Biophysical Chemistry 19 (1984) 57–68
Elsevier

BPC 00826

INTERACTION OF THE CATIONIC FORM OF AMPHIPHILIC DRUGS WITH PHOSPHATIDYLCHOLINE MODEL MEMBRANES

COMPETITION WITH LANTHANIDE IONS

Jan WESTMAN, L.E. Göran ERIKSSON and Anders EHRENBERG

Department of Biophysics, University of Stockholm, Arrhenius Laboratory, S-106 91 Stockholm, Sweden

Received 2nd August 1983
Revised manuscript received 16th September 1983
Accepted 5th October 1983

Key words: Model membrane; Drug; Europium; Ion binding; Potential

Model membranes (liposomes) of egg yolk phosphatidylcholine were exposed to the charged (cationic) form of amphiphilic drugs (procaine, tetracaine, metroprolol, alprenolol and propranolol). Drug analysis by ultraviolet light absorption of the bulk solution after centrifugation separation was used to determine the amount of drug bound to the membranes. Microelectrophoresis was employed to measure the change in the zeta-potential after drug adsorption. Binding constants were derived by simulating the experimental curves with a theoretical model which considers the electrostatic effects (Gouy-Chapman theory). Analogous experiments were carried out for the adsorption of Eu^{3+}. Metal analysis was made by three different methods. Good agreement between the centrifugation and electrophoresis experiments was obtained for reasonable positions of the plane of shear relative to the positional plane of the bound ions. Displacement of Eu^{3+} from vesicles upon addition of drug cations was followed by ^{31}P-NMR. The competition experiments were numerically simulated. The Eu^{3+} binding was assumed to obey a mass action type equilibrium, whereas the drug binding was described by a Henry's law partition. The binding constants for the drugs in the competition experiments followed the same order as in the absence of Eu^{3+}. However, the numerical values had to be reduced. The effect of anions was studied.

1. Introduction

Biological membranes are electrically charged. The surface charge on each side of the membrane is of great importance for many processes in, e.g., bioenergetics and pharmacology. The surface charge density may be altered by adsorption of ionic species, or changing the degree of dissociation of surface groups. According to the so-called diffuse double layer theory (Gouy-Chapman) the surface potentials generated respond to the valency and concentration of the electrolytes in the aqueous phase.

In order to appreciate the complex interactions in biological systems, a deeper understanding of the physico-chemical events in systems of model membranes may be helpful. The charged form of an amphiphilic drug is probably the species that is biologically active, and antagonistic effects between drugs and metal ions, e.g., Ca^{2+}, are probably of great importance. The competition need not be specific, but might primarily be ruled by electrostatic effects. In this context we have previously studied the interaction of amphiphilic drugs with phospholipid vesicles and liposomes [1,2], dealing with the electrostatic effects by the use of the Gouy-Chapman theory. Quantitative model studies of the binding of drugs, and drug-like molecules, have also been reported [3–9] by others, and the Gouy-Chapman theory has been considered in a few cases [1,3,7,8].

In this work we try to describe the binding of

the charged form of some local anesthetics and β-receptor blockers to model membranes of phosphatidylcholine in quantitative terms using a theoretical model which accounts for the electrostatic effects by the Gouy-Chapman theory. The binding was studied by three independent methods: Centrifugation separation of drug-lipid dispersions was used to determine the amount of drug left in the aqueous phase, the amount of drug bound to the liposomes being obtained by difference; microelectrophoresis was used to determine the surface potential (which is the crucial parameter in the electrostatic interaction) of liposomes as a result of drug binding; and competition experiments with Eu^{3+} were monitored by ^{31}P-NMR of the phospholipids. Local anesthetics were earlier observed to depress the binding of Pr^{3+} to phospholipid vesicles [10]. We use the same technique in a quantitative way to determine the binding constants for drugs to vesicles of phosphatidylcholine. We have chosen Eu^{3+} instead of Pr^{3+}, since its high degree of contact shift will minimize any effects of conformational changes in the zwitterionic part of the phospholipid [11]. In order to interpret quantitatively the competition experiment, we first had to determine the binding parameters for Eu^{3+} itself. We have previously studied the interaction of various lanthanide ions with vesicles of phosphatidylcholine in great detail with ^{31}P-NMR [11,12]. Here we use centrifugation separation and microelectrophoresis (the same techniques as used for the drugs) to determine the binding for Eu^{3+}.

2. Materials and Methods

The drugs studied were the three β-receptor blockers metoprolol, alprenolol and propranolol, and the two local anesthetics procaine and tetracaine (fig. 1). Propranolol, procaine and tetracaine were purchased as hydrochlorides from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Metoprolol (base) and alprenolol (hydrochloride) were kind gifts from AB Hässle (Mölndal, Sweden), Eu_2O_3 (99.99%) was obtained from Alfa Products (Danvers, MA, U.S.A.). The purity refers to the content of Eu in comparison to the total amount

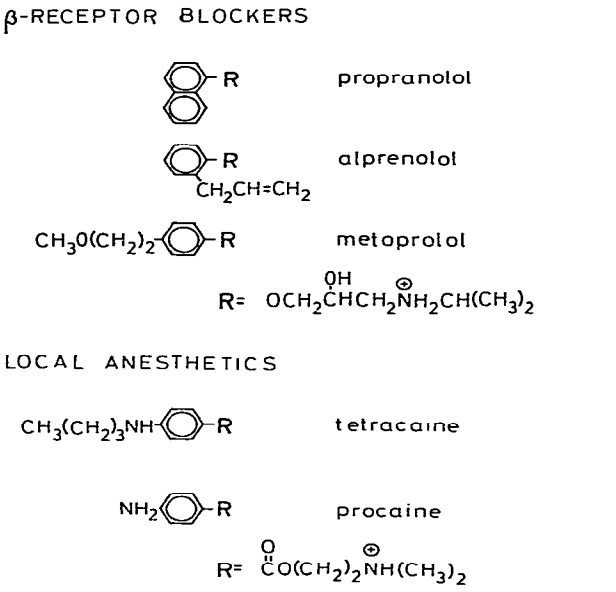

Fig. 1. The structures of the cationic forms of amphiphilic drugs.

of rare earths present. The oxide was heated at 900°C in a platinum crucible for about 1 h and then dissolved in dilute HCl by gentle heating. The slight excess of acid was neutralized to a pH value of about 4. $EuCl_3 \cdot H_2O$ from Alfa Products (Danvers, MA, U.S.A.) was used in some experiments. The Eu content of the latter product was standardized against the oxide. Deuterium oxide (99.8 atomic %) was obtained from Norsk Hydro (Norway) and delivered in a glass bottle. (By avoiding a plastic bottle the deuterium oxide was free from phthalate esters.) Phosphatidylcholine (grade 1) of egg yolk was purchased from Lipid Products (South Nutfield, Surrey, U.K.) and was used without further purification.

Multilamellar liposomes were prepared as follows: Lipid in chloroform/methanol was added to glass centrifuge tubes and the solvent was evaporated with nitrogen or argon gas. Remaining solvent was removed by vacuum pumping overnight. The drug or Eu^{3+} dissolved in 0.1 M NaCl was added to the dried lipid. No buffer was used. The tubes were then shaken in a vortex mixer until

all lipid was dispersed. If necessary the pH was adjusted with 1.0 M NaOH or HCl and the sample was vortex mixed again. The total vortex time was usually 6–7 min. All samples of multilamellar liposomes were freeze-thawed in the following way [2]: The samples were frozen in a dry ice/ethanol cold bath and thawed in lukewarm water, usually five repeated cycles with vortex mixing in between. Vesicles, for the competition experiments, were prepared by sonication under nitrogen for about 1 h. A Heat Systems Model 350 A Sonifier with a microtip was used at a low power output control (control setting 4) and 50% duty cycle. In order to minimize any effects of negatively charged contaminations produced by the sonication [12], the pH in the solution was kept at about 4 when sonicated vesicles were used. Phosphorus analyses were done with the modified Fiske-Subbarow method [13].

Binding of the drugs or Eu^{3+} to multilamellar liposomes of phosphatidylcholine was determined in the following way: Equilibrated dispersions of approx. 30 mM lipid in 0.1 M NaCl in the presence of millimolar concentrations of Eu^{3+} or the drug were centrifuged at $48\,000 \times g$ for 30 min at 20°C, in a Sorvall type RC2B centrifuge. For the drugs, deuterium oxide (99.8%) was used as solvent, since the lipid separated at the top of the solution leaves a much clearer aqueous phase compared to the supernatant in the case of H_2O dispersions. Samples were taken from the aqueous phase for determination of the drug concentration by ultraviolet absorption on a Cary model 219 spectrophotometer. The ultraviolet samples were diluted to 5 ml with phosphate buffer (pH 7.5). Procaine concentration was measured at 291 nm, tetracaine at 311 nm, metoprolol at 272 nm, alprenolol at 270 nm and propranolol at 288 nm. The Eu^{3+} concentration was determined by a colorimetric method using alizarine sulfonate as reagent [14], or by flame emission. For the latter method we used a type 251 spectrophotometer from Instrumentation Laboratory Inc. (Lexington, MA, U.S.A.). An air/acetylene flame at an emission wavelength of 459.4 nm was used. The Eu^{3+} concentration in the supernatant was also measured from the ^{31}P-NMR shift induced by the ion, when 100 μl of the supernatant were added to 1.5 ml of a vesicle solution, 10 mM in lipid concentration. The centrifugation method is based on the assumption that there is equilibrium between all bilayers, the aqueous compartments entrapped between them and the surrounding bulk solution. Freeze-thawing is employed to facilitate this equilibration and we have strong indications that it is effective in this aspect [2]. The lipid concentration was determined for each sample.

The ζ-potential [15] of multilamellar liposomes of phosphatidylcholine dipersed in 0.1 M NaCl was measured with an electrophoresis technique. Dilute lipid dispersions (0.24–0.60 mM lipid) containing millimolar concentrations of the drug or Eu^{3+} were examined. The ζ-potential of the liposomes was also measured in 0.1 M NaCl in the absence of Eu^{3+} and drug at pH values of approx. 5.0 and 10.0. It was found to be slightly negative with absolute values less than 4 and 3 mV, respectively. Such small ζ-potentials are quite difficult to measure and it is doubtful whether they are significantly different from zero. In the calculations the liposomes could therefore be assumed to be uncharged. The small error that this might give rise to is in any case the same for all involved ions. The apparatus used for measurements of electrophoretic mobility was a model Mark II from Rank Brothers (Bottisham, U.K.). A thin-walled cylindrical capillary cell was used. Observations were made at the stationary levels of the cell, where there is no macroscopic net flow of the solvent. The cell was immersed in a thermostatted bath at 20°C. Experiments were performed at a constant current of approx. 3 mA and a field strength close to 10 V/cm. Palladium electrodes pretreated with hydrogen were used. The cell was equipped with platinum reference electrodes connected to a digital precision voltmeter. The average of many observations in both directions was used to estimate the electrophoretic mobility (u). Because of the size of the liposomes (they are 'large' in the present context) the ζ-potential can be calculated from the Smoluchowski formula [15]

$$\zeta = \frac{u\eta}{\varepsilon_0 \varepsilon_r} \tag{1}$$

where η is the viscosity of the medium, ε_0 the permittivity of free space, and ε_r the relative per-

mittivity of the medium. If u is expressed in μm/s in a field of 1 V/cm in water at 20°C the following formula gives ζ in mV

$$\zeta = 14.08 \cdot u \quad (2)$$

The ζ-potential is assumed to be the potential at the so-called plane of shear [15,16], located a few ångstroms away from the surface ($x = 0$). If the surface potential is ψ_S, the potential $\psi(x)$ at a distance x from the surface can be calculated from the following formula in the case of a monovalent salt solution [16]

$$Kx = \ln\left\{\frac{\exp[zF\psi(x)/2RT]+1}{\exp[zF\psi(x)/2RT]-1} \cdot \frac{\exp[zF\psi_s/2RT]-1}{\exp[zF\psi_s/2RT]+1}\right\} \quad (3)$$

K is the inverted Debye length:

$$K = \left(\frac{2z^2F[Na^+]_B}{\epsilon_0\epsilon_r RT}\right)^{1/2} \quad (4)$$

where z is the absolute value of the charge of the monovalent salt and $[Na^+]_B$ the salt concentration in the bulk phase.

Compatition between Eu^{3+} and various drugs was followed by ^{31}P-NMR of the phospholipids using a Varian XL-100A spectrometer operating at 40.5 MHz. Spectra were run with proton noise decoupling at 20°C. 12-mm sample tubes containing 1.5 ml of 60 mM sonicated vesicles in 0.1 M NaCl were used. To the solutions was added $EuCl_3$ to a final concentration of 0.6 mM, causing a chemical shift on the phosphate groups on the outside of the vesicles. Titration with the drugs was done by adding small volumes of a concentrated drug solution (250 or 500 mM) with pH adjusted so that the pH in the samples remained about 4, i.e., well below the pK values of the drugs.

3. Theory

The experimental results were simulated by solving numerically the system of equations below. The treatment is similar to that applied earlier for the binding of Eu^{3+} [11,12] and drugs [1,2] to vesicles or liposomes of phosphatidylcholine. We assume that Eu^{3+} binds according to a mass action equilibrium. The drug adsorption follows a simple partition equilibrium. The surface potential due to the surface charge density and the ionic composition of the water phase (bulk phase) is assumed to be accounted for by the Gouy-Chapman theory.

The partition equilibrium for the cationic form of the drug is

$$DH^+(I) \rightleftarrows DH^+(S) \quad (5)$$

where I denotes the interface, i.e., the water phase in the immediate vicinity of the surface S. Since the pH was well below the pK values for the drugs, only the cationic species had to be considered. For Eu^{3+} the adsorption is described by

$$Eu^{3+}(I) + nP(S) \rightleftarrows Eu^{3+}P_n(S) \quad (6)$$

where n is the number of phospholipids constituting a binding complex. The partition coefficient for the drug is given by

$$K_{DH^+} = [DH^+]_S/[DH^+]_I \quad [1\ \text{Å}^{-2}] \quad (7)$$

and the binding constant for Eu^{3+} by

$$K_{Eu^{3+}} = [Eu^{3+}P_n]_S/[Eu^{3+}]_I[P]_S^n \quad [1\ (\text{Å}^2)^{n-1}\ \text{mol}^{-n}] \quad (8)$$

Surface concentrations are given in mol/Å^2 and interface concentrations in mol/l. The relationship between the interfacial and bulk concentrations is given by the Boltzmann equation

$$[DH^+]_I = [DH^+]_B \exp(-F\psi_s/RT) \quad [\text{mol l}^{-1}] \quad (9)$$

$$[Eu^{3+}]_I = [Eu^{3+}]_B \exp(-3F\psi_s/RT) \quad [\text{mol l}^{-1}] \quad (10)$$

$RT/F = 25.3$ mV at 20°C. The mixture of surface and volume concentrations used above has been discussed earlier [12]. The surface charge density at a membrane exposed to both Eu^{3+} and drug is given by

$$\sigma = Z_0 + N[DH^+]_S + 3N[Eu^{3+}P_n]_S \quad [\text{electronic charges Å}^{-2}] \quad (11)$$

where Z_0 is the initial surface charge density and N Avogadro's number. The relationship between surface charge density, surface potential and the ionic composition of the bulk phase is given by the Grahame equation

$$\sigma = \pm\left\{2\epsilon_r\epsilon_0 RT\sum_i [i]_B[\exp(-Z_iF\psi_s/RT)-1]\right\}^{1/2} \quad [\text{electronic charges Å}^{-2}] \quad (12)$$

where $2\varepsilon_r\varepsilon_o RT = 1.35 \times 10^{-5}$ l mol^{-1} Å^{-4} at 20°C. The summation should run over all ionic species in the bulk phase. The relationship between bulk concentrations and surface concentrations can be obtained from

$$V[P]_o = S[P]_{oS} \quad [\text{mol}] \tag{13}$$

where V denotes the total sample volume (in l) and S the total lipid surface (in Å^2). The small difference (< 5%) between the total sample volume and the water volume has been neglected in the calculations. $[P]_o$ is the total lipid concentration in mol/l and $[P]_{oS}$ the lipid surface concentration in mol/Å^2. If we assume that the area occupied per phosphatidylcholine molecule is 70 Å^2 we have $[P]_{oS} = (70\ N)^{-1}$ mol/Å^2. The amount of drug (or Eu^{3+}) in the bulk phase is corrected for the portion bound by using

$$V[DH^+]_o = V[DH^+]_B + S[DH^+]_S \quad [\text{mol}] \tag{14}$$

where $[DH^+]_o$ is the total drug concentration in mol/l. The bulk concentration is assumed to be constant all the way into the surface, i.e., the difference between the drug concentration in the double layer and the bulk phase is neglected [2].

Combining eqs. 13 and 14 one obtains

$$[DH^+]_o/[P]_o = [DH^+]_B/[P]_o + [DH^+]_S/[P]_{oS} \tag{15}$$

and similarly for Eu^{3+}. All relations were expressed in this dimensionless form for the calculations.

Instead of using the constants in eqs. 7 and 8 we have found it practical to do the following conversions

$$K^*_{DH^+} = K_{DH^+}/[P]_{oS} \quad [1\ \text{mol}^{-1}] \tag{16}$$

$$K^*_{Eu^{3+}} = K_{Eu^{3+}}/[P]^{1-n}_{oS} \quad [1\ \text{mol}^{-1}] \tag{17}$$

For the competition experiment, where both Eu^{3+} and the drugs are involved, the full set of equations is solved. The competition is assumed to take place only via the electrostatic interaction. The numerical simulations were done on a DEC 10 computer.

4. Results

4.1. Drug binding studied by centrifugation and electrophoresis

Dispersions of approx. 30 mM lipid in the form of multilamellar liposomes in 0.1 M NaCl containing propranolol (2.5 mM), alprenolol (2.5 mM) or metoprolol (10.0 mM) were centrifuged. The partition of the drugs was analysed spectrophotometrically. The experimental results were numerically simulated, yielding the intrinsic binding constants

Table 1

Intrinsic binding constants ($K^*_{DH^+}$, $K^*_{Eu^{3+}}$, l/mol) obtained for the binding of cations to the egg yolk phosphatidylcholine membranes

The experiments were carried out with 0.1 M NaCl at pH 4–6 and 20°C. For comparison the results from a related competition experiment are included [1]. The cationic spin label *N,N*-dimethyl-*N*-nonyl-*N*-tempoylammonium bromide was used as a surface probe in this case.

Cation	Centrifugation	Electrophoresis		Competition experiment	
		$x = 0$	$x = 2$ Å	With Eu^{3+} [b]	With spin label [c]
Propranolol	600	300	600	65 140	80
Alprenolol	190	100	150	45 100	48
Metoprolol	24	7	10	16 33	15
Tetracaine	60 [a]	90	135	42 90	27
Procaine	1.5 [a]	0.8	1.0	5 10	–
Eu^{3+}	1200	900	2500	(900)(2500)	–

[a] From ref. 2.
[b] For each column the binding constant of Eu^{3+} cited in the bottom row was used.
[c] From ref. 1.

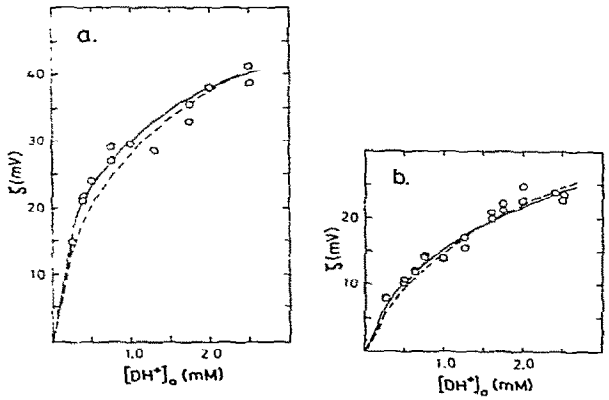

Fig. 2. The experimental ζ-potential (O) for phosphatidylcholine multilamellar liposomes measured in the presence of various total concentrations, $[DH^+]_0$, of (a) propranolol and (b) tetracaine. The solutions contained 0.1 M NaCl at pH about 5. The phospholipid concentration was 0.24–0.60 mM. Each sample was freeze-thawed repeatedly before measurement. The temperature was 20°C. The binding constants used for the drugs ($K^*_{DH^+}$, l/mol) are included in table 1. The binding curves are simulated for $x = 0$ (- - - - -) and $x = 2$ Å (———) for the location of the plane of shear relative to the plane of the bound charges.

($K^*_{DH^+}$) for metoprolol, alprenolol and propranolol as 24, 190 and 660 l/mol, respectively (table 1). The same type of experiment has previously been carried out [2] for tetracaine and procaine and their values for the binding constant are also included in table 1.

The ζ-potential was measured as a function of drug concentration. Multilamellar liposomes (0.24–0.60 mM phosphatidylcholine) in 0.1 M NaCl solution were freeze-thawed (five to ten times) in the presence of various drug concentrations. The pH was measured after the procedure and was between 5.0 and 6.0. The concentration of the strongly binding drugs, i.e., alprenolol, propranolol and tetracaine, was varied between 0.1 and 2.5 mM. For metoprolol addition of 5–20 mM drug was studied. The weakly binding procaine was only measured at 40 mM. Since the liposome particles are large the ζ-potential may be calculated directly from eq. 2. The results for propranolol and tetracaine are presented in fig. 2a–b. The dashed curve shows a simulation assuming that the plane of shear is located at $x = 0$ Å and the continuous line assuming that it is located at $x = 2$ Å from the plane of the bound ions. Different values of $K^*_{DH^+}$ had to be used for the two cases. The experimental points for the other drugs had a similar appearance, the only difference being the magnitude of the ζ-potential. (For procaine the ζ-potential measurement was limited to one drug concentration due to the very weak binding of the drug.) A distance of 2 Å from the surface to the plane of shear has earlier been demonstrated to apply to the adsorption of mono- and divalent cations to phospholipid membranes [17]. The partition coefficients derived from the electrophoresis experiments are given in table 1 for $x = 0$ and $x = 2$ Å. As can be seen, the agreement between the centrifugation and ζ-potential experiments is quite satisfactory. The strength of binding of the β-blockers to the phosphatidylcholine bilayers follows the order propranolol > alprenolol > metoprolol. Of the two local anesthetics, tetracaine has a much stronger binding than procaine, as expected [2].

4.2. Eu^{3+} binding studied by centrifugation and electrophoresis

For quantitative interpretation of the competition experiments (section 4.3) the interaction of Eu^{3+} alone with the phosphatidylcholine membrane was investigated. The binding of this ion to vesicles has previously been studied by NMR [11,12,18,19]. We have now studied the Eu^{3+} binding to multilamellar liposomes in 0.1 M NaCl using two of the present methods, analyses after centrifugation separation and electrophoresis. The samples were freeze-thawed in the presence of Eu^{3+} and centrifuged. The Eu^{3+} concentration in the supernatant was measured by three different methods: flame emission, colorimetry and NMR. The methods gave results in reasonable agrement. However, the NMR method was found to be the most convenient and was used for the final determination of the binding constant. Lipid remaining in the supernatant was determined by phosphorus analyses and the small amount of Eu^{3+} bound to this lipid fraction was accounted for.

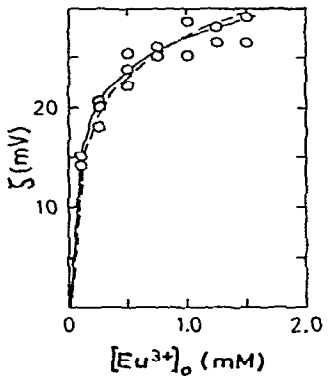

Fig. 3. Measured ζ-potential (O) for the binding of Eu^{3+} to phosphatidylcholine multilamellar liposomes. The samples (freeze-thawed) contained 0.1 M NaCl at pH ≈ 4 and 0.24 mM lipid. The temperature was 20°C. Simulations were done for $x = 0$ (- - - - -) and $x = 2$ Å (———) for the location of the plane of shear relative to the plane of the bound charges. The respective binding constants are given in table 1.

Simulation of the centrifugation experiment was done by assuming eq. 8 to be valid for Eu^{3+} binding and with $n = 2$. Since only a single salt concentration (0.1 M NaCl) was used we did not include any anion binding (cf. section 4.3 for a discussion). The best fit for the centrifugation experiment was obtained with $K^*_{Eu^{3+}} = 1200$ l/mol.

The binding of Eu^{3+} to liposomes in 0.1 M NaCl at pH ≈ 4 was also studied by electrophoresis. The ζ-potential as a function of added Eu^{3+} concentration is shown in fig. 3. As for the drugs, the experimental results for Eu^{3+} were simulated for $x = 0$ (dashed line) and $x = 2$ Å resulting in 900 and 2500 l/mol, respectively, for the binding constant (table 1).

4.3. Experiments to elucidate the question of anion participation

The binding of lanthanide ions to phosphatidylcholine vesicles has been observed by NMR to become specifically enhanced depending on the anion present [11,19]. The binding in salt solutions was reported to follow the order $Cl^- < Br^- < NO_3^-$. By the centrifugation method we have now found that the binding of Eu^{3+} to multilamellar liposomes exhibits significant differences in 0.1 M NaCl, NaBr and $NaNO_3$, following the same order as was found by NMR.

No significant differences between the salts (0.1 M) could be observed when the electrophoretic mobility of liposomes was followed in the absence of drug, or Eu^{3+}. This finding indicates that any anion binding to pure phosphatidylcholine must be very weak, since the ζ-potential is close to zero. A specific enhancement of the anion binding seems to take place in the presence of Eu^{3+} or propranolol. In preliminary experiments the ζ-potential of multilamellar liposomes of phosphatidylcholine in the presence of 0.5–8.0 mM propranolol in 0.1 M salt was found to decrease in the order NaCl > NaBr > $NaNO_3$. The ζ-potentials at 8 mM propranolol were found to be approx. 48.5, 44.5 and 41.5 mV, respectively. We have earlier [1,11,12] considered an anion binding by introducing a weak specific complex with the choline moiety. Since we are not able to determine the exact mechanism behind the anion effect, and since only a single salt at a given concentration (0.1 M NaCl) was used, we have in the present work carried out all the simulations without an anion binding. If a weak anion binding is introduced the binding parameter for the cation has to be adjusted accordingly (slightly reduced).

4.4. Competition between Eu^{3+} and drugs at phosphatidylcholine vesicles observed by ^{31}P-NMR

Addition of Eu^{3+} (0.6 mM) to vesicles (60 mM) in 0.1 M NaCl at pH = 4, caused an upfield shift for resonance of the outside ^{31}P groups. This caused a separation of about 5 ppm between the inside and outside signals. This shift becomes depressed by the addition of a concentrated drug solution (fig. 4a and b). The experimental points were corrected for the dilution resulting from the small volume of added drug. No leakage of the vesicles was detected at the drug concentrations in fig. 4. From the area of the ^{31}P resonances we estimate that the outer surface of the vesicles contains 65% of the total lipid molecules. This value was used to calculate the total concentration of active phosphorus sites, $[P]_o$.

In the theoretical model applied in the simula-

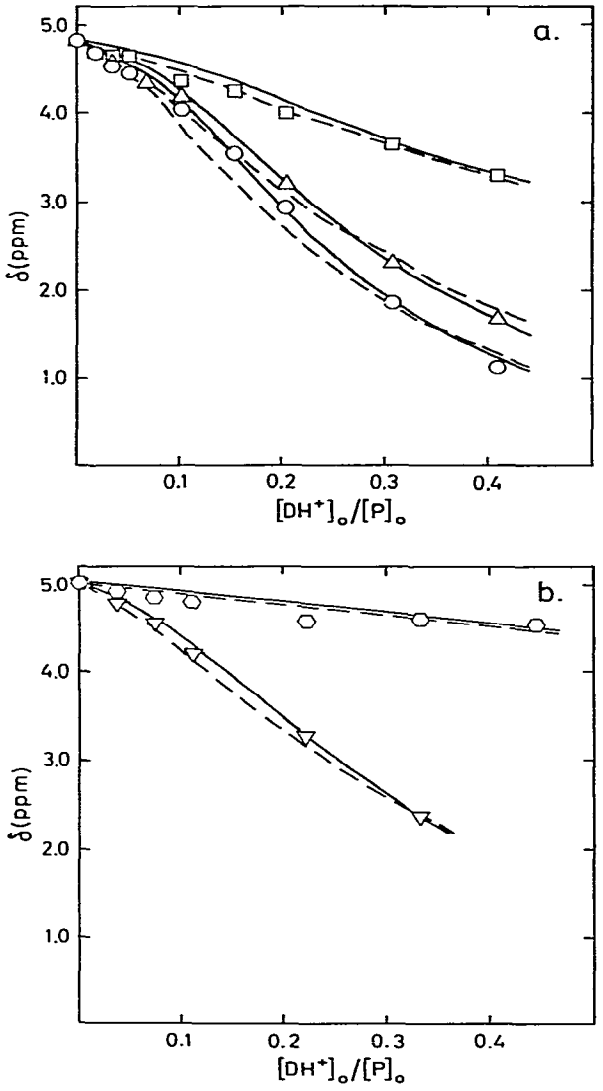

Fig. 4. Experimental points and simulated curves for the competitive binding to phosphatidylcholine vesicles of Eu^{3+} and the cationic form of amphiphilic drugs. The Eu^{3+} binding was monitored by the ^{31}P-NMR chemical shift between the out- and inside phosphorus groups. The initial Eu^{3+} concentration was 0.6 mM. The drug concentration was varied by additions. The solution contained 0.1 M NaCl at pH = 4. The total lipid concentration was about 60 mM. 65% of the total vesicle surface was estimated to be exposed to ions. $[P]_o$ is the total lipid concentration of these outside groups. The temperature was kept at 20°C. Two sets of simulations are demonstrated for each drug (binding constants $k^*_{DH^+}$, 1/mol, are given in table 1) corresponding to 900 (- - - - - -) and 2500 1/mol (———), respectively, for $K^*_{Eu^{3+}}$. (a) ○, propranolol; △, alprenolol; □, metoprolol. (b) ▽, tetracaine; ○, procaine.

tions we have assumed that the competition is simply electrostatic in nature. The shift curves in fig 4a and b were simulated with $n = 2$ using the binding constants determined for Eu^{3+} from the electrophoresis experiment, 900 (broken lines) and 2500 (continuous lines), and without any anion binding included. The best-fit binding constants for the drugs are given in table 1 for the two different values of the Eu^{3+} binding constant. In this table we have also included our results [1] from the competition between an amphiphilic cationic spin label molecule (*N*,*N*-dimethyl-*N*-nonyl-*N*-tempoylammonium bromide) and some drugs. The vesicle and drug concentrations used in the earlier study were about the same as with the present Eu^{3+} experiment. There is a fair agreement between the two competition experiments for the β-blockers. However, for the strongly binding β-blockers these values of the binding constants are substantially lower than those independently found by centrifugation or electrophoresis. For tetracaine all three methods used in this work give similar values for the binding constants, whereas the spin label method gave a somewhat lower value. Procaine is found to have a very weak binding by all three methods.

5. Discussion

In this study we have applied three different methods to follow equilibria for cationic drugs and lanthanide ions between the aqueous phase and the surface of a membrane. Real biological membranes are negatively charged. However, pure phosphatidylcholine which we have used in this study is zwitterionic and forms electrically neutral membranes. Specific interaction with ions, inorganic and organic, can charge the surface making it necessary to account for electrostatic phenomena. In spite of the doubts [16] about the inherent assumptions behind the Gouy-Chapman theory, it seems possible to predict the essential

features of charge interaction. The treatment and understanding of ion adsorption phenomena are far from trivial considering the complexity even of a phosphatidylcholine membrane. A common approach is the so-called Stern equation, i.e., a combination of the Langmuir isotherm with the Gouy-Chapman theory [20]. For a stoichiometry with $n = 1$ the Langmuir isotherm and the mass action law used here are essentially equivalent but for $n > 1$ they differ. The Langmuir isotherm assumes a surface consisting of immobile binding sites whereas the mass action law allows movement of the molecules in the surface. We prefer to use a mass action formalism for Eu^{3+} as the lipid molecules are moving quite freely in the surface and as it is clear that Eu^{3+} binds to the phosphate group of the phospholipid molecules. In our first work [12] on Eu^{3+} binding to phosphatidylcholine vesicles we found a stoichiometry of $n = 3$. Later [11] we could obtain results that at least partly were freed from the effect of conformational changes. Those results suggested $n = 2$ rather than $n = 3$. $n = 2$ has also been used by others [19]. Hence, we have chosen to use the value of $n = 2$ in the present work. The difficulties in determining stoichiometry in these types of systems have been discussed in detail elsewhere [23]. For ions that are mainly hydrophobic and when a direct binding to the phospholipid molecules is questionable, a Langmuir isotherm or a mass action law type treatment might both seem rather unnatural. For the drugs we therefore prefer to use a Henry's law type treatment as most of the drugs are mainly hydrophobic and as it is not clear whether a direct binding to phosphate groups of the phospholipids takes place. A Henry's law type of equation (eq. 7) allows a nonspecific dilution of the surface lipids by intercalating drug molecules, which may be physically more realistic.

The agreement between the binding constants from the centrifugation and electrophoresis experiments is satisfactory for both Eu^{3+} and drugs (table 1). The fact that the surface potentials predicted by the binding constants from the centrifugation experiments agree so well with the ζ-potentials, at a reasonable position of the plane of shear, suggests that the theory is essentially correct. The 'surface' of the liposomes is of course not mathematically well defined. If the charges of the bound ions are located at the plane of shear x equals 0, and if they are closer to the lipid part of the membrane x differs from 0. Thus, we have tested both $x = 0$ and $x = 2$ Å for the location of the plane of shear relative to the plane of the bound ions (which define the membrane surface in our theoretical model). The fit for $x = 2$ Å gives a somewhat better curve form in all cases. The plane of shear at $x = 2$ Å has earlier been applied by McLaughlin et al. [17]. For simplicity we neglected activity and anion effects. The value $K^*_{Eu^{3+}} = 1200$ l/mol was calculated for a 2 : 1 complex from the centrifugation experiment. By electrophoresis, 900 and 2500 l/mol at $x = 0$ and $x = 2$ Å, respectively, gave a good fit (fig. 3). By ^{31}P-NMR we earlier obtained 340 l/mol for the binding constant to vesicles [11] in 0.1 M NaCl, where an anion binding of Cl^- with a binding constant equal to 0.6 l/mol was also used. These values represent the effective binding constants in 0.1 M NaCl, i.e., the intrinsic binding constants times the activity factors in 0.1 M NaCl. However, determination of values for the binding parameters from NMR is hampered by the uncertainty in the absolute values of the limiting shift parameter [11]. Surewicz and Leyko [9] have recently determined a partition coefficient for the propanolol binding to egg yolk phosphatidylcholine liposomes. They did not consider potential effects. Nevertheless, if we neglect such effects at the low surface occupation used (1 propranolol per 61 lipids) we can compare their results with ours. Their partition coefficient [9] then has to be multiplied by a conversion factor of about 1.28, giving a value of 354 compared to our value of 660 l/mol for the centrifugation experiment and 300–600 l/mol from the electrophoresis experiment. With negatively charged membranes much higher partition coefficients were obtained [9], due to the neglect of potential effects.

For the strongly binding drugs, the competition experiments both with Eu^{3+} and the spin label give substantially smaller values for the binding constants than the two other types of experiments (centrifugation and electrophoresis). The discrepancies seem to increase the stronger the drug binds. We therefore tried to include the effect of membrane expansion caused by intercalated drug

molecules (assuming a surface area of 70 Å^2 for the drugs). This could not resolve the discrepancies between the different types of experiments. In the competition experiment small sonicated vesicles were used whereas large multilamellar liposomes were used in the other two types of experiments. One reason for the discrepancy could be that the small sonicated vesicles become sterically strained when drug molecules are introduced into the membrane only on the outer surface of the vesicles. A recent ^{1}H-NMR study of vesicles in the presence of drugs is in accordance with this (G. Eriksson, unpublished data). Such a sterical effect should be more important the stronger the binding of the drug and would result in lower binding constants from the competition experiment. The larger multilamellar liposomes, on the other hand, can probably accommodate drug molecules on both sides of the membrane and are therefore much less strained. Another reason could be that the local equilibrium conditions inside the multilamellar liposomes are different from those at a vesicle surface.

It is interesting to note the similar results with such different surface probes as Eu^{3+} and the spin label amphiphile (table 1). Eu^{3+} with its stronger charge is supposed to form some kind of complex with lipid phosphorus groups. The spin label cation we assume to be intercalated. Only low surface concentrations are estimated for both probes.

We have assumed that the competition is mainly governed by a common electrostatic surface potential. In the competition experiments an about 10-fold higher drug concentration was used than in the other experiments. When we extended the electrophoresis experiments for propranolol to these higher drug concentrations ($\approx$ 15 mM) we found a plateau for the ζ-potential at approx. -48 mV (in 0.1 M NaCl) beginning at approx. 10 mM propranolol. This plateau is not predicted by the Henry law type equation, with or without membrane expansion included, or by a Vollmer isotherm.

The theory used in this work (Gouy-Chapman) assumed an overall smeared-out surface charge density and an overall dielectric constant of 80. It might be that the electrostatic interactions within the surface are underestimated in this treatment as the dielectric constant at the surface has been found to be 20–40 [9]. We have therefore tried a theoretical model in which the potential at the inner Helmholtz plane was used to estimate the electrostatic contribution to the chemical potential in the surface, and the potential at the outer Helmholtz plane was used in the Gouy-Chapman equation. The dielectric constant was assumed to be 20 at the inner Helmholtz plane, and the distance between the two planes was assumed to be 2 Å. The potential at the outer Helmholtz plane was supposed to be equivalent to the ζ-potential [21]. Such a model predicts a ratio of the surface potential to the ζ-potential which is much larger than for the model mainly used in this work and a somewhat greater tendency for the ζ-potential to level out for larger total concentrations of added drug. Again, this model was not able to give agreement between the competition experiments (section 4.4) and the experiments with centrifugation and electrophoresis (sections 4.1 and 4.2). Akutsu and Seelig [22] have studied La^{3+} binding to phosphatidylcholine liposomes up to very high La^{3+} concentration (1 M) and observed complete saturation. They claimed that the Gouy-Chapman theory was unsuccessful in describing this observation. However, by using an empirical interaction potential and activity coefficients it became possible to fit a theoretical curve to the NMR experiments. A more sophisticated model for the binding of ions and/or drugs should include contributions caused by any changes in, e.g., the orientation of the zwitterionic head groups [22], as well as the lateral density of the lipids. Moreover, in the present model we have assumed that all the charges created by 'bound' species are located in a single plane parallel to the surface. Nevertheless, we have not found it necessary to include these types of effects in our theoretical model as we could satisfactorily account for our experimental results with the simple model used here.

The effect of anions is not merely a result of their screening of the surface potential in the diffuse double layer theory. In the case of the lanthanide ions their binding to the phosphatidylcholine membrane is enhanced in a specific manner in the presence of monovalent anions [11,12,19]. We also had to consider the anion

effect with the binding of amphiphiles to vesicles at different NaCl concentration [1]. In the present study a single salt concentration was employed. We have therefore refrained from explicitly introducing an anion binding into the model. The binding constants reported here will thus be apparent in the sense that any anion effect refers to 0.1 M NaCl. Moreover, we have not used activity factors or complex constants. Barsukov et al. [24] have determined from electrophoresis experiments specific anion adsorption to phosphatidylcholine. Such adsorption was questioned by McLaughlin et al. [25]. From our electrophoresis measurements we now reject a sizeable direct binding of anions (Cl^-, Br^-, NO_3^-) to pure phosphatidylcholine membranes. However, we have found a difference in the ζ-potential of liposomes in 0.1 M NaCl, 0.1 M NaBr and 0.1 M $NaNO_3$ in the presence of propranolol. We suggested [11] that the anion binding accompanied the cation binding. This has now been confirmed by the ζ-potential measurements of liposomes in the presence of propanolol and by centrifuging liposomes in the presence of Eu^{3+} and the monovalent salts. The order of Eu^{3+} binding enhancement was $Cl^- < Br^- < NO_3^-$, or the same as found by NMR [11]. A ^{35}Cl-NMR study has also indicated an interaction of Cl^- with vesicles accompanying the propranolol binding (G. Eriksson, unpublished data). The centrifugation results should be more unambiguous than those from the electrophoresis experiments. In the former the equilibrium bulk concentration can be determined directly, whereas in the latter an overall contribution to the ζ-potential, which might be due to only positive charges or to a sum of positive charges and compensating negative charges, is determined. We have previously [1,11,12] discussed the anion effect accompanying cations in terms of a specific anion binding constant. The size, polarisation and hydration energy for an anion will contribute to its specificity. It now seems that a synergism exists between the bound cations and the surrounding anions. The specificity observed for the anions could be due to a close parallel binding directly to positive sites at the surface.

6. Conclusions

Three different experimental techniques (electrophoresis, centrifugation and ^{31}P-NMR) were used to study the interaction of Eu^{3+} and some β-blockers and local anesthetics with lipid model membranes. A theoretical model, based on the Gouy-Chapman theory in combination with an equation to describe the adsorption equilibria to the membrane, was used to simulate the experimental results. There is good agreement between the results from the centrifugation and the electrophoresis experiment for Eu^{3+} and all the drugs. However, for the strongly binding drugs substantially lower binding constants had to be used in the simulations of the competition experiment. We believe this to be at least in part due to the fact that the small vesicles (used in the competition experiments) become sterically strained when large amounts of drug molecules are introduced on the outside of the vesicles. The larger multilamellar liposomes used in the two other types of experiments can probably accommodate drug molecules on both sides of the membrane and this become much less strained.

An anion effect observed earlier has been confirmed also in this work. We believe that this effect is due to a binding of anions to positive sites formed by drug molecules or Eu^{3+} already bound to the membrane. The binding constant for the lanthanide ion found here is about two orders of magnitude larger than for Ca^{2+}. Hence, the drugs used in this work (except perhaps procaine) should very effectively compete with Ca^{2+} at a biological membrane.

Acknowledgements

We would like to thank Dr. Hans Grasdalen, University of Trondheim, Norway, for valuable discussions. This work was supported by grants from the Swedish Natural Science Research Council, the Wenner-Gren Foundation and the Magn. Bergvall Foundation. The skilful assistance with the experiments by Maria Dalin and Lars Mittermaier is acknowledged.

References

1 L.E.G. Eriksson and J. Westman, Biophys. Chem. 13 (1981) 253.
2 J. Westman, Y. Boulanger, A. Ehrenberg and I.C.P. Smith, Biochim. Biophys. Acta 685 (1982) 315.
3 S. McLaughlin, in: Molecular mechanism of anesthesia, Progress in anestheiology, vol. 1, ed. B.R. Fink (Raven Press, New York, 1975) p. 193.
4 D. Papahadjopoulos, K. Jacobson, G. Poste and G. Shepherd, Biochim. Biophys. Acta 394 (1975) 504.
5 A.G. Lee, Biochim. Biophys. Acta 448 (1976) 34.
6 A.G. Lee, Mol. Pharmacol. 13 (1977) 474.
7 A.G. Lee, Biochim. Biophys. Acta 514 (1978) 95.
8 E. Goormaghtigh, J. Caspers and J.M. Ruysschaert, J. Colloid Interface Sci. 80 (1981) 163.
9 W.K. Surewicz and W. Leyko, Biochim. Biophys. Acta 643 (1981) 387.
10 M.S. Fernández and J. Cerbón, Biochim. Biophys. Acta 298 (1973) 8.
11 J. Westman and L.E.G. Eriksson, Biochim. Biophys. Acta 557 (1979) 82.
12 H. Grasdalen, L.E.G. Eriksson, J. Westman and A. Ehrenberg, Biochim. Biophys. Acta 469 (1977) 151.
13 G.R. Bartlett, J. Biol. Chem. 234 (1959) 466.
14 Colorimetric determination of traces of metals, Chemical analysis (1959), vol. 3, p. 748.
15 R.J. Hunter, Zeta potential in colloid science (Academic Press, London, 1981).
16 R. Aveyard and D.A. Haydon, An introduction to the principles of surface chemistry (Cambridge University Press, London, 1973).
17 S. McLaughlin, N. Mulrine, T. Gresalfi, G. Vaio and A. McLaughlin, J. Gen. Physiol. 77 (1981) 445.
18 H. Hauser, M.C. Phillips, B.A. Levine and R.J.P. Williams, Eur. J. Biochem. 58 (1975) 133.
19 H. Hauser, C.C. Hinckley, J. Krebs, B.A. Levine, M.C. Phillips and R.J.P. Williams, Biochim. Biophys. Acta 468 (1977) 364.
20 S. McLaughlin, Curr. Top. Membranes Transp. 9 (1977) 71.
21 J. Plaisance, J. Colloid Interface Sci. 56 (1976) 33.
22 H. Akutsu and J. Seelig, Biochemistry 20 (1981) 7366.
23 J. Westman, A study on the interaction of inorganic ions and amphiphilic drugs with phospholipid model membranes, Ph.D. Thesis, Stockholm (1981).
24 L.I. Barsukov, Yu.E. Shapiro, A.V. Viktorov, V.I. Volkova, V.F. Bystrov and L.D. Bergelson, Bioorg. Khim. 3 (1977) 1355.
25 A. McLaughlin, C. Grathwohl and S. McLaughlin, Biochim. Biophys. Acta 513 (1978) 338.