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CHARGE AND pH DEPENDENT DRUG BINDING TO MODEL MEMBRANES

A ^{2}H-NMR AND LIGHT ABSORPTION STUDY

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The binding of the local anesthetics tetracaine and procaine to model membranes of egg phosphatidylcholine and bovine phosphatidylserine has been studied by ^{2}H-NMR and light absorption. Dispersions of drug-lipid mixtures in 0.1 M NaCl were centrifuged and the concentration of drug in the supernatant was measured by ultraviolet light absorption. Several freeze-thaw cycles of the sample were used before centrifugation to facilitate equilibration of the drug between the bilayers. Binding curves for the drug were obtained as a function of pH. The results were simulated by a theoretical model based on the Gouy-Chapman theory, in which both the charged and the uncharged forms of the drug, and the equilibrium between them, were included. Two deuterated forms of the drugs, [$^2H_6$]tetracaine and [$^2H_4$]procaine, were used for the ^{2}H-NMR experiments. In most cases the ^{2}H-NMR spectrum contained a broad central resonance and an underlying quadrupolar pattern. However, after five freeze-thaw cycles only a single broad resonance was observed under most conditions. Particle size measurements showed that freeze-thawing resulted in a more uniform population of liposomes of smaller average diameter than those obtained by simple vortex mixing. The single broad resonance observed in both cases is interpreted as due to rapid exchange of the anesthetic between lipid and bulk solution. In the absence of freeze-thawing, the quadrupolar pattern is attributed to anesthetic species in exchange with only a limited amount of water. The data suggest that a true equilibrium between lipid, water and anesthetic is only attained after freeze-thawing.

1. Introduction

Many biological processes involve reactions at the surface of, or within, cell membranes. Explanation of these functions in structural terms requires a physico-chemical understanding of the various interactions taking place. In many cases the processes concern small amphiphilic molecules with pK values close to physiological pH, and therefore both charged and uncharged forms may interact with the membranes.

As a simple model system we have studied the interactions between the local anesthetics procaine and tetracaine, and aqueous dispersions of the neutral lipid phosphatidylcholine (PC) and the negatively charged mixture of phosphatidylcholine and phosphatidylserine (PS), in 0.1 M NaCl at various pH values. The binding was followed by determination of the drugs through their ultraviolet light absorption in supernatants of centrifuged samples and by ^{2}H-NMR of deuterated

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Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; DPPC, dipalmitoylphosphatidylcholine.

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forms of the drugs directly in the lipid-water mixtures. These techniques have already been used by Boulanger et al. [1] on the same type of system. Here we try to fit the experimental results within a theoretical framework of a general character which should be applicable to many similar systems.

The theory used is based on the Gouy-Chapman treatment of the electrical double layer in which the surface charge density and surface potential, and their relation to the ionic composition of the solution, are the main features. The effect of the surface potential has been considered previously by McLaughlin [2,3] and Lee [4,5] in model studies with phospholipids and drugs. McLaughlin and Harary [6] have used the so called Stern equation, which essentially is based on the Gouy-Chapman theory, to describe the adsorption of the amphiphilic ion 2,6-toluidinylnaphthalenesulphonate to model membranes. One of us has used this approach to describe the competitive binding of the charged forms of some drugs with a positively charged spin label in vesicles of phosphatidylcholine [7].

The theoretical approach to describe the binding of an uncharged and charged form of an amphiphile with an acidic or basic function to a membrane surface was outlined by Lee [4] and applied to the binding of a number of drugs to model membranes of the uncharged lipid dipalmitoylphosphatidylcholine (DPPC). However, Lee used an indirect technique to measure the binding and specific assumptions had to be made. His results differ in some respects from those of this and an earlier study [7].

In the earlier study using the present experimental techniques [1], the samples were centrifuged after vortex mixing. However, we have found that true equilibrium with all bilayers of the liposomes so formed is probably not reached. We have tried several methods to reach equilibrium, such as increasing vortex time, increasing the number of centrifugations, letting the sample stand overnight and freeze-thawing the sample several times. We found that the best method to achieve equilibrium is a series of repeated freeze-thaw cycles. This also results in a more uniform distribution of liposome sizes. The results obtained after freeze-thawing may be simulated using a theoretical model based on the Gouy-Chapman theory with different binding constants for the charged and the uncharged forms of the drugs. The same set of binding constants could be used for both the neutral PC and the negative PC/PS bilayers.

2. Materials and Methods

Egg yolk phosphatidylcholine (Grade I) and bovine phosphatidylserine (Grade I) were purchased from Lipid Products (South Nutfield, Surrey, U.K.) and used without further purification. The HCl salts of procaine and tetracaine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Deuterium-depleted water was purchased from Aldrich Chemical Company Inc. (Milwaukee, WI, U.S.A.). The deuterated forms of the drugs [$^2H_4$]procaine and [$^2H_6$]tetracaine (Fig. 1) were synthesized according to Boulanger and Leitch [8]. Lipid concentration was determined by phosphorus analysis [9] or by weighing.

Ultraviolet light absorption measurements were made on Cary 14, Beckman DB or Aminco DW-2 spectrophotometers. pH was measured with a glass electrode on a standard pH-meter. Centrifugations were done at 48000 × g on a Sorvall RC2B centrifuge for 30 min at 30°C. ^{2}H-NMR experiments were performed on a Varian XL-100 NMR spectrometer (15.35 MHz) with an external proton field-frequency lock and an internal standard of C^2HCl_3, or on a Bruker CXP 300 NMR spectrometer (46.1 MHz) without lock or internal standard, and with quadrature detection. All ^{2}H-NMR experiments were performed at 30°C, with a standard pulse sequence, and all samples were prepared with deuterium-depleted water. ^{31}P-NMR

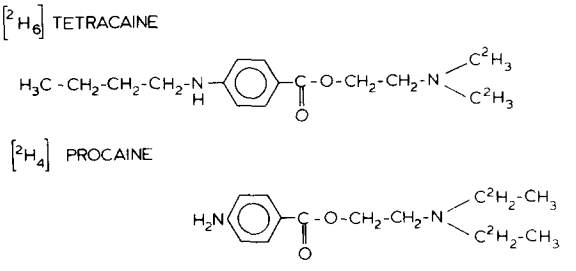

Fig. 1. The deuterated molecules used in the ^{2}H-NMR experiments.

experiments were performed on a Varian XL-100 NMR spectrometer (40.48 MHz) at 30°C. An Elzone particle counter (Particle Data Inc.) was used to determine the size distribution of the liposomes.

Lipid samples were prepared as follows. Lipids in chloroform were added to glass centrifuge tubes and the chloroform was evaporated with N_2 or Ar. The remaining solvent was removed by vacuum pumping overnight. The drug dissolved in 0.1 M NaCl was added to the dried lipids. No buffer was used. The tubes were then shaken in a vortex mixer until all lipid was dispersed. pH was adjusted with 1.0 M NaOH or 1.0 M HCl and the sample was vortexed again. The total vortex time was usually 6–7 min. Freeze-thawing experiments were made in the following way: the sample was frozen in a dry ice/ethanol mixture and thawed in lukewarm water; usually this was repeated five times with vortexing between each cycle.

3. Theory and simulation of drug binding to a phospholipid bilayer

The experimental results were simulated by solving the system of equations below. The treatment of the influence of surface potential is similar to that used earlier for the binding of lanthanide ions to vesicles of PC [10,11], and for the binding of charged forms of drugs at low pH to vesicles of PC [7], and also outlined earlier by Lee [4]. We assume that the drug binds to the lipid surface according to a simple partition equilibrium, and that the potential on the surface created by the surface charge and by the ionic composition of the water phase (bulk phase) is given by the Gouy-Chapman theory.

The acid base equilibria for the drug D in the bulk phase (B) is

$$DH^+(B) \rightleftarrows D(B) + H^+(B) \tag{1}$$

The partition equilibria for the uncharged and charged forms of the drug are

$$D(I) \rightleftarrows D(S) \tag{2}$$

$$DH^+(I) \rightleftarrows DH^+(S) \tag{3}$$

where I denotes interface, i.e. the water phase in the immediate vicinity of the surfaces S. The acid base equilibrium for the drug on the surface is

$$DH^+(S) \rightleftarrows D(S) + H^+(S) \tag{4}$$

The relation between interphase and bulk phase concentrations of the charged form is given by the Boltzmann equation

$$[DH^+]_I = [DH^+]_B e^{-\frac{F\psi}{RT}} \tag{5}$$

where ψ denotes surface potential, F is the Faraday constant, R the gas constant and T the absolute temperature ($RT/F = 26.1$ mV at 30°C).

The mass action laws for Eqns. 1–4 are:

$$K_1 = \frac{[D]_B \cdot [H^+]_B}{[DH^+]_B} (\text{mol/l}) \tag{6}$$

$$K_2 = \frac{[D]_S}{[D]_I} (1/\text{Å}^2) \tag{7}$$

$$K_3 = \frac{[DH^+]_S}{[DH^+]_I} (1/\text{Å}^2) \tag{8}$$

$$K_4 = \frac{[D]_S \cdot [H^+]_S}{[DH^+]_S} (\text{mol/Å}^2) \tag{9}$$

The relation between $[H^+]_S$ and $[H^+]_I$ can be treated in a formal way as

$$K_S = \frac{[H^+]_S}{[H^+]_I} (1/\text{Å}^2) \tag{10}$$

The Boltzmann equation should also be applied to the protons and gives

$$[H^+]_I = [H^+]_B e^{-\frac{F\psi}{RT}} \tag{11}$$

The mixture of surface and volume concentrations used above have been discussed earlier [10,11]. The surface charge density created on the surface is given by

$$\sigma = Z_0 + N \cdot [DH^+]_S \, (\text{electronic charges/Å}^2) \tag{12}$$

where Z_0 is an initial surface charge density and N is the Avogrado number.

The relation between surface charge density, surface potential and the ionic composition of the bulk phase is given by the Grahame equation

$$\sigma = \pm \left\{ 2\epsilon\epsilon_0 RT \sum_i [i]_B \left(\exp\left(-\frac{z_i F \psi}{RT} \right) - 1 \right) \right\}^{1/2} \tag{13}$$

where $(2\epsilon\epsilon_0 RT)^{-1/2} = 274.0$ at 30°C.

The summation should run over all ionic species in the bulk phase. The relation between bulk concentration and surface concentration can be deduced using

$$v \cdot [P]_O = s \cdot [P]_{OS} \tag{14}$$

The small difference (< 5%) between the total sample volume and the water volume has been neglected in the calculation. In Eqn. 14 $[P]_O$ denotes total lipid concentration in mol/l, v total sample volume in litres, $[P]_{OS}$ lipid surface concentration in mol/Å^2 and s total lipid surface in Å^2. If we assume that the area per phospholipid molecule is 70 Å^2 we get $[P]_{OS} = (70\,N)^{-1}$ mol/Å^2. The amount of drug in the bulk phase can then be corrected for that bound by using

$$v \cdot [D]_O = v \cdot [D]_B + v \cdot [DH^+]_B + s \cdot [D]_S + s[DH^+]_S \tag{15}$$

$[D]_O$ is total drug concentration in mol/l. The bulk concentration is assumed to be constant all the way into the surface in this formula (Eqn. 15), i.e. the difference between the drug concentration in the double layer and the bulk is neglected. This assumption can in the present case lead to a maximal error in $[DH^+]_B$ of 2% for the PC case and 14% for the PC/PS case.

Using Eqn. 14 one gets

$$\frac{[D]_O}{[P]_O} = \frac{[D]_B}{[P]_O} + \frac{[D]_S}{[P]_{OS}} + \frac{[DH^+]_B}{[P]_O} + \frac{[DH^+]_S}{[P]_{OS}} \tag{16}$$

All relations were expressed in this dimensionless form for the calculations.

For Eqn. 12 we obtain for example

$$\sigma = Z_0 + \frac{[DH^+]_S}{[P]_{OS}} \cdot \frac{1}{70} \tag{17}$$

From Eqns. 7 and 8 we have found it practical to calculate the partition coefficients expressed in l/mol by the conversions

$$K_2^\star = \frac{K_2}{[P]_{OS}} \text{ (l/mol)} \tag{18}$$

$$K_3^\star = \frac{K_3}{[P]_{OS}} \text{ (l/mol)} \tag{19}$$

It is easily shown that

$$K_4^\star = \frac{K_4}{K_S} = \frac{K_2^\star \cdot K_1}{K_3^\star} \quad \text{(mol/l)} \tag{20}$$

noting that the dissociation constant K_1 is valid also at the interface. Hence, knowing K_1 from separate experiments and inserting values for $K_2^\star$ and $K_3^\star$, it is easy to solve the total equation system on a computer. $K_2^\star$ and $K_3^\star$ are adjusted to give a best fit to the experimental curves.

According to Eisenberg et al. [12] and McLaughlin et al. [13] sodium ions bind to PS liposomes. We have thus introduced into the formalism a binding of sodium ions to the liposomes to compete with the binding of the drug, using the same binding constants as Eisenberg et al. [12]. The sodium ion binding is supposed to take place only to PS^- molecules according to a Langmuir adsorption isotherm.

$$Na^+ + PS^- \rightleftarrows NaPS \tag{21}$$

$$K_x = \frac{[NaPS]_S}{[Na^+]_1 \cdot [PS^-]_S} \tag{22}$$

In Eqn. 22 $[PS^-]_S$ represents all the PS molecules that have no sodium ion bound, i.e. also the small amount of PS^{2-} formed at higher pH values.

The binding of the sodium ions will give a contribution to the total surface charge according

to

$$Z_{tot} = Z_0 + \frac{[DH^+]_S}{[P]_{OS}} \cdot \frac{1}{70} + \frac{[NaPS]_S}{[P]_{OS}} \cdot \frac{1}{70} \quad (23)$$

where

$$[P]_{OS} = [PS]_{OS} + [PC]_{OS} \quad (24)$$

Z_0 is set to zero for the case of pure PC and to

$$Z_0 = -\frac{1}{70}\left(\frac{[PS^-]_S}{[P]_{OS}} + 2\frac{[PS^{2-}]_S}{[P]_{OS}}\right) \quad (25)$$

for the case of PC/PS. With 50% PC and 50% PS one gets

$$Z_0 = -\frac{1}{70}\left(0.5 + \frac{0.5}{(10^{(pK_a - pH_B)} + 1)}\right) \quad (26)$$

where K_a is the equilibrium constant for the reaction $PS^- \rightleftharpoons H^+ + PS^{2-}$ on the surface. The value used for pK_a was 10.0 [14]. In Eqn. 26 pH_B is used instead of pH_I as the investigation in Ref. 14 used the bulk pH for the determination of the pK_a value. In Ref. 14 another ionic strength and surface charge density was used and our application of their pK_a value is thus not strictly correct. However, pK_a is certainly close to 10.0 and the error will anyway be very small in our simulations.

TABLE I

EQUILIBRIUM CONSTANTS USED FOR THE SIMULATIONS OF FIGS. 2(a and b) AND 3(a and b)

The K_2^* values for the uncharged form of the drug can be directly compared with the partition coefficient K_p of Ref. 1 using the formula $K_p = K_2^* \, 1000/MV_L \approx 1.1 \cdot K_2^*$, where MV_L is the molecular weight of the lipid. The K_3^* values are not directly comparable with the K_p values for the charged form of the drug of Ref. 1 as the influence of the surface potential is included in this investigation but not in Ref. 1.

Equilibrium constant	Tetracaine	Procaine
K_1 [a] (mol/l)	$1 \cdot 10^{-8}$	$1 \cdot 10^{-9}$
K_2^* [b] (l/mol)	350	65
K_3^* [c] (l/mol)	60	1.5
K_4^* [d] (mol/l)	$1 \cdot 10^{-7.23}$	$1 \cdot 10^{-7.36}$
K_x [e] (l/mol)	0.6	0.6

[a] Dissociation constant for the drug in water.
[b] Binding constant for the uncharged form of the drug.
[c] Binding constant for the charged form of the drug.
[d] Dissociation constant for the drug in the membrane determined through the simulations.
[e] Binding constant for the sodium ions to phosphatidylserine.

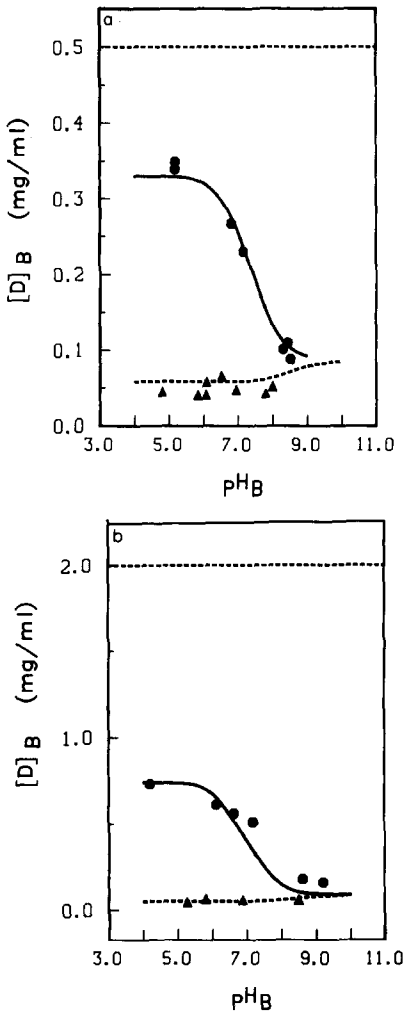

Fig. 2. Drug concentration $[D]_B$, in the supernatant as a function of pH_B. Five freeze-thaw cycles were performed on each sample before centrifugation. The binding constants in Table I were used for the simulations. The total amount of drug added is indicated by the straight horizontal dashed line. The medium was 0.1 M NaCl. (a) 0.5 mg/ml tetracaine with 10 mg/ml PC, ultraviolet absorption results (●) and simulation (————); or with 5 mg/ml PC+5 mg/ml PS, ultraviolet absorption results (▲) and simulation (- - - - -). (b) 2.0 mg/ml tetracaine with 50 mg/ml PC, ultraviolet absorption results (●) and simulation (————); or with 25 mg/ml PC+25 mg/ml PS, ultraviolet absorption results (▲) and simulation (- - - - -).

In the case of PC there will most likely be a binding of chloride ions to either the PC molecules or to the drug molecules already bound, as discussed earlier [10,11]. Since this binding is rather weak, and as we do not know the binding constant, we have not introduced this into our equations.

The same sets of constants (Table I) were used to simulate the binding of the anesthetics to either PC or PC/PS. The K_1 values are those determined experimentally, $K_2^\star$ and $K_3^\star$ are found from the simulations, and $K_4^\star$ is calculated from K_1, $K_2^\star$ and $K_3^\star$ via Eqn. 20. The values of the binding constants that were used for the simulations are compromises to give a best fit to all four experimental curves for tetracaine (Fig. 2) and procaine (Fig. 3), respectively. For tetracaine a deviation of ± 0.05 mg/ml at pH = 4.0 to the simulated curve for the PC case in Fig. 2b is achieved by choosing $K_3^\star$ between 50 and 70 mol/l and the same deviation at pH = 9.0 is achieved by choosing $K_2^\star$ between 225 and 750 mol/l. For the procaine/PC case in Fig. 3b a deviation of ± 0.1 mg/ml at pH = 4.0 is the result of changing $K_3^\star$ from 0.85 to 2.3 mol/l, and at pH = 9.0 the same deviation is produced by varying $K_2^\star$ from 58 to 73 mol/l. A binding constant of $K_x = 0.6$ for sodium to PS was used. For both tetracaine and procaine different values of the binding constants have been used for the charged ($K_3^\star$) and the uncharged ($K_2^\star$) forms. The results of the various simulations are shown in Figs. 2, 3.

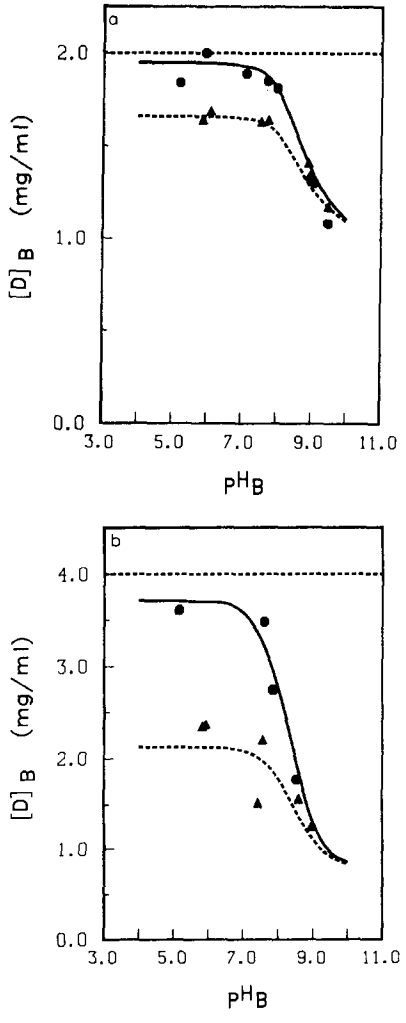

Fig. 3. The same conditions as for Figs. 2a and 2b but procaine was used instead of tetracaine. (a) 2.0 mg/ml procaine and (b) 4.0 mg/ml procaine.

4. Experiments

4.1. Dissociation and solubility of the drugs

The solubility of both drugs in 0.1 M NaCl was determined by measuring the concentration of drug in the supernatant after centrifugation of equilibrated mixtures for 30 min at $48000 \times g$ at 30°C. The solubility is known to decrease considerably with increasing pH [15]; at pH 9.5 in 0.1 M NaCl it was 0.35 mg/ml for tetracaine and 4.0 mg/ml for procaine. In all subsequent experiments the total concentration of drug was selected to ensure that the aqueous concentration was below saturation.

The pK_1 value is highly concentration-dependent for tetracaine, and slightly so for procaine [15,16]. Plausible explanations for this would be the low solubility of the uncharged forms of the drugs, as weil as micelle formation. For the simulations we chose $pK_1 = 8.0$ for tetracaine and $pK_1 = 9.0$ for procaine. Those pK_1 values correspond to those for the lowest aqueous concentrations of the drugs used in the experiments.

4.2. Determination of the partition equilibria between drug and liposomes as a function of pH by light absorption after centrifugation

The partition equilibrium for the drug between water and lipid was studied by measurement of the

drug concentration by ultraviolet light absorption in the supernatant of samples vortexed or vortexed and freeze-thawed before centrifugation. In the less concentrated lipid dispersions at low and medium pH the supernatant was usually rather clear and sampling was simple. However, for the more concentrated lipid dispersions the pellet was sometimes not so well defined and sampling became less accurate since the supernatant was easily contaminated by lipids from the pellet. There was always some lipid in the supernatant, probably in the form of small liposomes (vide infra).

When necessary the ultraviolet absorption spectra were corrected for the light scattering background. The drug bound to the contaminating lipids will lead to an overestimate of the actual drug concentration in the aqueous phase. To estimate this error the lipid concentration in the supernatant was measured by phosphorus analysis. For procaine the error was negligible in all cases, because of the relatively low binding of procaine to the lipids; for tetracaine it was found to be a few percent at the lower lipid concentration at low pH and about 10% for the higher lipid concentration at low pH. At high pH the error in the case of tetracaine could be a factor of two, due to a very low aqueous concentration of drug (Fig. 2b).

pH was measured after the final centrifugation. For $pH < pK_1$, the pH values before and after centrifugation were always very similar. However, if before centrifugation the pH was adjusted to $> pK_1$, the pH after centrifugation was often of the order of one unit lower. This is what one would expect when more of the drug molecules in the uncharged form bind to the membrane and no buffer is used. To test whether the procedure above resulted in a stable partition equilibrium of the drug between water and lipid phase, the procedure was modified in several ways. The number of repeated vortexing-centrifugation cycles (up to 6) or the length of the vortexing period (1 to 8 min) had no significant effect on the drug concentration in the supernatant, whereas equilibration for 24 h at 20°C before the final centrifugation had a small but significant effect. However, when the sample was freeze-thawed several times before centrifugation a strong effect was observed. In the more concentrated lipid dispersions the drug concentration in the supernatant decreased with the number of freeze-thawings for both the charged and the uncharged membranes, the strongest effect being for the charged ones. After approximately five freeze-thaw cycles the concentration was stabilized. The most pronounced difference between the various treatments was found at low pH. At high pH all the treatments gave roughly the same results.

The samples taken for ultraviolet absorption analysis were always diluted in buffer of pH = 5 immediately after sampling. This was done to ensure well defined conditions during the ultraviolet absorption measurements, as the ultraviolet absorption spectra of the drugs are slightly pH dependent, and also to minimize hydrolysis [17]. The absorption maxima were at 310 nm for tetracaine and at 290 nm for procaine. Concentration was calculated by reference to a standard sample of the anesthetic.

4.3. Investigation of the lipid-drug system using ^2H-NMR

For the ^{2}H-NMR experiments the deuterated forms [$^2H_6$]tetracaine and [$^2H_4$]procaine of the drugs (Fig. 1) were used. These samples were prepared in the same way as those in the centrifugation experiments described above. After the centrifugation the sample was vortexed and examined in the NMR spectrometer. To conserve deuterated material each sample was used for measurement at up to three pH values.

In one type of experiment [$^2H_4$]procaine/lipid dispersions were examined in the XL-100 spectrometer. A pulse delay of 2.0 s was used (T_1 was approximately 0.1 s). A single line was the only signal seen, the intensity of which was determined by comparison of the area under the line with that of an external standard of C^2HCl_3, previously calibrated versus an aqueous drug sample of known concentration. This was done at several pH values with phosphatidylcholine liposomes. The results, Fig. 4, demonstrate that there is a close correlation between the area under the single line and the concentration of bulk drug determined by ultraviolet light absorption.

Most of the experiments were performed on a Bruker CXP 300 spectrometer which has the capability to observe broad resonances. A pulse delay of 0.75 s was used. In addition to the broad central

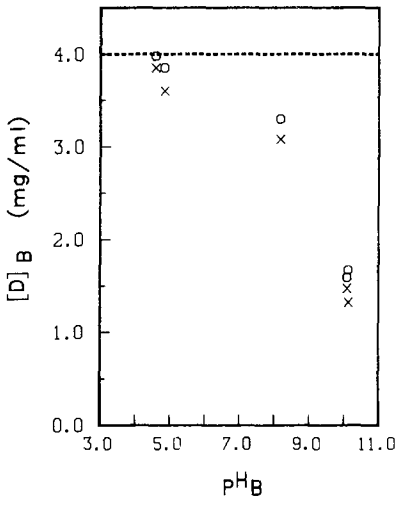

Fig. 4. Comparison of [$^2H_4$]procaine concentration in the aqueous bulk phase determined by ^{2}H-NMR (15.35 MHz) and by ultraviolet light absorption as a function of pH. 4.0 mg/ml [$^2H_4$]procaine + 20 mg/ml PC in 0.1 M NaCl was used. ^{2}H-NMR results (×) and ultraviolet absorption results (○).

resonance, a quadrupole pattern was usually observed. The decomposition of the spectra into 'central resonance' and 'quadrupole resonance' components is somewhat arbitrary and was made with a slightly curved line at the foot of the central resonance. The total areas under the signals were determined by integration in the spectrometer computer, and the relative areas under the central resonance and the quadrupole pattern, respectively, were determined by cutting and weighing. No internal or external standard could be used under the experimental conditions. The total area was found to be independent of pH. This indicates that all deuterated species are observed. The amount of the drug corresponding to the central resonance was calculated in proportion to the total amount present.

Figs. 5 and 6 show spectra of dispersions which were examined in the CXP 300 after one centrifugation. Spectra were taken at pH $< pK_1$, at pH close to pK_1 and at pH $> pK_1$ (pK_1 values refer to the respective drug). Spectra were also taken on the same samples after 24 h under N_2 which did not differ much from those in Figs. 5 and 6. In Fig. 7 results from the ultraviolet absorption determinations of the drug concentration in the su-

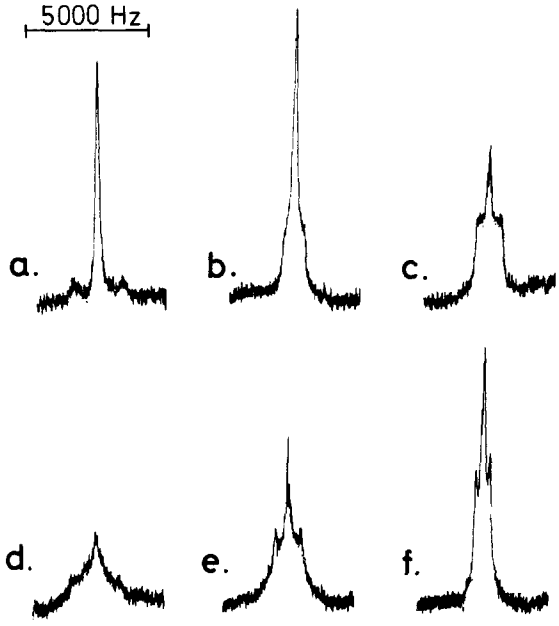

Fig. 5. Typical ^{2}H-NMR spectra (46.1 MHz) of 2.0 mg/ml [$^2H_6$]tetracaine in lipid dispersions in 0.1 M NaCl after one centrifugation and revortexing, with 50 mg/ml PC, pH $< pK_1$ (a), pH $\approx pK_1$ (b), and pH $> pK_1$ (c); or with 25 mg/ml PC + 25 mg/ml PS, pH $< pK_1$ (d), pH $\approx pK_1$ (e), and pH $> pK_1$ (f). K_1 is the dissociation constant for the drug in water.

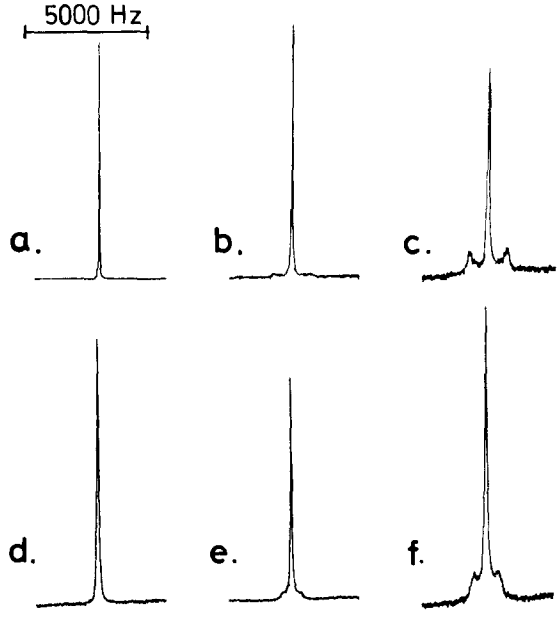

Fig. 6. The same conditions as in Fig. 5 but 4.0 mg/ml [$^2H_4$]procaine was used instead of [$^2H_6$]tetracaine.

pernatant of vortexed and centrifuged drug-lipid dispersions are compared with an estimation of the drug concentration corresponding to the central resonance of the ^{2}H-NMR spectra.

Fig. 7a shows a correlation between ultraviolet absorption and NMR data for [$^2H_6$]tetracaine for both the PC and the PC/PS case, indicating that the central resonance corresponds mainly to the water phase concentration of drug molecules and that hence the broad quadrupole pattern mainly corresponds to the population of drug molecules which are bound to the membrane phase. For [$^2H_4$]procaine both Fig. 4 and Fig. 7b show that for the PC case there is a close correlation between NMR and ultraviolet absorption data. For the PC/PS case however, some of the procaine is membrane bound at low pH according to the ultraviolet absorption measurements, but no quadrupole pattern is observed in the ^{2}H-NMR spectra at low pH. As the pH is increased, a quadrupole pattern appears with both PC and PC/PS, and at higher pH values there is a good correlation between ^{2}H-NMR and ultraviolet absorption data in both cases.

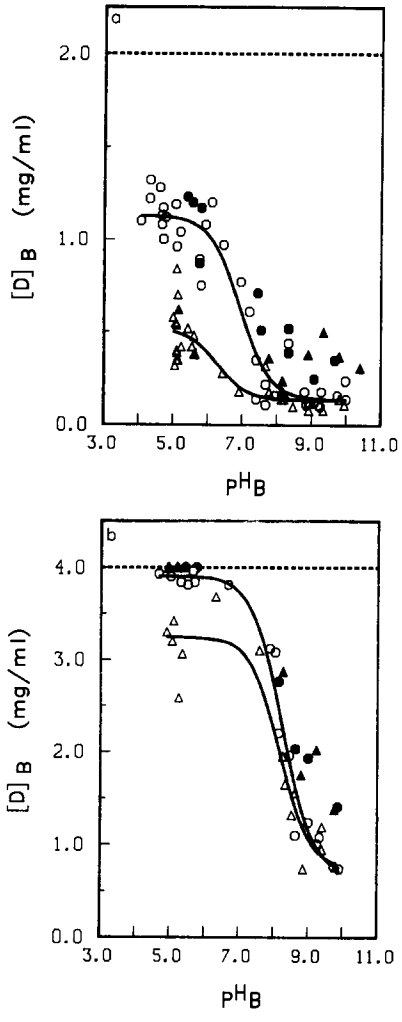

Fig. 7. Comparison of [$^2H_6$]tetracaine and [$^2H_4$]procaine concentrations in the aqueous bulk phase determined by ^{2}H-NMR (46.1 MHz) and by ultraviolet light absorption as a function of pH. The medium was 0.1 M NaCl. The full drawn lines are the results of a non-linear regression fit of the ultraviolet light absorption data to a function of the form $[D]_B = c + (a/(1.0 + 10^{(b-pH_B)}))$, where a, b and c are constants. The total amount of drug added is indicated by the straight horizontal dashed line. (a) 2.0 mg/ml [^{2}H]tetracaine with 50 mg/ml PC, ultraviolet light absorption results (○) and ^{2}H-NMR results (●); or with 25 mg/ml PC + 25 mg/ml PS, ultraviolet light absorption results (△) and ^{2}H-NMR results (▲). (b) The same conditions as in (a) but 4.0 mg/ml [$^2H_4$]procaine was used instead of [$^2H_6$]tetracaine.

TABLE II

QUADRUPOLE SPLITTINGS D_q OF [$^2H_6$]TETRACAINE AND [$^2H_4$]PROCAINE (SEE FIG. 1) IN LIPID DISPERSIONS AT LOW AND HIGH pH

The lipid concentrations were 50 mg/ml PC and 25 mg/ml PC + 25 mg/ml PS, respectively, and the drug concentrations were 2.0 mg/ml tetracaine and 4.0 mg/ml procaine, respectively, in 0.1 M NaCl. The D_q values in the table are mean values from the number of experiments given in the respective parentheses, and the indicated errors are maximal deviations from the mean values.

Drug/lipid dispersions	D_q at low pH (Hz) pH = 5.0–6.0	D_q at high pH (Hz) pH = 8.5–10.5
Tetracaine/PC	1920 ± 80 (5)	910 ± 60 (2)
Tetracaine/PC/PS	1760 ± 30 (3)	720 ± 220 (6)
Procaine/PC	–	1480 ± 100 (4)
Procaine/PC/PS	–	710 ± 240 (5)

Table II shows the quadrupole splittings for the two deuterated drugs, Fig. 1, in the two lipid systems at high and low pH. At high pH the quadrupole pattern of [$^2H_4$]procaine is broader with PC than with PC/PS, whereas at low pH no quadrupole pattern could be detected.

4.4. Characterization of the freeze-thawing process

Several experiments were designed to explore the effect of the freeze-thaw process on the

lipid/water suspensions. The particle counter was used to measure the sizes of the liposome particles in vortexed PC and PC/PS samples both before and after freeze-thawing. Freeze-thawing made the particle size distribution more homogeneous and the average diameters markedly smaller. It was confirmed by ^{31}P-NMR, light scattering and phosphorus analyses of supernatants that the liposomes obtained by freeze-thawing are much larger than sonicated vesicles.

The effect of added $Gd(NO_3)_3$ on the ^{31}P-NMR spectra of the liposomes was studied. Vortexing in presence of $Gd(NO_3)_3$ gave only small effects on the spectra. If, however, $Gd(NO_3)_3$ was present during the freeze-thawing the spectra were extremely broadened which indicates that freeze-thawing facilitates the contact between cations in the water phase and all lipid bilayers.

By sedimentation tests in 50% ^2H_2O and in H_2O it was found that after freeze-thawing 'the lipid phase' reaches a higher density which is close to that of the bulk solution.

^{2}H-NMR measurements were also performed on samples of both drugs in the two types of lipid mixtures before and after freeze-thawing. At high pH the quadrupole pattern disappeared on freeze-thawing to yield a single broad line. At low pH the quadrupole pattern disappeared for tetracaine/PC, but remained for tetracaine/PC/PS. The mean diameters of the liposomes after freeze-thawing, determined by the particle counter to be 5 and 2 μm for PC and PC/PS, respectively, are large enough to give rotational correlation times sufficiently long so that no averaging of tensor elements of either ^{31}P chemical shift or ^{2}H quadrupole coupling would be effected.

It has been shown [15] that, due to exchange between molecules in one state giving rise to a quadrupole pattern and another giving rise to a single line, the resultant spectrum can be a powder pattern of reduced splitting, or a single broadened resonance depending on the rate of the exchange between the two states and their relative populations. The collapse of the quadrupole splitting after freeze-thawing is thus most reasonably explained as due to rapid exchange between lipid bound and free anesthetic molecules.

In the incompletely hydrated, vortexed samples there must exist a variety of lipid environments, varying from complete hydration with exposure to a large volume of bulk solution to low hydration with exposure to much less bulk solution. The former sites would lead to a collapsed quadrupole splitting, whereas the latter ones would yield a quadrupole splitting of reduced magnitude relative to that of the bound drug molecules. The observed reduced quadrupole splitting would thus yield only a lower limit for the order parameter of the lipid bound drug molecules.

Consequently in Figs. 5 and 6 the broad central line is due to a relatively large volume of unbound drug molecules in fast exchange with a relatively small amount of bound drug molecules. The broad quadrupole pattern is due to a large amount of bound drug molecules in fast exchange with a relatively small volume of free drug molecules.

A limiting situation pertains in the case of tetracaine with PC/PS at low pH where the drug concentration in the aqueous phase is very small (Fig. 2b), the quadrupole pattern is hardly affected and the central resonance is much broadened (Fig. 5d).

In conclusion, freeze-thawing induces the formation of liposomes which are smaller, have a more homogeneous size distribution, and are more completely hydrated than liposomes of samples which have been only vortexed.

5. Discussion

The results from the different centrifugation experiments suggest that vortexing and centrifugation alone are not sufficient to ensure equilibration between the aqueous phase and all bilayers at low pH. At high pH all methods of sample preparation give roughly the same result which indicates that the uncharged form of the drug equilibrated more easily with the liposomes than does the charged form. This behaviour is probably due to the fact that the diffusion across the bilayers is faster for the uncharged than for the charged form of the drug. The increased binding of the drugs to the liposomes at low pH, resulting from the freeze-thawing of the dispersions, is probably equilibration through a breaking and reforming of the liposomes. At high pH, where the drug is uncharged, the binding for each drug is nearly the same for both PC and PC/PS. Thus the dif-

ferences in binding to the two lipids at low pH, where the drug is charged, are due to the differences in surface charge of PC and PC/PS. The largest difference between the freeze-thawing experiments and the other type of experiments is for the case of PC/PS at low pH. If the reasoning above, implying that freeze-thawing gives a true equilibrium, is correct, this difference could be explained by an even more difficult diffusion for the charged drug molecules across a negatively charged membrane, possibly as a result of the formation of ion pair complexes between the drug and the PS molecules. The transmembrane motion of the large lipid molecules is known to be very slow. Freeze-thawing has been employed earlier by Cullis and Verkleij [18] to facilitate the equilibration of dibucaine with phosphatidylserine dispersions at pH = 7.0. They concluded that the anesthetic was unable to traverse the outer membrane of the liposomal structure unless the membrane was made leaky by the freeze-thaw procedure.

The ^{2}H-NMR experiments which are shown in Figs. 5 and 6 were performed on samples that were vortexed and centrifuged but not freeze-thawed. Thus according to the arguments above a true equilibrium between the drug molecules and all the bilayers is probably not attained at low pH. Nevertheless, as ultraviolet absorption measurements were done on the same samples that were used for the NMR experiments these results can still be used to qualitatively study the different populations of the drug. Thus the comparison between ^{2}H-NMR and ultraviolet absorption experiments shown in Fig. 4 and Fig. 7a is meaningful. For the case of tetracaine, the ^{2}H-NMR and the ultraviolet absorption results suggest that the quadrupole pattern corresponds to the population of drug that is bound to the lipid phase and is exchanging rapidly with drug in a small volume of water, while the central line corresponds to those drug molecules which are present in the water phase and exchange rapidly with a small amount of lipid bound species. The quadrupole pattern at low pH is wider than that at high pH, indicating a lower degree of ordering in the lipid phase for the uncharged form of the drug provided the same volumes of water are accessible for exchange. ^{2}H-NMR studies of the effect of these anesthetics on the degree of ordering of the fatty acyl chains of PC have indicated that the uncharged forms penetrate more deeply into the bilayer to a region of inherently lower molecular order [19]. At intermediate pH values the ^{2}H-NMR drug spectrum that is seen is either a superposition of the spectra of the charged and the uncharged forms or the result of fast exchange between the two forms in the membrane.

For [$^2H_4$]procaine no quadrupole pattern can be detected at low pH even at high lipid and drug concentration. This is probably due to the low partition coefficient for procaine at this pH. The correlation between the amount of drug measured in aqueous phase and the area under the central ^{2}H-NMR spectral component is similar to that discussed above for tetracaine.

For [$^2H_4$]procaine at higher pH the width of the quadrupole pattern is broader for the PC case than for the PC/PS case (Table II). This is in contrast to tetracaine, for which no significant difference was found between the PC and the PC/PS cases (Table II). This suggests that procaine and tetracaine could be located at different depths in the membrane. At the tails of the lipids one would not expect much difference in mobility between the PC and the PC/PS membranes. Close to the head groups a difference in mobility for the two systems would, however, be plausible. A difference in quadrupole splitting between PC and PC/PS, in which the *N*-methyl groups of PC were labelled, has been detected by ^{2}H-NMR [20].

The results of the present and earlier [1,15] ^{2}H-NMR experiments on these systems can be readily interpreted in view of the above data. The type of spectra shown in Figs. 5 and 6, comprising a central resonance and a quadrupole pattern, have been observed previously [1]. In the earlier study the width of the central resonance was proportional to the lipid content of the sample. This was interpreted as the effect of a population of lipid bound anesthetic in rapid exchange with that in bulk solution, in accordance with the present results. The quadrupole pattern was earlier assigned to another population of anesthetic also bound to the membrane, but in slow exchange with that in bulk solution. However, we have now shown that all lipid bound anesthetic is in rapid exchange with the aqueous phase but because of

the structure of the liposomes some of the lipid bound anesthetic has access only to a limited volume of the aqueous phase. In a sense this lipid population is in 'slow exchange' with the main bulk solution.

Lee [4] has earlier determined the binding constants for tetracaine and procaine to liposomes of dipalmitoyl phosphatidylcholine (DPPC) by measuring the change in the lipid transition temperature as drug was added to the membrane. The change in transition temperature was monitored by the fluorescence probes chlorophyll *a* and *N*-phenylnaphthylamine. He found the binding constants for the uncharged form of the drug to be 1000 l/mol and 143 l/mol for tetracaine and procaine, respectively. These figures are comparable with the binding constants 350 l/mol and 65 l/mol determined by us for the same drugs ($K_2^\star$ in Table I). For tetracaine in the charged form Lee found the same binding constant as for the uncharged form, i.e. 1000 l/mol. This is considerably larger than our value of 60 l/mol ($K_3^\star$ in Table I). It should be added that for the dissociation of tetracaine in the aqueous phase Lee [4] used $pK_1 = 8.5$ whereas we have used $pK_1 = 8.0$, considering its concentration dependence. For procaine Lee [4] reports that the pK of the drug is increased upon binding to the membrane. However, from Fig. 6 of Ref. 4 we note that the binding constant is larger in the uncharged than in the charged form, which requires that pK decreases upon binding. Hence, assuming that the ΔpK of Ref. 5 merely has got the wrong sign we calculate for $\Delta pK = -1.0$ that the binding constant of charged procaine is 14.3 l/mol (With $\Delta pK = 1.0$ a binding constant of 1430 l/mol would be obtained.). This should be compared with our value of 1.5 l/mol ($K_3^\star$ in Table I). In both cases a $pK_1 = 9.0$ for procaine was used. Hence the major difference between our direct determinations of binding constants and Lee's indirect determinations is larger binding constants for the charged drugs in the latter case. Lee has based his results upon observation of how bound drug decreases the transition temperature from the liquid crystalline to the gel state. Träuble and Eibl [21] have pointed out that the transition temperature of a charged membrane should decrease with increasing surface charge. If the change in transition temperature observed is not corrected for this effect it would lead to an overestimate of the binding of charged drug molecules. There are also other possible sources for the differences between the two sets of results. However, results calculated by partition equilibria, used by us, and Langmuir isotherms, used by Lee, would be expected to deviate appreciably only at high amounts of bound anesthetic (cf. Ref. 7). A more likely reason for differences in the results is that Lee's data are valid for dipalmitoylphosphatidylcholine in Tris buffer at approx. 40°C whereas we used egg phosphatidylcholine without any buffer and measured at 30°C.

Our simulations show that the experimental results can be reasonably well explained by the type of theoretical treatment used here. The results for tetracaine obtained in this work are in fair agreement with an earlier investigation [7] where a binding constant of 30 l/mol was found for tetracaine to vesicles of phosphatidylcholine at low pH.

It is worth noting, see Table I, that the ratio $K_2^\star/K_3^\star$ is approx. 7.5-times larger for procaine than for tetracaine. This means according to Eqn. 20 that the ratio $K_4^\star/K_1$ between the dissociation constants in the membrane and in the aqueous phase also are larger for procaine. Hence, in spite of a pK_1 difference of one unit, the $pK_4^\star$ values are nearly the same for the two drugs, 7.36 and 7.23, respectively.

When for a specific drug the binding constants to the lipid bilayer of the charged and uncharged forms as well as the dissociation constant in the aqueous phase have been determined, it is possible, for a system of known drug-lipid-salt composition and pH, to calculate the fractions of the drug in its charged and uncharged form present in the aqueous phase and bound to the membrane. As an example we show in Fig. 8 the pH dependence of the fractions of charged and uncharged tetracaine bound to PC and PC/PS bilayers. The drug concentrations in the aqueous phase for the same system are shown in Fig. 2b.

From Fig. 8 it is seen, from the crossing points of the curves, that the apparent $pK_4^\star$ values of the membrane bound drug is 7.0 for PC and 8.3 for PC/PS bilayers, while the intrinsic $pK_4^\star$ value is 7.23. The lower apparent $pK_4^\star$ value in PC is due to the positive surface charge because of bound charged tetracaine and the higher value in PC/PS

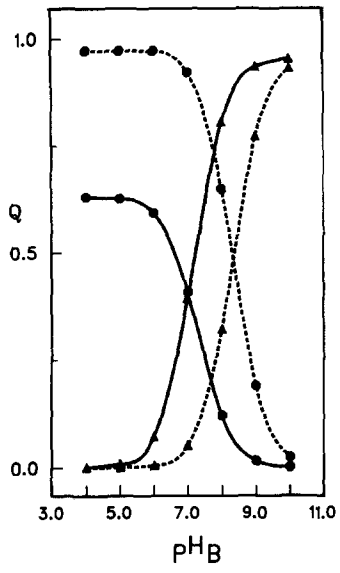

Fig. 8. The fractions Q of the total amount of the drug, tetracaine, bound in charged (●) and uncharged (▲) form to PC (————) and PC/PS (- - - - -) liposomes bilayers as function of pH. Data were simulated for the systems described in Fig. 2b using parameters presented in Table I.

is due to the negative surface charge because of PS. The pH profiles of the curves for uncharged tetracaine in PC and PC/PS bilayers are similar but not identical. In essence there is a shift between the two curves of approx. 1.1 pH unit with the curve for the charged bilayer at the higher pH. A major cause for this shift is the relatively large binding constant for the charged form of tetracaine. For procaine the corresponding binding constant is very small and in this case the two curves are separated only by approx. 0.2 pH units.

From Fig. 8 we see that the amount of uncharged tetracaine in the membrane at pH 7 decreases by a factor of approx. 10 when going from uncharged PC to negatively charged PC/PS. In the PC bilayer there is approx. 7.5% of the total amount of the drug bound in the uncharged form at pH 6, i.e. two pH units below the pK_1 of the drug in the aqueous phase.

Obviously the ratio K_2^*/K_3^* as well as the absolute values of the binding constants and the surface concentration of charged lipids are important parameters determining the amount of the uncharged drug in the membrane. The concentration of uncharged drug in the lipid bilayer of a biological membrane is further determined by the local pH and ionic strength. Diffusion of drugs and similar molecules through membranes probably is possible only for the uncharged molecules. The mentioned parameters determine the concentration of uncharged drug molecules in the membrane and hence the diffusion rate through the membrane. In a biological membrane, uncharged areas could function as 'channels' through the membrane for drug molecules since the concentration of the uncharged form of the drug could be markedly increased in such microenvironments. The effect would be enhanced by a local increase in pH. When considering the biological effect of a drug, e.g. local anesthesia, the rate limiting step might be either the transport rate over to the other side of the lipid bilayer, or (also include) the desorption rate from the membrane 'backside' surface. It appears important to be able to measure these rates separately.

6. Conclusions

The attainment of equilibrium between lipid, water, and local anesthetic is strongly dependent on the method of sample preparation. Freeze-thawing leads to rapid attainment of equilibrium and to a more uniform distribution of particle size. When fully hydrated vesicles are used as model membranes, lipid bound anesthetic is in rapid exchange with anesthetic in the aqueous phase, leading in most cases to disappearance of the quadrupole splitting observed in the ^{2}H-NMR spectra of less completely hydrated systems. The modified Gouy-Chapman theory for interactions of amphiphiles with charged surfaces, reproduces very well the distribution of local anesthetic between water and lipid phases at various pH values.

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