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Halothane Changes the Domain Structure of a Binary Lipid Membrane

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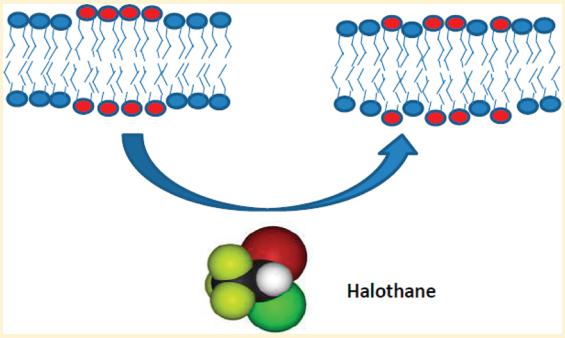
Supporting Information

ABSTRACT: X-ray and neutron diffraction studies of a binary lipid membrane demonstrate that halothane at physiological concentrations produces a pronounced redistribution of lipids between domains of different lipid types identified by different lamellar *d*-spacings and isotope composition. In contrast, dichlorohexafluorocyclobutane (F6), a halogenated nonanesthetic, does not produce such significant effects. These findings demonstrate a specific effect of inhalational anesthetics on mixing phase equilibria of a lipid mixture.



INTRODUCTION

The molecular mechanisms of volatile anesthetic action remain obscure despite an impressive history of research and clinical use for over a century and a half. The remarkable correlation between anesthetic solubility in oil and anesthetic potency,[1] the Meyer-Overton rule, strongly suggested a lipid membrane-mediated mechanism or binding to a hydrophobic protein pocket. Paradoxically, structural studies of lipid bilayer membranes in the presence of anesthetics yielded negligible effects. In classic experiments 30 years ago employing X-ray and neutron diffraction from dimyristoylphosphatidylcholine (DMPC)/cholesterol membranes, Franks and Lieb[2] found that for inhalation anesthetics "...at surgical concentrations, however, there are no significant changes in bilayer structure".

The conceptual view of cell membranes has shifted from relatively homogeneous lipid bilayers with interspersed proteins to complex lipid mixtures, with laterally separated membrane domains formed as a result of lipid demixing.[3] Accumulating evidence indicates that certain membrane proteins are clustered in domains such as cholesterol-rich "lipid rafts".[4] Potentially important effects of inhalational anesthetics on lipid domains have been proposed[5] and illustrated by both model calculations that suggest distinct effects at domain boundaries,[6] and by demonstrations of lipid reorganization using nearest-neighbor recognition techniques.[7] None of these effects have yet been verified by structural methods. On the other hand, modulation of ion channel function by lipid domains and mechanical properties of bilayers has been demonstrated in a number of systems.[8]

Using X-ray and neutron diffraction, we studied a binary lipid mixture of dipalmitoylphosphatidylcholine (DPPC) and dilauroylphaphatidylcholine (DLPC) 1:1 to demonstrate that halothane, but not dichlorohexafluorocyclobutane, produces a pronounced redistribution of lipids between different domains at physiologically relevant concentrations. This lipid mixture is a well characterized system with highly nonideal mixing that forms distinct DPPC-rich ordered and DLPC-rich fluid phases over a wide temperature range (30 °C).[9] (See Note 1 in Supporting Information regarding nomenclature.) In addition to a convenient temperature range where this mixture exhibits two phases, it is resistant to oxidation and radiation damage at ambient temperatures. Previous studies with electron spin resonance probes[5a] and spectrophotometry[10] demonstrated that halothane produced a temperature shift in the mixing transition of DMPC/DPPC mixtures, which have been characterized as being miscible but with slightly nonideal mixing and having only a narrow temperature range (5 °C) with fluid and solid phase coexistence. The mechanism of these shifts and specificity to anesthetics remained unclear.

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EXPERIMENTAL SECTION

Lipids were obtained as powders from Avanti Polar Lipids, halothane, and F6 from Sigma. Highly oriented multilamellar stacks of lipid bilayers at 1 to 2 mg/cm^2 were formed on thin microscope cover glass substrates by slow evaporation of solvent from solutions in ethanol or 80% ethanol/20%water at 37 to 40 °C in air, followed by 15 min in vacuum. Neutron diffraction was obtained with the Advanced Neutron Diffractometer/Reflectometer[11] at the Center for Neutron Research with momentum transfer perpendicular or parallel to the bilayer planes for lamellar or in-plane diffraction, respectively. Humidity was maintained at 98% with saturated salt solution[12] and stable vapor concentrations of anesthetic agents were obtained by adding solutions of anesthetics with hexadecane. Such solutions are very close to ideal, and vapor pressures follow Raoult's law, so the solution provides a reservoir of anesthetic at essentially constant chemical potential.[13] Vapor concentrations were sampled with gas syringes and measured with an Agilent 6850 chromatograph.

X-ray diffraction was performed with a Rigaku Ultima-III diffractometer fitted with a sealed chamber. Samples were prepared as for neutron diffraction. Introduction of halothane and F6 solutions with hexadecane into the sealed chamber with a syringe did not change temperature by more than 0.1 °C and humidity remained constant to within 0.5%. We measured at 28 °C, a temperature midway along the mixing transition for a series of X-ray diffraction studies on DPPC/DLPC multilayers.

RESULTS

Lamellar neutron diffraction data from a DLPC/d62-DPPC mixture are shown in Figure 1. Two series of lamellar diffraction peaks, which index on two different *d*-spacings, are present and indicate two lamellar phases. This requires separate stacking of d62-DPPC-rich and DLPC-rich 2-dimensional (2D) domains into 3D-domains. Such stacking is also readily observed by diffraction for mixtures of DOPC/DPPC/cholesterol, but not for all lipid mixtures,[14] and may depend on 2D-domain size, lipid molecular area, and hydration in addition to such conditions as composition and temperature.[15] Intensities of the two series of neutron diffraction peaks result from the different deuterium compositions of the two domains. For the H_2O hydration here, the intensities from the d62-DPPC-rich domains are strong, due to high contrast between H_2O and CD_2, whereas diffraction from DLPC-rich domains is much less intense since H_2O and CH_2 have about the same neutron scattering length density and so contrast is very small. Thus, the larger *d*-spacing is identified as a d62-DPPC rich phase and the smaller *d*-spacing is the DLPC rich phase. With increased temperature, the d62-DPPC-rich ("solid") phase intensities decrease dramatically, while the disordered DLPC-rich ("liquid") phase intensities increase, demonstrating transfer of d62-DPPC to the DLPC-rich liquid phase and changing the contrast by mixing of the hydrogen and deuterium-containing chains. Lateral diffusion constants for DPPC and DLPC in multilayers (>10^3 nm^2/s)[16] are sufficient to mix submicrometer-sized domains in a few minutes. At 15 °C, there are only small liquid phase peaks, whereas at 35 °C, only the liquid phase peaks remain and have become much more intense. Addition of halothane at 25 °C shifts the intensities of the solid and liquid peaks so that the intensities of the smaller *d*-spacing predominate.

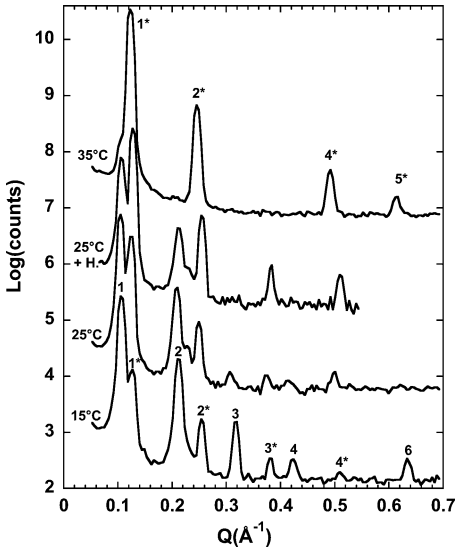

Figure 1. Neutron diffraction of 1:1 d62-DPPC/DLPC oriented multilayers on glass at different temperatures and after addition of halothane. $Q = 4\pi \sin(\theta)/\lambda$ is the momentum transfer directed perpendicular to the membrane plane. θ is half the scattering angle and λ is the neutron wavelength. Traces from top: 35 °C, 25 °C + halothane 1.5 mol %, 25 °C, 15 °C. Note the progressive decrease of the first Bragg diffraction peak (DPPC-rich ordered phase) with increasing temperature and upon addition of halothane. *d*-spacings for the 15° trace are 59.470 ± 0.015 Å (DPPC-rich phase) and 49.357 ± 0.145 Å (DLPC-rich phase). The *d*-spacing for DLPC-rich phase is larger than that of pure DLPC under similar conditions,[34] indicating a proportion of DPPC in this phase, as predicted by Jorgenson et al.[21]

We also measured in-plane neutron diffraction on oriented d62-DPPC/DLPC multilayer membranes (Figure 2). In-plane neutron diffraction by noncrystalline phospholipids in the region of the chain diffraction (Q = 1.4 Å^{-1} to 1.5 Å^{-1}) depends strongly on the deuterium content of the fatty acid chains. The negative neutron scattering length of hydrogen makes the net scattering length of the CH_2 group small and negative, whereas CD_2 is large and positive. As a result, in-plane neutron diffraction from the chains of noncrystalline phospholipids is only observed with chain-perdeuterated phospholipids. For a mixture of H and D chains, such as in a d62-DPPC/DLPC mixture, the intensity of the chain diffraction depends on the size and number of domains consisting of primarily d62-chains and on the order of d62-chains within these domains. Mixing of the H and D lipids decreases the intensity, while separation of d62-DPPC into 2D domains increases the intensity. This in-plane neutron diffraction method, with H/D chain mixing, is especially effective for observing changes in composition of lateral domains,[17] since it does not require vertical alignment of the lipid domains into 3D domains as lamellar diffraction does. We found that the mixing transition for d62-DPPC/DLPC is broad, beginning at about 22 °C and extending to 31 °C. Other techniques have found similarly broad mixing transitions in other lipid mixtures.[5a] The breadth of these transitions is not apparent in phase diagrams for these mixtures.[18] Halothane at 1.5 mol %, about twice the minimum alveolar concentration for anesthesia, produces a marked shift of about 5 °C in the mixing transition toward lower temperatures (an order of magnitude larger than the shift in the main melting transition of pure DPPC induced by anesthetic concentrations of octanol[19]), while 7.5 mol % F6 produces a shift of about half this magnitude. We obtained similar in-plane data for binary lipid bilayers supported in pores of anodisc filters, where the geometry is very different from that of planar multilayers (Supporting Information Figure 1a). In-plane neutron diffraction measurements were also performed for a 1/1 mixture of dimyristoylphosphatidylcholine (DMPC) and d70-

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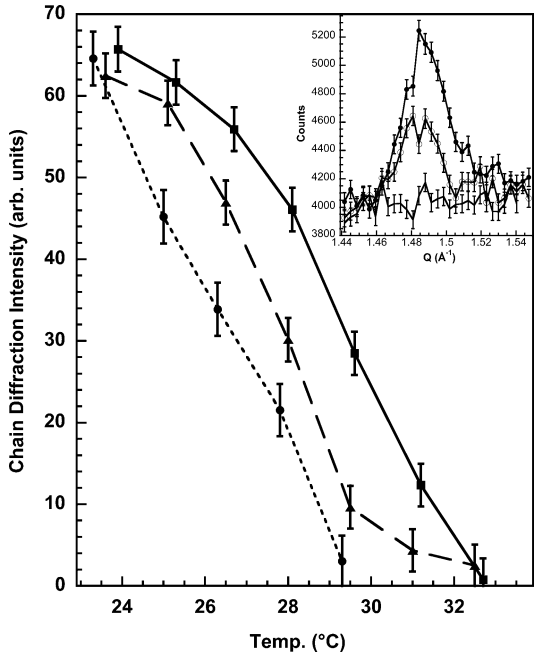

Figure 2. Neutron diffraction of 1:1 d62-DPPC/DLPC oriented multilayers on glass. Q [1.4 Å^{-1}−1.5 Å^{-1}] is directed parallel to the plane of the membrane. Neutron diffraction in this plane is generated by the d62-lipid chains and is greatly reduced by mixing of d62-lipid chains with undeuterated lipid chains so that d62-chains are no longer adjacent. The midpoint of the phase mixing transition for the native lipid mixture is about 29 °C (squares) and corresponds well to the phase diagrams established by calorimetry.[9a] Addition of 1.5 mol % halothane (measured at 27 °C) decreases the transition temperature to 25 °C (circles), but 7.5 mol % F6 decreases the transition only to 27 °C (triangles). Inset: Neutron counts collected in $\theta/2\theta$ scans across the chain diffraction peak for the 1:1 d62-DPPC/DLPC oriented multilayers for three temperatures: at the beginning, midpoint and end of the temperature scan (top, middle and bottom traces, respectively) in the absence of halothane. Peaks were integrated from Q = 1.46 to 1.52 Å^{-1} and background counts outside this region were subtracted to obtain the plotted chain diffraction intensity. Error bars represent one standard deviation, from counting statistics.

distearoylphosphatidylcholine (DSPC) as planar multilayers, with a midpoint transition temperature of 51 °C (Supporting Information Figure 1b).

Because the domains of d62-DPPC/DLPC mixtures stack separately as 3-D structures with different *d*-spacings, we were able to study the coexistence of the two domains at a range of concentrations of halothane and F6 using X-ray diffraction. Sample preparation and measurement procedures were as for neutron diffraction. Figure 3 illustrates separation of phases measured by X-ray diffraction. Two series of peaks, corresponding to the solid and liquid phases, are seen, as in the neutron diffraction studies. Introduction of halothane increases the intensity of the liquid phase peaks and decreases the intensity of the solid phase peaks. This change was evident within 5 min, remained stable at fixed halothane concentrations, and was reversible on halothane removal. In contrast, F6 produced only minor effects on the relative intensities. Note that the diffraction pattern is qualitatively different from that obtained using neutron scattering and d62-DPPC/DLPC because both lipid phases scatter X-rays in proportion to their similar electron densities, whereas for neutrons, the phase

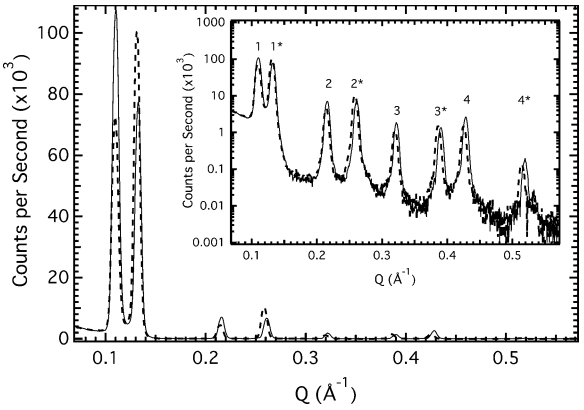

Figure 3. Change in Bragg X-ray diffraction for a 1:1 mixture of DPPL/DLPC multilayers on a glass before (solid line) and after (dotted line) addition of halothane 2.9 mol %. The first peak corresponds to the DPPC-rich "solid" phase, and the second to the DLPC-rich "liquid" phase. Inset illustrates the diffraction pattern displayed in logarithmic scale. The trace after halothane addition has been normalized to account for nonspecific attenuation of X-ray intensity by the halothane vapor.

containing mainly d62-DPPC has a very different neutron scattering length density than the mainly DLPC phase.

To quantify the difference between halothane and F6, we measured the ratio of the intensities of the first-order diffraction peaks for each phase as an estimate of the ratio of the lipid mass in the two phases. Halothane and F6 both produce nonspecific changes in X-ray Bragg diffraction. The intensity of a diffraction peak depends upon the structure factor, and the geometrical and mosaic spread factors. We did not observe any change in θ scans after addition of halothane or F6, suggesting that the mosaic spreads remained constant. Thus, both halothane and F6 appear to affect the level of disorder in the lipid multilayers, but only halothane produces the large shift in phase transition temperature. While the initial ratio of solid to liquid lipid phases varied between samples, the relative content of solid phase always decreased upon halothane addition. Figure 4 illustrates the change in the ratio of solid/liquid phases plotted against concentrations of halothane and F6. There is a marked difference between the slopes of the linear regression lines for halothane, −17.6 ± 3.5, and for F6, −0.6 ± 0.75 (s.d.). Even

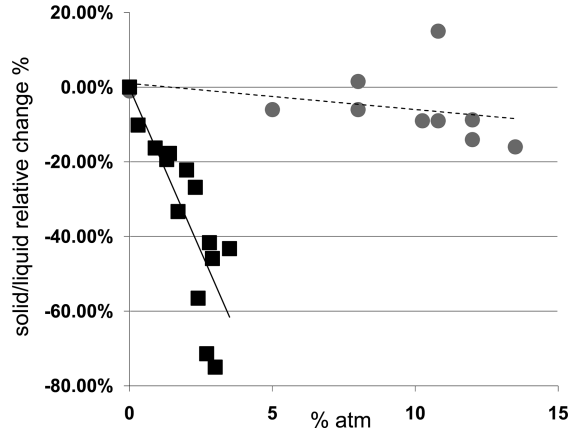

Figure 4. Relative change in the intensity of first-order solid/liquid peaks in 1:1 mixtures of DPPC/DLPC at 28 ± 1 °C (s.d.) as a function of halothane (squares) and F6 (circles) concentrations.

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corrected by the relative solubility of halothane and F6 (halothane has a 5-fold higher oil/gas partition coefficient than F6—see Supporting Information Note 5), the average slope for the halothane effect is about 5-fold higher than that for F6, significantly different at $p < 0.05$.

We obtained very similar data for the effects of halothane on the phase mixing of ternary mixtures of DOPC/DPPC (1:1) and DOPC/sphingomyelin (1:1) with 20% cholesterol. Figure 5 demonstrates the effect of 4 mol % halothane on the ratio of

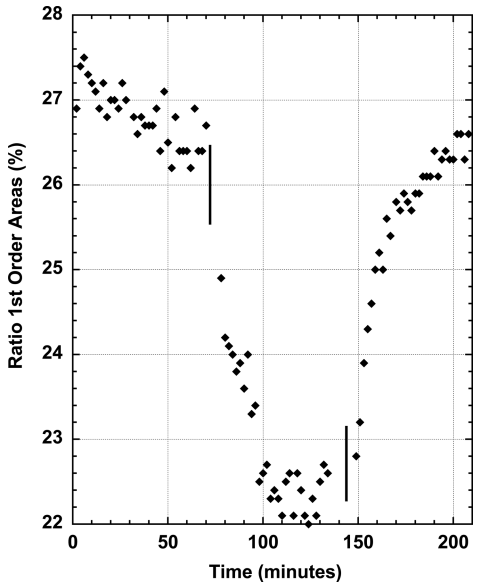

Figure 5. Ratio of first-order X-ray diffraction peak areas for a DOPC/sphingomyelin (1:1) with 20% cholesterol mixture as a function of time, at 27 °C. (Porcine brain sphingomyelin obtained from Avanti.) Humidity maintained at 99% throughout. This mixture demonstrates more complex phase behavior than the DPPC/DLPC system, but for these conditions exhibits two phase equilibria of the liquid-ordered/liquid-disordered type. First vertical bar indicates the introduction of halothane/hexadecane (1:10) into the chamber with resultant gas concentration of 4.0 + 0.2 mol %. Second vertical bar indicates removal of halothane solution and introduction of pure hexadecane to deplete gas in the chamber.

first-order peak areas in a mixture of DOPC/sphingomyelin (1:1) with 20% cholesterol. Note that the ratio declines by about 20% and quickly reverses after the halothane is withdrawn. Further description of the phase behavior of these mixtures and their responses to different anesthetics will be presented elsewhere.

To compare our methods with those of previous investigations,[2] we recorded X-ray diffraction (Supporting Information Figure 2) from multilayer samples of DMPC with 40 mol % cholesterol, in the absence and presence of 7.3 mol % halothane (about ten times the minimum alveolar concentration). In accord with the earlier findings, the headgroup to headgroup distance in the DMPC/cholesterol membrane (Figure 6) does not shift significantly (<0.03 Å) in the presence of this large anesthetic concentration. However, repeating the measurements for membranes composed of dioleoylphosphatidylcholine (DOPC), an unsaturated lipid (Supporting Information Figure 3), we found that 3.4 mol % halothane produces a small, but measurable change (−0.65 Å) in the thickness of the bilayer between headgroups with a −0.23 Å change in *d*-spacing (Figure 7). We have scaled the

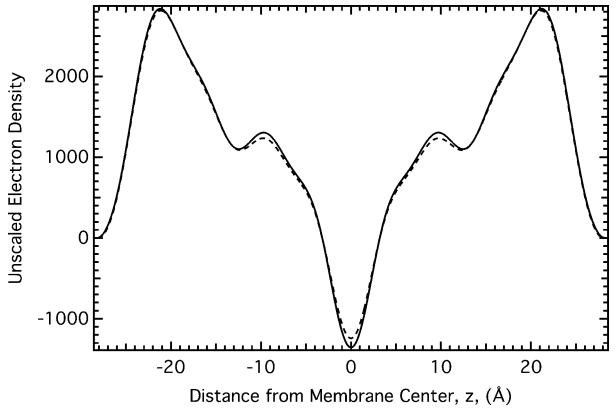

Figure 6. Electron density profiles for DMPC+40% cholesterol mulitlayers at 28 °C before (solid) and after (dashed) the addition of halothane at 7.3 mol %. No significant shift in location of headgroups is observed, only the slight changes toward the center of the membrane, as previously described.[2]

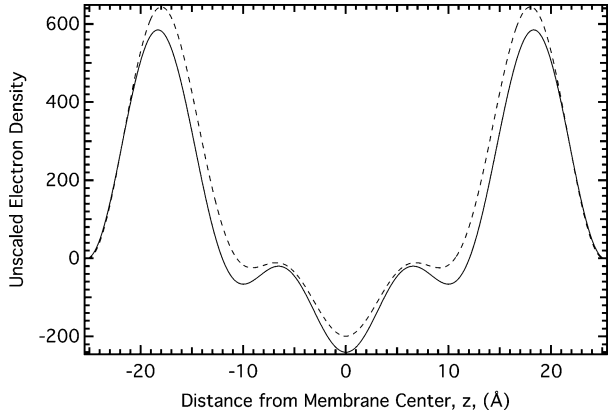

Figure 7. Electron density profiles for the DOPC multilayers at 28 °C before (solid) and after (dashed) the addition of 3.4 mol % halothane. Small perturbations in the headgroup to headgroup distance (−0.65 Å) across the bilayer are evident in the presence of halothane.

plots to match the electron density at the center of the water layers. The change in electron density in the interfacial area is consistent with the preferential partitioning of halothane into this region of the membrane, as described in simulations.[20] (See Supporting Information Note 4 for further details on the relationship between the diffraction plots and electron density profiles). Concentrations of F6 up to 7.5 mol % produced no significant change (<0.03 Å) in headgroup to headgroup distance (data not shown).

DISCUSSION

The effects of volatile agents on the cooperative interactions between lipid molecules in bilayers seem to depend not only on the amount of gas partitioning into the membrane, but also on where these molecules distribute in the bilayer.[21] Our data support this conjecture. The partition coefficient (see Supporting Information for methods) of halothane vapor into DLPC (168 ± 7) agrees well with values obtained for liquid phase bilayer vesicles[22] and is over twice the partition coefficient for gel phase DPPC (68 ± 6). However, differential partition coefficients alone will not explain the shifts in mixing temperatures demonstrated in the present study. The corresponding entropic component of the free energy of

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halothane absorbed from 2 mol % vapor by the domains in a 1:1 DPPC/DLPC membrane relaxing into a uniformly distributed bilayer (about −0.2 kJ/mol lipid) is less than 1% of the excess heat capacity exhibited by binary lipid mixtures at the transition temperature.[23] Moreover, even 5-fold higher concentrations of F6 did not produce solid to liquid phase shifts comparable to those produced by halothane, even though the amounts of these agents absorbed by the bilayers would have been roughly equivalent.

The importance of membrane-modifying agent distribution in the bilayer is also supported by our diffraction data on DOPC membranes demonstrating that halothane changes the headgroup-to-headgroup spacing significantly, while F6 does not, and by numerical simulations from another lab showing that halothane preferentially resides in the interfacial area, while another nonimmobilizer (hexafluoroethane) distributes evenly across the hydrocarbon chains.[20] Recently, in a separate study using gramicidin channels as a probe of membrane mechanics, we have concluded that addition of nonlamellar lipids or cholesterol to DOPC bilayers can reduce partitioning of halothane into the regions of bilayer responsible for its interaction with the channels by as much as 3-fold.[24] These data are also consistent with recent work, by very different methods, demonstrating preferential distribution of anesthetics into a liquid disordered phase in DPPC/cholesterol (2.5 mol %) bilayers at 45 °C.[25]

While the halothane-induced change in DOPC membranes is easily measured as a sub-Angstrom effect on membrane thickness, this change alone may not be a significant influence on ion channels. An upper estimate of the corresponding free energy for the "hydrophobic mismatch"[26,27] experienced by a trans-membrane protein is given by the product of water/oil surface tension, protein outer circumference, and the change in the membrane hydrophobic thickness. For DOPC membranes, this estimate is close to 3 k_BT. However, the actual energy changes involved via this mechanism are usually much smaller than the upper estimate.[27]

Differential distribution of anesthetics across the membrane bilayer may produce changes in lateral pressures (membrane tension) as suggested by Gruner and Shyamsunder[28] and Cantor.[29] The present data do not allow us to rigorously evaluate this hypothesis. However, changes in phase mixing in multicomponent membranes would provide substantial effects on membrane properties such as area per lipid. Halothane at 3.4 mol % produced slightly less than 2% change in membrane thickness in DOPC. Since membranes are incompressible, this should correspond to a 2% increase in membrane area per lipid, comparable to the effects predicted by simulations.[20b] Halothane at 2.9 mol % produced about a 40% decrease in the solid/liquid phase ratio of DPPC/DLPC (Figure 4). Given the areas/lipid molecule of DPPC in solid[30] and liquid[31] phases of 47 and 64 Å^2, respectively, at an initial ratio of 1:1 solid to liquid phases, this change in phase ratio corresponds to an increase of about 4% in membrane area per lipid molecule. Estimates for ternary systems containing cholesterol will be more complex, due to the pronounced effects of cholesterol on bilayer thickness and area per lipid molecule.[32]

The X-ray and neutron scattering methods used here do not involve any probe molecules and, therefore, do not disturb the model membrane phase behavior. They demonstrate effects of the inhalational anesthetic halothane on membrane organization and in particular on mixing transitions which can be quite pronounced. This analysis is consistent with a growing body of evidence[8b−d,33] showing that conformational dynamics of transmembrane channels are very sensitive to the parameters of the lipid bilayer within which they reside. The generalizability of these findings to other lipid mixtures and to other anesthetics remains to be established.

■ ASSOCIATED CONTENT

Supporting Information

Additional information and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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