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Interaction of charged amphiphilic drugs with phosphatidylcholine vesicles studied by NMR

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Small unilamellar vesicles from egg phosphatidylcholine in NaCl solutions were exposed to some amphiphilic pharmaca. The aromatic drugs (chlorpromazine, dibucaine, tetracaine, imipramine and propranolol) were in their cationic form of the amines. By ^{1}H- (100 and 400 MHz) and ^{31}P- (40.5 and 161.7 MHz) NMR the membrane signals were observed. In particular, the *N*-methyl choline proton signals were followed upon drug addition. The intrinsic chemical shift difference (0.02 ppm) between the inner (upfield) and outer choline signals was influenced by the drug concentration. Packing properties of the lipid head groups and ring current shift probably contributed. At very high drug concentration, the vesicles are destroyed. A transformation into a micellar state with a high sample viscosity took place in a narrow concentration range of drug. The anion effects of Cl^- were studied from the ^{35}Cl-NMR linewidth at 9.8 and 39.1 MHz. A continuous increase in the signal linewidth followed upon drug addition to the vesicles. Only chlorpromazine produced a broadening in the absence of vesicles (NaCl blank). The linewidth reflected a critical micelle concentration of this drug around 7 mM in 0.1 M NaCl. The ^{35}Cl-NMR experiments demonstrated the existence of an anionic counterion effect. This phenomenon should be accounted for when quantitatively analysing drug-membrane interactions in electrostatic terms.

1. Introduction

Many pharmaca convey their activity through receptors. Nonspecific interactions with the lipidic matrix may also be crucial due to the large membraneous 'antenna'. Depending on the hydrophobic character of a molecule, and the way it is administered, a substantial enrichment into the membrane may occur. It has been claimed that only model membranes produced from extracted lipids, free of proteins, are able to bind hydrophobic drugs [1]. These findings have not been substantiated by others [2–5]. It seems rather that model membranes produced from phospholipids alone (extracted or synthetic) in an aqueous system have many inherent properties in common with authentic biological membranes. Whether this also holds for small unilamellar vesicles has been debated. Nevertheless. regions which have a small radius of curvature often occur in biological cells or organelles, either permanently or induced as a result of membrane activation.

The molecular mechanism behind the function of, e.g., local anesthetics, has been discussed extensively in the literature. There seems to be no rigorous discrimination between the anesthetic effect and other physiological responses caused by amphiphilic pharmaca. Hence, for example, some psychotropic drugs and adrenergic antagonists may also have effects in common with anesthetics. Altered physical properties (dynamics and order)

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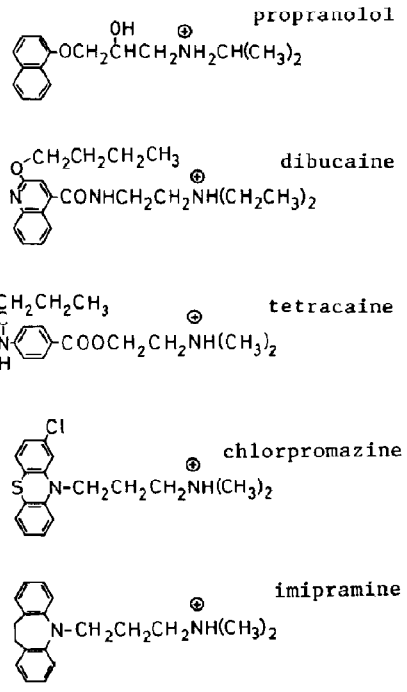

Fig. 1. Structures of the cationic form of the amphiphilic molecules studied.

of the membrane itself may contribute to the physiological response.

In the present paper NMR has been applied to follow the interaction of five rather lipophilic drugs (fig. 1) with small unilamellar vesicles, formed by phosphatidylcholine (lecithin) from mainly egg yolk. The selected drugs represent local anesthetics (dibucaine, tetracaine), β-adrenergic receptor blockers (propranolol), neuroleptics (chlorpromazine), as well as trycyclic antidepressants (imipramine). The study was restricted to the charged (cationic) form of the amine drugs, dominating at physiological pH values. We have previously tried to interpret quantitatively the binding of some of the drugs to lecithin vesicles, also considering the electrostatic effects [6,7]. Such bindings are here further delineated with respect to two problems, which obscured the earlier analysis. At first, molecular constraints in the vesicles caused by the drugs are studied with ^{1}H- and ^{31}P-NMR. Secondly, NMR from $^{35}Cl^{-}$ is applied to determine whether this anion accompanies the drug ions at the membrane surface.

2. Materials and methods

Egg yolk phosphatidylcholine (grade I) in chloroform/methanol was purchased from Lipid Products (South Nutfield, Surrey, U.K.), and used without further purification. DL-Dipalmitoylphosphatidylcholine was from Sigma (St. Louis, MO). All the drugs as hydrochlorides were obtained from Sigma. Deuterium oxide (99.97 atom%) was obtained from Studsvik, Sweden. The sodium salt of 3-trimethylsilyltetradeutropropionic acid (TSP) was delivered by Wilmad Glass (Buena, NJ).

Vesicles were prepared by sonication under nitrogen and ice cooling for about 1 h (50% duty cycle) by a Heat Systems model 350 A sonifier. A microtip at a control setting of about 4 was used. Titanium particles were removed by centrifugation (48 000 $\times$ g). No buffer was used, but the clear solution was pH adjusted to around 4.5 with ^{2}HCl. Phosphorus analyses were done with the modified Fiske-Subbarow method [8]. Microelectrophoresis was carried out as described elsewhere [7].

^{1}H-NMR spectra were recorded by JEOL JNM-GX400 and Varian XL-100 spectrometers at 400 and 100 MHz, respectively. 1-ml samples in 5-mm tubes were measured at 25°C. ^{31}P- and ^{35}Cl-NMR spectra were obtained at 40.5 and 9.8 MHz, respectively, with a Varian XL-100 spectrometer using 12-mm sample tubes at 20°C. The corresponding spectra were also measured at 161.7 and 39.1 MHz, with the high-field spectrometer using 10-mm sample tubes at 25°C. The ^{31}P spectra were recorded with complete proton decoupling. Drugs dissolved to 0.5 M in $^{2}H_2O$ (or $^{1}H_2O$) were added directly into the sample tubes.

Electron microscopy was performed with a Philips EM 400T instrument, using copper grids with carbon films. Negative staining with 2% uranyl acetate was applied, without further dilution of the samples.

3. Results

3.1. Lipid ^1H- and ^{31}P-NMR signals

With sonicated egg phosphatidylcholine relatively well resolved ^{1}H-NMR spectra can easily be recorded at room temperature. At 400 MHz (9.4 T) the *N*-methyl protons from choline gave individual resonances from the interior and exterior head groups (fig. 2). The intrinsic chemical shift difference was about 0.02 ppm for the actual average size of the vesicles. From the choline signal intensities an approximate outer/inner monolayer molecular ratio of 1.9 was estimated. This ratio corresponds to an average outer vesicle radius of about 17 nm with a bilayer thickness of 4.5 nm, provided that the hydrated molecular area is assumed to be identical within the two monolayers.

^{1}H-NMR spectra were recorded upon addition of the various amphiphilic drugs to samples of egg lecithin vesicles. The interest was focused on the choline signals. As seen (figs. 2 and 3), the shift between the components from the two sides is influenced by an increasing concentration of drug,

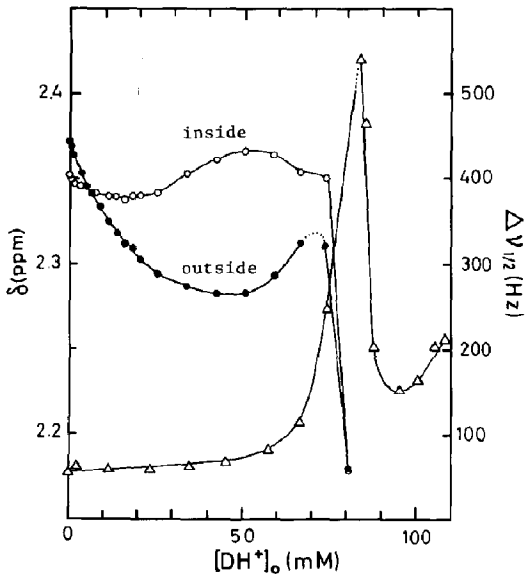

Fig. 3. ^{1}H-NMR chemical shift (○, ●) of the two *N*-methyl choline signals in fig. 2 plotted as a function of the total propranolol concentration, $[DH^+]_o$. Variation of the ^{31}P-NMR (161.7 MHz) linewidth (at half height) (△) upon addition of propanolol to vesicles; 34.5 mM egg phosphatidylcholine in 0.1 M NaCl at 25 °C.

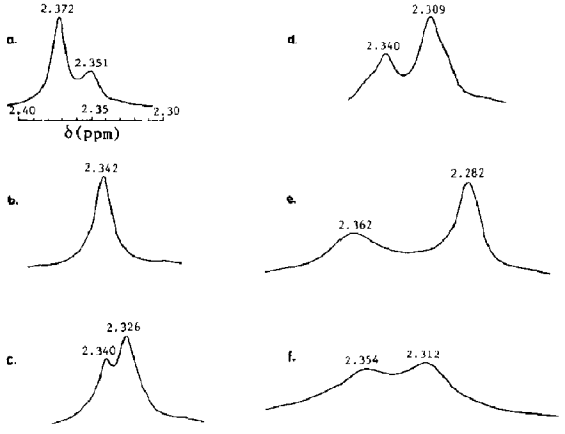

Fig. 2. Effect of propranolol concentration on the 400 MHz ^{1}H-NMR signals from the outer and inner choline groups of vesicles. The chemical shifts indicated are referred to the signal from the ω-CH_3 groups of the lipid acyl chains. 35.6 mM egg phosphatidylcholine in 0.1 M NaCl at pH 4.7 and 25 °C. Propranolol concentration (mM) was (a) 0, (b) 6.4, (c) 11.2, (d) 18.3, (e) 42.5, (f) 67.7.

as demonstrated for propranolol. The shifts (ppm) in the spectra are referred to the shift of the internal ω-CH_3 acyl signal of the phospholipid. This reference signal was preferred since an internal, as well as external, TSP standard was sensitive to the addition of drugs. The shift numbers indicated in the figures may be converted to the TSP reference scale by addition of 0.86 and 0.90 for the internal and external standard, respectively. Those values are only valid in the absence of any drug. The spectra at the very highest drug concentrations are not illustrated (see below). In fig. 3 the peak values for the two lines are plotted as a function of the total propranolol concentration. The shifts plotted are derived without any curve fitting, in case of overlapping lines. With due consideration of any overlap the shift of the inner choline line remains practically constant around 2.34 ppm up to a total propranolol concentration of about 30 mM. At still higher concentration, the inner signal responds to the drug

addition and a downfield shift is observed. A monotonic upfield shift is observed for the outer signal up to about 50 mM propranolol. At this drug concentration a maximum chemical shift separation of about 0.09 ppm between the outer and inner choline signals is recorded. At even higher drug concentration the separation is gradually diminished, resulting in a complete collapse of the signals. The final narrow signal is shifted upfield to 2.18 ppm. At this stage (80 mM drug; drug/lipid ratio about 2.2) the samples become completely transparent. At just slightly higher drug concentration, within a narrow concentration interval, the sample turns into a highly viscous, clear solution. Upon further addition of drug the sample returns to a more fluid state.

Of the other ^{1}H-NMR signals the one assigned to the lipid CH_2CO-acyl protons, located 1.5 ppm downfield from the ω-CH_3 signal, was particularly sensitive to the effect of drug. A linear upfield shift of this signal was observed up to a drug/lipid mole ratio of about 0.3, where the induced shift became approx. 0.17 and 0.13 ppm for propranolol and dibucaine, respectively. The shift with chlorpromazine was of the same order. No evidence for a split signal was seen. The drug-induced shift of the lipid moieties followed the order $CH_2CO > CH_2N^+ > (C=CCH_2C=C;\ CH_2OP) > CH_2C=C$.

The other amphiphilic drugs studied (dibucaine, chlorpromazine, imipramine) principally exhibited the same ^{1}H-NMR effects as those shown in figs. 2 and 3. Addition of about 10 mM dibucaine to vesicles (30 mM lecithin) resulted in a coincidence of the choline outer and inner resonances. After the addition of slightly more of this drug a reversal of the shifts for the two topical signals took place. However, some shoulders from unidentified spectral components were seen at this stage with dibucaine. At about 30 mM dibucaine only two separated and shifted choline resonances were seen. With higher concentration the signal from the terminal ω-CH_3 acyl protons exhibited some structure. The sample became clear, and viscous, at about 69 mM dibucaine present. The local anesthetic tetracaine was only able to remove the 0.02 ppm shift separation of the *N*-methyl resonances from the two monolayers. In contrast to the other drugs investigated, tetracaine did not cause a reversed shift separation.

With ^{31}P-NMR at 40.5 MHz, and complete proton spin decoupling, no obvious resolution of the inner and outer head group signals was discernible with egg lecithin vesicles. However, at 161.7 MHz an upfield shoulder from the inner head groups was seen, indicating about 0.1 ppm separation between the two signals. In the presence of a drug the phosphorus resonances from the vesicles immediately turned into a single symmetrical line. The linewidth at half-height of the ^{31}P-NMR signal remained almost constant provided the vesicles were intact (fig. 3). Concomitantly with the collapse of the shift separation for the ^{1}H-NMR choline signals an increase in the ^{31}P linewidth took place (fig. 3). The drug transformed the sample into a clear liquid with a high viscosity. Upon further drug addition the ^{31}P linewidth diminished and the sample viscosity decreased. The ^{31}P-NMR signal remained symmetrical all the time, with no indication of a chemical shift anisotropy.

Upon addition of the paramagnetic shift ion Pr^{3+} separate ^{31}P-NMR signals can be observed from the inner and outer surfaces, as long as the vesicles are not permeable to the ions. The charged drugs and Pr^{3+} compete electrostatically for the membrane surface, which may complicate the interpretation. Nevertheless, in the presence of 27 mM propranolol and 36 mM Pr^{3+} separate surface signals were observed with vesicles at 30 mM lipid. With 46 mM drug it was not possible to observe an unshifted (interior) signal, even when increasing the Pr^{3+} concentration to 90 mM. Tentatively, this effect is interpreted as a more even exposure of the head groups to Pr^{3+} in the drug-lipid system at this high drug concentration.

Some preliminary results from electron microscopy demonstrated that the vesicles were converted into a new intermediate phase at high drug concentration. The viscous samples exhibited a textured structure, without indication of vesicles. Regions with a corrugated pattern were observed. They consisted of an ordered lamellar-like structure with an approximate thickness of a bilayer. The contrast of the micrographs did not allow estimation of the dimensions of the mixed micelles.

The concentrated viscous material was applied directly on the grids, in order to preserve authentic samples. This may cause some artifacts by the negative staining. A comparison with the freeze-fracture technique may be helpful in this context.

3.2. ^{35}Cl-NMR study of counterion effects

In the absence of vesicles (blank samples) the $^{35}Cl^-$-NMR linewidth at half height ($\Delta\nu_{1/2}$) for a 0.1 M NaCl solution (50% ^2H_2O) at 20°C was 12.5 Hz, at the resonance frequency 9.8 MHz. When applying an exponential weight function the width was increased to 13.5 Hz. $\Delta\nu_{1/2}$ remained almost constant, or increased only to a very small extent upon addition of the hydrochloride form of the drugs up to a total concentration of about 20 mM, $[DH^+]_o$ (fig. 4). However, with chlorpromazine the situation was different, since a dramatic increase in the linewidth from the blank sample was observed at drug concentration higher than 7 mM (fig. 4, circles). Light absorption and spectrofluorometric studies of chlorpromazine solutions indicated a nonlinearity in the concentration dependence around the critical value found with NMR.

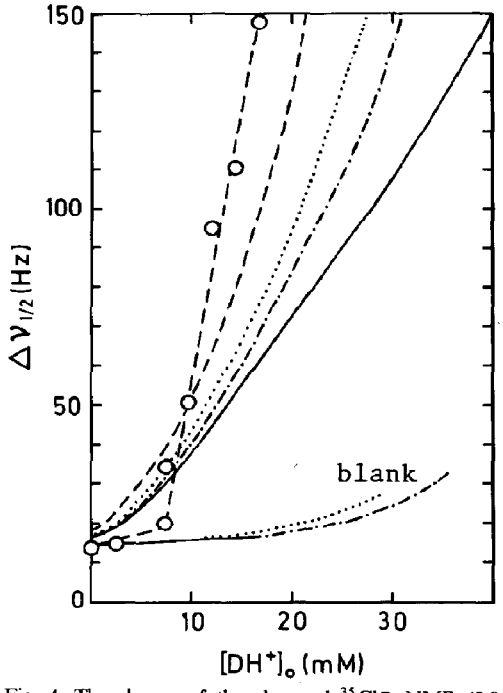

Fig. 4. The change of the observed $^{35}Cl^-$ NMR (9.8 MHz) signal linewidth (at half height), upon addition of various drugs to samples containing vesicles, or 0.1 M NaCl alone (blank). The concentration of phosphatidylcholine was about 30 mM in 0.1 M NaCl (pH about 4.5) at 20°C. (— — —) Chlorpromazine, blank values denoted by circles; (· · · · · ·) dibucaine; (· — · — ·) imipramine; (- - - - - -) propranolol and tetracaine.

In the presence of egg lecithin vesicles (about 30 mM lipid) the $^{35}Cl^-$ linewidth was about 17.5 Hz and increased monotonically upon addition of the drugs (fig. 4). This was also the case for chlorpromazine. The enhancement followed the order chlorpromazine > dibucaine > imipramine > propranolol = tetracaine. The lineshapes correspond most closely to a Lorentzian one.

In fig. 5a the dependence of $\Delta\nu_{1/2}$ on lipid concentration is indicated in the case of propranolol binding at 0.1 M NaCl. As can be seen, a higher vesicle concentration induces a stronger linewidth broadening. The effect of salt concentration on $\Delta\nu_{1/2}$ is illustrated in fig. 5b for propranolol binding at a lecithin concentration of 60 mM. At the lowest NaCl concentration the linewidth was most sensitive to the presence of drug. It was not meaningful to measure below an NaCl concentration of about 50 mM, since the strong line broadening made the signal-to-noise ratio unsatisfactory.

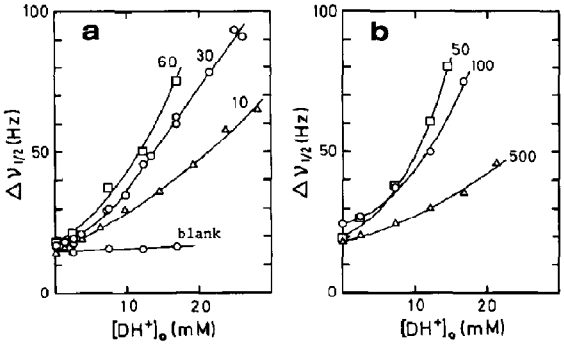

Fig. 5. Propranolol effect on the linewidth of the $^{35}Cl^-$ NMR (9.8 MHz) signal. (a) Dependence of variable egg phosphatidylcholine concentration (mM) in 0.1 M NaCl at 20°C and pH about 4.7. (b) Influence of NaCl concentration (mM) in samples containing about 60 mM lipid as vesicles.

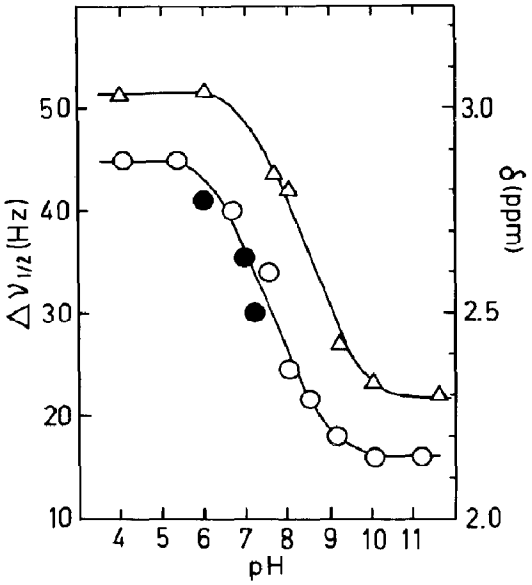

Fig. 6. The pH dependence of NMR signals from a sample containing vesicles in the presence of tetracaine. $^{35}Cl^-$ NMR (9.8 MHz) linewidth (○, ●) from 0.1 M NaCl at 20°C with 30 mM egg phosphatidylcholine and 9.8 mM drug (30 atom% ^2H_2O). (●) Values measured after neutralization of the solution from the extreme alkaline value. ^{1}H-NMR shift (△), referred to external TSP, for the *N*-methyl signal of tetracaine (4 mM) in the presence of 10 mM phosphatidylcholine; 0.1 M NaCl (99 atom% ^2H_2O) at 30°C.

The pH dependence of the $^{35}Cl^-$ linewidth was investigated in the case of tetracaine (9.8 mM) interacting with vesicles (about 30 mM lipid). In fig. 6 it is seen that the value of $\Delta\nu_{1/2}$ decreases when raising the bulk pH to alkaline values. The linewidth drops to about 16 Hz at pH 11. This value should be compared to 17–18 Hz recorded with vesicles at acidic pH values, in the absence of any drug. An upfield shift of the ^{1}H-NMR signal from the *N*-methyl groups of tetracaine (4 mM) is observed (fig. 6) when increasing the pH value in a vesicle sample (10 mM lipid).

It was observed that the linewidth of the $^{35}Cl^-$ signal from samples containing egg lecithin vesicles and drugs became narrower at an elevated temperature. The temperature dependence was also studied with a sample of dipalmitoylphosphatidylcholine (30 mM) vesicles dissolved in 0.1 M NaCl in the presence propranolol (16 mM). A continuous decrease of $\Delta\nu_{1/2}$ from 51 Hz at 30°C to 24 Hz at 60°C was observed.

4. Discussion

4.1. The effects of drugs on lipid organization

Aqueous samples of sonicated egg lecithin were directly employed, without an elaborate preparative size discrimination of the vesicle. By using size-fractionated vesicles (15–27 nm in average Stokes diameter) it has been demonstrated [9] that the ^{1}H-NMR shift between the *N*-methyl choline signals is dependent on the vesicle size. An upfield linear shift of the inner *N*-CH_3 resonance was reported, whereas the resonance from the outer monolayer choline groups remained unshifted, when the vesicle size decreased. The actual shift of 0.02 ppm between the two monolayers seems to be intrinsic for vesicles with an average outer (Stokes) radius of about 11 nm. This small vesicle size requires that the hydrated head group surface area of the outer monolayer is about 0.87 nm^2, compared to only 0.53 nm^2 at the inner surface [9]. The thermal lipid phase transition has been followed by ^{1}H-NMR, using sonicated distearoylphosphatidylcholine vesicles [10]. The chemical shift difference between the *N*-methyl protons was 0.07 ppm below the transition (< 50°C), but decreased to 0.03 ppm above the melting point. It was concluded that an upfield shift of the inner choline signal is responsible for the effect, whereas the resonance from the outer layer exhibits a constant shift. The phase transition is commonly characterized by an altered lateral molecular packing density. Hence, one may assume that the exterior head groups can be accommodated without influencing their ^{1}H-NMR shift. From the above-discussed reports one would expect the NMR signals from the interior *N*-methyl choline groups to be particularly sensitive to inherent geometrical constraints in the membrane.

At the inner vesicle surface the phosphorylcholine head group has to adopt a more extended configuration, making the thickness of the polar region larger than in the outer layer [9,11]. The intrinsic asymmetry of a highly curved bilayer

vesicle is manifested by a chemical shift nonequivalence between the head groups of the two monolayers [12]. In the unperturbed planar phospholipid bilayer the average orientation of the zwitterionic dipoles is parallel to the membrane surface, with the charges preferentially compensating each other [13]. However, in the fully hydrated liquid-crystalline state the two-dimensional network becomes loose. Interaction of the membrane with amphiphilic drugs may influence its properties in various ways: (i) intercalation of spacer molecules will separate the phospholipid head groups; (ii) more specific interaction of the hydrophilic/charged moieties of the drug molecules with the lipid polar groups may take place; (iii) changing the electrostatic (including dielectric) conditions at the surface by drug binding may influence the interfacial conditions with respect to ions and dipoles; (iv) transmission of effects to the unoccupied opposite layer of the membrane.

From fig. 3 it is seen that the *N*-methyl ^{1}H-NMR signals of the two layers in the vesicle respond to the drug addition in a complicated way. Loading the outer layer with drug molecules gives rise to an initial progressive upfield shift of the exterior *N*-methyl resonances of lecithin. The effect from outside is not transduced to the inner layer at this stage. This indicates that the drug molecules can be accommodated in the outer layer without any major supramolecular rearrangement. The initial shift responses of the two choline resonances may be compared with reported effects of size and temperature on vesicles. Hence, it seems that drug intercalation into the outer layer induces a choline NMR shift there, similar to that experienced in the inner layer if the size of the vesicle decreases, or a phase transition to a gel state takes place. The inner signal becomes sensitive to drug addition at high concentration, when a downfield shift sets in for the inner choline heads.

^{1}H-NMR spectra of the same character as that demonstrated in fig. 2 have been indicated with the amphiphilic fluorescence probe 1-anilino-8-naphthalenesulfonate (ANS) interacting with vesicles [14]. An upfield shift of the (unresolved) choline signal from egg lecithin vesicles has also been reported with benzyl alcohol [15] and indole-3-acetic acid [16], and claimed to be due to ring currents. Nuclear Overhauser effect (NOE) measurements [17] have indicated that the phenothiazine ring of chlorpromazine is located near the α-methylenes of the fatty acid chains. Hence, the observed upfield chemical shift at the exterior surface (fig. 3) may be induced, at least partly, by the average ring currents of the intercalated aromatic drugs. The same mechanism should then also be responsible for the observed upfield shifts of the α-CH_2CO acyl protons.

When the outer layer becomes saturated and laterally compressed, translocation of molecules between the membrane layers becomes more likely. The intensity ratio between the outer and inner choline proton signals was about 1.9 for vesicles in the absence of drugs (fig. 2a). This ratio was reduced to only about 1.1 (with due consideration of the problem of locating the baseline), when a maximum proton shift separation was caused by the drug (cf. fig. 2e). Mere dilution of the vesicle lipid surface concentration by the presence of drug molecules should not affect the original intensity ratio. From the NMR spectral features there is no evidence for a major increase in particle size upon drug exposure. However, the ^{31}P-NMR lineshape is expected to be symmetrical from vesicles with a radius as large as 150 nm [18].

From ^{1}H-NMR experiments it was claimed that when adding chlorpromazine [19] and tetracaine [20] to egg lecithin vesicles, containing Pr^{3+} inside, the drugs rapidly pass the bilayer by transverse diffusion. We have instead applied ^{31}P-NMR to observe any displacement of Eu^{3+} on the inside of the vesicles. No evidence for competition between the shift-ion and tetracaine was observed over several hours. A similar conclusion was reported [21] from displacement experiments with Pr^{3+} and a tetracaine/lipid ratio of 1 : 2. A change in the shift, caused by the interior paramagnetic ions, is not necessarily an effect due to the presence of drug molecules having diffused into the inner layer. ^{31}P-NMR indicates that the binding characteristic of Eu^{3+}, even in the absence of drug, is not identical at the two sides of the vesicle. Hence, the evidence for transverse diffusion ('flip-flop') of the drug based on paramagnetic shift suppression will be obscured by any changes in ion affinity and spectroscopic parameters of the head groups.

Recently, it has been reported [22,23] that the uptake of the cationic forms of dibucaine and chlorpromazine into large unilamellar egg lecithin vesicles requires a transmembrane potential (interior negative) established by K^+ diffusion. This is interesting since it is a common opinion that local anesthetics exert their nervous effects in the cationic form on the inside of the excitable cells.

In this work fabricated vesicles were strained by drugs, which affected the intrinsic properties of the head group regions in the membrane. Small unilamellar vesicles can withstand high concentrations of charged amphiphilic (lipophilic) drugs. At extreme drug concentrations vesicles, as well as multilamellar liposomes, probably become converted into small mixed micelles, which should be further characterized with respect to existence and supramolecular structure.

4.2. *Effects of anions*

The phosphatidylcholine (egg lecithin) used is in a zwitterionic form and should be electrically neutral in the absence of amphiphilic drugs. The electrophoretic mobility of liposomes was practically zero, or had just a small mobility representing a hardly significant negative ζ-potential. We have previously suggested that any anion effects are weak in the case of pure phosphatidylcholine vesicles [6]. The amphiphilic drugs studied here are all in the cationic form below neutral pH values, with reported pK_a values between 8.2 (tetracaine) and 9.5 (propranolol). A positive surface (ζ)-potential is created with the membrane by drug adsorption, as measured by microelectrophoresis [7]. An enhanced anion contribution should occur when the membrane surface becomes electrically charged by adsorption (binding) of cations.

In the present work the chemical specificity in any anion effects was not unravelled, and the study was restricted to the biologically relevant Cl^-. Since $^{35}Cl^-$ is a quadrupolar nucleus ($I = 3/2$) one can use its NMR linewidth at half height ($\Delta\nu_{1/2}$) as an assay for any interaction of the ion with membrane particles. Assume for simplicity that only two sites, in rapid chemical exchange, contribute to a composite linewidth according to

$$\Delta\nu_{1/2} = \Delta\nu_f p_f + \Delta\nu_m p_m = \Delta\nu_f(1 - p_m) + \Delta\nu_m p_m$$

where p_f and p_m are the probabilities that Cl^- is free in solution or 'bound' in the membrane vicinity, respectively. Since the population of free Cl^- in the bulk phase dominates ($p_m \ll p_f$), a single line with a Lorentzian shape is observed. When $p_m \ll 1$ the broadening of the linewidth is governed by

$$\delta = \Delta\nu_{1/2} - \Delta\nu_f \simeq \Delta\nu_m p_m$$

where $\Delta\nu_f$ is the width for a blank sample ($p_m = 0$). The linewidth of the bound Cl^- is expected to be several orders of magnitude larger than for the free width. It may be expressed in the extreme narrowing case as

$$\Delta\nu_m = \frac{2\pi}{5}\left[\frac{e^2qQ}{h}\right]^2 \tau_c(1 + \eta^2/3)$$

where (e^2qQ/h) is the quadrupole coupling constant and q the electric field gradient at the 'membrane site'. τ_c is the correlation time for the field fluctuation and η the asymmetry parameter. Unfortunately, it is not possible from the NMR experiment to separate the effect of the coupling constant from that of the correlation time.

Increase in the temperature narrowed the $^{35}Cl^-$ signal, measured in the presence of vesicles charged by drug adsorption. This indicates that the $^{35}Cl^-$ linewidth was dominated by the quadrupole relaxation, as determined by τ_c. The temperature experiment with dipalmitoylphosphatidylcholine vesicles in equilibrium with a drug was more complex, since other parameters may depend on the phase transition. Below the temperature corresponding to the phase transition of the pure lipid (41° C), $\delta > 0$. This indicates that the vesicles are positively charged due to drug adsorption, in agreement with experiments with microelectrophoresis. Above the phase transition temperature an increased surface (ζ)-potential was observed as a result of a higher drug adsorption to liposomes in the liquid crystalline state, compared to the gel state.

That the $^{35}Cl^-$ NMR linewidth is really affected by anions interacting with a positively

charged membrane surface is demonstrated in fig. 6. When the surface charge, due to bound tetracaine, is neutralized by raising the bulk pH value above the pK_a value of the drug, the linewidth drops to a value even somewhat below that measured in the absence of the drug. The effect is reversible when lowering the pH value. It seems that the vesicles exposed to pH values above 10 acquire a slightly negative charge, and then repel Cl^-. A related effect we have also observed with a cationic amphiphilic surface potential probe, which was observed to bind more tightly to phosphatidylcholine vesicles at pH around 10 [6].

Several variables may influence the fraction (p_m) of Cl^- contributing to the line broadening. Equilibrating the vesicles to an increasing total concentration of drug creates a growing surface charge density. By increasing the vesicle (lipid) concentration, at a given drug concentration, more molecules become adsorbed. The surface charge density should increase, but concomitantly the surface concentration of the drug becomes diluted. The first effect dominates, provided the membrane is not saturated. Hence, $\Delta\nu_{1/2}$ grows with lipid concentration (fig. 5a). A dual effect is also expected in the experiment where the total NaCl concentration is varied (fig. 5b). Higher salt concentration promotes the drug adsorption according to the diffuse double layer theory. However, the contribution due to the electrostatic effect is masked by the ionic dilution, which reduces p_m. This means that the strongest enhancement in $\Delta\nu_{1/2}$ is seen at low salt concentration.

This study has convincingly demonstrated that the $^{35}Cl^-$ line-broadening effect is closely correlated to the adsorbed drug cations. From our earlier studies [6,7] we have indirectly found that the anion effect cannot be properly accounted for by the Gouy-Chapman theory alone. Since the anion effect is specific it can most easily be described by a weak binding constant following the order $Cl^- < Br^- < NO_3^-$. With liposomes of dimyristoylphosphatidylcholine in solutions of various monovalent potassium salts, specific anion adsorption was reported from studies of electrophoresis [24]. With egg lecithin vesicles, anion dependent *N*-methyl choline shifts were observed by ^{1}H-NMR [25]. The anion 'counterion' effect will influence the drug binding in a synergistic manner due to the electrostatic coupling between the two ions. The NMR active $^{35}Cl^-$ are attracted by surface charges from drug moieties. The specifically bound anions are located in a strong electric field gradient. Although the fraction of those ions is small it should govern $\Delta\nu_{1/2}$, if a rapid exchange exists.

Assuming that relaxation parameters (e.g., τ_c and η) in the $^{35}Cl^-$ line-broadening mechanism are the same for the various amphiphilic drugs, δ can be used as a measure of the surface concentration of the drug. Hence, the hydrophobicity of the drugs should follow the order propranolol = tetracaine < imipramine < dibucaine < chlorpromazine (fig. 4). In the absence of vesicles (blank solution) the drugs have only a small effect on $\Delta\nu_{1/2}$. A remarkable exception is chlorpromazine, which produces a dramatic increase in the $^{35}Cl^-$ linewidth above 7 mM drug. In the presence of vesicles, chlorpromazine does not cause the same abrupt increase in $\Delta\nu_{1/2}$. Evidently, the $^{35}Cl^-$ linewidth reflects the micellar properties of chlorpromazine itself. The critical micelle concentration (CMC) has been reported to be 7.0 mM in 0.1 M NaCl, with an aggregation number of 35 [26]. The possibility cannot be excluded that micelles partially contribute to $\Delta\nu_{1/2}$ at high chlorpromazine concentration, even in the presence of vesicles. The CMC for tetracaine has been reported to be about 70 mM [27], and it is likely that the rest of the drugs studied here also exhibit high CMC values.

Experimentally, the $^{35}Cl^-$ NMR study is straightforward. However, a quantitative analysis of the present results is hampered by the interdependence of the linewidth parameters, as well as the complexity in the interfacial phenomena. Nevertheless, this study has unambiguously demonstrated the participation of the anions upon binding of drug cations. The effect of anions, including the electrostatic contribution, should therefore be considered when deriving intrinsic binding constants of ions to a membrane.

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