Biochimica et Biophysica Acta 812 (1985) 268–276
Elsevier

BBA 72370

Multiequilibrium binding of a spin-labeled local anesthetic in phosphatidylcholine bilayers

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(Received May 28th, 1984)

Key words: Local anesthetic; Anesthetic-membrane interaction; Phosphatidylcholine bilayer; Spin label; ESR

The equilibria among spin-labeled amine local anesthetic species in dioleoylphosphatidylcholine liposomes at an anesthetic: lipid mole ratio of 1 : 100 are investigated. Electron spin resonance (ESR) spectra demonstrate that anesthetic mobility within the bilayer is charge-dependent, with the uncharged species the more mobile. Partition coefficient measurements confirm ESR evidence that changes in anesthetic mobility represent anesthetic-phospholipid interaction and not changes in bilayer fluidity. Spin-exchange attenuation experiments show that anesthetics within the bilayer are accessible to the aqueous medium. Dependence of tertiary-amine anesthetic p*K* on dielectric constant has been used to estimate the interfacial p*K*. We propose a model of equilibria among species of the tertiary amine anesthetic in the aqueous medium and those intercalated in the bilayer, including a species electrostatically bound to the lipid phosphate. Using experimentally determined equilibrium constants, the model provides the binding constant between the electrostatically bound and unbound cationic anesthetics within the bilayer. The model simulates the pH dependence of the mobile fraction of total anesthetic population determined by subtraction techniques on experimental ESR spectra.

Introduction

The mediating role of membrane lipids in the phenomena of local anesthesia has long been appreciated [1–4]. Understanding the equilibria among local anesthetic species in bilayers is necessary for understanding more complex equilibria in membrane-protein systems, including native and reconstituted membranes. The equilibria between charged and uncharged species are especially important because the charged form is the more potent blocker of sodium conductance [5].

In this investigation, we determine the equilibria among different states of the charged and uncharged forms of a tertiary amine local anesthetic in dioleoylphosphatidylcholine (DOPC) liposomes. The states include the charged and uncharged anesthetics in the aqueous phase and the charged and uncharged membrane-bound species. Our model of the charged membrane-bound phase includes both an intercalated species which is not electrostatically bound and one which is so bound to the anionic phosphate of the DOPC. We characterize the location of membrane-bound species within the bilayer.

Previous investigations have studied phenomena involving equilibria among the various states of tertiary amine local anesthetics in dispersions of phosphatidylcholine (PC). Phenomena include fluidization of PC bilayers [6,7], anesthetic partition into PC bilayers from the aqueous phase [8],

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position of the anesthetic molecules relative to phospholipid bilayer components [7,9], and electrostatic binding of charged anesthetics to monolayers [10].

Because of the nature of the phenomenon, such as fluidization, or of the method of investigation, such as NMR, previous investigations of anesthetic-bilayer interactions have often been carried out at high anesthetic : lipid mole ratios, including 1 : 8 and higher. These high ratios can contribute charge effects [11]. In addition, many of the anesthetics used have relatively low partition coefficients which can vary greatly over pH. In the case of tetracaine, the coefficient for the uncharged form is nearly 30-times that for the charged. This results in large differences in the amount of anesthetic in the bilayer for the same aqueous concentration as pH is varied [8].

We have chosen to avoid such complexities by studying anesthetic-bilayer interactions at a low anesthetic : lipid mole ratio (ca 1 : 100). We will demonstrate in Results that the small amount of anesthetic allows study of anesthetic-lipid interactions without fluidizing or solubilizing effects that accompany higher anesthetic concentrations. In addition, all anesthetic species in our investigation reside almost completely in the bilayer phase, with very little change in partition between the bilayer and water over all experimental conditions.

We are able to use such a low anesthetic : lipid mole ratio because our anesthetics are spin-labeled (Fig. 1) for electron spin resonance (ESR). Due to the greater sensitivity of ESR, a much smaller amount of spin label is required compared to that needed for nuclear magnetic resonance spectroscopy. We use ESR spectroscopy to distinguish component populations within the total anesthetic in our model membrane system on the basis of their differing mobilities. We emphasize that the spin-labeled local anesthetics used in this investigation are potent blockers of sodium conductance at micromolar concentrations [12]. They contrast with spin-labeled compounds used to monitor membrane fluidity in specific regions of the membrane. The latter compounds include spin-labeled fatty acids or their esters [8,13]. For these spin-probes, the reporter group is attached to the methylene chain, which resides in the hydrophobic interior of the bilayer. For the spin-labeled anesthetics in this study, the nitroxide reporter group is attached to the hydrophilic tertiary or quaternary amine (Fig. 1). This position allows the mobility of the anesthetic head-group itself and its environment to be monitored.

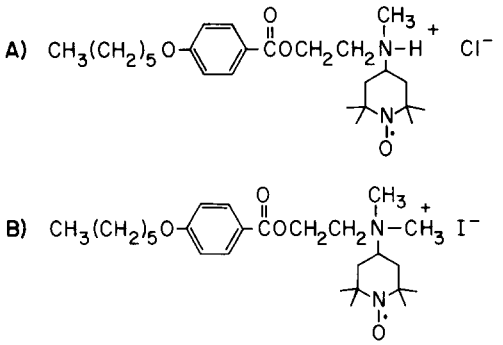


Fig. 1. Chemical structures of the spin-labeled local anesthetics abbreviated (A) C6SL and (B) C6SLMeI.

Both anesthetics have very high partition coefficients, whose measurement we give in Results. This property allows study of equilibria of anesthetic species which are restricted almost completely to the membrane, due to the small exchange with the aqueous phase. The high partition coefficients also facilitate measurements of the equilibrium constants by maintaining a nearly constant anesthetic : lipid ratio within the bilayer.

These spin-labeled anesthetics have been used previously in our laboratory to study in situ membrane proteins and reconstituted lipid systems [14,15].

Materials and Methods

Dioleoylphosphatidylcholine (DOPC) was purchased from Avanti Polar Lipids. All solvents were reagent grade. The spin-labeled anesthetic 2-[*N*-methyl-*N*-(2,2,6,6-tetramethylpiperidinooxyl)]-ethyl-*p*-hexyloxybenzoate (C6SL), its methylated quaternary analog (C6SLMeI) (Fig. 1), and unlabeled C6SL were synthesized in this laboratory [16].

Liposomes were prepared according to the method of Szoka and Papahadjopoulos [17] and filtered using a 1.2 μm Millipore filter. For C6SL experiments in which the pH of the medium was varied, liposomes were prepared with 100 mM

KCl and 10 mM Tris adjusted to the appropriate pH. In similar experiments, 45 mol% cholesterol was included with the DOPC or 7.5 or 10.0 mM $CaCl_2$ was added to the preparation buffer. The spin label was added at a mole ratio of one spin label per 100 lipids or as indicated. The samples were incubated for 30 min then centrifuged at $32\,000 \times g$ for 30 min. The supernatant was saved for pH determination. Samples from the pellet were sealed in 100 μl capillary pipettes. Where C6SLMeI was used, the aqueous phase was 200 mM KCl and 20 mM Tris.

For the spin-exchange attenuation experiments, liposomes were prepared in 20 mM Tris buffer at pH 10 and 100 mM $K_3Fe(CN)_6$ or 200 mM KCl. After incubation with C6SL or C6SLMeI and centrifugation as above, the supernatant was replaced with a similar buffer at the appropriate pH and the samples were freeze-thawed a total of 6 times. These were then centrifuged for 60 min at $32\,000 \times g$ and samples taken from the pellet as above.

For experiments where C6SLMeI and unlabeled C6SL were used together, liposomes were prepared in 100 mM KCl, 10 mM Tris at pH 7.5 with both anesthetics included in the vacuum-dried lipid, each at an anesthetic : lipid mole ratio of 1 : 100. After centrifugation, the preparation was divided equally and the supernatants were replaced with similar buffer at the appropriate pH and freeze-thawed in liquid nitrogen 6 times. After centrifugation, samples were taken as above.

The phospholipid content of the liposome preparations for the partition coefficient experiments was determined by phosphate assay using the method of Ames [18], modified by reading the final absorbances at 710 nm rather than 820 nm.

The partitioning of C6SL into DOPC liposomes was determined by a modification of the method of Papahadjopoulos and coworkers [2]. Liposome suspensions (1 ml with 10 mg DOPC) were added to 32 ml of buffer and C6SL at the appropriate pH. For pH 5 determination, the buffer consisted of 100 mM KCl and 10 mM citrate. The buffer for pH 9.2 determination consisted of 100 mM KCl and 10 mM Tris buffer. These mixtures were equilibrated for 1 h at room temperature then centrifuged at $50\,000 \times g$ for 40 min. The supernatant was assayed for C6SL at the 259 nm absorption band on a Hitachi 110 spectrophotometer. The partition coefficients (P) were calculated using the equation [19]

$$P = \frac{(C_t - C_s)(1 - m)}{(C_s)(m)} \quad (1)$$

where

C_t, absorbance of buffer and anesthetic without DOPC liposomes;

C_s, absorbance of buffer and remaining anesthetic after removal of DOPC liposomes;

m, weight ratio of DOPC to entire suspension.

Dependence of C6SL pK on the fraction of ethanol in solutions of ethanol and buffer was measured by a procedure described by Tencheva et al. [20]. Because of the low solubility of C6SL, actual measurements were made on analogs with two- and four-carbon methylene chains and the values are expected to be the same as that of C6SL.

The ESR spectra were recorded with a Varian E-3 ESR spectrometer interfaced to a PDP 11/10 computer with 12-bit resolution at the analog to digital conversion. Samples were recorded at 20°C, 0.1 s filter time constant, modulation amplitude of 1.0 G, and a scan width of 130 G.

The composite spectra were resolved by spectral subtraction. The reference subtrahend was obtained by appropriate experimental manipulations (see Results). The end point of the spectral titration is defined as the point in the titration where no negative inflection is detectable due to over subtraction [14].

Results

ESR spectra of C6SL in the presence of liposomes (Fig. 2) contain no discernible solution component and indicate that the anesthetic resides almost exclusively in the bilayer. This is consistent with the high partition coefficients at high and low pH, which we report below. Like other amine local anesthetics, C6SL is a weak base due to the ability of the tertiary amine head group to acquire a proton and a positive charge (Fig. 1A). The pK in the aqueous phase has been measured earlier in this laboratory [14] and found to be 7.2–7.4. We estimate a somewhat lower value at the bilayer/

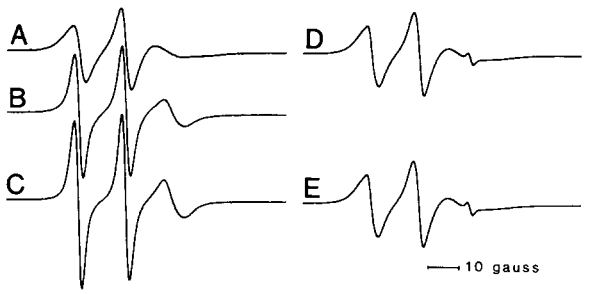


Fig. 2. Comparison of the effect of varying pH on C6SL and C6SLMeI in DOPC liposomes. The anesthetic to lipid mole ratio is 1:100. C6SL: pH (A) 5.2, (B) 7.0, (C) 10.1. C6SLMeI: pH (D) 6.5, (E) 10.0.

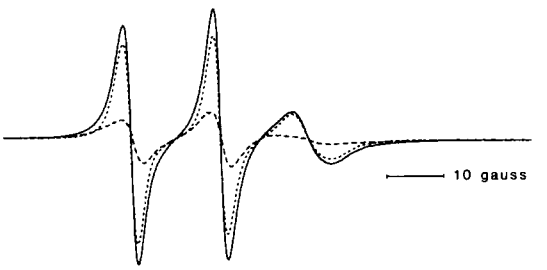


Fig. 3. Typical resolution of a spectrum of C6SL in DOPC liposomes using spectra B and C in Fig. 2. The data spectrum at pH 10.1 (dotted line; from C in Fig. 2) represents the mobile population component and is subtracted from the spectrum at pH 7.0 (solid line; from B in Fig. 2). The difference spectrum (dashed line) represents the constrained population component.

water interface, whose derivation we describe in Discussion.

The pH dependence of C6SL behavior in bilayers is also demonstrated by ESR. In DOPC liposomes the spectra of C6SL vary over pH from 5.2 to 10.1 with the higher pH spectra showing much more head group mobility than the lower pH (Fig. 2). C6SLMeI is a quaternary amine anesthetic whose headgroup charge is unaffected by pH (Fig. 1B). Over the same pH range, C6SLMeI spectra show no change in mobility and closely resemble the constrained C6SL spectra at low pH. The constancy of the C6SLMeI spectra over the same pH range shows that the observed pH-dependent changes in the spectra of C6SL are consequences of anesthetic charge and not due to structural changes in the membrane.

Our interpretation of the pH and charge dependence of C6SL spectral shape is that (1) at either of the two extreme pH values the spectrum represents a nearly pure population of charged and constrained, or uncharged and mobile anesthetic molecules, and (2) at an intermediate pH, the spectrum represents the sum of the spectra generated by each of these two populations within the sample. A confirmation of this interpretation would be our ability to resolve intermediate spectra into two components, each of whose shape resembles the extremes. Using spectral subtraction techniques, we have been able to resolve C6SL spectra from samples between pH 5 and 10 into these two components. For these resolutions, we model the mobile component with a spectrum with pH near 10. An example of resolution is shown in Fig. 3 for C6SL at pH 7.0, showing the data spectrum with its mobile and constrained components. When corrected to represent the same number of anesthetic molecules, the spectral shapes of the constrained components obtained in this manner at different pH values are nearly identical within the accuracy of our subtraction technique. This supports our interpretation that each intermediate spectrum can be considered the sum of the same two components. (We note that the constrained component is slightly less mobile than the lowest pH value. This is consistent with the presence of a small remaining mobile population which we attribute to a surface pK which is substantially lower than that of C6SL in the bulk phase. See Discussion for further details.)

We have quantified the pH dependence of the relative sizes of mobile and constrained populations by computing the fraction of the total anesthetic population which is mobile for samples at various pH. This fraction is equal to the ratio of the double integrals of the mobile component and of the total composite spectrum for each pH. The equality arises because the double integral of each spectrum is proportional to the population of nitroxide labels which generated it. Computed values for the fraction, mobile population/total population, are plotted vs. pH in Fig. 4. We see that below pH 5 the anesthetic molecules are nearly all constrained and the mobile fraction increases with increasing pH until half are mobile at pH 6.6 and all are mobile above 9. The inclusion of 45 mol% cholesterol in the DOPC liposomes or addition of 7.5 or 10 mM $CaCl_2$ to the

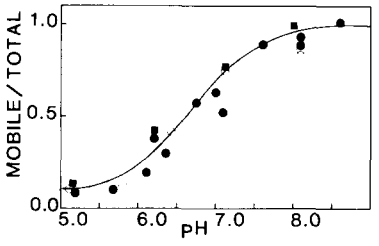


Fig. 4. Mobile fraction of total anesthetic in liposomes plotted against pH. Computation of the fractions from experimental ESR spectra is explained in Results. Liposome preparations are 100% DOPC (●); 55 mol% DOPC, 45 mol% cholesterol (■); 100% DOPC with 7.5 or 10.0 mM $CaCl_2$ included in the buffer (○). Simulation (———) of 100% DOPC data is explained in Discussion.

buffer does not substantially affect this dependence.

The two-component interpretation of the pH dependence of C6SL spectral shape is further supported by the availability of an underlying mechanism. We propose a model for such a mechanism which is described in detail in the Discussion. It is sufficient to say here that the mobile population consists of both uncharged and charged molecules, and the constrained population consists solely of positively charged anesthetics whose motion is restricted by ionic interaction with the anionic phosphate groups of membrane lipids. The relative sizes of the two components within the total anesthetic population are dependent on the availability of charged anesthetic molecules, which, in turn, is dependent on the pH of the environment and the surface pK of C6SL. This model has provided a simulation of the mobile fraction of the total anesthetic population which is shown as a solid line in Fig. 4. The close fit of this model to the points provided by spectral subtraction supports the treatment of the experimental data we have described.

We have evidence that the pH-dependent mobility of the C6SL headgroup is not the result of fluidization of the bilayer from increased partition of the uncharged anesthetic. If fluidization is the cause of increasing reporter group mobility, it should be possible to increase the mobility of the nitroxide reporter without changing the head group charge on C6SL. Fluidity should be increased by increasing the amount of uncharged anesthetic. We have looked for changes in the environment of the charged anesthetic head group, while varying the amounts of uncharged anesthetic. To test the environment of the charged headgroup for such changes in fluidity, we used DOPC liposomes with C6SLMeI and unlabeled C6SL, each at an anesthetic : lipid mole ratio of 1 : 100. Samples at pH 5.0 and 9.2 produced nearly identical C6SLMeI spectra (not shown). To test the environment of the uncharged anesthetic headgroup, we compared spectra (not shown) from a wide range of C6SL : DOPC mole ratios at pH near 10. At this pH, the anesthetic is nearly all uncharged. As with the charged C6SLMeI, the uncharged C6SL head group environment showed no significant differences in fluidity over anesthetic : lipid ratios of 1 : 400 to 1 : 12.

Bilayer fluidization from increased partition of the uncharged species also predicts that pronounced changes in the size of the solution component will accompany fluidity changes. At low pH, C6SL spectra would be dominated by a large solution component, accompanied by a much smaller less mobile component. Our data does not fulfill this prediction since, as we have indicated, C6SL spectra from low pH preparations display no dominant solution component (Fig. 2). Bilayer fluidization also implies that as the pH and mobility increase, the membrane-bound component must also increase, at the expense of the solution component. Our data (not shown) does not demonstrate such an effect and the total number of spin labels detected is independent of the pH and the mobile fraction detected.

Finally, our system does not produce the conditions required for pH-dependent fluidization of the bilayer by an amine local anesthetic. In order for anesthetics to fluidize the membrane at high pH more than at low pH, there must be a large difference between the partition coefficients of the uncharged (high pH) and charged forms (low pH), and the charged form coefficient must be very low. Schreier and coworkers [8] have demonstrated pH-dependent fluidization of egg lecithin bilayers by tetracaine and measured an approximately 30-fold difference between the partition coefficients of the high and low pH forms, with the low pH coefficient near 25. We have measured the partition coefficients of C6SL at pH 5.0 and 9.0 and

obtained values of 2625 ± 375 and $10\,800 \pm 800$, respectively. (Both measurements are $\pm$S.D., $n = 3$.) This means that the fraction of the anesthetic in the bilayer varies by no more than 0.03% over the pH range shown in Fig. 4. The absence of fluidization in our system is consistent with the report by Papahadjopoulos and coworkers [2] that dibucaine does not change the fluidity of dipalmitoylphosphatidylcholine bilayers at an anesthetic : lipid mole ratio of 1 : 30.

In addition to the dependence of anesthetic mobility on its charge, we have also investigated the anesthetic accessibility to the aqueous medium. Our method was to study the spin-exchange attenuation of ESR spectra of the charged and uncharged spin-labeled anesthetics using potassium ferricyanide. In this process, the ESR spectrum is attenuated by interaction of spin labels with ferricyanide without chemical modification.

DOPC liposomes were prepared at appropriate pH in 100 mM potassium ferricyanide or 200 mM KCl as control of equal ionic strength. C6SL or C6SLMeI was introduced to the samples at an anesthetic : lipid mole ratio of 1 : 100. C6SL spectra (not shown) from preparations at pH 9.5 in potassium ferricyanide compared to a similar preparation with KCl showed that only 2–3% of the spin-labeled anesthetics were detected after attenuation by the ferricyanide ion. For similar preparations at pH 6.5, 8–9% of the population was detected after attenuation. When C6SLMeI was introduced into the liposomes instead of C6SL, 50–60% of the anesthetic molecules were detected after attenuation by potassium ferricyanide. Since C6SL is a weak base, the pH 9.5 preparations contain fewer charged species than the sample prepared at pH 6.5. Both contain smaller charged populations than the C6SLMeI population, which is permanently charged. From comparison among these populations, it is clear that the charged anesthetics are less attenuated.

Estimation in this laboratory of the pK for C6SL (see above) was made by a procedure [20] which requires measuring the dependence of the pK on the fraction of ethanol in solutions of ethanol and buffer. We report here that the dependence is -0.2 pH units per 10% increase in ethanol, which permits us to estimate the value of the pK at decreased dielectric environment.

Discussion

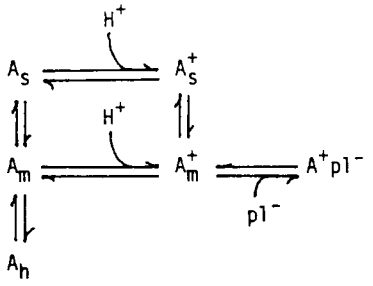
Identification of charged and uncharged populations of C6SL in DOPC liposomes, whose ratio is dependent on pH, extends earlier studies in our laboratory of C6SL in liposomes from extracted erythrocyte lipids [14]. The pH dependence is not substantially affected by the inclusion of 45 mol% cholesterol in the liposomes nor by 7.5 or 10 mM $CaCl_2$ in the aqueous medium. The lack of effect on anesthetic binding in response to calcium is consistent with the findings of Schlieper and Steiner [21] and Giotta et al. [22]. The high anesthetic partition coefficients determined in this study, as well as other evidence presented above, discount the possibility that the pH-dependent changes in mobility are due to fluidization of the membrane in this system.

The accessibility of both charged and uncharged anesthetics to potassium ferricyanide in the aqueous environment indicates that most of the headgroups of both species reside near the membrane/water interface. Ferricyanide ions have been demonstrated to attenuate spin labels at the surface of bilayers but not deeper into the hydrophobic region [23].

The more limited access of ferricyanide ions to C6SLMeI partitioned into the bilayer compared to C6SL suggests possible localization of cationic C6SLMeI at a protected site near the interface. The anionic phosphate of DOPC may represent such a location. The anionic ferricyanide ion may experience charge repulsion in this region and be sufficiently depleted to attenuate less. The uncharged anesthetic with its greater mobility may be more accessible to the ferricyanide ions at the membrane/buffer interface.

Location of the charged anesthetic in the lipid phosphate region of the bilayer is consistent with the findings of other workers using different methods to study the position of tetracaine in phosphatidylcholine bilayers [7,9,24,25].

Our findings in this study and our previous work with erythrocyte lipid cited above suggest the following Reaction scheme (2) for the distribution of a tertiary amine anesthetic in DOPC liposomes. The charged and uncharged anesthetic species in bulk solution are designated A_s^+ and A_s. A_m^+ and A_m represent the anesthetic molecules intercalated



(2)

in the membrane with the substituted amine headgroups at the membrane/water interface. A_h represents the uncharged anesthetic situated completely in the hydrocarbon region of the membrane bilayer. pl^- signifies the phospholipid and A^+pl^- represents the species of charged anesthetic undergoing electrostatic interaction with the anionic phosphate of DOPC.

The mass action laws for this scheme are expressed below. Solution components are given in molarity and species within the bilayer have concentration units of mol/mol of DOPC. (Because the anesthetic to lipid ratio is small, $[pl^-]$ is effectively one and does not appear after its expression in (5).) P^o is the partition coefficient of the tertiary amine anesthetic at a pH sufficiently above the pK_a for nearly all the molecules to be uncharged. Due to the formation of cationic anesthetic-phospholipid complexes (A^+pl^-), the partition coefficient for the uncomplexed A^+ may not be directly determined. The partition coefficient determined at low pH is only an apparent partition coefficient P^+ for the cationic anesthetics. It should also be pointed out that the partition coefficients in Eqns. 7 and 8 are defined with different units than those in Eqn. 1. However, since only the ratio P^+/P^o is used in our simulation, its value is unaffected because the units cancel.

For C6SL in this investigation, we interpret the ferricyanide spin exchange attenuation data to mean that the uncharged species (A_m) is nearly completely accessible to the aqueous phase which implies K_h is effectively zero. For that reason we do not include it in the quantitative expression of our model. However, for more hydrophobic anesthetics, the uncharged anesthetic dissolved in the hydrocarbon region may be significant. Because DOPC is neutral, the hydrogen ion concentration $[H^+]$ is the same for bulk and surface equilibria. The pH difference between the bulk phase and the surface due to positively charged anesthetics at an anesthetic to lipid ratio of 1 : 100 is well below 0.1 pH unit and is therefore neglected.

$$K_a = \frac{[H^+][A_s]}{[A_s^+]} \tag{3}$$

$$K_m = \frac{[H^+][A_m]}{[A_m^+]} \tag{4}$$

$$K_b = \frac{[A^+pl^-]}{[A_m^+][pl^-]} \tag{5}$$

$$K_h = \frac{[A_h]}{[A_m]} \tag{6}$$

$$P^o = \frac{[A_m]}{[A_s]} \tag{7}$$

$$P^+ = \frac{[A_m^+]+[A^+pl^-]}{[A_s^+]} \tag{8}$$

The partition coefficients are sufficiently large, so that solution phase components do not significantly contribute to the ratio of mobile to total plotted in Fig. 4. We propose that the membrane phase components contribute to the mobile and constrained populations in the following relationships:

$$\text{mobile population} = A_m + A_m^+ \tag{9}$$

$$\text{constrained population} = A^+pl^- \tag{10}$$

This relationship and the mass action laws given above allow us to model the data presented in Fig. 4. For these data, the independent variable is pH we have assigned the dependent variable as the mobile fraction of the total anesthetic population represented by the experimental spectrum. The dependent variable is the ratio of the double integral of the subtrahend in a spectral subtraction to that of a total data spectrum.

From the principles underlying ESR spectral subtraction [14], it can be demonstrated that the populations comprising the fraction are each proportional to the double integral of the ESR spectrum it generates. The subtrahend spectrum is a

spectrum from a sample at high pH whose anesthetic molecules are effectively all mobile. For correct subtraction, the subtrahend spectrum is scaled to represent the actual mobile component within the total data spectrum. For that reason, the ratio of the double integrals of the two spectra represents the ratio of the mobile component population to the total as given in Eqn. 11.

$$\frac{\text{mobile population}}{\text{total anesthetic population}} = \frac{\text{double integral subtrahend}}{\text{double integral total}} \quad (11)$$

From Eqns. 4, 5 and 9–11, we can express the ratio of mobile to the total population in the following equation, where the total equals the sum of Eqns. 9 and 10.

$$\frac{\text{mobile population}}{\text{total population}} = \frac{(10^{pH-pK_m}+1)}{(10^{pH-pK_m}+1+K_b)} \quad (12)$$

From Eqns. 3–8 and the definition of pK, the following expression may be derived for the K_b.

$$K_b = \frac{P^+}{P^o}(10^{pK_a-pK_m})-1 \quad (13)$$

Substituting this expression for K_b into Eqn. 12 we obtain the following expression which models the ratio of the C6SL mobile population to the total population in a data spectrum at a particular pH. We have represented this ratio in terms of the constants pK_a, pK_m, P^+/P^o, and the experimental variable, pH.

$$\frac{\text{mobile population}}{\text{total population}} = \frac{(10^{pH-pK_m}+1)}{[10^{pH-pK_m}+(P^+/P^o)(10^{pK_a-pK_m})]} \quad (14)$$

An estimate of pK_m may be made utilizing the dependence of anesthetic pK on the dielectric constant of the environment. We have estimated the dependence of the pK of C6SL on % ethanol in a solution of ethanol and buffer, which is −0.2 pH unit per 10% ethanol (see Results). Since the dielectric constants of ethanol-water solutions are known [26], the dependence of the pK of the C6SL analog can be translated into a dependence of its pK on dielectric constant. Shinitzky [27] estimated the dielectric constant of the membrane/water interface for egg-phosphatidylcholine liposomes and egg-phosphatidylcholine/cholesterol liposomes to be approx. 28. Fernandez and Fromherz [28] have measured this dielectric constant for Triton X-100 micelles, which also have neutral surface charge, and found it to be close to 32. We have averaged these two values and assigned a dielectric constant of 30 for the headgroup environment of C6SL. This corresponds to a pK_m of 5.57. Using this pK_m, pK_a of 7.3, and our measurement of apparent partition coefficients for uncharged and charged C6SL, we can substitute Eqn. 13 and obtain a K_b value of 12.

Substituting the experimentally determined values for pK_a = 7.3, pK_m = 5.57, and P^+/P^o = 0.24, we have used the expression in Eqn. 14 to simulate the data in Fig. 4 (solid line). We stress that all constants in our simulation are experimentally derived and no adjustment of constants was made. We see that the simulation gives quite a good fit to the data and strongly supports the model we have proposed in Reaction scheme 2 of the equilibria for the tertiary amine anesthetic C6SL in DOPC liposomes.

Acknowledgements

We are indebted to Andrew L. Palma for synthesizing the spin-labeled anesthetics used in this study, and to James Warner and George Hare for their engineering assistance. We thank Dr. Julie Earnest for her comments on the manuscript. The work is supported in part by NIGMS(28087) and by Faculty Research Grants from the University of California, Santa Cruz.

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