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Effects of Isoflurane, Halothane and Chloroform on the Interactions and Lateral Organization of Lipids in the Liquid-Ordered Phase

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Abstract

The first quantitative insight has been obtained into the effects that volatile anesthetics have on the interactions and lateral organization of lipids in model membranes that mimic "lipid rafts". Specifically, nearest-neighbor recogntion measurements, in combination with Monte Carlo simulations, have been used to investigate the action of isoflurane, halothane and chloroform on the compactness and lateral organization of cholesterol-rich bilayers of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) in the liquid-ordered (l_o) phase. All three anesthetics induce a similar weakening of sterol-phospholipid association, corresponding to ca. 30 cal/mol of lipid at clinically-relevant concentrations. Monte Carlo lattice simulations show that the lateral organization of the l_o phase, under such conditions, remains virtually unchanged. In sharp contrast to their action on the l_o phase, these anesthetics have been found to have a similar *strengthening effect on* sterol-phospholipid association in the liquid-disordered (l_d) phase. The possibility of discrete complexes being formed between DPPC and these anesthetics, and the biological relevance of these findings, are discussed.

INTRODUCTION

General anesthesia represents one of the most important advances in the history of medicine. Despite numerous mechanistic studies, which have revealed that general anesthetics disrupt the flow of ions across the cell membranes, their *primary targets* continue to be debated.[1–3]

A classic view of general anesthetics is that lipids in cell membranes are the main targets. Early evidence in support of this hypothesis was based on correlations between anesthetic potency and olive oil/gas partitition coefficients—the so-called Meyer-Overton rule.[1,2,4,5] A more modern version of this hypothesis assumes that general anesthetics alter the compactness, lateral organization, or lateral pressure profile of "lipid rafts"; that is, regions that are presumed to be rich in cholesterol and high-melting lipids.[6–11] Such changes are then thought to modify the conformation and activity of neighboring integral proteins involved in signal transduction.[12] Another model assumes that general anesthetics displace cholesterol from its weak associations with phospholipids, thereby releasing it in an "active" form, which changes the functioning of susceptible membrane proteins such as ion channels.[13] Alternatively, it has been proposed that membrane proteins, themselves, are the

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Supporting Information.
Experimental procedures and tables of raw data. This material is available free of charge via the Internet at http://pubs.acs.org.

primary targets of general anesthetics.[2,14] More recently, it has been posited that general anesthetics may disrupt the flow of ions across *lipid-based* ion channels.[15] Given the diversity of these hypotheses, and the absence of definitive evidence that can rule out any of them, a fair statement is that *the mechanism by which general anesthetics operate is unknown*.

The primary aim of this work was to quantify the effects that volatile general anesthetics have on *the compactness and the lateral organization of lipid bilayers* in the liquid- ordered (l_o) phase—what is considered to be the best working model for lipid rafts.[16] Specifically, we sought to determine how three volatile anesthetics (isoflurane, halothane and chloroform) influence nearest-neighbor free energies of interaction in cholesterol-rich membranes derived from 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC). We also sought to determine how these effects may influence the lateral organization of such membranes.[17] Because the liquid-disordered phase (l_d) is thought to mimic less compact regions of cell membranes that are complementary to the rafts in biological membranes, it was also of interest to examine the effects that these anesthetics have on this phase.

Our reason for choosing these particular anesthetics was two-fold. First, isoflurane, halothane and chloroform vary, significantly, in their molecular volumes. More specifically, they are 144 $Å^3$, 110 $Å^3$ and 90 $Å^3$, respectively.[18] Because it has previously been suggested that molecular volume is a critical factor in the biological action of general anesthetics, we sought to determine whether they would act differently on the l_o phase.[19] Second, all three of these anesthetics have been used, clinically, although only isoflurane remains in broad use.

RESULTS AND DISCUSSION

Quantifying Nearest-Neighbor Interactions in Lipid Bilayers

In this work, we have made extensive use of the nearest-neighbor recognition (NNR) method for quantifying free energies of interactions.[20] In contrast to other methods that have been used for such a purpose (i.e., differential scanning calorimetry, fluorescence resonance energy transfer, isothermal titration calorimetry, and analyses of phase diagrams), NNR analysis does not require any matching of experimental data with theoretical curves.[21] Moreover, the NNR method can detect changes in free energies of interaction down to tens of calories per mole—a magnitude that has been found to significantly affect the lateral organization of certain membranes.[22]

In essence, the NNR method is a chemical technique that probes lipid mixing at the molecular level.[20] Thus, NNR measurements take molecular-level snapshots of bilayer organization by detecting and quantifying the thermodynamic tendency of exchangeable monomers to become nearest- neighbors of one another. Typically, two lipids of interest (**A** and **B**) are converted into exchangeable dimers via the introduction of disulfide bonds (i.e., homodimers **AA** and **BB**, and heterodimer **AB**), which are then allowed to undergo monomer interchange via thiolate-disulfide exchange (Figure 1). The resulting equilibrium that is established is governed by an equilibrium constant, $K = [\mathbf{AB}]^2/([\mathbf{AA}][\mathbf{BB}])$. When lipid monomers **A** and **B** mix ideally, this is reflected by an equilibrium constant that equals 4.0. When homo-associations are favored, the equilibrium constant is less than 4.0; favored hetero-associations are indicated by a value that is greater than 4.0. Taking statistical considerations into account, nearest-neighbor interaction free energies between **A** and **B** are then given by $\omega_{AB} = -1/2\ RT\ \ln(K/4)$.[21] Values of ω_{AB} are the primary information that is sought from NNR measurements.

The exchangeable lipids **1** and **2** that were chosen for this work bear the same diacylglycerol and sterol frameworks as DPPC and cholesterol, respectively (Chart 2). As shown elsewhere, when used at low concentrations (ca. 2.5 mol% of each lipid), NNR measurements with **1** and **2** not only reflect their own interactions but also those of host membranes made from DPPC and cholesterol.[20d,f] Thus, despite the presence of a negative charge in **1**, and a disulfide bridge in their head group region, the mixing properties of these exchangeable lipids bear a striking resemblance to those of DPPC and cholesterol. That the presence of a disulfide bridge does not in any way influence the packing properties of these exchangeable lipids has already been demonstrated through monolayer measurements. Specifically, limiting areas and condensing properties were found to be indistinguishable from lipid analogs that are devoid of the such a bridge.[20d] Additionally, **1** and DPPC have nearly identical gel to liquid-crystalline phase transition temperatures, which are 41.9°C and 41.5°C, respectively.[23] Thus, **1** and **2** *are excellent surrogates for DPPC and cholesterol, respectively*. For this reason, changes in ω_{AB} can be used to follow the conversion from the liquid-disordered to the liquid-ordered phase.[20f] In other words, ω_{AB} for **1** and **2** reflects the compactness of host membranes made from DPPC and cholesterol, becoming more negative as the compactness of the membrane increases.[24]

Measurement of Anesthetic Binding and Nearest-Neighbor Recognition

To measure the binding of volatile general anesthetics by lipids, we used a specially-designed reaction vessel in which a liposome dispersion was physically separated from a buffer phase (Figure 2). Nearest-neighbor recognition measurements were carried out in similarly configured vessels. Volatilization of the anesthetic was then made by direct injection into an open tube within this vessel that was sealed with a Teflon-coated rubber septum. Quantification of the amount of anesthetic that became bound to the liposomes was determined by measuring the excess that was present in the dispersion relative to the liposome-free buffer *via* gas-liquid chromatography. Detailed experimental procedures that have been developed for such analyses have previously been described. [17] For all of the NNR experiments that are reported herein, an equimolar mixture of **A** and **B** (2.5 mol% of each lipid) was included in host membranes (95 mol%) made from a mixture of DPPC/cholesterol or pure DPPC. Thus, *cholesterol-rich membranes* were made from DPPC/cholesterol/**1**/**2** (57.5/37.5/2.5/2.5, mol/mol/mol/mol), and *cholesterol-poor* analogs were made from DPPC/**1**/**2** (95.0/2.5/2.5, mol/mol/mol). At the temperature used in this work (i.e., 45°C), the former is in the l_o phase, and the latter is in the l_d phase, as previously confirmed by fluorescence measurements.[24]

Influence of Isoflurane

In Figure 3A are shown the binding data that was obtained for isoflurane, where the number of moles of anesthetic that are bound per mole of phospholipid, $X_{A/P}$, have been plotted as a function of the concentration of isoflurane present in solution.[25] For the cholesterol-rich bilayers, $X_{A/P}$ increased, exponentially, as the isoflurane concentration increased (saturation in buffer occurs at ca. 12 mM). In contrast, the cholesterol-poor membranes showed a *linear* increase in $X_{A/P}$ over a similar concentration range. In addition, the extent of binding for the cholesterol-poor membranes was significantly greater than that measured for the cholesterol-rich bilayers at all isoflurane concentrations examined.

Nearest-neighbor recognition measurements that were made under similar conditions mirrored these exponential and linear dependencies (Figure 3B). Thus, for the cholesterol-rich membranes, the value of ω_{AB} became less negative with increasing isoflurane concentrations in the membrane, reflecting a *weakening* effect on sterol-phospholipid association. For the cholesterol-poor membranes, a linear dependence on the isoflurane concentration was observed. In this case, incremental addition of the anesthetic led to *a*

strengthening of sterol-phospholipid association. Although ω_{AB} appears to reach a plateau in the cholesterol-rich membranes when $X_{A/P}$ is ca. 0.3, the strengthening effect that isoflurane has on the cholesterol-poor membranes continues up to the point of saturation in buffer.

Influence of Halothane

Results that were obtained with halothane showed, qualitatively, similar trends as that found with isoflurane, except that a slight positive deviation from linearity was apparent for the cholesterol-poor membranes when $X_{A/B}$ was greater than ca. 1.0 (Figure 4A). The values of ω_{AB} also mirrored these exponential and linear dependencies. In contrast to isoflurane, however, the effects of halothane on ω_{AB} appear to be maximized for the cholesterol-rich and cholesterol-poor membranes when $X_{A/P}$ is ca. 1.0. At this point, ω_{AB} comes close to a convergent value of ca. −100 cal/mol.

Influence of Chloroform

The behavior that was observed for these cholesterol-rich and cholesterol-poor membranes membranes with respect to chloroform was very similar to that of halothane (Figure 5). Thus, the observed decrease in sterol-phospholipid association in the presence of the anesthetic for the cholesterol-rich membranes, and the increase in association for the cholesterol-poor membranes appear to level off when $X_{A/P}$ is ca. 1. With chloroform, convergence appears to be complete when $\omega_{AB} = -130$ cal/mol.

Formation of Anesthetic--Phospholipid Complexes?

The finding that the addition of chloroform and halothane to cholesterol-rich and cholesterol-poor membranes leads, essentially, to the same value of ω_{AB} indicates that sterol-phospholipid associations are also becoming similar in both membranes. The fact that these values converge when the anesthetic concentration in the membrane is close to that of DPPC is intriguing. What does this mean?

A simple explanation that can account for these findings is that DPPC forms a 1/1 complex with chloroform and halothane in both membrane types. This complex would interact with cholesterol in a similar manner in both l_d and l_o phases. In previous work it has been shown by Raman spectroscopy that the presence of an excess of chloroform increases the number of gauche conformers of DPPC in cholesterol- rich and cholesterol-poor membranes.[6] With more gauche conformers present, there is a reduction in the number of hydrocarbon contacts that can be made with cholesterol; hence, the cholesterol-phospholipid association is weakened in the l_o phase. In the l_d phase, where all nearest-neighbor associations are much weaker to begin with, we posit that a further weakening of cholesterol-phospholipid association is offset by a *relatively larger weakening* of phospholipid-phospholipid association, resulting in a net increase in cholesterol-phospholipid association. (Note that ω_{AB} is the difference in energy between cholesterol-phospholipid and the average of cholesterol-cholesterol and phospholipid-phospholipid interactions).

It is also worth noting that at low anesthetic concentrations, the anesthetic partition coefficient between the l_d and l_o phases clearly favors the l_d phase, which is in agreement with freezing point depression induced by halothane in pure DPPC.[26] However, as ω_{AB} between phospholipid and cholesterol converge towards a common value in l_d and l_o phases, the partition coefficient of the anesthetic *between* the two phases approaches 1. That this is the case is evident from plots made of K_p versus the anesthetic concentration in buffer, where $K_p = [X_{A/P}]_{\text{liquid-disordered}}/[X_{A/P}]_{\text{liquid-ordered}}$. Such a plot is shown for chloroform in Figure 6; similar plots are given in the Supporting Information for halothane and isoflurane.

These results are fully consistent with anesthetic- phospholipid interactions becoming similar in both phases.

The simplest explanation for this behavior is that both phases are becoming saturated with anesthetic, and that this saturation occurs close to anesthetic/phospholipid ratios between 1–3.[27] However, that the l_d and l_o phases are not becoming identical (which is suggested for chloroform, Figure 5B), is indicated by previous Laurdan generalized polarization measurements. Specifically, these measurements have shown that the l_o phase remains significantly more ordered even at saturating amounts of the anesthetic.[6]

Biological Relevance

The present findings show striking similarities to what has recently been reported for the action of a variety of amphipaths on red blood cells (RBCs).[13] Before discussing these similarities, we wish to point out that the weakening effect that these anesthetics have on sterol-phospholipid association in cholesterol-rich bilayers and their strengthening effect in cholesterol-poor membranes is exactly analogous to cholesterol's fluidizing effect on the gel phase and its condensing effect on the liquid-crystalline phase.[28] This analogy suggests that these anesthetics may act as "partial replacements" for cholesterol in lipid bilayers.

A major conclusion that was reached in the RBC study was, in fact, that these amphipaths displace and replace cholesterol from its weak association with phospholipids. Evidence in support of this conclusion was based on the finding that certain of these amphipaths led to the forestalling of the lysis of *cholesterol-depleted* RBCs.[13] It was further concluded that this replacement was stoichiometric, in which approximately one mole of any of these amphipaths in the RBC membrane displaces and activates one mole of cholesterol. This one-for-one exchange suggests the existence of a competitive complexation process, which is consistent with the hypothesis that DPPC and chloroform (as well as halothane) are forming 1/1 complexes. However, it should be noted that the effects of cholesterol and anesthetic on the excess heat capacity of DPPC mixtures are known to be very different.[26] Both cause freezing point depressions at low concentrations, but the effect of anesthetic is much more pronounced.[26] More noticeably, whereas cholesterol causes the appearance of a high-temperature broad transition,[21] which has been interpreted within the context of the complex model as thermal melting of the complexes,[21] the anesthetics cause a pronounced general broadening. Although a molecular-level explanation for the latter remains lacking, it does indicate that a reduction in the phospholipid cluster sizes in the transition region has occurred. [26] Most importantly, the present findings clearly show that *the chemical activity of cholesterol increases in the presence of anesthetics because the interactions between phospholipid and cholesterol become weaker in the l_o phase*. This is why cholesterol is released from the RBC membrane, as shown by increased activity of cholesterol oxidase.[13]

Finally, the fact that isoflurane, halothane and chloroform show, essentially, the same effect on the phospholipid-cholesterol interaction when $X_{a/p}$ is less than ca. 0.50, despite their differences in molecular volumes, is consistent with the limited specificity found for the association of amphipaths with phospholipids (Figure 7).

Influence of Isoflurane, Halothane and Chloroform on the Lateral Organization of the Liquid-Ordered Phase

The clincally-relevant concentration of general anesthetics has been estimated to be ca. 3 mol% relative to the plasma membrane bilayer lipids.[29] To a first approximation, the loosening effect that isoflurane, halothane and chloroform have on these cholesterol-rich membranes is ca. 30 cal/mol (Supporting Information). To judge the effects that such a change in ω_{AB} would have on the lateral organization of DPPC/cholesterol (60/40, mol/

mol), we carried out Monte Carlo simulations of the membrane, modeled as a triangular lattice, where each site represents a phospholipid or a cholesterol molecule. It should be noted that in these simulations the nearest-neighbor interactions between anesthetic and the lipids are not explicitly included. Instead, addition of anesthetic was modeled by taking into account its effect on the phospholipid-cholesterol interactions. Thus, ω_{AB} was set at −270 cal/mol in the l_o phase in the absence of anesthetic, and to −240 cal/mol in the presence of 3 mole % anesthetic. Such small differences do not lead to any noticeable difference in the lateral organization of the l_o phase (See Fig. SI-7 in the Supporting Information).

CONCLUSIONS

This study has provided the first quantitative insight into the effects that volatile general anesthetics have on the compactness and lateral organization of the liquid-ordered phase. Thus, isoflurane, halothane and chloroform, despite their differences in molecular volumes, have been found to have, essentially, the same weakening effect on sterol-phospholipid association in cholesterol-rich membranes in the l_o phase. Additionally, these anesthetics were found to have a similar *strengthening* effect on sterol-phospholipid association in the l_d phase. This weakening and strengthening effects are exactly analogous to cholesterol's long-known fluidizing and condensing effects on lipid bilayers. Striking similarities have also been found between these results and those obtained for the action of a variety of amphipaths on RBCs.

Anesthetics have been suggested to alter the conformation and activity of susceptible membrane proteins (e.g., ion channels) by (i) changing the compactness or lateral pressure profile of the lipid matrix or (ii) causing the release of an "active" form of cholesterol.[7,8,13] They have also been suggested to alter the packing of lipid-based ion channels. [15] What we have found is that they do not cause a significant change in lipid organization in the l_o phase. Thus, the idea that anesthetics may act by changing the lipid packing within a single phase seems unlikely. Alternatively, it has previously been found that a change of only 50 cal/mol in the interaction parameter between ordered and disordered lipids has a large effect on the sizes of the l_d/l_o domains in ternary lipid mixtures of cholesterol with ordered and disordered phospholipids.[22] Thus, a primary effect of general anesthetics may be to alter the overall lateral organization of the l_d and l_o domains in membranes. Finally, we have found that the chemical activity of cholesterol in the l_o phase increases in the presence of clinically-relevant concentrations of anesthetics; in the l_d phase, however, that activity actually decreases. Thus, whether cholesterol is released or not depends on the composition and state of the original membrane.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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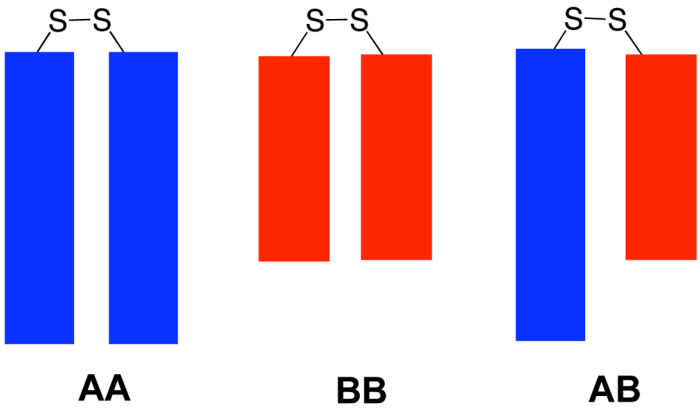

$$AA + BB \underset{}{\overset{K}{\rightleftharpoons}} 2\,AB$$

$$K = \frac{[AB]^2}{[AA]\,[BB]}$$

K=4 for random mixing of A and B

$$\omega_{AB} = -1/2\ RT\ \ln(K/4)$$

Figure 1.

A stylized illustration showing the exchangeable homodimers, **AA** and **BB** and the corresponding heterodimer, **AB**, plus the equations that describe the dimer equilibrium and the relationship between the equilibrium constant, *K*, and the corresponding nearest-neighbor interaction free energy, ω_{AB}, between **A** and **B**.

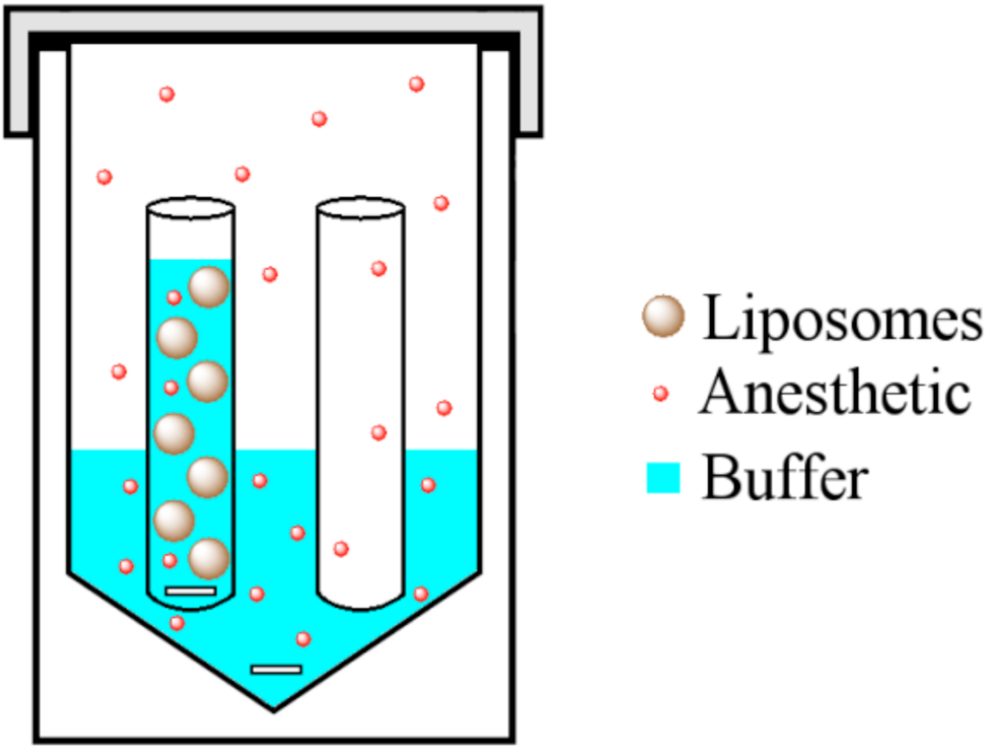

Figure 2.
Reaction vessel used for carrying out binding measurements.

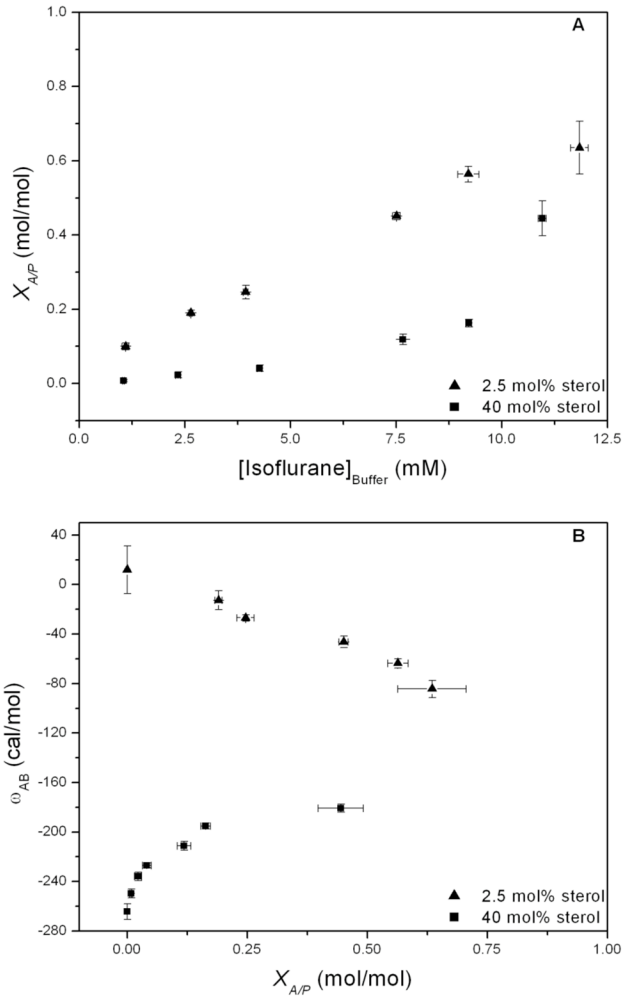

Figure 3.

(A) Plot of $X_{A/P}$ versus isoflurane concentration in buffer at 45°C for cholesterol-rich and cholesterol-poor bilayers. (B) Plot of free energy of interaction between **1** and **2** (i.e., ω_{AB}) as a function of $X_{A/P}$. Error bars that are not visible lie within the symbols themselves. For each of these measurements, the total concentration of lipid was 4.1 mM.

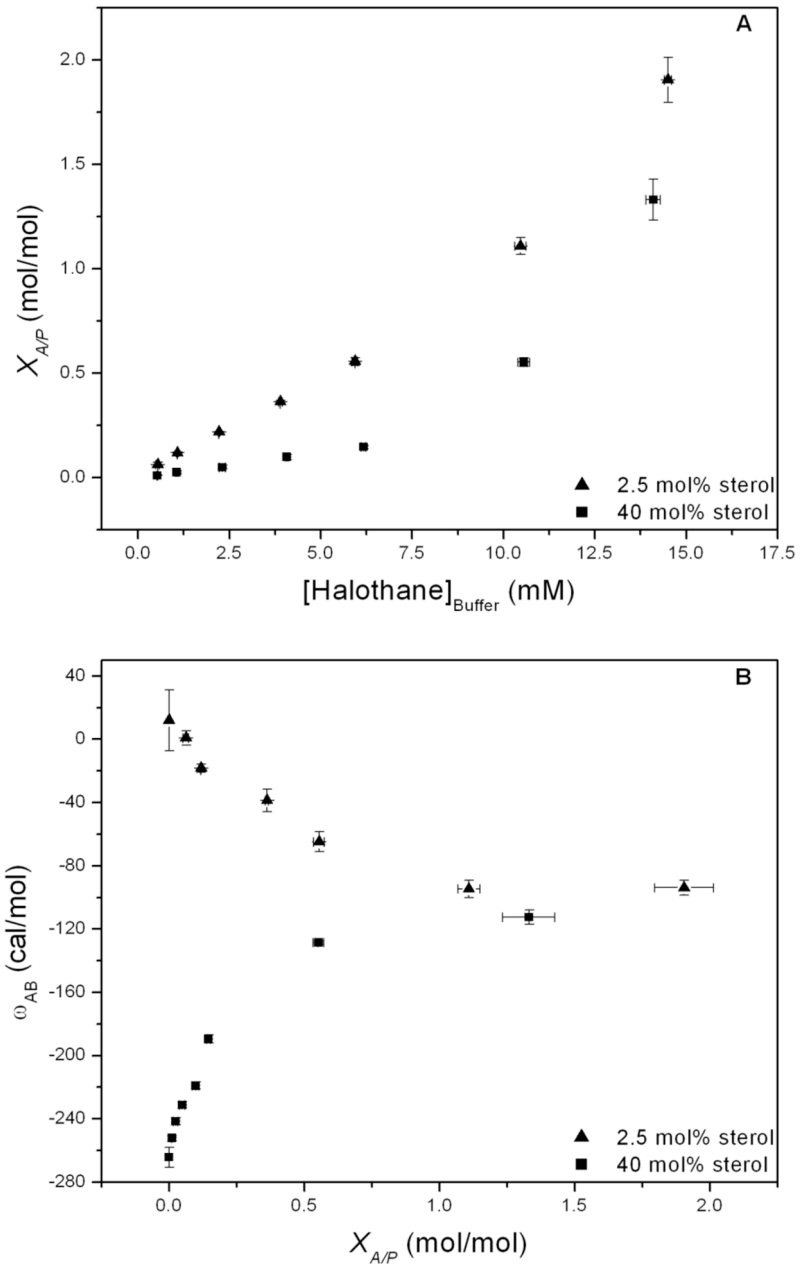

Figure 4.

(A) Plot of $X_{A/P}$ versus halothane concentration in buffer at 45°C for cholesterol-rich and cholesterol-poor bilayers. (B) Plot of free energy of interaction between **1** and **2** (i.e., ω_{AB}) as a function of $X_{A/P}$. Error bars that are not visible lie within the symbols themselves. For each of these measurements, the total concentration of lipid was 4.1 mM.

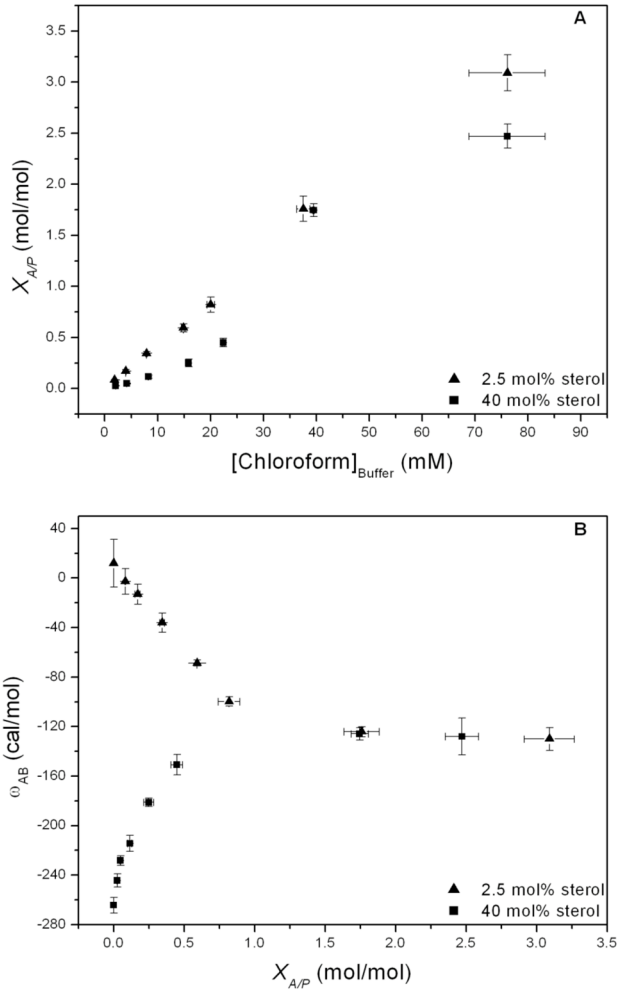

Figure 5.

(A) Plot of $X_{A/P}$ versus chloroform concentration in buffer at 45°C for cholesterol-rich and cholesterol-poor bilayers. (B) Plot of free energy of interaction between **1** and **2** (i.e., ω_{AB}) as a function of $X_{A/P}$. Error bars that are not visible lie within the symbols themselves. For each of these measurements, the total concentration of lipid was 4.1 mM.

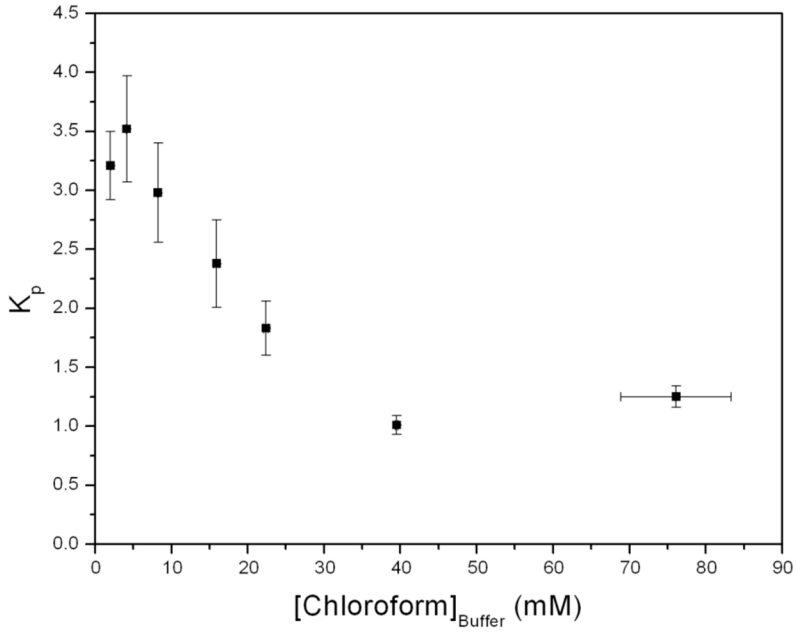

Figure 6.

Plot of Kp versus the concentration of chloroform in buffer, where $K_p = [X_{A/P}]_{liquid\text{-}disordered}/[X_{A/P}]_{liquid\text{-}ordered}$.

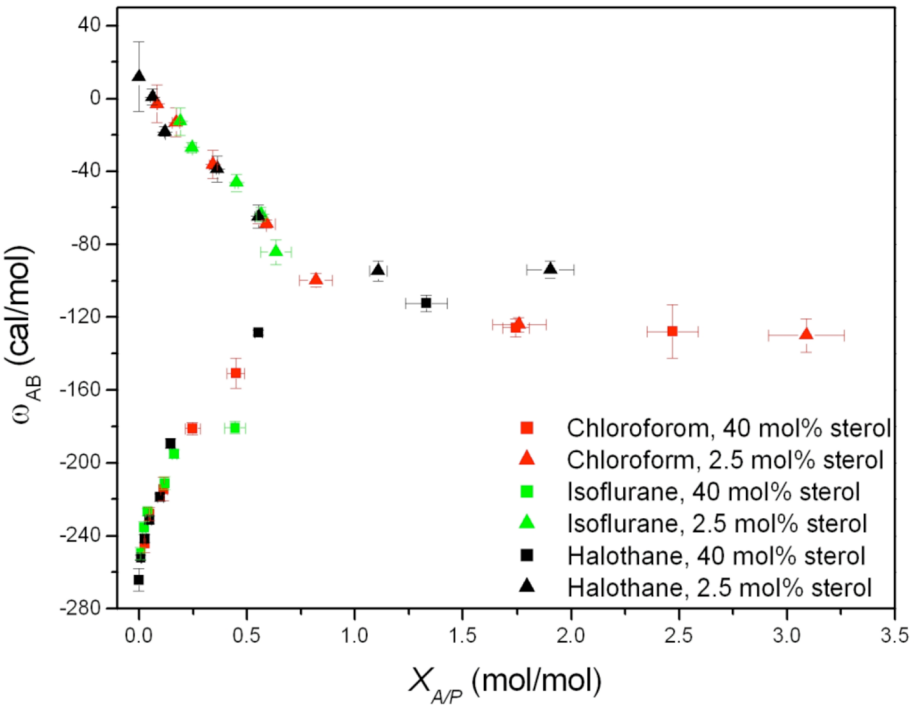

Figure 7.
Composite plot of ω_{AB} as a function of $X_{A/P}$ for isoflurane, halothane and chloroform (data taken from Figures 3,4 and 5).

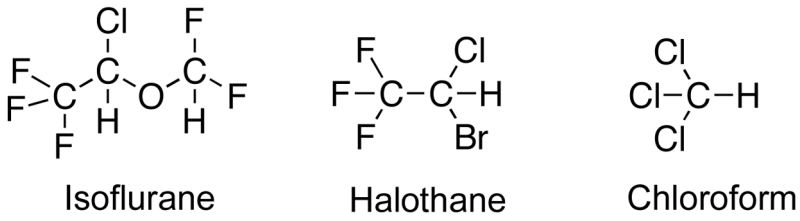

Chart 1.

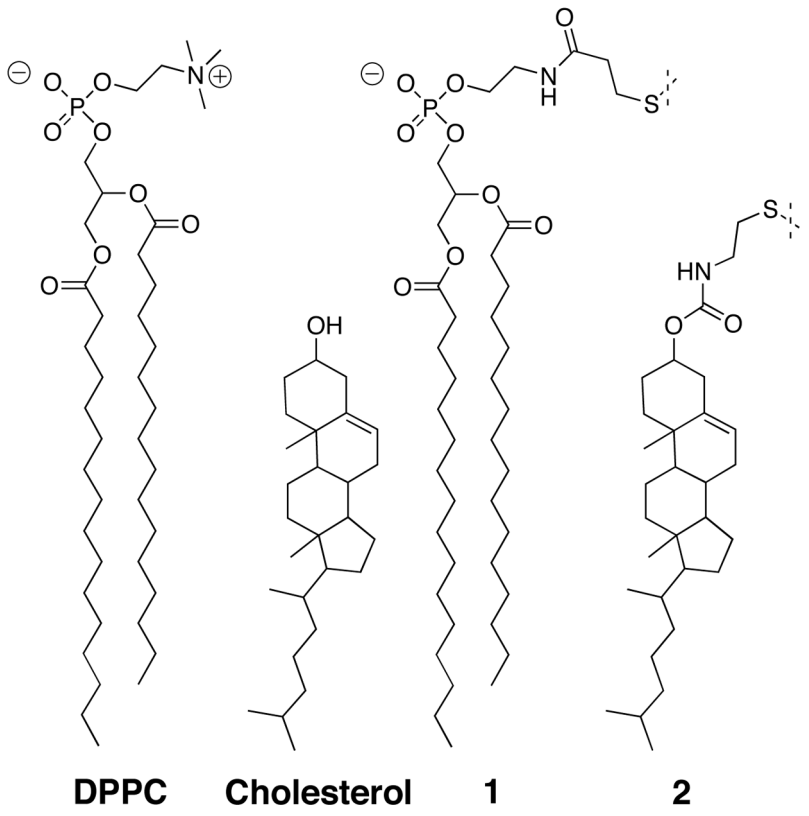

Chart 2.