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Infrared spectra of phospholipid membranes: interfacial dehydration by volatile anesthetics and phase transition

Yeong-Shou Tsai, Shao-Mu Ma, Shinya Nishimura and Issaku Ueda

Department of Anesthesia, University of Utah School of Medicine, and Anesthesia Service, Veterans Affairs Medical Center, Salt Lake City, UT (U.S.A.)

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Fourier-transform infrared attenuated total reflection (ATR) spectroscopy was used to study the effect of volatile anesthetics on fully hydrated dipalmitoylphosphatidylcholine (DPPC) vesicle membranes. The main phase transition was monitored by the change in the C-H_2 asymmetric stretching frequencies of the lipid tails. The surface property was analyzed by the changes in the P=O stretching, $(CH_3)_3$-N^+ stretching of the hydrophilic head, and C=O stretching of the glycerol skeleton. The partial pressures of those agents that decreased the transition temperature 1.0 C° were halothane 0.75, enflurane 1.90 and CCl_4 0.85 kPa. At a 2:1 lipid/anesthetic mole ratio, the polar anesthetics, halothane and enflurane, increased the ratio of (P=O stretching band area)/($(CH_3)_3$-N^+ stretching band area) by 26.3% and 21.1%, respectively, whereas apolar CCl_4 increased it 10.5%. The water molecules bound to the P=O moiety are apparently replaced by the anesthetic molecules. The deconvoluted C=O spectra showed two peaks: free *sn*-1 that is closer to the lipid core and hydrogen-bonded *sn*-2 that is closer to the polar head. Addition of halothane and enflurane, but not CCl_4, increased the number of peaks to three. The third peak is free *sn*-2, formed by disrupting hydrogen-bonding to water. Because the temperature-induced spectral change was limited to C-H_2 stretching at the main phase transition, the effects of anesthetics on the lipid membrane structure are not identical to temperature elevation. Among anesthetics, the effects of apolar and polar molecules on the interfacial properties are different.

Introduction

Volatile anesthetics disorder biological and model membranes and depress the main phase-transition temperature. Because the order-disorder phase transition is a property expressed by the conformation of the hydrocarbon chains, depression of the main phase-transition temperature by volatile anesthetics is generally attributed to their direct perturbing action on the lipid core. Nevertheless, a distinctive feature of modern volatile anesthetics is the presence of acidic protons in their molecular structures. These molecules are polar and amphipathic, and two-dimensional proton nuclear Overhauser effect NMR spectroscopy [1] showed that only the hydrophobic end of a volatile anesthetic methoxyflurane interacted with hydrophilic part of phospholipid membranes with no other cross-peaks, demonstrating the preference of the volatile anesthetic to stay at the interfacial region. The preferred residence of volatile anesthetics at the membrane/water interface has been demonstrated by ^{1}H-NMR [2], ^{19}F-NMR [3], and gas chromatography [4]. Because lipid membranes are supported by the matrix of hydrogen-bonded water molecules, the disordering effect of amphipathic anesthetics on lipid membranes may be invoked by disengaging the support of the water matrix.

Apolar molecules such as xenon or cyclopropane, on the other hand, penetrate into the lipid core and directly perturb the lipid tail conformation. The mode of depression of the main transition temperature may differ between polar and apolar anesthetics.

This study compared polar halothane and enflurane with apolar carbon tetrachloride (CCl_4) concerning their effect on the water–membrane interaction of dipalmitoylphosphatidylcholine (DPPC) membranes at the order-disorder phase transition in a temperature range 25 to 50 °C.

Among various spectroscopic methods available for

Correspondence: I. Ueda, Anesthesia Service 112A, VA Medical Center, Salt Lake City, UT 84148, U.S.A.

monitoring molecular order, infrared and Raman are sensitive methods to assess dynamics of each part of the lipid molecules in the bilayer matrix. Craig et al. [5] used Raman C-C and C-H_2 spectra of the lipid tail to study halothane effect on the thermotropic phase transition of phospholipid vesicle membranes. By Fourier-transform infrared spectroscopy, we [6] have shown that the primary hydration site of DMPC molecules is the phosphate moiety, and volatile anesthetics decreased the hydration in a water-in-oil reversed micellar system. When combined with the attenuated total reflection (ATR) system, Fourier-transform infrared spectroscopy is a powerful method in analyzing the vibrational states of fully hydrated DPPC molecules in vesicle membranes.

Experimental

Synthetic 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine (L-α-dipalmitoylphosphatidylcholine, DPPC) was obtained from Sigma (St. Louis, MO), and its purity was checked by thin-layer chromatography using chloroform/methanol/water (65 : 25 : 4, v/v) to confirm single spot. Halothane was obtained from Ayerst (New York), and enflurane from Anaquest (Madison, WI). The stabilizer (thymol) contained in the halothane preparation was removed by passing through activated aluminum oxide (Fluka, Ronkonkoma, NY) columns. Spectroscopy grade carbon tetrachloride and chloroform (contains 0.7% ethanol as a stabilizer) were obtained from EM Science (Cherry Hill, NJ). Triply distilled water was used for the vesicle preparation.

DPPC was dissolved in chloroform and then dried in a rotary evaporator under the flow of nitrogen. The dried DPPC sample was kept in a desiccator overnight at reduced pressure to remove remaining chloroform. The vesicle suspension (40 mM) was prepared by adding an appropriate amount of water to the dried DPPC and vortexed. The multilamellar suspension was then sonicated in a cup-horn of a Branson Model 185 Sonifier (Danbury, CT) for 20 min at a temperature few degrees above the main transition temperature of the phospholipid. The vesicle suspension typically showed pH 6.7 and was stored in a refrigerator for a week before use to obtain relatively uniform vesicle size distribution [7].

Infrared spectra were obtained by a Perkin-Elmer (Norwalk, CT) Model 1750 FTIR spectrophotometer equipped with a Perkin-Elmer Model 7300 computer, and each spectrum was signal-averaged after 30 scans. Attenuated total reflection system was a Spectra-Tech (Stamford, CT) Model 0005-133 CIRCLE liquid cell with ZnSe crystal, and the cell volume was 0.5 ml [8]. The cell was equipped with a Spectra-Tech Model 0005-420 heating-cooling jacket to control the cell temperature. A United Systems (Dayton, OH) Digitec Model 5810 thermometer was used to monitor the temperature with 0.01 C° resolution. The IR spectra of the vesicle were scanned between 25 and 50 °C at 1 C° intervals, except at temperatures above the main phase transition where the interval was 2 C° in some cases. The spectra in the presence of anesthetics were obtained at the same temperature range.

Anesthetics were added to the vesicle suspension by a microsyringe. The anesthetic concentrations in the vesicle suspension and in the gas phase in equilibrium with the suspension were measured by gas chromatography. The vesicle suspension containing anesthetic was placed in a 1.0 ml glass bottle and closed airtight by a Teflon-lined silicone rubber cap. The gas and liquid samples were collected with a microsyringe. A Shimadzu (Columbia, MD) gas chromatograph with a flame ionization detector and a 1/8 in × 6 ft stainless steel tubing containing Porapak Q 80/100 mesh was used to determine the anesthetic concentrations in the gas and liquid phases. The chromatography system was run under isothermal condition at 160 °C using helium as the carrier gas with a flow rate of 10 ml/min.

The difference spectrum was acquired by subtracting the spectrum of water from that of the vesicle. Because the temperature dependence of the water spectrum is large [9], care was taken to match the temperature to the vesicle suspension in obtaining the water spectrum for subtraction.

The peak positions in the infrared spectra were analyzed with a computer program according to the method by Cameron et al. [10] The accuracy of the peak position was ± 0.1 cm^{-1}. Deconvoluted spectra of the C=O stretching band were obtained by a software (Perkin-Elmer) with a half-width of smoothing function of 18 cm^{-1} and a narrowing factor of 2.25.

Results

The difference spectrum shown in Fig. 1 was obtained after subtraction of water bands from the vesicle spectrum. The frequencies of the major functional groups were: C-H_2 stretching of the hydrocarbon chains at 2920 cm^{-1} for asymmetric and 2851 cm^{-1} for symmetric, C=O stretching of the ester linkage at 1730 cm^{-1}, P=O stretching at 1230 cm^{-1}, and $(CH_3)_3$-N^+ stretching of the choline head at 970 cm^{-1}. These assignments on the major infrared bands agree with the corresponding bands in phospholipid spectrum reported in the literature [9,11–13].

1. Phase transition temperature

The main phase-transition temperature of the vesicle, T_m, was estimated from the shift of the wavenumber of the C-H_2 stretching. Of the symmetric and asymmetric stretching bands, the latter at 2920 cm^{-1} was mainly used to monitor the phase transition because the change

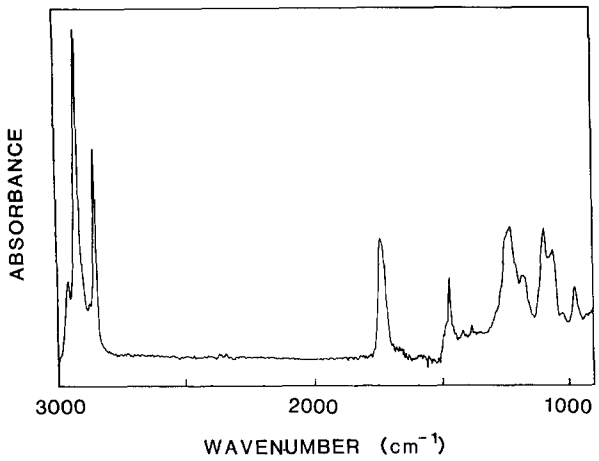

Fig. 1. The infrared difference spectrum of dipalmitoylphosphatidylcholine vesicle at 25°C after subtraction of water absorption bands.

in this band was larger than the symmetric band. Fig. 2 shows the temperature dependence of the 2920 cm^{-1} band of the vesicle in the absence and presence of halothane, enflurane, and CCl_4. The mole ratio between DPPC and the anesthetics was 2.0. The values for the transition temperature, T_m, were taken at the midpoint of the transition region. The C-H_2 stretching frequency from the DPPC vesicle showed a sharp upward shift at the main transition temperature, 39.6°C (Fig. 2). The slightly lower transition temperature compared with multilamellar preparations appears to be caused by the size of the sonicated single-shelled vesicles. Our proton-NMR data on sonicated vesicles [14] also showed 39.6°C for the main transition temperature. A small change was also observed between 34 and 36°C, that corresponds to the pretransition temperature of DPPC membranes [9,15] The results agree well with those reported in the literature [12]. All three anesthetics

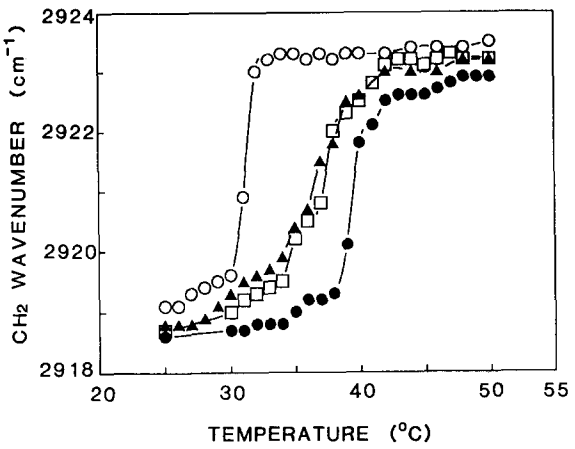

Fig. 2. CH_2 asymmetric stretching frequencies as a function of temperature for dipalmitoylphosphatidylcholine vesicle samples. Symbols: ●, controls without anesthetics; ▲, carbon tetrachloride; □, enflurane; and ○ halothane. Phospholipid/anesthetic mole ratio = 2:1.

increased the C-H_2 asymmetric stretching frequency at all temperatures (Fig. 2). Halothane, enflurane and CCl_4 lowered the main transition temperature of DPPC by 8.6 C°, 2.4 C° and 3.3 C°, respectively. The halothane result is in good agreement with the Raman data by Craig et al. [5] on DPPC liposomes. From their figure, a decrease of 8.7 C° is found at the mole ratio of DPPC/halothane 2:1.

Anesthetic partial pressure in the gas phase was used to compare their effects on the transition temperature with clinical potency. At 2:1 lipid/anesthetic mole ratio, the anesthetic partial pressures in the gas phase in equilibrium with the vesicle were halothane 6.40 kPa, enflurane 4.75 kPa, and CCl_4 2.68 kPa. Because the decrease of transition temperature depends linearly on the concentration of anesthetics [16,17], the partial pressures of these agents that depressed the transition temperature 1.0 C° were halothane 0.75, enflurane 1.9, and CCl_4 0.85 kPa. The minimal alveolar concentrations (MAC) of halothane and enflurane to anesthetize 50% of patients are, respectively, 0.75% and 1.7% in the gas phase (in dogs, the values are 0.86% for halothane and 2.2% for enflurane) [18]. These two anesthetics showed comparable effects in depressing the phase transition temperature of DPPC at the equivalent anesthetizing concentrations. The MAC value for CCl_4 is not available.

2. Interfacial effects: phosphate hydration and choline head

We [6] reported previously that the main hydration site of DPPC membranes is the phosphate moiety. Despite its positive charge, the choline head contributed little to membrane hydration. The ratio of the P=O stretching band area to that of $(CH_3)_3$-N^+ stretching band area is used to evaluate the hydration of the vesicle membranes. This is because the band-area values vary among experiments, but the signal strength of $(CH_3)_3$-N^+ stretching remains unaffected by temperature variation [9] or by anesthetic perturbation [6]. The ratio represents the change in P=O stretching.

Temperature did not appreciably affect the ratio between P=O and $(CH_3)_3$-N^+ stretching signals in the range of 25–50°C. The mean value of the phosphate/choline band-area ratios for the pure vesicles, measured at various temperatures, was 3.8 with a standard deviation of 0.1. Cameron and co-workers [11] also reported that there were no significant changes in the absorption bands of these two groups during the phase transition of DPPC multilamellar dispersions.

Addition of anesthetics increased the phosphate/choline band-area ratio. Thus, the temperature-induced disordering and anesthetic-induced disordering are not identical at the interfacial properties. At the lipid/anesthetic mole ratio 2:1, the mean value of the phosphate/choline band-area ratio increased from 3.8 of the

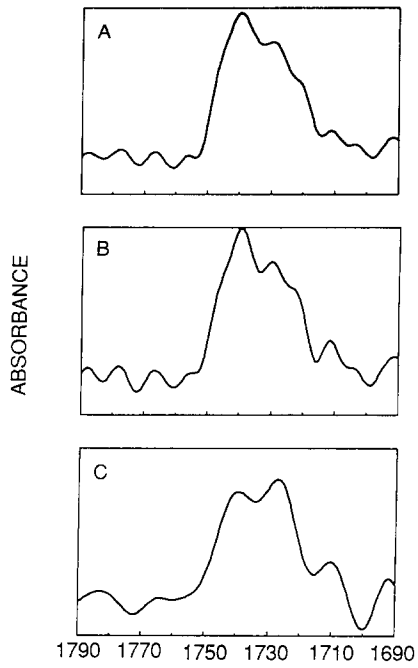

Fig. 3. Deconvoluted spectra of the C=O stretching bands of the pure vesicle samples at; (A) 25 ° C, (B) 35 ° C, and (C) 44 ° C.

control to halothane 4.8 (26.3% increase), enflurane 4.6 (21.1% increase), and CCl_4 4.2 (10.5% increase) with standard deviation of 0.1. Halothane and enflurane have greater effect on the absorption band-area ratio than CCl_4.

3. Deconvoluted carbonyl spectrum

Though the DPPC molecule contains two ester groups linking to the two lipid tails, the infrared spectra of the vesicle samples showed only one C=O stretching band at about 1730 cm^{-1}. A deconvoluted carbonyl spectrum for the fully hydrated diacyl phospholipid membrane, however, resolved two components at 1742 cm^{-1} and 1727 cm^{-1} [9]. The 1742 cm^{-1} peak has been assigned to the non-hydrogen-bonded free *sn*-1 C=O group in the interior hydrophobic region of the membrane, and the 1727 cm^{-1} to the hydrogen-bonded *sn*-2 C=O group close to the polar head region [19,20]. Fig. 3 shows the deconvoluted C=O spectra at 25, 35 and 44 ° C for the pure vesicle. Two bands were resolved at approximately 1740 and 1729 cm^{-1}. When the temperature was increased, the relative peak heights of these two bands varied, but the wavenumbers of these two components remained essentially the same; phase transition did not affect the wavenumber (Table I).

Fig. 4 shows the deconvoluted C=O spectra of the vesicles with the anesthetics. Three peaks were resolved for the halothane sample (at 1744, 1735 and 1728 cm^{-1}) and for the enflurane sample (at 1740, 1734 and 1729 cm^{-1}). In contrast, only two peaks were found for the

TABLE I

Frequencies of deconvoluted C=O stretching bands at 25, 35 and 44 ° C

Phospholipid : anesthetic mole ratio = 2 : 1.

	Component frequency (cm^{-1})		
DPPC			
25 ° C	1740		1729
35 ° C	1740		1730
44 ° C	1740		1727
DPPC/halothane			
25 ° C	1744	1735	1728
35 ° C	1742	1735	1729
44 ° C	1743	1738	1728
DPPC/enflurane			
25 ° C	1740	1734	1729
35 ° C	1741	1735	1728
44 ° C	1743	1734	1728
DPPC/carbon tetrachloride			
25 ° C	1739		1728
35 ° C	1739		1729
44 ° C	1740		1728

CCl_4 sample (at 1739 and 1728 cm^{-1}). Wong and Mantsch [20] suggested that the peak at 1744.3 cm^{-1} is the C=O stretching mode of the free *sn*-1 carbonyl group, and the peaks at 1737.5 and 1728.2 cm^{-1} are, respectively, the C=O stretching modes of the free and the hydrogen-bonded *sn*-2 carbonyl moieties. The present data showed that the perturbation by halothane or

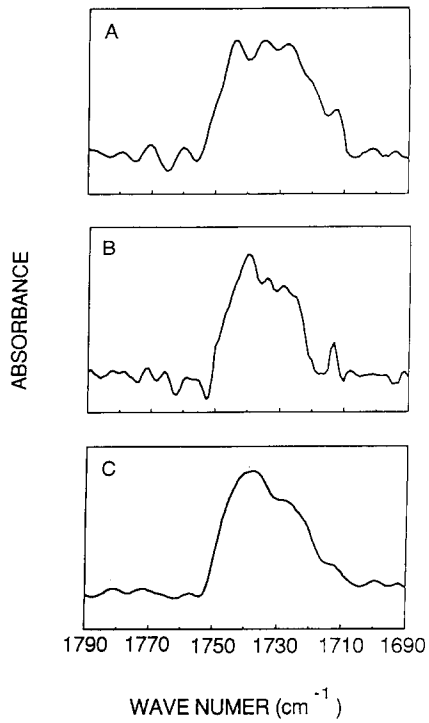

Fig. 4. Deconvoluted spectra of the C=O stretching band of the vesicle/anesthetic samples at 25 ° C; (A) halothane, (B) enflurane, and (C) carbon tetrachloride.

enflurane resulted in the formation of a free *sn*-2 carbonyl component. No free carbonyl peak was identified in the CCl_4 sample, suggesting that the ester linkage region was not disturbed. Table I lists the effects of halothane, enflurane and CCl_4 on the carbonyl bands in the deconvoluted spectra at 35 and 44 °C. These data and those obtained at 25 °C demonstrate that polar anesthetics break hydrogen bonds at the glycerol skeleton at temperatures below and above the main phase transition.

Discussion

The thermotropic phase transition without anesthetics was characterized by a sharp increase in the frequency of $C\text{-}H_2$ stretching of lipid tails without significant changes in the frequencies of the interfacial moieties, represented by P=O stretching mode of the phosphate moiety, $(CH_3)_3\text{-}N^+$ stretching mode of the hydrophilic head, or C=O stretching mode of the glycerol skeleton. Addition of anesthetics, on the other hand, affected the interfacial P=O stretching frequency and C=O stretching frequency as well as the lipid core $C\text{-}H_2$ frequency. Among anesthetics, polar molecules increased the P=O band area of the phosphate moiety and dehydrated the glycerol C=O adjacent to the hydrophilic head region. Apolar CCl_4 did not affect glycerol hydration and the interfacial effect was limited to the phosphate moiety. The mechanism of perturbation of the phospholipid membrane structure induced by CCl_4 obviously differs from polar anesthetics. Thus, anesthetic effects on phospholipid membranes are complex, and the mode of their action is not identical to temperature elevation. The difference between clinical anesthetics and CCl_4 in hydrogen-bond breaking activity agrees with the report by Sandorfy, Urry and co-workers [21] who proposed from their FTIR data that clinical anesthetics, but not CCl_4, disrupted the hydrogen-bonded head-to-head dimerization of gramicidin molecules incorporated into phospholipid bilayer membranes.

Hydrogen-bond forming activity of the phosphate groups of lipid membranes is well documented [6,19,21,22]. By Fourier-transform infrared spectroscopy, we [6] have shown in partially hydrated DMPC membrane that the main hydration site was the phosphate moiety, and volatile anesthetics displaced the water molecules from the phosphate moiety. Another study with differential scanning calorimetry [24], also in partially hydrated DMPC suspension, showed that volatile anesthetics decreased the amount of water molecules hydrogen-bonded to the phospholipid surface. In the present study, all anesthetics increased the phosphate/choline band-area ratio. The increase is attributable to the formation of a competitive proton donor-acceptor complex between the phosphate moiety and anesthetics, releasing the hydrogen-bonded water molecules (see below). The weak effect of the nonpolar CCl_4 molecules on the P=O stretching band area may be caused by its high polarizability.

The importance of water structure in anesthesia mechanisms was first mentioned by Pauling [25] and Miller [26]. They proposed that volatile anesthetics promote hydrogen bonding to form clathrates. The clathrate theory was refuted by the poor correlation between the anesthetic potencies and the hydrate dissociation pressures [27]. Many volatile anesthetics, such as halothane, methoxyflurane, etc., do not form clathrates at 0 °C.

Hydrogen-bond breaking action of anesthetics was proposed by Eyring et al. [28] based on the large positive entropy and enthalpy values associated with the anesthetic inhibition on the solubilized firefly light-emitting enzyme [29]. Meanwhile, Di Paolo and Sandorfy [30] found that the intensity of the stretching band of the hydrogen-bonded N-H group of secondary aliphatic amines, dissolved in brominated fluorocarbon solvents, decreased when the temperature was lowered, and disappeared completely at −190°C with concomitant increase in free N-H stretching band. This means that lowering temperature melted the ice. This paradoxical ice-melting phenomenon was further demonstrated with halothane and other fluorinated anesthetics. Although in non-aqueous media, a good correlation was found between the hydrogen-bond breaking ability and anesthetic potency of fluorinated anesthetics [30]. It should be mentioned that all volatile anesthetics presently in clinical use are fluorinated. Sandorfy and co-workers [31,32] used quantum chemical and statistical mechanical methods and demonstrated that volatile anesthetics break the hydrogen-bonds even in H_2O dimer by forming a competitive water-anesthetic complex.

The mechanism by which the state of interfacial hydrogen bonds affects membrane functions remains speculative. But it may not be unreasonable to assume that possible loss of the water molecules that cover the surface of electrogenic ion channels may retard the passage of current-carrying ions. Other possibilities include conformational change of channel proteins by disruption of intra- or inter-molecular hydrogen-bonds among protein subunits [21] or between lipid and proteins, or both.

Acknowledgments

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