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PRESSURE REVERSAL OF INHALATION ANESTHETIC-INDUCED DISORDER IN SPIN-LABELED PHOSPHOLIPID VESICLES

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SUMMARY

The effects of anesthetics in luminous bacteria, newts, tadpoles, and mice are reversed by application of 150–200 atm of helium or hydrostatic pressure. The disorder induced by the inhalation anesthetic halothane in spin-labeled phospholipid vesicles is also reversed by similar pressures of helium. If pressure reversal of anesthesia occurs in intact nerve systems, then all theories of anesthesia must accommodate the phenomenon and nerve membrane model systems demonstrate it as well. The occurrence of pressure reversal in spin-labeled phospholipid vesicles supports their use as models for the hydrophobic region of nerve membranes and suggests that the primary site of pressure reversal is in the lipid phase of the nerve membrane. This finding is in agreement with the theory that anesthetics act by disordering the lipid region of the membranes.

INTRODUCTION

Reversal of the anesthetic state by pressure has been demonstrated *in vivo* with luminous bacteria, tadpoles, newts and mice[1–3]. These studies indicate that the depressant effects of clinical concentrations of inhalation anesthesia are reversed following the application of 150–200 atm of helium or hydrostatic pressure. A useful experimental approach for testing certain molecular theories of anesthesia would be to determine whether a state corresponding to anesthesia induced in an artificial model system is also reversible by the application of pressure. It has been shown that the electron spin resonance (ESR) spectra of phospholipid vesicles (labeled with phosphatidylcholine molecules containing a nitroxide spin label on the β-fatty acid chain) are sensitive to concentrations of both local[4] and inhalation[5] anesthetics. Interpretation of these spectra indicates that the degree of order of the phospholipid vesicle bilayer decreases with increasing concentration of anesthetic in a dose-related fashion.

Among the theories of anesthesia is the postulate that inhalation anesthetics exert effects by dissolving in the lipid regions of cell membranes[6,7]. An aqueous dispersion of phospholipid–cholesterol vesicles serves as a model system for the hydrophobic region of cell membranes. The disordering of the vesicle bilayer by anesthetics suggests that these compounds may act *in vivo* by disordering nerve cell membranes, and on this

basis one would predict that increased pressure must reorder the lipid region of the nerve membrane. If the phospholipid vesicle bilayer is indeed a good model for the lipid region of a nerve membrane, the disorder produced in the vesicle by anesthetics must be reversed by pressure. Indeed, any model system proposed for nerve membranes must stand the test of having induced anesthetic effects reversed by pressure. The present study describes the effects of pressure on anesthetic-disordered phospholipid vesicles.

METHODS

Spin-labeled phosphatidylcholine (1β(7,8)) was prepared by replacing the β-fatty acid chain of egg phosphatidylcholine with an 18-carbon fatty acid which has a nitroxide spin label rigidly attached to the C-10 positon utilizing the method of Hubbel and McConnell[8]. The label was diluted to 1 part in 100 by addition of egg phosphatidylcholine prepared according to the method of Singleton *et al.*[9]. A vesicle suspension of phosphatidylcholine, cholesterol, and water (10:3:87, by wt) was prepared as described previously[5]. Halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) was added to aliquots of the suspension to produce concentrations of 80 and 240 mmoles of halothane per mole of lipid. These concentrations are approximately twice and six times the lipid concentration predicted to produce anesthesia by the Meyer–Overton theory[7] and slightly more than ten fold that present in a nerve bathed in a solution containing 10 mg halothane in 100 ml water[5]. Equilibration of this solution with the superior cervical ganglion of the rat has been shown to produce a 50% depression of the synaptic action potential (J. Kendig and E. N. Cohen, unpublished).

The measurement of the a'_N value during a phase transition was made in a vesicle suspension of dipalmitoyllecithin. The spin label Iβ (7,6) (0.002 g) was added to 0.2 g dipalmitoyllecithin (Cal Biochem, San Diego, Calif.) and dissolved in 10 ml of chloroform. After thorough evaporation of the solvent on a rotary evaporator, water (0.8 g) was added, and the mixture vigorously vortexed to produce a vesicle suspension. Spectra were recorded after 5 min. of equilibration at each temperature increment.

The anesthetic and vesicle suspensions were placed in a specially constructed glass ESR tube capable of being pressurized with helium to 4000 lb/inch^2. The ESR spectra were measured after 5 min of equilibration following pressure increase as well as decrease, although the ESR spectra appeared to change instantaneously with changes in pressure. Remeasurement of spectra at various pressure increments following repeated compressions and decompressions performed by venting the pressurizing helium indicated no change in the S'_n parameter.

All ESR spectra were measured on a Varian series 4500 instrument operated in the X-band with the cavity temperature thermostated at 20 $\pm$ 0.1 °C by a Varian V4500 temperature control unit. A Harvey–Wells proton probe was used for sweep calibration and field measurement.

RESULTS

Fig. 1 shows the ESR spectra of the Iβ (7,8) spin label in egg phosphatidylcholine –cholesterol vesicles measured at atmospheric pressure, at 2000 and at 4000 lb/inch^2 of

helium. The spectra show well-resolved hyperfine extremes which can be interpreted as parameters of the motion of the hydrocarbon chains within the bilayer[8]. As $2T'_{\parallel}$ increases and $2T'_{\perp}$ decreases, the mean angular deviation ($\bar{\theta}$) of the nitroxide $2p\pi$ orbital axis from the unique axis of magnetic symmetry decreases. This is interpreted as a decrease in the anisotropic motion of the fatty acid chains and thus a decrease in bilayer fluidity. The degree of fluidity of a bilayer is expressed in terms of the order parameter (S'_n) given by

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

Figs 1 and 2 show that the order parameter (S'_n) for the bilayer is increased by the application of helium pressure. Fig. 2 reveals the striking linearity of this effect.

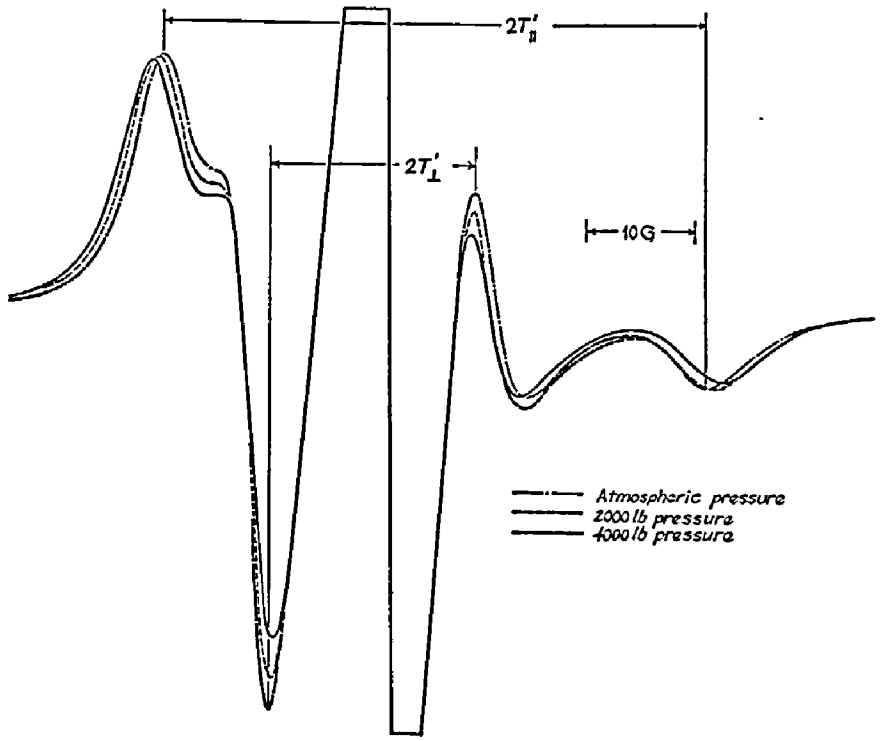

Fig. 1. ESR spectra of Iβ (7,8) spin label in a phosphatidylcholine vesicle containing no anesthetic observed at various pressures of helium. The shift of the $2T'_{\parallel}$ and $2T'_{\perp}$ spacings indicates an increase in bilayer order with increasing pressure.

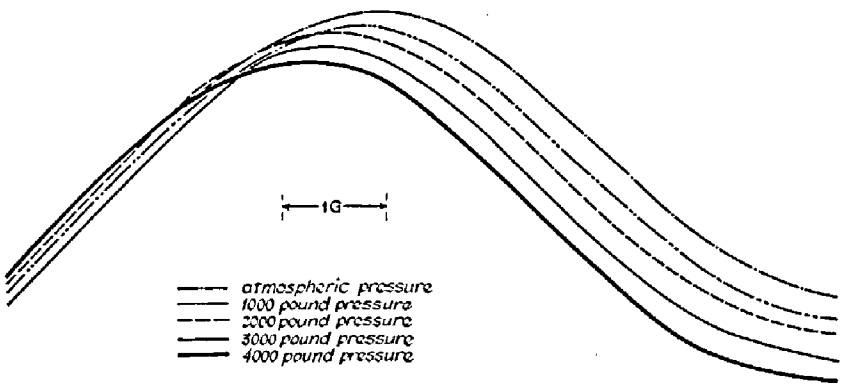

Fig. 2. Expanded-scale drawing of the top of the low-field signal seen in the left peak in Fig. 1. The linearity of spectral change *versus* helium pressure is striking.

Fig. 3 shows a spectrum of a Iβ (7,8) spin label in a vesicle at atmospheric pressure containing no halothane. Superimposed on this are three spectra of a Iβ (7,8) spin label in vesicles containing 0.18 mole of halothane per mole of lipid at atmospheric pressure and at 2000 and 4000 lb/inch^2 of helium. It has been previously shown[5] that halothane decreases S_n' in a linear, concentration-dependent manner. This change is seen in Fig. 3 in the spectrum labeled "halothane, atm pressure". The remaining two spectra demonstrate that helium pressure can partially reverse the disorder created in the bilayer by halothane. In the expanded-scale spectra in Fig. 4, the complete reversal of the disorder created by 120 mmoles of anesthetic per mole of lipid is demonstrated.

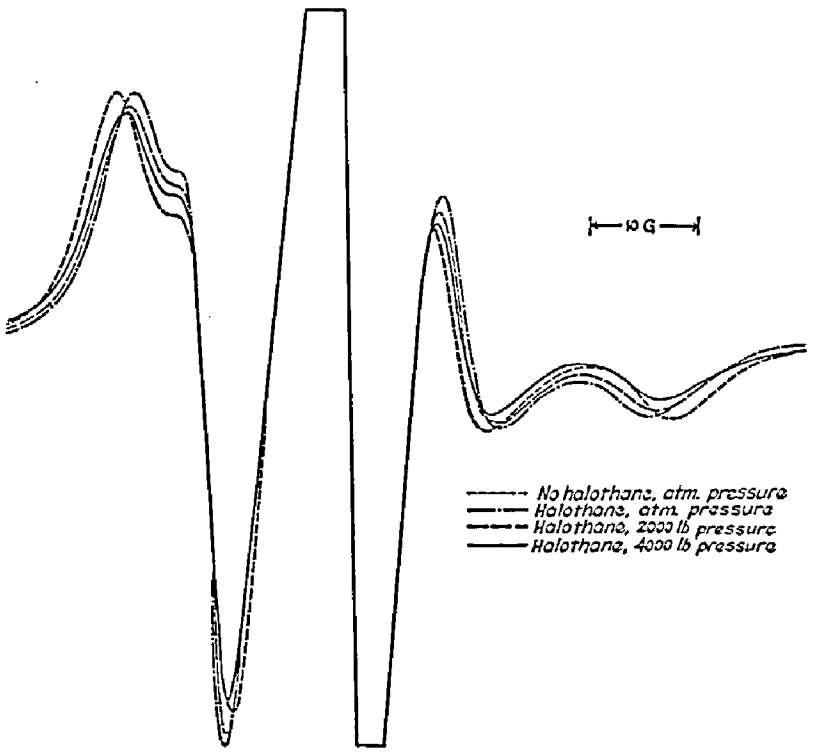

Fig. 3. ESR spectra of Iβ (7,8) spin label in a phosphatidylcholine vesicle containing 0.18 mole of halothane per mole of lipid at increased helium pressures. A spectrum of the Iβ (7,8) spin label in a vesicle without anesthetic is superimposed. At the highest pressure, the order of the bilayer containing a three-fold clinical concentration of anesthesia was nearly restored to that of a non-anesthetic treated bilayer at atmospheric pressure.

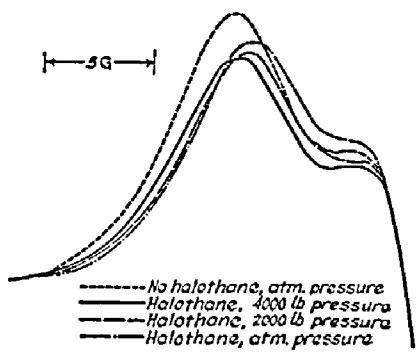

Fig. 4. An expanded-scale drawing of the entire low-field signal similar to that seen at the left of Fig. 3. The anesthetic concentration is only 0.12 mole per mole of lipid, and in this instance the order of the anesthetic-treated bilayer is completely restored to that of the untreated bilayer by application of 4000 lb/inch^2 of helium.

The spectra obtained in Figs 1–4 could be reproduced at will by varying the helium pressure and re-recording the spectra. Therefore, the effects observed are reversible changes in the bilayer and are not caused by destruction of the vesicles or loss of halothane.

Fig. 5 shows the dependence of S'_n on helium pressure and anesthetic concentration. From these data one can calculate that 2500 lb/inch^2 or 170 atm. of helium totally reversed the disordering effect of 80 mmoles of halothane. Since the change of S'_n with anesthetic concentration has been shown to be linear[5], and the slopes of the 80 and 240 mmoles of anesthetic per mole of lipid lines in Fig. 5 are parallel, it can be estimated that 1875 lb/inch^2 or 128 atm. of helium would reverse the effect of a clinical concentration of 60 mmoles of halothane per mole of lipid.

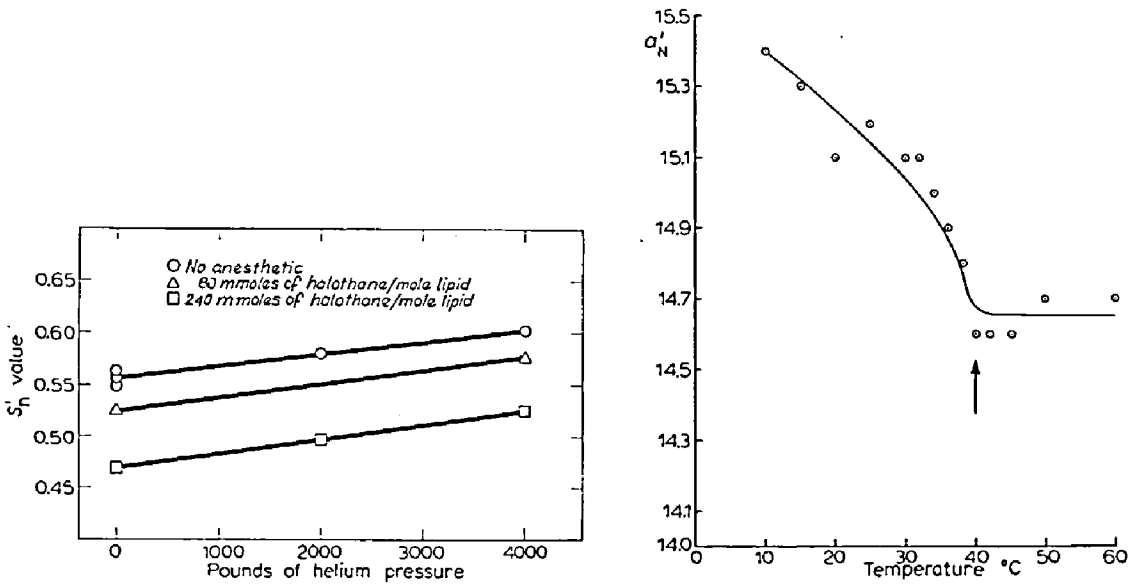

Fig. 5. The order parameter (S'_n) of Iβ (7,8) spin-labeled phosphatidylcholine vesicles at various halothane concentrations and helium pressures.

Fig. 6. The change in the isotropic nitrogen hyperfine coupling constant a'_N in Iβ (7,6) spin-labeled dipalmitoyllecithin vesicles over the region of their phase transition temperature.

The isotropic nitrogen hyperfine coupling constant (a'_N) may be calculated from the equation $a'_N = 1/3\ (2T'_\perp + T'_\parallel)$. The value of a'_N depends on solvent polarity and ranges from 14.1 G when the niroxide spin label is dissolved in hexane to 15.1 G when in aqueous solution. Although the effect is small and the scatter large, the trend is for a'_N to increase, and thus the environment of the nitroxide spin label to become more polar with increasing pressure.

DISCUSSION

The order parameter (S'_n) of a spin label in a phopholipid vesicle is increased by the application of high pressure helium. Thus, under helium pressure the fatty acid chains of a phospholipid bilayer have less configurational freedom than at atmospheric pressure. Among the possible explanations for this phenomenon is the stabilization of hydrocarbon chains by dissolved helium molecules; stabilization of

the chains by water molecules forced into the bilayer; restriction of motion caused by interdigitation of the opposed hydrocarbon chains; or a decrease in the surface area of the bilayer, resulting in neighboring hydrocarbon chains restricting each other's motion.

It is unlikely that the hydrocarbon chains are stabilized by the dissolved helium molecules. Other gases such as zenon and nitrous oxide, which are more lipid soluble than helium, actually produce anesthesia at elevated pressures[10]. Presumably these gas molecules create more disorder as they enter the bilayer. In fact, it seems that it is the property of helium of exerting pressure without having significant solubility in the lipid phase which allows it to reverse anesthesia.

It is possible that high pressure alters the equilibrium distribution of water molecules in the lipid region and forces more water into the space between the fatty acid chains and decreases their motion. Certainly the trend of the change of the isotropic nitrogen hyperfine coupling constant (a'_N) with pressure indicates that the environment of the nitroxide spin label became more polar at higher pressures and higher bilayer order.

The possibilty of restricted bilayer motion due to interdigitation of the hydrocarbon chains must be considered. It could be possible that the exterior pressure, not fully compensated for by the dissolution of helium in the lipid region, actually compresses the membrane and forces the ends of the chains together, or causes a net average tilt of the hydrocarbon chains, resulting in a closer packing. X-ray diffraction analysis of bilayers under high pressure remains to be done. However, Träuble and Haynes[11] have shown from X-ray diffraction data that as bilayers fall below their phase transition temperature and increase in order, they actually expand in thickness. They attribute this to a straightening or unkinking of the hydrocarbon chains.

Restriction of molecular motion caused by the phospholipid molecules moving closer together remains the most reasonable explanation. Seeman and co-workers[12,13] have shown that the addition of anesthetics to erythrocyte membranes expands their surface area. Presumably this must result in more room between the fatty acid chains. Since Figs 3 and 4 show that helium pressure is capable of reversing the effects of anesthetics on a bilayer, it would seem that pressure must cause the bilayer surface area to contract. It would, of course, produce this effect in the absence of anesthetics as well. Thus the application of pressure to a non-anesthetic treated bilayer would contract its surface area to less than normal, causing the hydrocarbon chains to pack together and the membrane to become less fluid.

The direction of change of the a'_N value indicates that the region surrounding the nitroxide spin label becomes more polar with an increase in applied helium pressure. Therefore, the decrease in bilayer polarity caused by the presence of inhalation anesthetics[5] is also reversed by pressure.

A separate experiment was performed to determine if an increase in the a'_N value with an increase in the S'_n value of a lipid bilayer represents a general phenomenon. Dipalmitoyllecithin undergoes a phase transition at 40 °C. Below this temperature it is in a more highly ordered gel phase. Vesicle suspensions of spin-labeled dipalmitoyllecithin have been shown to undergo an abrupt increase in the S'_n order parameter as they fall below their phase transition temperature[8]. Fig. 6 indicates that the a'_N value of these vesicles shows an abrupt increase as the temperature of the vesicles decreases to their transition temperature.

The above result duplicates that of the pressure-reversal experiment in which the polarity of the environment of the spin label increased coincident with the increase in the order of the bilayer. An interpretation of this data suggests that as the order of the bilayer increases, the nitroxide spin labels become more exposed to water molecules. The latter could be the result of an increase in the internal hydration of the bilayer with pressure. Another possible explanation may be that as the membrane surface area contracts, the surface charge density increases, and the nitroxide is affected by the net charge increase.

The demonstration of pressure reversal of inhalation-anesthetic induced disorder in phospholipid vesicles allows two conclusions. Phospholipid vesicles would appear to be good models with which to define the primary site of action of anesthetics. Disorder is produced in the vesicles at clinical concentrations of anesthetics, and this disorde. is reversed at increased helium pressures similar to those which produce reversal of anesthesia *in vivo*. Secondly, the close agreement of this phenomenon produced in the pure lipid and animal systems lends support to the theory that anesthesia is first produced in the lipid phase. These data also suggest that pressure reversal occurs in the lipid rather than in the protein regions of nerve membranes. It remains likely that these effects are transmitted to proteins essential to nerve impulse propagation which are included in and solvated by the lipid bilayer[5].

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