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Anesthetic Interaction with a Model Cell Membrane:

Expansion, Phase Transition, and Melting of the Lecithin Monolayer

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A synthetic L-α-dipalmitoyl lecithin monolayer at an air–water interface was used as a model to study the effects of volatile anesthetics on cell membranes. Methoxyflurane, chloroform, halothane, enflurane and fluroxene were used in this study. When the surface pressure of the monolayer was kept constant, anesthetics at clinically effective tensions expaned the area 0.5 per cent. When the area of the monolayer was kept constant, the surface pressure was increased about 1.0 dyn/cm by anesthetics. This increase of the surface energy was caused by the addition of 2.70×10^{13} anesthetic molecules to one square cm of the interface. The surface pressure–surface area curve shows a discontinuity, and a transition from liquid-expanded phase to liquid-condensed phase occurs at this point. The liquid-expanded phase is regarded as the "melted" state of the monolayer. Anesthetics shifted the phase transition point towards a more condensed region, indicating melting of the monolayer membrane. Anesthetics decreased the latent heat and entropy change of the phase transition, implying that anesthetics facilitate the melting of the membrane. The compressional modulus, a measure of the rigidity of the monolayer, was decreased by anesthetics. This decrease of rigidity, or increase of fluidity, was also disclosed by analysis of the hysteresis curve (surface pressure–surface area) obtained by compression and expansion of the monolayer. The results support the unfolding theory of anesthesia which postulates that disordering and expansion of the cell membrane with release of the structured water from the interface is the basis of general anesthesia. (Key words: Theories of anesthesia; Membrane: effect of volatile anesthetics; Anesthetics, volatile: chloroform; Anesthetics, volatile: methoxyflurane; Anesthetics, volatile: enflurane; Anesthetics, volatile; halothane; Anesthetics, volatile: fluroxene.)

JOHNSON AND EYRING and their co-workers[1–6] have shown that general anesthetics unfold and expand enzyme molecules and externally applied pressure reverses this unfolding concomitant with the reversal of anesthesia. Evidence to confirm this unfolding theory of anesthesia has accumulated in recent years.[7–11]

Ueda and Kamaya[12] demonstrated that anesthetics inhibit firefly luciferase with a large increase of entropy, and suggested that the state of the enzyme changed and that melting occurred. Eyring, Woodbury and D'Arrigo[13] postulated that this increase of entropy was caused primarily by release of the structured water initially bound to the hydrophilic site of the enzyme. This indicates melting of bound water at the interface.

It is well known that lipids and their congeners form monomolecular films when spread on the water surfaces and show surface activity. These compounds form an array of molecules at the interface, orienting lipophilic sites to the air and hydrophilic sites to the water. The water molecules at the interface form lattice structures and show crystalline characteristics very different from those of the bulk water. Interfacial monomolecular films have been used as a model for cell membranes. The system is useful for the analysis of a mechanism of anesthesia as it provides intramolecular lipophilic and hydrophilic sites and structured interfacial water.

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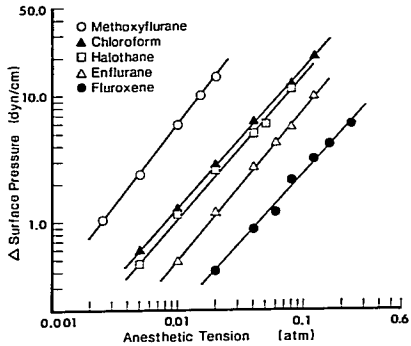

FIG. 1. Increase of the surface pressure of the lecithin monolayer by anesthetics. *Abscissa:* partial pressures of the anesthetics in the gas phase expressed at atmospheric pressure and plotted on a logarithmic scale. *Ordinate:* increases of surface pressures in dynes/cm plotted on a logarithmic scale. Each point is the average of three observations which produced almost identical values.

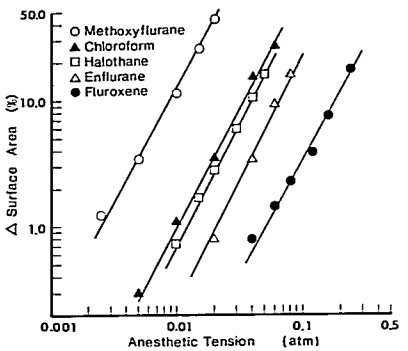

FIG. 2. Increase of the surface area of the lecithin monolayer by anesthetics. *Abscissa:* anesthetic tensions as in figure 1. *Ordinate:* percentage increases of surface area plotted on a logarithmic scale. Each point is the average of three observations. The variations among these three observations were negligible.

In 1953, Dean *et al.*[14] reported that nitrous oxide, ethylene, diethyl ether, and chloroform increased surface pressures of monolayers of stearic acid, palmitic acid, and sterole. Because saturated vapors of diethyl ether and chloroform were used, the pressures used were in excess of clinical ranges, and no attempt was made to correlate between these findings and the mechanisms of general anesthesia.

In 1962, Clements and Wilson[15] reported the effects of general anesthetics upon monolayers of stearic acid, cholesterol, and lecithin. They showed that the affinities of anesthetics to the lipid films correlate remarkably well with clinical partial pressures. They concluded that equal numbers of molecules of different anesthetics are adsorbed to the monolayers at the same depth of anesthesia.

The present communication reports a study wherein synthetic lecithin monomolecular films were employed as model cell membranes. It will be shown that anesthetics melt the monolayers, increase the film fluidity, and expand the surface area.

Method

When water-insoluble material is spread on the surface of water, it exerts pressure

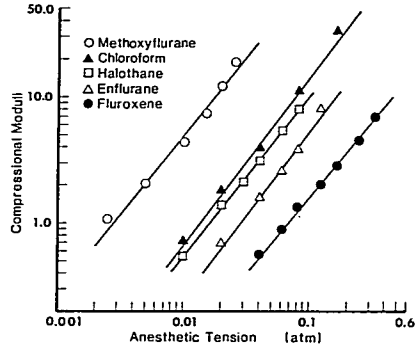

FIG. 3. Decrease of the compressional moduli by anesthetics. The anesthetics decreased the compressional moduli dose-dependently, indicating fluidization of the monolayer. The decrease was expressed as per cent of the control value, and the conventional dose–response curves are linearized by taking log (100 minus per cent of control)/(per cent of control) as the ordinate. *Abscissa:* anesthetic tensions as in figure 1. Each point is the average of three observations. The standard deviations were less than 5 per cent of the mean values.

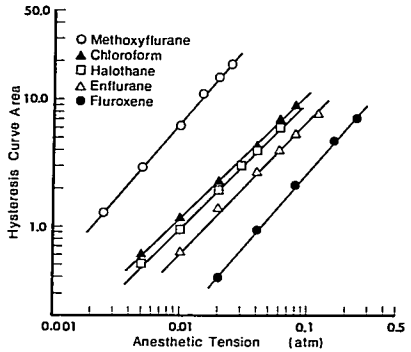

FIG. 4. Decrease of the hysteresis curve area by anesthetics. The areas under the hysteresis of π–A of the compression and expansion cycle in the control situation and with the anesthetics are compared. The anesthetics decreased the hysteresis area dose-dependently. The plotting is similar to that in figure 3 in linearizing the dose–response curves. Each point is the average of three observations. The standard deviations were less than 5 per cent of the mean values.

laterally. This lateral pressure is called film pressure (π), and because the depth of the film is unknown, the force is expressed as dynes/cm. The surface tension of water (γ_0) is decreased by the increments of film pressure; therefore, the following relationship holds:

$$\pi = \gamma_0 - \gamma$$

where γ is the surface tension measured while the film is present.

Surface tension was measured with a Cahn RG Automatic Electro-balance with a dynamic surface tension accessory. The dynamic surface tension accessory is so constructed that two teflon barriers slide on the inside of a Kel-F-lined sample trough, varying its contained surface area. Leakage of lecithin between the teflon barrier and the trough became significant when the fluidity of the lecithin monolayer was increased by anesthetics. Therefore, the teflon bars were remodeled so that the monolayer was enclosed by a continuous teflon tape. Surface-active materials were noted to be eluted from untreated teflon tape after repeated compression and expansion of the surface. This contamination was eliminated by washing the tape with ligroin followed by ethanol and then distilled water.

A platinum plate was used as the sensor

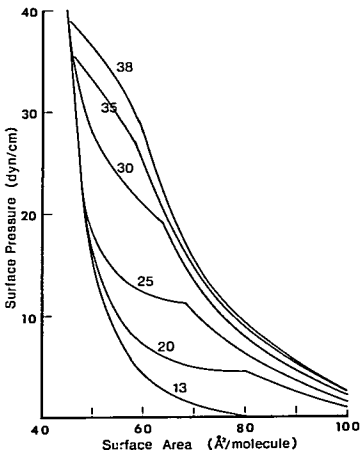

FIG. 5. The effects of temperature on the π–A curve. The lecithin molecules were spread at an area of 100 Å² per one molecule initially and then the area was compressed to 40 Å² per one molecule in 6 minutes. At least three observations were made at each temperature. Reproducibility was better than 0.5 dyn/cm in all compression studies. Representative curves are shown. *Abscissa:* surface area per one lecithin molecule. *Ordinate:* surface pressure in dynes/cm. Temperatures are degrees C.

for surface tension. The recommended method of flaming the plate by a Bunsen burner did not allow satisfactory wetting of the plate, presumably due to adsorption of some natural gas molecules on the platinum surface. To ensure the wettability of the sensor plate, the surface was roughened and the plate was kept in a detergent solution. Before use, it was washed 15 minutes with running water, and then rinsed thoroughly with distilled water.

The temperature of the trough was regulated by circulating water from a constant-temperature water bath which was accurate to ±0.01 C. The temperature of the surface monolayer was determined with a thermistor inserted into the water just beneath the monolayer.

The output from the Cahn electrobalance was fed into a Brown-Honeywell strip chart recorder, and was also recorded, together with the signal from the surface area potentiometer, on the Hewlett Packard Moseley XY-recorder.

The anesthetics were vaporized in a Copper Kettle and diluted with air. The concentration was calculated from the Kettle temperature and the flow of the diluent air, and confirmed by gas chromatography.

The surface tension accessory was encased in a metal box which had a capacity of about 1 liter. Analysis of the gas in the chamber by gas chromatography showed that its anesthetic concentration reached equilibrium with the inflow gas within 1 minute when anesthetics were introduced at a flow rate of 4 l/min.

Water was distilled in a Corning all-glass still, deionized, and then distilled again from an alkaline potassium permanganate solution. The water was then treated with acid-washed activated charcoal, filtered and again distilled. The quality of this triple-distilled water was checked by the dynamic surface tension balance which confirmed that it was free from contaminating surface-active material.

The purity of synthetic L-$\alpha(\beta,\gamma$-dipalmitoyl) lecithin (Calbiochem) was checked by thin-layer chromatography with chloroform–methanol–water 65:25:4 (v/v/v) as a developing solution and was found to give single spot. It was dissolved in a ligroin-ethanol 9:1 (v/v) mixture and was dispensed onto the water surface by a Hamilton microsyringe in a volume less than 20 μl. The solution was kept at −20 C and was renewed every week. Ligroin and ethanol were tested for surface-active materials and were found to be free from these contaminants when measured by the surface-tension balance.

Results

Increase of Surface Pressure at Constant Area

When general anesthetics were introduced into the gas phase, surface tension was depressed (surface pressure was increased). The change was completely reversible when the anesthetics were purged from the trough chamber with air. Figure 1 shows the increase of surface pressure ($\Delta\pi$) by anesthetics at a lecithin concentration of 3×10^{-10} mol/cm²

at 20.0 C. The partial pressures of anesthetics which increased surface pressure 1.0 dyn/cm correlated with the clinical tensions and were: methoxyflurane 0.22×10^{-2} atm, chloroform 0.76×10^{-2} atm, halothane 0.85×10^{-2} atm, enflurane 1.6×10^{-2} atm, fluroxene 4.5×10^{-2} atm.

The surface concentration of adsorbed anesthetics was estimated by Gibbs' adsorption isotherm according to the approximation of Dean *et al.*[14,16] At the above anesthetic tensions, their surface concentrations were: methoxyflurane 0.50×10^{-10} mol/cm², chloroform 0.42×10^{-10} mol/cm², halothane 0.44×10^{-10} mol/cm², enflurane 0.45×10^{-10} mol/cm², fluroxene 0.44×10^{-10} mol/cm².

Expansion of the Monolayer at Constant Surface Pressure

When the anesthetics were introduced to the gas phase while keeping surface pressure constant, the area of the monolayer expanded. Figure 2 shows relative expansion of the monolayer as a function of the partial pressures of anesthetics. The surface concentration of lecithin was 3×10^{-10} mol/cm² and the temperature was 20.0 C. The partial pressures of anesthetics which expanded the monolayer 0.5 per cent showed good correlation with clinical tensions; they were: methoxyflurane 0.17×10^{-2} atm, chloroform 0.74×10^{-2} atm, halothane 0.82×10^{-2} atm, enflurane 1.4×10^{-2} atm, fluroxene 3.5×10^{-2} atm.

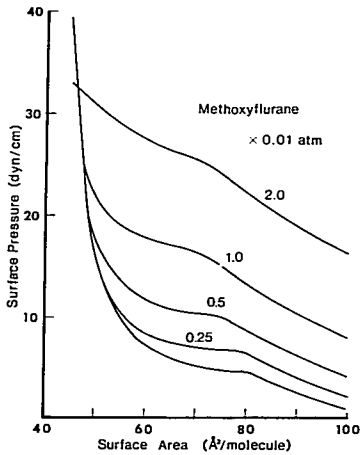

FIG. 6. The effects of methoxyflurane on the π–A curve. The lecithin monolayer was compressed as in figure 5 at 20 C. The tensions of methoxyflurane are indicated in the figure as $\times 10^{-2}$ atm. Three observations were made at each tension of anesthetic, with reproducibility better than 0.5 dyn/cm. Representative curves are shown.

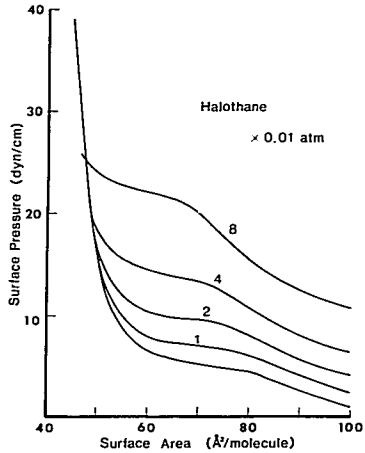

FIG. 7. The effects of halothane on the π–A curve, shown as in figures 5 and 6. The tensions of halothane are indicated as $\times 10^{-2}$ atm.

Compressional Moduli

The surface compressibility of a monolayer is expressed as

$$C_s = -\frac{1}{A}\left(\frac{\partial A}{\partial \pi}\right)_T$$

where C_s = surface compressibility, A = available surface area for the monolayer molecule, π = surface pressure, T = absolute temperature. The reciprocal of surface compressibility, C_s^{-1}, is called the "compressional

TABLE 1. Latent Heat (Q) and Entropy Change (ΔS) of the Phase Transition of the Lecithin Monolayer

Temperature		Area at Phase Transition	Latent Heat (Q)	Entropy Change (ΔS)
C	K	(Å²)	(Kcal/mol)	(Entropy Unit)
20.0	293	80	19.4	66.1
25.0	298	68	11.8	39.6
30.0	303	63	8.7	28.6
35.0	308	58	5.4	17.6
38.0	311	57	4.8	15.4

modulus" and has an advantage in that its dimension is dyn/cm. A smaller figure indicates easier compressibility.

The compressional moduli decreased with elevation of the temperature, showing an increase in the fluidity of the monolayer. The fluidizing effect of anesthetics on the lecithin monolayer is illustrated in figure 3. The surface concentration of lecithin was 3×10^{-10} mol/cm² and the temperature was 20.0 C and the compressional modulus without anesthetics was 179 ± 12 dyn/cm (SD, n = 15). At larger available surface areas of lecithin molecules in the liquid-expanded state, however, the compressional moduli were slightly increased by anesthetics.

The Hysteresis Area of Compression and Expansion of the Monolayer

When the monolayer is compressed and expanded, the surface pressure–surface area curve (π–A curve) shows hysteresis. The initial several compressions and expansions showed changes in the shape of the pressure–area curves, but after a dozen cycles or so the curves became stabilized and almost identical curves were obtained at succeeding compression and expansion cycles. The area inside the hysteresis curve has a dimension of dyn × cm = erg and represents the work delivered to the monolayer. When the monolayer was compressed and expanded between 50 Å² and 25 Å² per a lecithin molecule at a rate of 8 min per cycle, the work delivered for the change of 1.0 cm² of the monolayer was estimated as about 23.3 ± 1.9 erg (SD, n = 15) at 20.0 C. The elevation of temperature decreases this work and indicates the increase of fluidity of the monolayer. Figure 4 demonstrates the dose-dependent decrease of this work by anesthetics, indicating the increase of monolayer fluidity.

Again, there was good correlation between the anesthetic tensions which decreased the area under hysteresis 10 per cent and the clinical pressures: methoxyflurane 0.20×10^{-2} atm, chloroform 0.90×10^{-2} atm, halothane 1.0×10^{-2} atm, enflurane 1.7×10^{-2} atm, fluroxene 4.3×10^{-2} atm.

Compression of the Monolayer and Phase Transition

Figure 5 shows π–A curve of the lecithin monolayer at several temperatures. Discontinuity of the π–A curve is apparent and the transition between liquid-expanded and liquid-condensed phases occurs at this point. In the area to the left of the discontinuity, the monolayer is in the liquid-condensed phase and to the right, it is liquid-expanded. Moving across this point from left to right melts the monolayer. Elevation of the temperature moves this point to a higher surface pressure and a smaller available surface area per lecithin molecule.

Figures 6 and 7 show the effects of methoxyflurane and halothane on the π–A curve of the lecithin monolayer at 20.0 C. The point of phase transition shifted dose-dependently to the higher surface pressure and smaller surface area, showing that plasticizing of the monolayer is similar in its effect to elevation of the temperature.

Discussion

The unfolding theory of anesthesia of Eyring and Johnson[1,2,13] postulates that the anesthetics expand lipoproteins vital to neural activity, presumably in the cell membrane, leading to dysfunction.

In 1942, from the effects of temperature and pressure on the light intensity of luminous bacteria, Johnson, Eyring and their coworkers[1,2] estimated that the active form of the light emission enzyme (luciferase) is larger by 50 cm³/mol than the resting state. The light intensity decreases above or below this optimal volume. They estimated that the anesthetics increased the volume of luciferase by 20 cm³/mol above the optimal active state. This apparent unfolding of the enzyme, pre-

TABLE 2. Effects of Methoxyflurane on Latent Heat (Q) and Entropy Change (ΔS) of the Phase Transition of the Lecithin Monolayer

	Methoxyflurane Tension					
	0.0025 atm		0.005 atm		0.01 atm	
Temperature (C)	Q Kcal/mol	ΔS Entropy Units	Q Kcal/mol	ΔS Entropy Units	Q Kcal/mol	ΔS Entropy Units
13.0	—	—	14.1	49.4	12.0	41.8
20.0	10.8	36.7	8.5	29.1	7.1	24.1
25.0	7.9	26.6	7.2	24.1	4.9	16.5
30.0	5.8	19.0	4.6	15.2	3.1	10.0

sumably as a result of anesthetic binding to the lipophilic site, reduced the light intensity. Pressures of several hundred atm reversed this volume expansion concomitant with recovery of the intensity of anesthetic-depressed luminescence of the bacteria.

This pressure reversal of anesthesia was further demonstrated with tadpoles anesthetized with alcohol.[4,5] The alcohol-anesthetized tadpoles started swimming again when a hydrostatic pressure of 200 atm was applied. In recent years these observations have been confirmed with newts and mice anesthetized with modern anesthetics.[7,8]

The numerical values given above for the anesthetic-induced expansion of luciferase are deduced from the temperature and pressure effects on the light intensity of the bacteria, and are indirect. Seeman and Roth[17] calculated that this expansion amounts to about 0.03 per cent, assuming the volume of the bacterial luciferase to be about 60,000 cm^3/mol. This calculation is again approximate, and subject to correction pending the elucidation of the conformation of the luciferase molecule.

The surface area of a lecithin monolayer is sensitive to general anesthetics and responds by increasing its area. The direct measurements done in the present study show that clinical tensions of the anesthetics increase the surface area of the monolayer by 0.5 per cent.

Seeman *et al.*[17,18] demonstrated that general anesthetics prevented hypotonic hemolysis of washed human erythrocytes and increased the cell volume. From these data they calculated that the anesthetics expand the area of the erythrocyte membrane about 0.4 per cent at clinical tensions. From the study of the pressure reversal of anesthesia in newts and mice, Lever *et al.*[8] calculated that 0.4 per cent volume reduction occurs at the pressure which reverses the anesthesia. These values are in good agreement with the values obtained in the present study.

Clements and Wilson[15] reported that 0.16 $\times 10^{-10}$ mol of several different anesthetics were adsorbed on 1 sq cm of the interfacial monolayers at clinical tensions. We calculated the average surface density of anesthetic molecules at clinical tensions as about 0.45×10^{10} mol/cm^2. The discrepancy may have arisen as a result of underestimation of clinical tensions by Clements and Wilson.[15] They assumed the clinical anesthetic tension of nitrous oxide to be 0.58 atm, according to the figure given by Carpenter,[19] and calculated the isonarcotic pressures of other anesthetics from this value.

The discontinuity of the π–A curve indicates that a transition between liquid-expanded and liquid-condensed phases occurs at this point. In the liquid-expanded phase, the lecithin molecules are lying flat on the water surface,[20] the long axis of the molecule parallel to the surface. As the surface area of the monolayer is decreased, their long axis tends to become vertical to the water surface. The change in molecular orientation occurs at the discontinuity point on the π–A curve. Schematically, during further compression of the area, the surface pressure does not increase until the phase transition is complete, *i.e.*, all lecithin molecules stand upright. After completion of the phase transition, the surface pressure begins to rise again in response to the compression of the surface area. During actual

compression, however, there is a gradual increase of surface pressure in the intermediate region. The discontinuity disappears when the lecithin monolayer was compressed at above 41 C. In this case the π–A curve shows a liquid-expanded phase only. The discontinuity is also not observed at temperatures below 13 C. In this case the π–A curve rises from the baseline in a liquid-condensed phase. These phenomena indicate that the lecithin monolayer "melts" at high temperature and "freezes" at low temperature. The occurrence of the phase transition of lecithin at 41 C was also observed with lecithin bilayers.[21,22]

The melting of the monolayer with methoxyflurane and halothane is demonstrated in figures 6 and 7. Addition of the anesthetics shifted the phase transition point towards more condensed area and higher surface pressure. The effects are similar to adding heat to the system. With supraclinical tensions, the liquid-condensed phase disappeared, indicating complete melting of the monolayer.

The reversible phase transition of the monolayer can be treated by the Clapeyron-Clausius equation to calculate the latent heat of melting of the lecithin monolayer:

$$\frac{d\pi_C}{dT} = \frac{1}{T}\frac{Q}{(A_1 - A_2)}$$

where π_C = surface pressure at the phase transition, T = absolute temperature, Q = latent heat of the phase transition, A_1 = molecular area of lecithin at the phase transition, A_2 = molecular area of lecithin at the liquid-condensed phase. The applicability of this equation and its limitations were discussed by Phillips and Chapman.[23] By dividing the latent heat (Q) by the corresponding absolute temperature, entropy is obtained for the phase transition. Table 1 shows the latent heat and entropy changes of the monolayer at several temperatures and table 2 shows the effect of methoxyflurane on these values. The values are for the reaction proceeding from the left to the right in the π–A curve, *i.e.*, melting.

The increases of the fluidity of the monolayer by anesthetics are demonstrated by the decreases of the compressional moduli and the hysteresis curve area of the compression and expansion of the monolayer. In all cases, clinical tensions of anesthetics showed remarkable correlations with the tensions which affected these modalities.

These results support our earlier conclusion,[12] from a study of firefly luciferase, that anesthetics melt the enzyme.

The increased fluidity of the monolayer may be brought about by two factors, 1) relaxation and expansion of the lipid portion of the lecithin molecule, and 2) liberation of bound water attached to the hydrophilic site. Proton nuclear magnetic resonance studies (to be reported elsewhere) of lecithin bilayer spherules dispersed ultrasonically in heavy water (D_2O) showed that the anesthetics first increased the proton signal of the hydrophilic region of the lecithin molecules. As anesthetic tension increased, the proton signal from the lipid portion started to show activity. The increase of the proton motion in the hydrophilic regions is indicative of the melting of the structured water (bound water) attached to the hydrophilic sites of the molecules. Gottlieb *et al.*[24] estimated that six water molecules are attached to each lecithin hydrophilic site. These structured water molecules may be released sequentially according to the depth of anesthesia.

This examination of the properties of pure lipid membranes together with studies of the enzyme reactions of luminescence[1–3,12] show that the properties of both are profoundly modified by the addition of general anesthetics. The mode of action is combination of the anesthetic with the hydrophobic parts of the lipid and with the hydrophobic groups on the protein. The resulting effects are a plasticizing of the lipid and a change in the conformation of the protein, respectively. The lipoproteins of naturally occurring membranes would accordingly be expected to respond to addition of a general anesthetic by a change of conformation of the protein, greatly facilitated by the plasticizing and swelling of the lipid portion of the membrane.

The large effect of hydrostatic pressure in counteracting anesthesia in the lower pressure range ordinarily tends to be reversed at higher pressures.[4,8] This is not surprising when one considers the multiplicity of states at about the same free energy but with different volumes and different properties which are accessible

in a complex membrane, with each of these states stabilized most in its appropriate hydrostatic pressure range. A large part of such volume changes arises from changes in electrostriction accompanying differences in the extent of ionization of hydrated acidic and basic groups of each conformation. To a lesser extent, changes in clathrate and other structures contribute to these volume changes. The singling out of one of these effects of general anesthetics on the complex lipoprotein membrane as the sole cause of anesthesia is clearly an oversimplification.

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