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Membrane-buffer partition coefficients of tetracaine for liquid-crystal and solid-gel membranes estimated by direct ultraviolet spectrophotometry

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The membrane-buffer partition coefficient of tetracaine was measured by direct ultraviolet spectrophotometry in dimyristoylphosphatidylcholine unilamellar liposomes at temperatures above and below the main phase transition. The partition coefficients of uncharged tetracaine to solid-gel (18°C) and liquid-crystal (30°C) membranes were $6.9 \cdot 10^4$ and $1.2 \cdot 10^5$, respectively. Despite the general assumption that local anesthetic binding to the solid membrane is negligible, this study showed that the solid membrane binding amounts to 57.5% of the liquid membrane binding. Binding of the charged form to the liquid or solid membrane was not detectable under the present experimental condition of 0.03 mM tetracaine bulk concentration. The present method measures metachromasia of local anesthetics when bound to lipid membranes. Its advantage is that the separation of the vesicles from the solution is not required. A linearized equation is presented that estimates the partition coefficient or binding constant graphically from a linear plot of the absorbance data. The method is applicable for estimation of drug partition when a measurable spectral change occurs due to complex formation.

Introduction

The lipid theories of anesthesia attribute the anesthesia mechanism to the disordering effect of anesthetics on the membrane lipids. The anesthetic-induced depression of the temperature of the order–disorder phase transition has been implicated as a mechanism of anesthesia. The depression, however, is often analyzed by assuming complete exclusion of anesthetic molecules from the solid membrane (see, for instance, Refs. 1–6). The validity of this assumption is questionable because there are reports [7–9] on the anesthetic binding to lipid membranes at temperatures below the phase transition. The difference between the anesthetic binding to solid and liquid membranes determines the magnitude of the temperature depression, hence, the anesthetic potency. Despite its importance in elucidating anesthesia mechanisms, there have been few studies that addressed specifically the difference in the anesthetic binding to liquid and solid membranes.

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A number of methods are available for estimation of the membrane-buffer partition coefficients of local anesthetics. The conventional binding study consists of separating the bound and free anesthetics by dialysis, filtration, centrifugation, or column chromatography. The free anesthetic concentrations are measured by ultraviolet spectrophotometry, radioactive tracer counting, total organic carbon analysis, etc. The main disadvantage of these methods is the separation process. It often takes too long to prevent alkaline hydrolysis of the ester-type drugs, and is difficult to maintain the temperature identical to that of the binding assay during separation, especially during ultracentrifugation. The technical and theoretical problems inherent to the separation process will be discussed later.

When a measurable spectral change occurs due to anesthetic-membrane complex formation, binding can be estimated from the change by applying a suitable model. Thus, nuclear magnetic resonance spectroscopy, electron paramagnetic resonance spectroscopy, etc., have been used to estimate local anesthetic binding to membranes.

We report here a simple method of direct ultraviolet spectrophotometry, that does not require separation of the vesicles from the solution, to determine tetracaine binding to phospholipid vesicle membranes. The study is aimed at estimating the difference in anesthetic interaction with phospholipid membranes between the solid-gel and liquid-crystal states. Zwitterionic dimyristoylphosphatidylcholine (DMPC) was chosen for the model membrane because the main phase-transition temperature is in the convenient range (about 24 °C) to obtain separate data on the liquid-crystal and solid-gel states.

Materials and Method

Synthetic DMPC and tetracaine hydrochloride were obtained from Sigma. Water was triply distilled in all glass stills, once from alkaline potassium permanganate solution.

The following buffer systems were used according to the pH range: 0.05 M KH_2PO_4/Na_2HPO_4 for the pH between 5.5 and 8.5 and 0.05 M $NaHCO_3/Na_2CO_3$ for the pH between 9 and 11.

DMPC vesicle suspension in a buffer solution was prepared by sonication in a cup-horn of a Branson Sonifier Model 185 (Danbury, CT) above the phase-transition temperature. The preparation was fused at 4 °C for 2 weeks to obtain homogeneous vesicle size distribution [10]. Sample solutions were prepared by mixing the DMPC suspension with tetracaine solution in the same buffer to give a desired concentration of DMPC. The concentration of tetracaine was kept at $3 \cdot 10^{-5}$ M throughout the experiment.

A Perkin-Elmer Model 554 spectrophotometer with a Peltier-type electronic temperature controlling system (Norwalk, CT) was used for the spectrophotometric measurements. Tetracaine showed an absorbance maximum at 306 nm that shifted slightly depending on the pH of the solution. In this work, the molar absorption coefficient at 306 nm was used to monitor the interaction of tetracaine with the DMPC vesicle membrane.

The molar absorption coefficients of tetracaine in buffer solutions were determined from the absorbance at four different concentrations of tetracaine. The identical buffer solutions were used as the reference. In the case of tetracaine in DMPC suspension, the absorbance was measured against the DMPC suspension of the same concentration to compensate for the light scattering. The 90 ° scattering of the liposomes in the absence and presence of tetracaine was compared by a Perkin-Elmer Model MPF 44B Spectrofluorometer, and the insignificance between the two was verified. The apparent molar absorption coefficient was calculated by dividing the apparent absorbance by tetracaine concentration.

In alkaline solutions, tetracaine undergoes nonenzymatic hydrolysis. Hence, in the high pH range, the experiment was started promptly after the pH adjustment. The tetracaine spectra were checked before and after the experiment, and corrections were made for the hydrolysis, if needed.

Experiments were performed at temperatures below (18 ± 0.1 °C) and above (30 ± 0.1 °C) the gel-to-liquid-crystal phase transition. The reproducibility of the results was confirmed by three series of independent runs. Data points were stored in an IBM PC microcomputer interfaced with a PDP 11/23 minicomputer. Linear regression analyses and error estimates were performed with a statistic program.

Theoretical consideration

This method estimates partition coefficients or binding constants from the change in the ultraviolet absorbance of local anesthetics when bound to vesicle membrane. Both partition and binding models have been used to analyze the anesthetic–lipid interaction [1–9,11–18]. In this section, a theory for the partition model is presented. The true partition equilibrium is defined as the ratio of the solute activity in two phases and consists of a single value, independent of the solute concentrations. The binding model analysis is similar to the partition model and is briefly presented in the Appendix.

Let K_{LH} and K_L designate the membrane-buffer partition coefficient of charged and uncharged local anesthetics, respectively. Then, under the condition of dilute anesthetic concentration.

$$K_i = \frac{X_i^M}{X_i^W} \tag{1}$$

where subscript i indicates charged or uncharged anesthetic, X is the mole fraction, M the membrane phase, W the aqueous phase.

In the case of charged local anesthetics, however, the binding incorporates positive charges to the membrane surface. The increase in the surface charge decreases the concentration of the charged anesthetic molecules near the surface. The partition coefficient, K_{LH}, defined in Eqn. 1, includes this surface potential effect. The intrinsic partition coefficient without the surface potential effects, K_{LH}^{int}, is written as

$$K_{LH} = K_{LH}^{int} \exp\left(-\frac{F\psi}{RT}\right) \tag{2}$$

where F is Faraday's constant, ψ is the surface potential of the lipid vesicles where charged anesthetics are bound.

The mole fractions of each component in aqueous and membrane phases are expressed as

$$X_i^M = \frac{n_i^M}{n_M + n_i^M + n_j^M} (i \neq j: \text{L or LH}) \tag{3}$$

$$X_i^W = \frac{n_i^W}{n_W + n_i^W + n_j^W} (i \neq j) \tag{4}$$

where n_i^M and n_i^W are the number of i-form local anesthetic molecules in the membrane and water, respectively, and n_M and n_W are the number of lipid molecules and water molecules, respectively. Let n_A represent the total local anesthetic molecules, then

$$n_A = n_L^W + n_{LH}^W + n_L^M + n_{LH}^M \tag{5}$$

We [19] have reported that the water solubility of tetracaine base was about $2.13 \cdot 10^{-4}$ M. Under the present experimental condition of $3 \cdot 10^{-5}$ M, tetracaine may be safely assumed to be fully dissolved as monomer in the solution. The apparent molar absorption coefficient, ϵ_{app}, becomes

$$n_A \epsilon_{app} = n_L^W \epsilon_L^W + n_{LH}^W \epsilon_{LH}^W + n_{LH}^M \epsilon_{LH}^M \tag{6}$$

where ϵ_i^j are the molar absorption coefficients of i (charged or uncharged) anesthetics in the j phase. When the local anesthetic concentration is in the millimolar range, n_i^W becomes much smaller than n_W. Under this condition, Eqn. 4 is approximated as

$$X_i^W = \frac{n_i^W}{n^W} \tag{7}$$

Further, when the number of local anesthetic molecules in the membrane is much smaller than the lipid molecules, Eqn. 3 becomes

$$X_i^M = \frac{n_i^M}{n_M} \tag{8}$$

From the Henderson-Hasselbalch equation and Eqns. 1 and 5–8, it follows

$$\epsilon_{app} = \left\{\left(\epsilon_L^W + \epsilon_L^M K_L \frac{n_M}{n_W}\right) + \left[\epsilon_{LH}^W + \epsilon_{LH}^M K_{LH}^{inf} \frac{n_M}{n_W} \times \exp\left(\frac{-F\psi}{RT}\right)\right] 10^{pK_a - pH}\right\} \times \left\{\left(1 + K_L \frac{n_M}{n_W}\right) + \left[1 + K_{LH}^{int} \frac{n_M}{n_W} \times \exp\left(\frac{-F\psi}{RT}\right)\right] 10^{pK_a - pH}\right\}^{-1} \tag{9}$$

where pH and pK_a are the values in the bulk solution. In the present case, assuming the partition coefficient of the charged tetracaine to be

about 900 [12] in mole fraction unit, the surface potential is at most +0.26 mV at 25 °C and at an ionic concentration of 0.05 M. Thus, the error caused by this approximation is less than 1%. When buffer salt concentration increases, the error will be further decreased. When the effect of the electrostatic double layer is small, Eqn. 9 reduces to

$$\epsilon_{app} = \frac{\left(\epsilon_L^W + \epsilon_L^M k_L \frac{n_M}{n_W}\right) + \left(\epsilon_{LH}^W + \epsilon_{LH}^M K_{LH}^{int} \frac{n_M}{n_W}\right) 10^{pK_a - pH}}{\left(1 + K_L \frac{n_M}{n_W}\right) + \left(1 + K_{LH}^{int} \frac{n_M}{n_W}\right) 10^{pK_a - pH}} \quad (10)$$

Eqn. 10 expresses the apparent molar absorption coefficient of local anesthetics at any pH value and at any lipid concentration. When only one form of local anesthetic is present, the equation can be reduced to a linear form.

When the pH is sufficiently higher than the pK_a, the concentration of the charged species of local anesthetics becomes negligible and Eqn. 10 reduces to

$$\epsilon_{app} = \frac{\epsilon_L^W + \epsilon_L^M K_L C_M / 55.5}{1 + K_L C_M / 55.5} \quad (11)$$

where C_M is the molar concentration of the phospholipid. In 1000 ml of solution, n_M/n_W approximately equals $C_M/55.5$. Then, Eqn. 11 is rearranged as

$$\frac{1}{1 - \frac{\epsilon_{app}}{\epsilon_L^W}} = \frac{1}{1 - \frac{\epsilon_L^M}{\epsilon_L^W}} \left(1 + \frac{55.5}{C_M} \frac{1}{K_L}\right) \quad (12)$$

When the reciprocal of $[1 - (\epsilon_{app}/\epsilon_L^W)]$ is plotted against the reciprocal of C_M, K_L and $\epsilon_L^M/\epsilon_L^W$ values are obtained from the slope and y-intercept, respectively.

At low pH where the pH is sufficiently lower than the pK_a, ϵ_{app} becomes

$$\epsilon_{app} = \frac{\epsilon_{LH}^W + \epsilon_{LH}^M K_{LH}^{int} C_M / 55.5}{1 + K_{LH}^{int} C_M / 55.5} \quad (13)$$

and

$$\frac{1}{1 - \frac{\epsilon_{app}}{\epsilon_{LH}^W}} = \frac{1}{1 - \frac{\epsilon_{LH}^M}{\epsilon_{LH}^W}} \left(1 + \frac{55.5}{C_M} \frac{1}{K_{LH}^{int}}\right) \quad (14)$$

From the same plot as used for Eqn. 12, K_{LH}^{int} and $\epsilon_{LH}^M/\epsilon_{LH}^W$ are obtained.

In the pH range where charged and uncharged species coexist, Eqn. 10 is used for estimating the partition coefficients.

Results

The pH-dependence of molar absorption coefficient of tetracaine in the buffer solution was measured at 25 °C between pH 5.5 and 11. The apparent molar absorption coefficient at 306 nm for charged and uncharged tetracaine were $(2.274 \pm 0.017) \cdot 10^4$ and $(2.097 \pm 0.014) \cdot 10^4$ $cm^{-1} \cdot M^{-1}$, respectively. These values were invariant between 18 and 30 °C. In the present small temperature range, these values apparently stayed constant. Since this is a pH titration, the ionization constant, pK_a, of tetracaine in aqueous solution can be estimated from the pH where the degree of ionization is 0.5. The value was 8.70.

We [19] reported pK_a values of several local anesthetics measured at an ionic strength of 0.05 M and at various temperatures by the non-logarithmic linear potentiometric method [20]. The value for tetracaine at 25 °C was 8.46. Boulanger et al. [12] reported a value of 7.5 by pH titration and ^{13}C-NMR chemical shift, but 8.5 appears to be the generally accepted value [21,22]. A comparison among reported values are listed in our previous report [19]. As commented by Boulanger et al. [12], the ionization constant of the tertiary amine side of the aromatic amine local anesthetics varies with the anesthetic concentration, ionic strength, buffer species, etc.

Fig. 1 shows the absorbance ratio of uncharged tetracaine between the presence and absence of the lipid vesicles at pH 10.50 to the liquid-crystal (30 °C) and solid-gel (18 °C) membranes. The absorbance decreased with the increase in the DMPC concentrations. A larger change was observed at 30 °C than at 18 °C as expected. At pH 5.50, the ratio of the absorbance did not change apprecia-

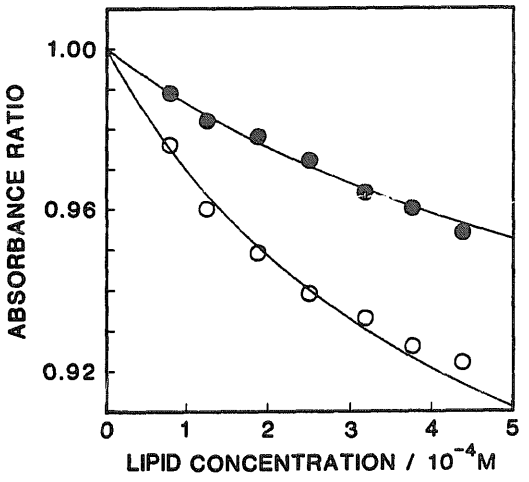

Fig. 1. Tetracaine partition at pH 10.50. The ordinate is the absorbance ratio in the presence and absence of the lipid vesicles, and the abscissa is the phospholipid concentration in 10^{-4} M. Tetracaine concentration was 0.03 mM. The lines are computer-generated according to Eqn. 11. Symbols: 18 °C ●, and 30 °C ○.

bly. It is possible that the molar absorption coefficient of charged tetracaine did not change significantly when bound to the membrane. It is also possible that the bound amount might have been too small to be detected because of the low tetracaine concentration in the bulk. At intermediate pH range, the absorbance change was decreased according to the decrease in the pH at 18 °C (Fig.

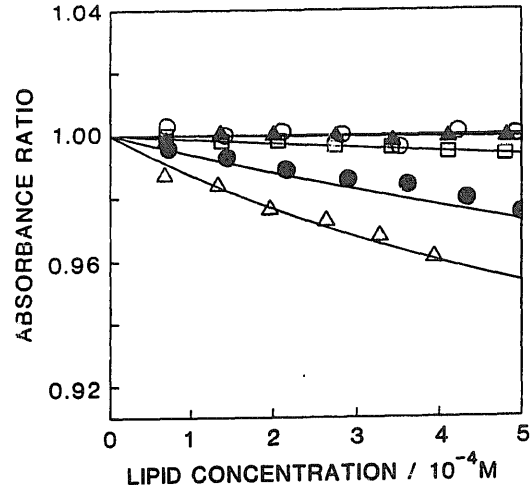

Fig. 2. Tetracaine partition at intermediate pH values and at 18 °C. The plot is the same as Fig. 1. The partition was decreased monotonously when the amount of uncharged species was decreased. The lines are computer-generated according to Eqn. 10. Symbols: pH 5.52 ○, pH 6.55 ▲, pH 7.50 □, pH 8.34 ●, and pH 9.47 △.

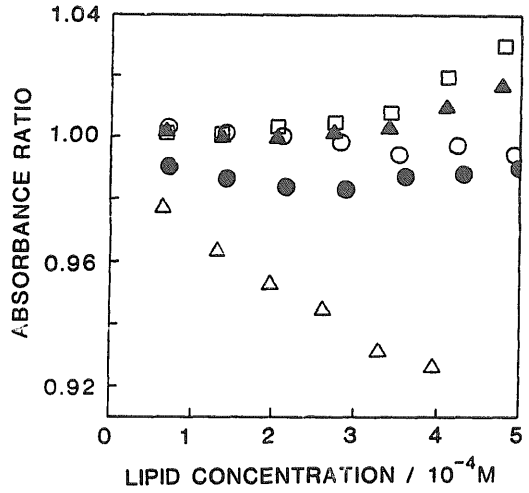

Fig. 3. Tetracaine partition at intermediate pH values and at 30 °C. The plot is the same as Fig. 1. Symbols: pH 5.52 ○, pH 6.55 ▲, pH 7.50 □, pH 8.34 ●, and pH 9.47 △.

2). At 30 °C, however, the absorbance pattern showed a biphasic effect where a maximum effect was observed at about pH 7.50 (Fig. 3).

Discussion

At pH 10.50, the absorbance ratio, $\epsilon_{app}/\epsilon_L^W$, showed a significant change when DMPC vesicles were added to tetracaine solution (Fig. 1). The data have been plotted in the double reciprocal mode according to Eqn. 12 in Fig. 4. From this figure, the partition coefficients, K_L, and $\epsilon_L^M/\epsilon_L^W$ were estimated. At 30 °C, $K_L = 1.2 \cdot 10^5$, $\epsilon_L^M/\epsilon_L^W = 0.828$, $r = 0.995$, where r is the correlation coefficient. At 18 °C, $K_2 = 6.9 \cdot 10^4$, $\epsilon_L^M/\epsilon_L^W = 0.876$, $r = 0.992$. The lines drawn in Fig. 1 are theoretical curves, constructed by using these values and Eqn. 11.

An anonymous reviewer of this manuscript commented that the present partition coefficients are two orders of magnitude larger than other reported values. The numerical value of the partition coefficient differs according to the unit that is used to express the concentration; mole fraction, molarity, molality, weight fraction, etc. We [23] reported the conversion factors among these concentration units by measuring the partial molal volume of DMPC, dipalmitoylphosphatidylcholine (DPPC), and distearoylphosphatidylcholine in water. The conversion factors for mole fraction versus molality and molarity are 37.5 and 36.6

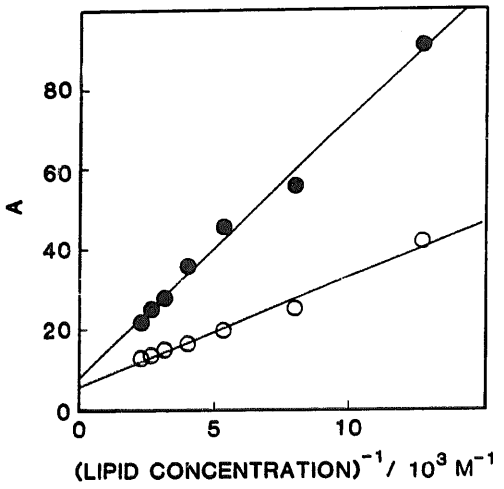

Fig. 4. Double-reciprocal plot of the data shown in Fig. 3 according to Eqn. 12. The ordinate, A, is the reciprocal of $(1-\epsilon_{app}/\epsilon_L^W)$ and the abscissa is the reciprocal of the phospholipid concentrations. From the slope, the partition coefficient, K, was estimated to be $1.2\cdot10^5$ at 30 °C ($r = 0.995$) and $6.9\cdot10^4$ at 18 °C ($r = 0.992$). The absorbance ratio, $\epsilon_{app}/\epsilon_L^W$, was 0.828 for 30 °C ○ and 0.876 for 18 °C ●.

(DMPC), respectively. The present values translate into $3.2\cdot10^3$ for 30 °C and $1.8\cdot10^3$ for 18 °C when expressed as the molality partition coefficient.

As described earlier, estimation of anesthetic partition into lipid membranes by the depression of the phase-transition temperature is often based on the assumption that anesthetics do not bind to solid membranes. The present study showed that the solid-membrane binding amounts to 57.7% of the liquid-membrane binding. Reports that compared anesthetic binding to liquid and solid membranes are few. Simon et al. [9] used tritium-labeled halothane to estimate its partition into DPPC vesicle membranes, and reported that the solid-membrane binding (20 °C) was about 25% of the liquid-membrane binding (55 °C). Lee [8] reported that the octanol binding to the solid DMPC membrane is about 70–80% of the liquid membrane at the transition temperature. Katz and Diamond [7] evaluated partition of 16 solutes into DMPC vesicle membranes. Their data showed that the partition coefficient of acetone to the solid membrane (18 °C) was about 44% of the liquid membrane (30 °C). Standard anesthetics were not included in their series.

We found several reports on the tetracaine binding to the solid phospholipid vesicle membranes at a single low temperature. Sikaris and Sawyer [11] used quenching of fluorescence of a fluorophore incorporated into DPPC membrane by tetracaine to estimate the partition coefficient at pH 9.5 and at 20 °C. Their value was $3.6\cdot10^2$ (molarity). Boulanger et al. [12] used ^{2}H-NMR to estimate ^{2}H-labeled tetracaine partition into spin-labeled egg phosphatidylcholine lamellar vesicles, and reported $6.6\cdot10^2$ (molality) for uncharged tetracaine at pH 9.5. They also reported that the value for charged tetracaine (pH 5.5) was 22. These reports are not directly comparable with the present data because of the differences in the lipid species, temperature, buffer species, tetracaine concentration, etc.

The present study could not estimate the partition coefficient of charged tetracaine. The possible causes for the insignificant absorbance change are discussed in the Results section. It has been known that charged drug binding to a neutral surface is weak and follows negative cooperativity caused by the electrostatic repulsion between the bound and free charged drugs [24]. The data from the transition-temperature depression of bilayer vesicles [3], and surface pressure [15–17] and surface potential [18] of lipid monolayers showed marginal binding of charged local anesthetics to zwitterionic lipid membranes. Lee [4] reported about 3 °C depression of the main phase-transition temperature of the DPPC vesicle membrane by 2 mM charged tetracaine. The tetracaine concentration, however, was two orders of magnitude above the nerve-blocking concentration or the present study. Butterworth et al. [25] reported that the ED_{50} value for tetracaine in blocking the frog sciatic nerve was 0.012 mM. Assuming linearity between the bulk anesthetic concentration and the depression of the transition temperature, the present tetracaine concentration of 0.03 mM translates into 0.045C° depression of the transition temperature of DPPC vesicles, which probably cannot be detected by the transition temperature method.

With the gel-state membranes, the pH-dependence of the interaction between tetracaine and the phospholipid showed a monotonous decrease according to the decrease in the pH (Fig. 2). With the liquid-state membranes, however, $\epsilon_{app}/\epsilon_A$, where ϵ_A is the apparent molar absorption coefficient of tetracaine in the absence of lipid vesicles,

increased according to the increase in the phospholipid concentration (Fig. 3), and showed a maximum at pH 7.50. This biphasic effect suggests that the charged and uncharged tetracaine molecules may interact with each other (such as a complex formation) in the liquid-crystal membrane. The accuracy of the experimental data, however, may not allow detailed analyses at present.

The advantage of the present method is the rapidity of the procedure by omitting the separation of the free from the bound drugs. Dialysis requires several hours for the anesthetic binding to reach an equilibrium. This invites errors in local anesthetic binding because the nonenzymatic alkaline hydrolysis of ester-type local anesthetics becomes significant in this time scale. The rate of the alkaline hydrolysis of procaine is about 10% per hour at 40 °C [26], and about 7% per hour at 25 °C [27]. Separation by centrifugation is difficult because unilamellar vesicles do not precipitate at $100\,000 \times g$ after 1 h [28]. Although multilamellar vesicles can be precipitated at this centrifugal force, penetration of local anesthetics through the multilayered membranes is slow and unpredictable. Westman et al. [29] reported that equilibrium partition of local anesthetics into multilamellar vesicles required freeze-thawing of the suspension five times. It is also difficult to maintain the temperature identical to that of the binding assay during ultracentrifugation.

The theoretical and technical problems of the trapped water and nonsolvent water associated with the centrifugation process are detailed by Katz and Diamond [30,31]. They emphasized that water cannot be completely separated by centrifugation. About 60% of the water by weight is trapped in the liposome pellet [30], and it is not shown whether the trapped water has the same physical properties as bulk water. Furthermore, surface-bound water molecules are strongly structured and exclude solute molecules [31]. They designated cluster of the surface-bound water molecules as nonsolvent water. For these reasons, they used tritiated water to estimate the size of the trapped water. Ligand molecules are ^{14}C-labeled, and this double-tracer technique was used to estimate partition coefficients between water and lipid vesicles [7].

Ultrafiltration is less time-consuming than the above methods. When the ultrafilter membrane retains all of the lipid molecules and none of the anesthetic, the estimation of the partition coefficient is straightforward. However, ultrafilter membranes are usually non-ideal. They retain some of the anesthetic molecules and leak out some of the lipid molecules. In addition, some of the anesthetic molecules adhere to the filter. These have to be corrected by control experiments.

The applicability of the present direct spectrophotometry method, however, is limited by the magnitude of the absorbance change of the ligand when bound to lipid vesicles.

Appendix: Binding model

We assume the following binding sequence.

$$A_i + M \rightleftarrows A_i \cdot M \tag{A.1}$$

Here A_i represents the charged (LH) or uncharged (L) forms of the local anesthetic molecule. Let M and $A_i \cdot M$ represent the phospholipid molecule and the binding complex of *i*-form local anesthetics and lipid, respectively. The binding constant of *i*-form local anesthetic, K_i, is written,

$$K_i = \frac{[A_i M]}{[A_i][M]} \tag{A.2}$$

where square brackets signify the concentration of each component.

The apparent molar absorption coefficient ϵ_{app} is

$$\epsilon_{app} C_A = \Sigma \epsilon_i^W [A_i] + \Sigma \epsilon_i^M [A_i M] \tag{A.3}$$

Here C_A is the total anesthetic concentration, and

$$C_A = [L] + [LH] + [L \cdot M] + [LH \cdot M] \tag{A.4}$$

The total concentration of phospholipid (C_M) is written

$$C_M = [M] + [L \cdot M] + [LH \cdot M] \tag{A.5}$$

From Eqns. A1–A5, together with the Henderson-Hasselbalch equation, we obtain,

$$\epsilon_{app} = \frac{(\epsilon_L^W + \epsilon_L^M K_L C^M) + (\epsilon_{LH}^M K_{LH} C_M) 10^{pK_a - pH}}{(1 + K_L C_M) + (1 + K_{LH} C_M) 10^{pK_a - pH}} \tag{A.7}$$

This equation is identical to Eqn. 10 in the main text, and describes the relation between the ultraviolet absorbance change and the anesthetic–membrane interaction. The binding constant is estimated by the identical procedure described for the partition coefficient in the text.

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