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# *Pressure Reversal of Anesthesia:*

## *The Extent of Small-molecule Exclusion from Spin-labeled Phospholipid Model Membranes*

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**Recent evidence suggests that anesthetic agents may act by disordering the lipid phase of nerve cell membranes, and that increased pressure reverses the anesthetic effect by increasing order among lipid molecules. It is conceivable that high pressure may simply lower the anesthetic concentration at its presumed site of action by displacing anesthetic molecules from the membrane, or alternatively, that high pressure may directly reorder lipid membranes, the concentration of the anesthetic agent in the membrane remaining the same. The latter alternative is supported by the results of the present study, in which electron spin resonance techniques were used to demonstrate that the small spin-labeled molecule TEMPO is only partially displaced from phospholipid membranes by pressures which reverse anesthesia *in vivo*. (Key words: Pressure reversal; Anesthesia; Model membranes; Phospholipid vesicles; Electron spin resonance.)**

PRESSURE REVERSAL of anesthesia has been demonstrated *in vivo* in luminous bacteria, tadpoles, newts, and mice.[1-3] Tadpoles exposed to 2–5 per cent ethyl alcohol until they fall motionless promptly resume swimming when the pressure is increased to 200–300 atmospheres, although the concentration of alcohol remains the same. Mice in a state of deep anesthesia at atmospheric pressure show spontaneous activity within seconds following exposure to increased pressures of helium. Such phenomena afford an important consideration in defining a uniform theory for the molecular action of anesthetics and, at the same time, this observed effect serves to limit the number of acceptable theories of anesthesia. Those theories which are unable to account for the phenomenon of pressure reversal must be revised. As a corollary, artificial model membrane systems used to investigate the anesthetic state should as well be able to demonstrate a pressure antagonism of the anesthetic effect. The pressure reversal of anesthesia in one such model system, the phospholipid vesicle bilayer, has been investigated previously in these laboratories through electron spin resonance (ESR) techniques.[4]

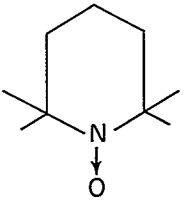


FIG. 1. The spin label TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl).

The ESR technique allows observation of the motion and polarity of the environment of a free electron. Hubbell and McConnell have demonstrated that phospholipid vesicles containing one part in 100 of nitroxide-spin-labeled phosphatidylcholine provide excellent model systems for the lipid bilayer region of nerve membranes.[5] These vesicles are produced by sonicating a phospholipid in water to produce microscopic spheres which have a lipid bilayer as a wall. More recently, it has

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been shown that ESR spectra obtained from such vesicles vary in a dose-related manner with both local[6] and inhalation[7] anesthetics. In such studies a nitroxide group containing a free electron is attached rigidly to the hydrocarbon chain of the $\beta$-fatty acid on a phosphatidylcholine molecule. When this spin-labeled phosphatidylcholine is allowed to tumble freely in solution, it gives a type of spectrum known as "isotropic." When it is rigidly constrained, as in a crystal, it gives a second type of spectrum, referred to as "anisotropic." When the spin-labeled molecule is included in a phospholipid bilayer, its motion is partially restrained. It is able to spin around the long axis of the hydrocarbon chain, but because of the structure of the bilayer in which it is included, it cannot tumble about its long axis. It is, however, able to wobble about its long axis. The extent of this wobble, actually the angular deviation of the electron spin vector from the magnetic field vector, is a measure of how constraining or immobile the bilayer is. The order parameter ($S'_n$) which may be calculated from these spectra is a measure of the conformational mobility of the fatty-acid chains which comprise the central hydrophobic region of the bilayer.[5] The value of ($S'_n$) is zero for complete disorder and one for a fully-ordered bilayer. Anesthetics serve to decrease the order parameter, indicating an increase in the fluidity of the hydrocarbon chain region. Halothane and methoxyflurane have been shown to produce this effect at lipid concentrations equivalent to those present during clinical anesthesia.[7]

Previous experiments indicate that the disorder created by halothane in the phospholipid-bilayer model-membrane system is antagonized by high pressures of helium.[4] These results have been interpreted to suggest that as anesthetics cause the membranes to expand, more room is allowed for motion of the fatty-acid hydrocarbon chains. Increased helium

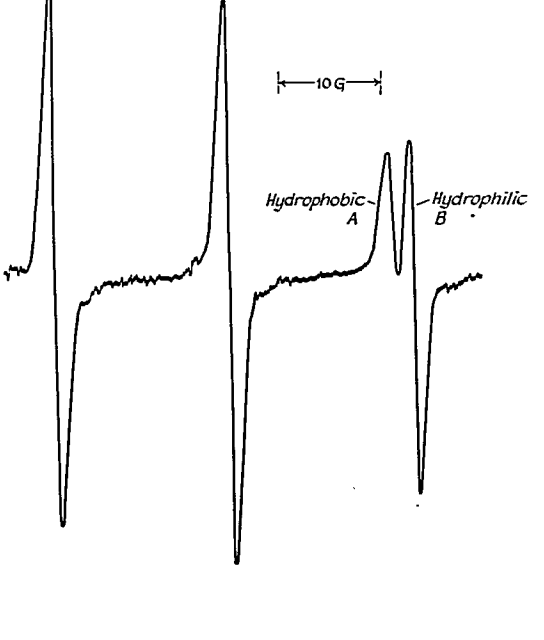


FIG. 2. ESR spectrum of TEMPO in a phospholipid vesicle suspension, showing the two high-field peaks due to TEMPO in a hydrophobic and hydrophilic environment.

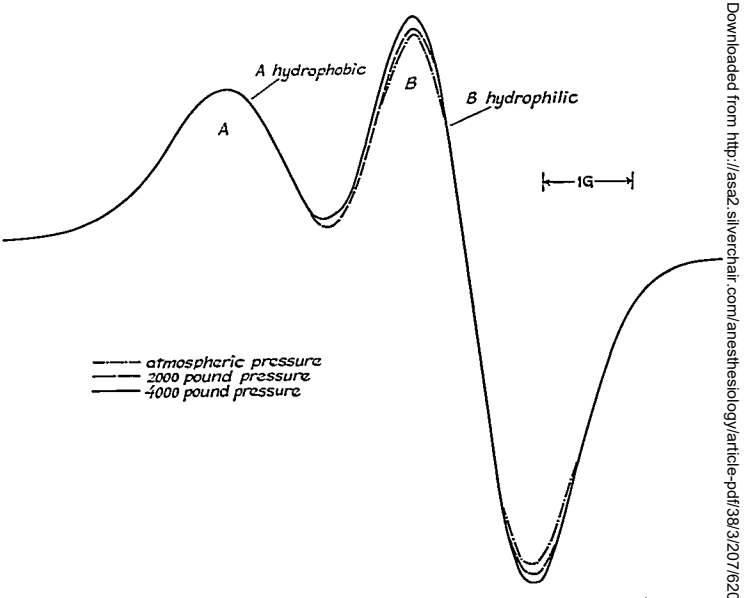


FIG. 3. Expanded ESR spectrum of TEMPO in a phospholipid vesicle suspension. The two high-field peaks indicate the effects of applied helium pressure on TEMPO distribution.

pressures may then compress the membrane, decreasing the surface area per fatty-acid chain and reducing the volume of the region in which each fatty-acid chain may move.

The second property observable by ESR is definition of the environment of the free electron present on the nitroxide spin label. The electron when dissolved in a hydrophobic phase experiences an electronic environment different from that of the spin label dissolved in water. The nuclear hyperfine coupling is greatly influenced by environmental change, producing separation of the two high-field peaks. Separation of these peaks allows direct observation of the partitioning of the nitroxide spin label between the lipid and aqueous phases within a phospholipid vesicle suspension.

An important question is whether pressure reversal of the anesthetic-induced disorder in the bilayer model corresponds to an exclusion of the small anesthetic molecule from the lipid region into the surrounding aqueous medium, or alternatively, to a compression of the phospholipid chains around the anesthetic molecule. The nitroxide spin label TEMPO [6] (2,2,6,6-tetramethylpiperidine-1-oxyl, fig. 1) was selected to answer this question. Its exclusion from the lipid bilayer was investigated under conditions of increased helium pressure.

## Methods

A mixture containing phosphatidylcholine, cholesterol, and water (2: 0.3: 97.7 by weight) was sonicated to produce a phospholipid vesicle suspension, as previously described.[7] The lipid/water distribution coefficient of TEMPO was found to be 50:1 by comparing ESR peak heights in a known mixture. A vesicle preparation containing 2.3 per cent lipid was prepared to produce approximately equal concentrations of TEMPO in the lipid and aque-

ous phases. The total concentration of TEMPO in the suspension was $5 \times 10^{-4}$ M. Using the 50:1 lipid/water distribution coefficient, the concentration of TEMPO in the lipid region of the vesicles is calculated to be $1.2 \times 10^{-2}$ M.¶ The spin-labeled vesicle suspension was then placed in a specially designed glass tube capable of being pressurized to 4,000 psig.

Electron spin resonance spectra were measured with a Varian Series 4500 instrument ** operated in the X band with the cavity temperature thermostated at 20 ± 0.1 C. A Harvey-Wells proton probe was used for sweep calibration and field measurement. The high-field region of the spectrum was scanned with the tube at atmospheric pressure and after 5 minutes of equilibration at 2,000 and 4,000 psig of helium. The spectrum recorded following release of the pressure was identical to the initial spectrum recorded at atmospheric pressure.

In parallel experiments, the anesthetic potency of TEMPO was examined by determining its ability to depress synaptic transmission in the isolated rat superior cervical ganglion. The preparation was perfused with oxygenated solutions containing TEMPO in various concentrations. Single stimuli, 0.2 msec in duration, were applied to the preganglionic nerve at a frequency of 0.1 per second, and both pre- and postganglionic compound action potentials recorded. In two experiments with ganglia from different rats, reversible 50 per cent depression of the postganglionic action potential was achieved at a concentration of $5 \times 10^{-5}$ M. The preganglionic action potential was unaffected at this concentration. The concentration of TEMPO in the lipid region of a nerve is calculated to be $2.5 \times 10^{-3}$ M when the nerve is equilibrated in a $5 \times 10^{-5}$ M bathing solution, assuming that the lipid/water distribution coefficient found for a vesicle obtains for the lipid region of a nerve.

## Results

The ESR spectrum of TEMPO present in an aqueous dispersion of phospholipids is shown in figure 2. Such a spectrum is readily interpreted as the sum of two spectra, one arising from TEMPO in a fluid hydrophobic environment (*A*), and the other from TEMPO present in an aqueous phase (*B*). Although this spectrum is a first-derivative presentation, the peak heights have been shown to provide a good estimate of relative TEMPO concentration.[9] The ratio of the two peaks corresponds to the distribution coefficient of TEMPO in these two phases. Thus, the change in the ratio of *A* to *B* indicates the extent to which TEMPO is excluded by helium at a pressure sufficient to reverse anesthesia completely (*in vivo*), and also to reverse the anesthetic-induced disorder in phospholipid vesicles. As is evident in figure 3, there is a change of approximately 5 per cent in the relative heights of peaks *A* and *B* as the helium pressure is increased from atmospheric to 4,000 psig.

## Discussion

Although alternative theories have been proposed, the preponderance of evidence indicates that anesthetics act by dissolving in the lipid portion of cell membranes. The phospholipid bilayer vesicle used in the present study closely resembles the currently accepted structure of the cell-membrane lipid matrix.[10] The application of helium pressure to phospholipid bilayers which have been disordered by anesthetics produces restoration of the order of the bilayer. This reordering of the lipid molecules may be brought about in one of two ways. Pressure increase may force the molecules of anesthetic out of the lipid bilayer, thereby reducing the concentration of anesthetic at its presumed site of action; alternatively, pressure may in some way reorder the bilayer without changing the number of anesthetic molecules in the lipid. The present experiments support the second alternative. With helium pressures at 4,000 psig, there was only a 5 per cent change in the distribution of the small molecule TEMPO between the lipid and the aqueous phases. Previous studies suggest that these helium pressures should be sufficient to reverse completely the disordering effect of the TEMPO.[4] The slight displacement observed is not sufficient by itself to account for the pressure reversal.

¶ This concentration of 12 mmoles/l lipid is somewhat less than that predicted to be an anesthetic concentration by the Meyer-Overton theory (30–60 mmoles anesthetic/l lipid).[8]

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It is important to note that while this result has been demonstrated for TEMPO, it is not an inhalation anesthetic. However, TEMPO does behave like an anesthetic, in that it produces reversible depression of synaptic transmission at concentrations lower than those necessary to block nerve conduction in rat sympathetic ganglion. Although the relationship between anesthesia and depression of synaptic transmission is still a matter for debate, sympathetic ganglia have often been used as models in the study of anesthesia.[11, 12] Nevertheless, it remains possible that certain molecular differences which make halothane a clinically useful anesthetic allow it to behave differently from TEMPO under conditions of pressure reversal. Since none of the anesthetics has the unpaired electron necessary to produce an ESR spectrum, TEMPO afforded the best opportunity to examine this phenomenon.

Elsewhere we have presented evidence that volatile anesthetics increase the fluidity of the hydrophobic region of lipid bilayers[7] and that pressure reverses this change in molecular order.[4] The conclusion to be drawn from the present work is that the phenomenon of pressure reversal is produced through a reordering of the hydrophobic region without regard to the presence of entrapped anesthetic molecules. Our studies further suggest that the lipid region is the primary site of anesthesia, and that the phenomenon of anesthesia may be associated with an increase in the fluidity of the lipid region of nerve membranes.

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