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THE EFFECT OF TWO INHALATION ANESTHETICS ON THE ORDER OF SPIN-LABELED PHOSPHOLIPID VESICLES

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SUMMARY

The order parameter (S'_n) of spin-labeled phosphatidylcholine vesicles has been shown to decrease in a concentration-dependent manner with two inhalation anesthetics, halothane and methoxyfluorane. Similar decreases of S'_n are observed in vesicles labeled adjacent to the polar head group and those labeled near the bilayer center. This suggests that inhalation anesthetics cause a generalized fluidization of the membrane rather than a disorder localized in a particular region of the bilayer. Measurements of the isotropic nitrogen hyperfine coupling constant (a'_N) show a decrease in polarity of the environment with increasing anesthetic concentrations. The experimental approach of plotting S'_n *versus* anesthetic concentration provides a test of whether anesthetics produce their effects on a per molecule or per volume basis.

INTRODUCTION

The effect of local anesthetics and certain other small molecules on the order of the hydrocarbon chain region of phospholipid vesicles has been studied by Hubbell and McConnell[1,2] and by Metcalfe *et al.*[3]. These investigators showed the degree of order in the phospholopid bilayer to decrease with increasing concentration of both local anesthetics and alcohols. The present study is concerned with the perturbation of similar phospholipid bilayers by volatile anesthetics. The extent to which this perturbation correlates with known anesthetic potencies provides a test for the validity for those theories of anesthesia which postulate that the lipid region of nerve membranes is the primary site of anesthetic action. The concept that a lipid region is involved in the anesthetic process is fundamental to many theories of anesthesia. Meyer[4] and Overton[5] first suggested a correlation between potency and the olive oil–water distribution coefficient of anesthetic compounds. Ferguson[6] subsequently pointed out that this correlation improved when the thermodynamic activity of the anesthetic was substituted for its concentration. Mullins[7] further modified Ferguson's theory to include the molecular volume of the anesthetic and concluded that "Narcosis by chemically inert molecules appears to take place when a constant fraction of the total volume of some non-aqueous phase in the cell is occupied by narcotic molecules." Seeman and co-workers have established extensive correlations of the concentration of

anesthetics with cell membrane expansion[8,9] and with membrane hydraulic permeability[10]. Träuble and Haynes[11] have used X-ray diffraction data and dilatometer measurements to formulate a model of membrane surface area expansion due to phase transition induced disorder.

Recently, the method of spin-labeling has found wide application in the study of biomembrane and phospholipid bilayer structures. Hubbell and McConnell[2] have presented an analysis of the paramagnetic resonance spectra of the spin-labeled phosphatidylcholine Iβ(m,n) (Fig. 1) in phospholipid bilayers, and have shown that spectral parameters can be quantitatively related to molecular motions of the hydrocarbon chains. In the present study, we have chosen to use the Iβ(m,n) spin label to investigate the perturbations induced in a phospholipid bilayer by two commonly used general anesthetics: methoxyfluorane (2,2-dichloro-1,1-difluoroethylmethyl ether), and halothane (2-bromo-2-chloro-1,1,1-trifluoroethane).

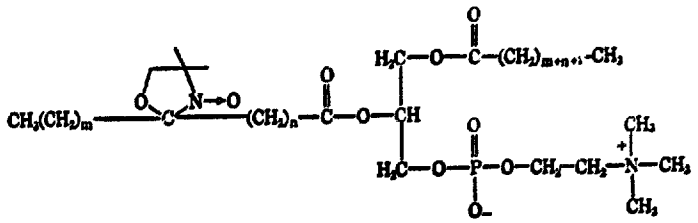

Fig. 1.

METHODS

Chromatographically pure phosphatidylcholine was prepared from egg yolks by the method of Singleton *et al.*[12]. Purity was checked by thin-layer chromatography on silica gel in a solvent system containing chloroform–methanol–water (65:25:4, by vol.). The spin-labeled phosphatidylcholines Iβ (m,n) were prepared by replacing the β-fatty acid chain of phosphatidylcholine with a nitroxide spin-labeled fatty acid[2].

Phosphatidylcholine (1 g) was dissolved in petroleum ether containing the Iβ (m,n) (0.01 g) and cholesterol (0.33 g). The solvent was removed on a rotary evaporator, and the mixture pumped at 10^{-3} mmHg for a minimum of 4 h. Water (8.7 ml) was then added and the mixture sonicated to produce a translucent vesicle suspension.

Partition coefficients for the anesthetics were determined by adding 1 μl of anesthetic to 1 ml of the phospholipid–cholesterol suspension in a glass tube drawn out at one end to minimize dead-space above the liquid. The tube was sealed, the mixture equilibrated overnight, and centrifuged at 3000 $\times$ g for 30 min. The anesthetic concentration in the supernatant was measured by gas chromatography on a 6 ft $\times$ 2 mm Poropac QS column at 205 °C. The supernatant concentration was determined to be less than one third of the anesthetic saturation concentration at 25 °C. The anesthetic mixture was then rapidly transferred to a small vial, 1 ml methanol added to solubilize the mixture, and the total anesthetic concentration again measured by gas chromatography. Three samples were measured for each anesthetic in triplicate determinations. The difference between the supernatant and total anesthetic concentrations gave the lipid concentration. Partition coefficients were expressed as mg anesthetic per g of lipid per mg anesthetic per g of water.

Samples were prepared in Pyrex Pasteur pipettes with the small end sealed and immersed in powdered dry ice. Using the determined halothane partition coeffi-

cient for phosphatidylcholine–cholesterol/water (13±3), a sufficient amount of liquid halothane was added to pipettes containing 0.5 ml of the phospholipid–cholesterol suspension to result in concentrations of 5.3, 15.9 and 50.3 ml halothane per mole of lipid, or equivalently, 49, 147 and 490 mmoles halothane per mole of lipid. The lipid molecular weight was taken as 800. Sufficient methoxyfluorane was added to produce concentrations of 6.9, 20.7 and 69 ml per mole of lipid or 58, 174 and 580 mmoles per mole of lipid based on a partition coefficient of 19±4. After purging the tubes with N_2 they were sealed and allowed to warm. Vigorous mixing on a vortex mixer restored the homogeneity of the samples. All samples were equilibrated at room temperature for at least 8 h before recording. After the spectra had been recorded, final concentrations of anesthetic in the suspension were confirmed by cooling the tube, introducing 0.5 ml methanol, mixing, and analyzing the resultant solution by gas chromatography utilizing a 6 ft × 2 mm Poropac QS column at 180 and at 205 °C.

Molal volumes for the anesthetics were calculated: halothane, 105.5, and methoxyfluorane, 115.7 ml/mole. Molecular volumes were estimated by measuring the volume of water displaced by silicon rubber sealed Cory–Pauling–Koltrum space-filling models. The displaced volumes were 138 and 151 ml for halothane and methoxyflurane, respectively.

Electron spin resonance (ESR) spectra were measured on a Varian series 4500 instrument operated in the X-band with the cavity temperature thermostated at 20 ± 0.1 °C by a Varian temperature control unit. A Harvey–Wells proton probe was used for sweep calibration and field measurements.

RESULTS

Figs 2 and 3 show the paramagnetic resonance spectra of Iβ (7,8) and Iβ (7,4) in egg phosphatidylcholine–cholesterol vesicles, and the effect of added halothane. The spectra show well-resolved hyperfine extremes and can be interpreted in terms of anisotropic motion of the labeled phosphatidylcholine within the phospholipid

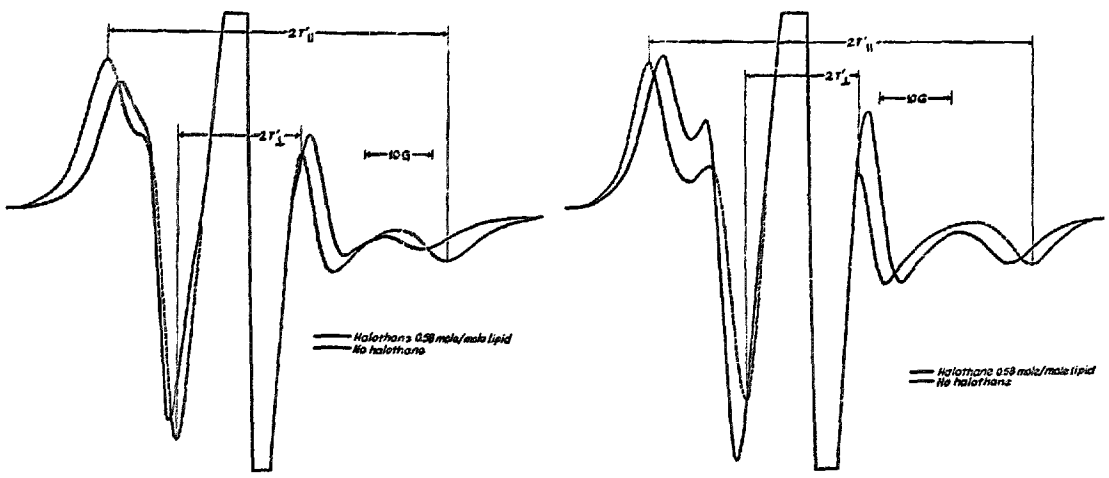

Fig. 2. ESR spectrum of the spin label Iβ (7,8) in phospholipid vesicles with and without halothane.

Fig. 3. ESR spectrum of the spin label Iβ (7,4) in phospholipid vesicles with an without halothane.

bilayer[1]. Under these conditions, the outer ($2T'_{\parallel}$) and inner ($2T'_{\perp}$) hyperfine extrema can be quantitatively related to the mean angular derivation ($\bar{\theta}$) of the nitroxide $2p\pi$ orbital axis from the unique axis of magnetic symmetry[2].

As the $\bar{\theta}$ increases, $2T'_{\parallel}$ decreases and $2T'_{\perp}$ increases. From Figs 2 and 3 it is clear that the presence of halothane markedly decreases $2T'_{\parallel}$ and increases $2T'_{\perp}$ indicating an increase in $\bar{\theta}$ for both Iβ (7,4) and Iβ (7,8).

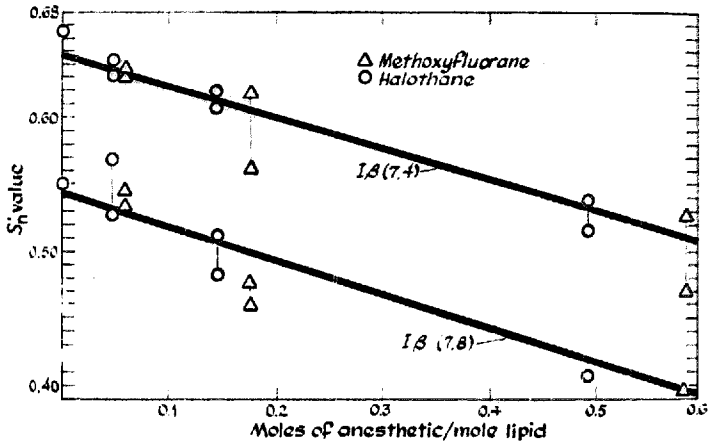

Fig. 4. Change in the order parameter (S'_n) of spin labels Iβ (7,4) and Iβ (7,8) in phospholipid vesicles containing varying amounts of halothane and methoxyfluorane. Bilayer order decreases and fluidity increases with increasing anesthetic concentration.

The degree of order as measured by the spin label is conveniently measured in terms of the order parameter, S'_n, given by

$$S'_n = \tfrac{1}{2}(3 < \cos^2 \theta > - 1)$$

Fig. 4 shows the dependence of S'_n as a function of the concentration of anesthetic in terms of moles anesthetic per mole of lipid. The heavy line through the

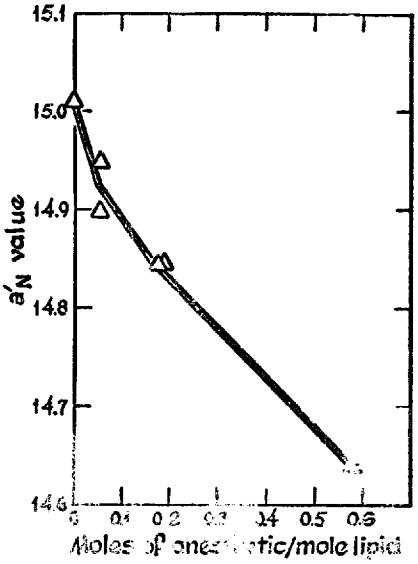

Fig. 5. Isotropic nitrogen hyperfine coupling constant (a'_N) as a function of anesthetic concentration. Environment of the spin label Iβ (7,8) becomes less polar with increasing methoxyfluorane concentration.

points is a least mean square fit. The points seem to be best fit by a straight line. The slopes of the two dose–response lines are identical within experimental error.

The isotropic nitrogen hyperfine coupling constants (a'_N) can be calculated from the measured values of $2T'_{||}$ and $2T'_{\perp}$ in that $a'_N = 1/3\ (2T'_{\perp} + T'_{||})$. The a'_N value of a nitroxide shows a small dependence on solvent polarity, ranging from $a'_N = 14.1$ G, when the nitroxide spin label is dissolved in hexane, to $a'_N = 15.1$ G when in aqueous solution. It is interesting to note that increasing concentrations of anesthetics produce reproducible decreases in a'_N, indicating a decrease in solvent polarity. This effect is shown in Fig. 5 for the case of methoxyfluorane. In general, calculated data for a'_N show considerable scatter due to the small magnitude of the shift, but the trend is consistent in all cases examined.

DISCUSSION

In vivo experiments by Kendig and Cohen (unpublished) with the superior cervical ganglion of the rat have shown that a bathing solution containing 10 mg of halothane in 100 ml H_2O at 23 °C will produce 50% depression of the synaptic action potential. In accord with the Ferguson[6] theory of anesthesia, the chemical potential of halothane in the bathing solution must equal its chemical potential in all parts of the ganglion. Therefore, any lipid phase in equilibrium with a solution of 10 mg of halothane in 100 ml of water will contain a chemical potential of halothane corresponding to this state of anesthesia. If the phosphatidylcholine–cholesterol/water partition coefficient determined for halothane is valid for the lipid region of a ganglion, this would correspond to 5.3 mmoles halothane per mole of lipid.

The concentrations of 49 mmoles of halothane and 58 mmoles of methoxyfluorane per mole lipid correspond to 61.3 and 72 mmoles anesthetic per l of lipid. This value is close to the 30–60 mmoles per l of olive oil which Meyer[4] and Overton[5] predicted would produce anesthesia. It is an important point that significant changes in the S'_n and the membrane fluidity occur at concentrations which correspond to both clinical anesthesia and to the theoretically predicted lipid uptake.

At higher anesthetic concentrations, the S'_n order parameter decreases in an approximately linear manner as shown in Fig. 4. These data clearly demonstrate a dose–response relationship between anesthetic concentration and membrane fluidity. It would be desirable to know whether the two anesthetics produce equal disorder at equal molar concentrations as postulated by Meyer[4] and Overton[5], or at equal molal volumes as postulated by Mullins[7,13]. Unfortunately, the calculated S'_n value would differ only by the ratio of the molal volumes (115.7/105.5 = 1.096), or by the displacement volumes of the Cory–Pauling–Koltrum space filling models (151/138 = 1.093). These differences were too small to detect in our current experiments, and appropriate experimental refinements are being attempted.

Least mean square fits of the data points for the Iβ (7,4) and Iβ (7,8) spin labels have identical slopes (Fig. 4). Although the region of the Iβ (7,8) label is always more disordered, identical increments of anesthetic concentration produce identical decreases in the S'_n value for each label. This finding suggests that the anesthetic does not produce local disorder at one level of the bilayer region, but rather causes a general fluidization of the entire hydrophobic region. Such a result is in accord

with the work of Seeman and co-workers[8,9] and Träuble and Haynes[11] who have demonstrated that an increase in the surface area of the bilayer is associated with a decrease in membrane order. An increase in surface area must then produce a larger volume for the wobble or wag of each fatty acid chain.

The change in the isotropic nitrogen hyperfine coupling constant a'_N with anesthetic concentration (Fig. 5) is small and must be interpreted with caution. The decrease in polarity in the region affecting the spin label could be the result of water displacement by the anesthetic. This possibility presents an interesting correlation with the theory of narcosis of Larsen *et al.*[14] suggesting that anesthesia is the result of a decrease in the dielectric constant of the nerve membrane caused by a decrease in its water content. Other possible interpretations are that the expansion of the surface area results in a lower surface charge density, or that anesthetics change the degree of tilt of the hydrocarbon chains in the bilayer which decreases the polarity of the spin label environment.

The increase in fluidity of the membrane bilayer may be related to anesthesia in at least two ways: the increased fluidity may directly change certain characteristics of the membrane essential to nerve impulse transmission, or it may cause a secondary change in a subunit such as in a protein which mediates nerve transmission. An example of a direct effect is the change in dielectric constant as postulated by Larsen *et al.*[11]. Similarly, Seeman *et al.*[10] have shown that the hydraulic permeability of erythrocyte membranes is increased by inhalation anesthetics. This affect, presumably due to the increased fluidity of the bilayer, could reduce nerve conduction and result in an anesthetic state. Roth and Seeman[15] have shown that clinical concentrations of anesthetics do not increase the passive permeability of erythrocyte membranes to K^+. However, Johnson and Bangham[16] have shown that in phosphatidic acid–phosphatidylcholine liposomes, with and without the K^+ carrier valinomycin, *in vivo* concentrations of anesthetics increase the small K^+ permeability. The latter authors interpret their results as suggesting that foreign molecules sterically impede the rearrangement of the groups near the surface of the lipid which produce the sudden increase in Na^+ permeability. Work by Papahadjopoulous[17] in which Ca^{2+} is added to the vesicle suspension shows that low levels of anesthetics reduce passive permeability to K^+ but high levels such as were used in this experiment increase passive permeability. The question of direct changes in ion mobility as a cause of anesthesis remains open.

Perhaps the more interesting possibility is the suggestion that the lipid region passes on the anesthetic effect to a protein. Recently, it has become evident that there are membrane proteins of highly hydrophobic nature that are "solvated" by the hydrocarbon chains of an organized lipid structure, presumably a bilayer[18]. The solute–solvent relationship of membrane proteins to membrane phospholipids has been clearly demonstrated in the case of rhodopsin in the rod outer segment membranes[19] and in the proteins of the erythrocyte membrane[20].

It is well known that the configuration of a water-soluble protein is highly dependent on solvent conditions. It is then possible that anesthetics, which produce perturbations in the hydrocarbon chains of the bilayers which "solvate" membrane proteins, may alter the tertiary structure of hydrophobic proteins essential to nerve impulse transmission in a manner analogous to the allosteric inhibition of enzymes.

ACKNOWLEDGEMENTS

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