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PRESSURE-INDUCED ELEVATION OF PHASE TRANSITION TEMPERATURE IN DIPALMITOYLPHOSPHATIDYLCHOLINE BILAYERS

AN ELECTRON SPIN RESONANCE MEASUREMENT OF THE ENTHALPY OF PHASE TRANSITION

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SUMMARY

The application of 136 atm of helium pressure to an aqueous dispersion of dipalmitoylphosphatidylcholine increased the temperature of the primary phase transition at 40.4±0.2 °C by 3.0 °C. The lower temperature pretransition at 30.5±0.5 °C, thought to be due to phosphate headgroup reorganization, was increased by 1.7 °C. Addition of 4 % dipalmitoylphosphatidic acid to the dipalmitoylphosphatidylcholine affected the phase transition in the head group region more than in the hydrocarbon chain region. The pressure and temperature data obtained, taken together with the literature value for the bilayer volume expansion during solid-fluid phase transition, and inserted into the Clausius–Clapeyron equation yield a ΔH value of 8.8 kcal/mole for this phase transition. This value is within experimental error of the ΔH value obtained from differential scanning calorimetry and serves to support the validity of the data and the experimental technique. Phase transition was observed by electron spin resonance measurement of the exclusion of the small spin label Tempo (2,2,6,6-tetramethylpiperidine-*N*-oxyl) from the solid domains of the bilayer. This result offers a possible explanation for the direct antagonism by high pressure of the effects of the inhalation anesthetics.

INTRODUCTION

The sharp phase transition of the hydrocarbon region of a pure phospholipid bilayer from a gel to a smectic liquid crystal is a phenomenon of growing importance in membrane research. This transition occurs at temperatures determined by the acyl chains. As a bilayer of a pure phospholipid changes from a solid-like phase to a liquid-like phase, the bilayer area increases, the thickness decreases, the total volume increases [1–4], and the hydrocarbon chains become more fluid [5]. It is likely that at physiological temperatures biological membranes are poised such that a percentage of

Abbreviation: TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl.

the total phospholipid content exists as domains of a pure solid phase within the otherwise fluid bilayer [3, 6, 7]. In a membrane composed of several phospholipids with different phase-transition temperatures, the ratio of phospholipid in the solid phase to the liquid phase serves to control many of the bilayer properties [8, 9].

For example, the cell membranes of *mycoplasma laidlawii* exhibit the phase transition phenomenon [10]. These bacterial membranes have been enriched with various fatty acids and corresponding changes were produced in phase transition or separation temperature. The aggregation of proteins in such membranes is sensitive to these altered phase transition temperatures. Freeze-fracture micrographs of membranes grown below these characteristic temperatures do not exhibit full protein aggregation until they have been exposed to temperatures at or above the phase separation (discontinuity) [11]. The aggregation of rhodopsin proteins in reconstituted rhodopsin-lipid vesicles is apparently controlled by the matrix of solid and fluid domains in the bilayer [12]. As the temperature of the bilayer containing randomly dispersed rhodopsin is lowered to its characteristic phase separation temperature, the freeze-fracture electron micrographs display circular regions from which rhodopsin particles have been excluded.

McConnell has previously described an electron spin resonance (ESR) technique for measuring the ratio of solid and liquid phases in the bilayer [13]. The exclusion of the spin label 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO) into the surrounding aqueous medium by the transition of a fluid phospholipid phase to a solid phase is measured by the ratio of the high-field hydrophobic (H) and hydrophilic (P) peaks in the ESR spectrum (Fig. 1). By means of this technique it is possible to draw a phase-transition curve for a pure phospholipid bilayer which corresponds very well with those obtained by differential scanning calorimetry [14] (Fig. 2). In addition, phase diagrams of binary mixtures of pure phospholipids may be constructed by this method [8].

MATERIALS AND METHODS

L-α-Dipalmitoylphosphatidylcholine was purchased from Calbiochem* and d,l-dipalmitoylphosphatidic acid was purchased from Applied Science Labs, Inc.**, each being used without further purification. In those experiments involving only dispersions of dipalmitoylphosphatidylcholine, a 10 % lipid dispersion was made up by weighing out 100 mg of powdered dry lipid into a pear-shaped 10-ml flask. To this was added 800 μl of 0.1 M sodium phosphate buffer at pH 7 and 100 μl of 5 mM TEMPO solution prepared in the same buffer. The flask was shaken vigorously on a Vortex mixer for 20 min while maintaining the sample temperature at least 5 °C above the transition temperature (40.4 °C) [8]. Moderate negative pressure was maintained in order to remove any gas bubbles formed. A similar procedure was followed for the dispersions dipalmitoylphosphatidylcholine–dipalmitoylphosphatidic acid, with the exception that the inner wall of a 10-ml pear-shaped flask was first coated with a film of phospholipid by evaporating a chloroform solution containing

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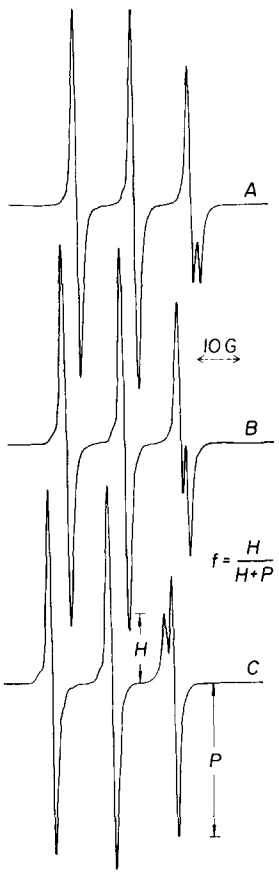
4 % (4 mg) dipalmitoylphosphatidic acid and 96 % (96 mg) dipalmitoylphosphatidylcholine.

The phospholipid dispersion was placed in a specially constructed quartz ESR tube (1 mm internal diameter) tested to withstand pressures of 200 atm. The ESR tube was placed vertically in a microwave cavity thermostated to within ± 0.1 °C over a



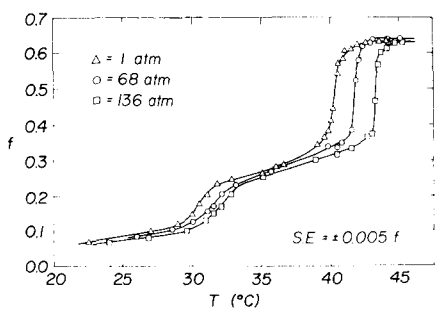


Fig. 1. ESR spectra of TEMPO in an aqueous dispersion of dipalmitoylphosphatidylcholine (100 mg/ml) at 42 °C measure dat atmospheric pressure (A), and helium pressures of 68 atm (B), and 136 atm. (C). Amplitude of the signal is plotted vs total scan width of 100 G (G). The amplitudes H and P are the high-field nitroxide hyperfine signals which to a first approximation are proportional to the amount of spin label dissolved in the hydrophobic phase and aqueous phase, respectively. Spectra A, B, and C are characteristic of a phospholipid bilayer in a fluid phase, at the fluid–solid transition, and in a solid phase, respectively. The parameter f is a measure of the fraction of total lipid which is in the fluid state.

Fig. 2. The TEMPO spectral parameter, f, vs temperature for an aqueous dispersion of dipalmitoylphosphatidylcholine (100 mg/ml) at atmospheric pressure (△), and helium pressures of 68 atm (○), and 136 atm (□). The spectral parameter f, is a measure of the fluidity of the membrane components, both of the fatty acid chains and the polar head groups. There is an abrupt decrease in the magnitude of f at 40.4 ± 0.2 °C corresponding to a transition from flexible and fluid fatty acid chains to more rigid ones. At 30.5 ± 0.5 °C a second transition occurs, less marked than the first, corresponding to a reordering of the polar head groups into a more rigid lattice.

range of 15–55 °C. The desired pressure was attained from a helium source* pressurized to 3500 lb/inch^2. Before beginning a series of pressure measurements, the ESR tube was pressurized and vented several times at 80 atm to remove any residual air in the system.

ESR spectra were measured on a modified Varian EM-500 spectrometer operating in the X-band region. All spectra were obtained as a function of temperature, beginning 5–10 °C above the transition temperature and then cooling the sample down through the temperature region of interest at 5 °C/h. After each increment of temperature decrease, the sample was equilibrated for 15 min before the next spectrum was measured. Each experiment was duplicated using a fresh phospholipid dispersion.

RESULTS

Fig. 1 shows the ESR spectra of TEMPO in an aqueous dispersion of dipalmitoylphosphatidylcholine at 42 °C and at three different pressures. Because TEMPO partitions between the aqueous and the hydrophobic phases of a phospholipid dispersion, the resulting spectrum can be considered to be a superimposition of the two spectra associated with the spin label in different environments. Partial resolution of the high field line into two components results from small differences in the isotropic g value and hyperfine coupling constants of TEMPO in the different environments. The labeled amplitudes (H and P, in Fig. 1) of the high field nitroxide hyperfine signals are a direct measure of the amount of TEMPO dissolved in the hydrophobic region of the membrane bilayer and the surrounding aqueous region, respectively. The relative variation in these amplitudes, as a function of bilayer internal fluidity, represents the change in TEMPO solubility in the respective environments [13]. The three spectra in Fig. 1 were measured at 1.6 °C above the phase-transition temperature of dipalmitoylphosphatidylcholine (40.4 °C in our system). Spectrum A, measured at 42 °C and atmospheric pressure, exhibits a large proportion of TEMPO dissolved in the lipid region of the bilayer, a characteristic of the fluid state. Spectrum B, measured at 42 °C and 68 atm, is identical to spectra measured at 40.4 °C at atmospheric pressure and exhibits the partial exclusion of TEMPO characteristic of a bilayer at its phase-transition temperature. Spectrum C, measured at 42 °C and 136 atm, exhibits the exclusion of TEMPO from the hydrophobic region characteristic of a gel phase.

Figs 2 and 3 show the TEMPO solubility parameter f, equal to $H/(H+P)$, which is proportional to the fractional solubility of TEMPO in the membrane bilayer, as a function of temperature, and at three different pressures for a dipalmitoylphosphatidylcholine (100 mg/ml) dispersion and a dipalmitoylphosphatidic acid (4 mg/ml) –dipalmitoylphosphatidylcholine (96 mg/ml) dispersion. At atmospheric pressure both figures indicate an abrupt increase in the solubility of TEMPO in the hydrophobic region at the temperature (40.4 ± 0.2 °C) where the phospholipid dispersion undergoes a transition from a gel phase to a lamellar smectic liquid crystalline phase. With increasing pressures the transition temperature is shifted to higher values. At 136 atm this shift amounts to 3.0 ± 0.2 °C. The transition temperature and the amount of shift shown in Fig. 2 is only slightly changed by the addition of 4% dipalmitoylphos-

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phatidic acid in Fig. 3. This demonstrates that the primary transition at 40.4 principally involves the fatty acid chains of the phospholipids, and these fatty acids are identical in dipalmitoylphosphatidylcholine and dipalmitoylphosphatidic acid.

On the other hand, the pretransition occurring at 30.5±0.5 °C is thought to

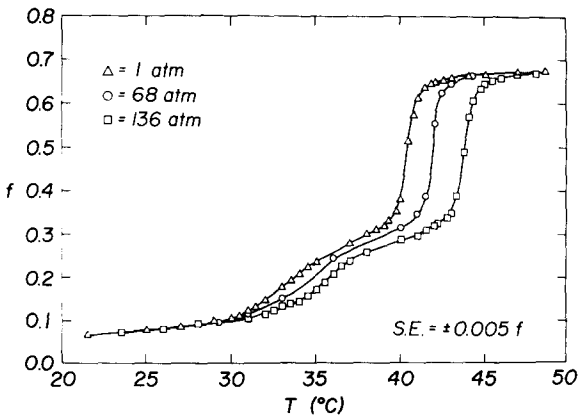

Fig. 3. The TEMPO spectral parameter, *f*, vs temperature for an aqueous dispersion of dipalmitoylphosphatidic acid (4 mg/ml)–dipalmitoylphosphatidylcholine (96 mg/ml) at atmospheric pressure (△), and helium pressures of 68 atm (○) and 136 atm (□). The spectral parameter *f*, is a measure of the fluidity of the membrane components, both of the fatty acid chains and the polar head groups. There is an abrupt decrease in the magnitude of *f* at 40.3±0.2 °C corresponding to a transition from flexible fatty acid chains to more rigid ones. At 32.5±0.5 °C a second transition occurs, less marked than the first, corresponding to a reordering of the polar head groups into a more rigid lattice.

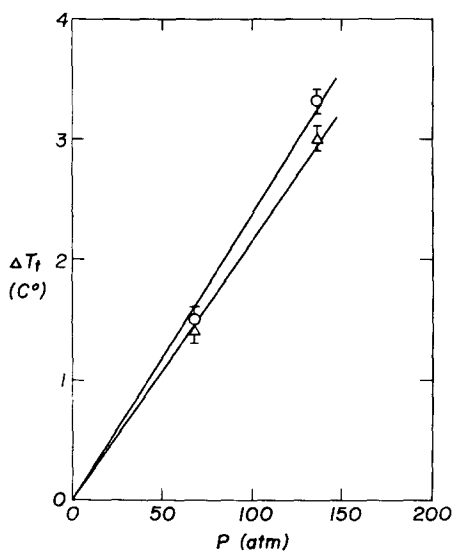

Fig. 4. The shift in the phase transition temperature, ΔT_t, vs helium pressure for aqueous dispersions of dipalmitoylphosphatidylcholine (100 mg/ml) (△), and dipalmitoylphosphatidic acid (4 mg/ml)–dipalmitoylphosphatidylcholine (96 mg/ml) (○).

be due to reordering of the polar head groups [8, 15]. Thus this transition should be influenced by the negative charge of the dipalmitoylphosphatidic acid. Indeed, as seen in Fig. 3, this pretransition is broadened and increased 1.5 °C in temperature by the addition of 4 % dipalmitoylphosphatidic acid.

Fig. 4 indicates the linearity of the increase in the primary phase transition temperature with pressure. There is a difference in the slopes of the lines corresponding to shifts in the pure dipalmitoylphosphatidylcholine (Δ) and the dipalmitoylphosphatidylcholine–dipalmitoylphosphatidic acid (○) phase-transition temperatures. This difference illustrates the small influence of a change in the head group region on the properties of the fatty acid chains. This finding is in accord with previous work of Shimshick [8].

DISCUSSION

The present study represents a continuation of our investigations of the pressure reversal of anesthesia. Studies by other workers have indicated that the depressant effects of clinical concentrations of inhalation anesthetics are completely antagonized by the application of 100–150 atm of helium to mammals, newts, and bacteria [16–18]. We have suggested that the primary site of this pressure reversal takes place in the lipid region of nerve membranes [19]. This concept is supported by the demonstration that inhalation anesthetics fluidize the hydrocarbon region of phospholipid bilayers [20], that the application of high pressure decreased the fluidity of bilayers, and that anesthetic and pressure effects are antagonistic [19].

There is ample evidence to suggest that the primary effects of anesthesia take place in the lipid layer. This effect may subsequently be transmitted to membrane-solvated proteins [20] or may result in the inability of bilayer lipids to change their internal organization. Several other models indicate the lipid region to be the primary site of anesthesia. For example, Papahadjopoulos [21] has proposed that nerve conduction results from the transposition of small patches of phosphatidylserine headgroups from the outside to the inside of the bilayer. Johnson and Bangham [22] have suggested that the sodium flux component of the action potential is caused by a surface rearrangement which is prevented when the surface is expanded by anesthetics.

A possible link between the pressure antagonism of anesthesia and changes in membrane structure may follow from the demonstration that pure phospholipid bilayers abruptly increase in volume as a result of increased internal motion of the acyl chains as they are warmed to their characteristic phase transition temperature [1, 2]. The thermodynamic result predicted by the Clausius–Clapeyron equation is that high pressure should raise the phase transition temperature. Thus, the isothermal application of high pressure to a bilayer consisting of mixed solid and fluid phases would result in a shift in composition toward the solid phase. The present study has shown that this predicted result occurs in phospholipid bilayer systems. The volume increase $(\Delta V)/V$ due to phase-transition-induced disorder in a dipalmitoylphosphatidylcholine vesicle at 40–42 °C has been measured as 1.4 ± 0.1 [1], 2.6 ± 0.1 [3], 3.5 ± 0.3 [2], and 4 % [4]. Substitution of the most recent value of 3.5 % $(\Delta V)/V$ obtained by Nagle [2] and $\Delta P/\Delta T$ from Fig. 2 into the Clausius–Clapeyron equation

$$\frac{\Delta P}{\Delta T} = \frac{\Delta H_{\text{phase transition}}}{T(V_F - V_S)}$$

yields an enthalpy of phase-transition ($\Delta H_{\text{phase transition}}$) value of 8.8 ± 0.8 kcal/mole. This calculated enthalpy assumes ΔH and $(V_F - V_S)$ are not greatly affected by pressure. Our ΔH is very close to those experimentally determined by differential scanning calorimetry (9.7 [14], 8.66 [24] kcal/mole) for the primary phase transition. A similar calculation with the Clausius–Clapeyron equation using a $(\Delta V)/V$ of 0.4 % [2] for the volume change of the pretransition and the $\Delta P/\Delta T$ from Fig. 2 (136 atm/1.70 °C) yields a ΔH of 1.7 ± 0.2 kcal/mole. This value is also near the 2.3 kcal/mole value derived from calorimetry [23].

The close similarity of the ΔH values obtained by differential scanning calorimetry with those derived from our $\Delta P/\Delta T$ measurements suggests that the assumptions about the small change of ΔH and $(V_F - V_S)$ with pressure are valid. Furthermore, the 3.0 °C change in phase transition temperature produced by 136 atm of helium pressure would seem to be reasonable. These values for pressure induced phase transition temperature changes may be used to predict the effect of pressure on the phase diagram of a binary mixture of pure phospholipids, or on the state of a *laidlawii* membrane near its characteristic phase separation temperature.

We have undertaken the present experiments to test the suggestion [20] that anesthesia is the result of an initial change in the state of the lipid region of a nerve membrane which then causes a change in the functioning of membrane-solvated proteins. We have previously investigated the effects of anesthesia [20] and the anesthesia-antagonizing effects of high pressure [19] on the bulk properties of heterogeneous egg phosphatidylcholine bilayers. Recent work, however, has suggested that the existence of domains of solid phase phospholipids within the otherwise fluid bilayer may be crucial to the function of membrane-solvated proteins. The present studies begin to test whether inhalation anesthetics act by changing the amount or distribution of these solid phase phospholipid domains.

We have previously shown that clinical concentrations of an inhalation anesthetic and 136 atm of pressure have equal and antagonizing effects on phospholipid bilayer mobility. In the present study we have shown that 136 atm of pressure produce a 3.0 °C change in the phase-transition temperature of dipalmitoylphosphatidylcholine. Other work [8] has shown that there is a temperature range of only 5 °C within which both fluid and solid domains exist in a bilayer of 1:1 dipalmitoyl- and dimyristoylphosphatidylcholine. Clearly, the 3.0 °C increase in the dipalmitoylphosphatidylcholine phase-transition temperature caused by pressure is sufficient to greatly alter the limits of the temperature range within which both the solid and fluid domains exist.

The present studies lead to the possibility that inhalation anesthetics act by displacing the balance of solid–fluid domains so far toward the all-fluid region that the properties of membrane bilayers are markedly changed. Under these conditions the membrane-solvated proteins responsible for nerve conduction would cease to function normally and anesthesia would result. Such a model would account for the pressure reversal of anesthesia [16–18] and the critical-volume hypothesis [25]. It may also explain why the primary effect of an anesthetic is accompanied by secondary effects such as changes in nucleotide transport and increased potassium leak rates,

since these functions would of course be affected by a general change in membrane properties.

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