obviously also holds reasonably well for the binding of Ca^{2+} ions to membranes of different original charge [7,8,21]. The competition that takes place between the charged drug and the spin label (SL-I) is primarily governed by a common electrostatic surface potential, and not by the number of vacant surface sites. It is likely that the same mechanism is operating when Ca^{2+} or lanthanide ions are displaced by the charged drug. In some preliminary experiment we have used SL-I to follow the lanthanide binding to vesicles. The precision was not encouraging and the cation binding is better studied directly by NMR. Since we have determined intrinsic binding constants for some paramagnetic lanthanide ions [7,8] it should be possible to use a lanthanide ion as a surface potential probe to determine intrinsic drug adsorption.

Adsorption of amphiphilic molecules to membranes is of great importance in many physiological phenomena. When studying charged species it is of particular concern to have a well-defined medium. For instance, investigators of microsomal preparations use different media, covering about one order of magnitude of the ionic strength. The binding of various β-receptor blockers to cytochrome P-450 has been stated to correlate with the membraneous concentration of the drug [23]. The activity of cytochrome P-450 has been found to respond to the surface charge density of the membrane [24]. It is necessary to be aware of surface potential effects, and when possible analyse the results in terms of intrinsic bindings.

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