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Site and Mechanism of Anesthetic Action

II. Pressure Effect on the Nerve Conduction-Blocking Activity of a Spin Label Anesthetic

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SUMMARY

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A pressure of 100 atm was found to enhance the nerve-blocking activity of a spin label anesthetic, 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO). This pressure-enhanced nerve conduction block was completely reversible. The mechanism of enhancement was investigated by monitoring the effect of pressure on the resonance spectrum of TEMPO in nerve membranes. Pressure caused only a small decrease in partition coefficient, and no change in binding site was detected. The enhanced activity could not be ascribed to any special property of spin label anesthetics. This finding suggests that the pressure effect on narcosis is not unique. Pressure can enhance, reverse, or have no effect on the anesthetized state. It is argued that different anesthetics may act on different sites which do not respond in the same way to pressure. These diverse effects of pressure on the anesthetized state can be rationalized in terms of anesthetic and pressure effects on membrane proteins.

INTRODUCTION

Reversal of the anesthetic state by pressure has been demonstrated in the isolated nerve (1, 2) and in whole animals (3, 4). These studies indicate that the nerve-blocking and depressant effects of clinical concentrations of inhalation anesthetics are reversed following the application of 100-200 atm of helium or hydrostatic pressure. Although the mechanism of pressure reversal of anesthesia is not yet well understood, there is considerable support for the theory that pressure counteracts the expansion produced by anesthetics in the nerve membrane (4). Indeed, several model studies have been used to test this hypothesis (5-7), assuming that the site of the expansion is the lipid bilayer.

However, the results presented in the preceding paper (8) showed that clinical concentrations of anesthetics have no detectable effect on the fluidity of a lipid

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bilayer model membrane, making it unlikely that the lipid bilayer is the site of the expansion observed in nerve membranes. Furthermore, while pressure causes a small decrease in fluidity, the amount of pressure does not correlate with the pressures required to reverse narcosis and nerve conduction block. It has been proposed that anesthetic-induced protein conformation changes may be responsible for the observed expansion (9). Furthermore, pressure has varying effects, depending on the anesthetic and the system studied. Pressure has no effect on the action of charged local anesthetics (10). It has been found that in a marine amphipod, *Marinogammarus marinus,* hydrostatic pressure appears to enhance the depressant effect of a volatile anesthetic, halothane (11, 12). The general phenomenon of nerve conduction block may be a result of different anesthetic agents binding to various regions within the nerve membrane. Pressure may not affect these different sites in the same manner.

In the present communication we show that (a) 100 atm pressure reversibly enhances the nerve-blocking effect of a water-soluble lipophilic spin label anesthetic, 2,2,6,6-tetramethylpiperidine-1-oxyl, (b) pressure causes no change in binding which can account for the enhanced activity, and (c) the diverse pressure effects on the anesthetized state can be rationalized according to the protein perturbation hypothesis of anesthesia (13).

MATERIALS AND METHODS

2,2,6,6-Tetramethylpiperidine-1-oxyl, m.p. 36.5°, was prepared as described (14).

Catalase from bovine liver was obtained from Sigma Chemical Company (18,000 Sigma units/mg), and L-ascorbic acid, from British Drug Houses (Toronto). Lidocaine HCl was a gift from Dr. P. Seeman, University of Toronto; procaine HCl was obtained from Sigma Chemical Company, and benzyl alcohol, from Fisher Scientific Company (Toronto). Helium (99.9%) was purchased from Matheson of Canada, Ltd. (Toronto), and contained 2200 psi. Solvents used were reagent grade.

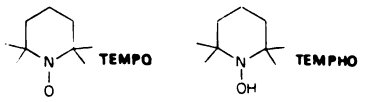
Preparation of membranes. Lipid dispersions were prepared as described in the preceding paper (8). Rat phrenic nerve and desheathed frog sciatic nerve were freshly dissected and immediately transferred to Ringer's solution.

Action potential measurements. The conduction-blocking action of the spin label and anesthetics was studied on rat phrenic nerve and desheathed frog sciatic nerve using the method of Staiman and Seeman (15). The nerve was placed on top of a 1-cm-wide cavity in a lucite block. On each side of this central cavity the nerve rested over a pair of slots containing platinum electrodes (for stimulating and recording) and filled with physiological solution. Drug solutions were placed in the central well (1 ml) by means of a Pasteur pipette. The nerve was stimulated supramaximally using a Grass SD-9 stimulator. The compound action potential was recorded from the platinum electrodes using a Tektronix storage oscilloscope (model 564B) triggered by the stimulator.

The effect of 100–150 atm of helium on the action potential was measured according to Roth *et al.* (10). The nerve chamber was placed in a cylindrical brass pressure vessel with electrical connections to the inside and connected to the helium tank. The temperature was monitored by a miniature glass bead thermistor inside the pressure vessel. Helium was added to the desired pressure and measurements were made of the height of the action potential. In order to observe the effect of pressure on the blocked action potential in the absence of excess aqueous phase (to reproduce experimental conditions used in measuring the ESR spectrum), the drug solution could be removed from the center well after block had occurred, and pressure was then applied. In control experiments, in which no drug was used, pressure had no effect on the action potential.

Reduced form of TEMPO.[5] To measure the blocking activity of the reduced form of TEMPO, 2,2,6,6-tetramethylpiperidine-1-hydroxyl, TEMPO in solution was reduced by adding ascorbate (TEMPO to ascorbate

[5] The abbreviations used are: TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; TEMPHO, 2,2,6,6-tetramethylpiperidine-1-hydroxyl.



ratio, 1:2). This procedure reduced 99.95% of the TEMPO. Since ascorbate solution irreversibly blocked nerve conduction, catalase (0.5 mg/ml) was added to protect the nerve from peroxide oxidation (16). The ascorbate-catalase solution had no effect on the height of the action potential. In a control experiment with procaine, the dose-response curves were the same in the presence and absence of ascorbate and catalase.

Drug combination. In order to measure the effects of combinations of other drugs with TEMPO on the action potential, the same nerve was used throughout the experiment, since the dose-response curve varied slightly for different nerves. In order to remove one drug, the center well was washed repeatedly until the action potential had returned to its control height and stabilized. The second drug was then added. For the combination, a solution containing one-half the concentration of each drug used previously was made by adding equal volumes of the individual drug solutions.

ESR spectral measurements. To measure the ESR spectrum a 1-cm piece of the frog sciatic nerve was equilibrated in a TEMPO solution of desired concentration in a glove bag filled with helium and transferred to a 50-μl micropipette, which was placed in a thick-walled quartz cell and immediately connected to the high-pressure system under positive helium pressure to eliminate oxygen from the system. Application of helium pressure on top of a column of air in the ESR cell resulted in a high partial pressure of oxygen at the surface of the nerve membrane as a result of lack of mixing of the gases. This high pressure of oxygen resulted in the broadening of the spectrum reported earlier (17). Under pure helium, 100 atm pressure does not cause broadening of the ESR spectrum of TEMPO.

The rate of reduction of TEMPO by the nerve was measured by monitoring the decrease in height of the membrane peak *a* (Fig. 3) with time before and after the pressure was changed. However, in order to measure the rate of reduction of TEMPO by the nerve membrane under conditions like those experienced in the pressure chamber during action potential measurements, it was necessary to have some oxygen present. In order to prevent broadening due to excess oxygen dissolving in the nerve, a column of buffer (5 cm) was placed in the cell above a small air pocket surrounding the nerve and its adherent buffer. When pressure was applied, the column of buffer prevented a high concentration of oxygen from diffusing into the nerve. The small pocket of air around the nerve did not contain enough oxygen to broaden the signal.

RESULTS

TEMPO has been reported to reversibly block nerve conduction (15) and synaptic transmission (6). The ED_{50} for rat phrenic nerve is 6.4 mM (15), and for frog sciatic nerve 13 mM (Fig. 2).

Figure 1 shows the effect of 100 atm of helium on the depressed action potential of frog sciatic nerve anesthetized with TEMPO at a concentration sufficient to decrease the height of the action potential

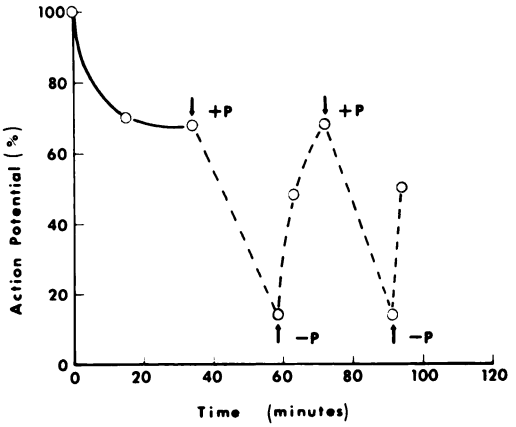


FIG. 1. *Effect of application and release of 100 atm of helium pressure on action potential of frog sciatic nerve anesthetized with TEMPO*

The data are from a single experiment. ±P indicates application or release of pressure at the time indicated by an arrow. Measurements were made after transient changes in temperature (±2°) had subsided.

to 68% of its control height. Upon application of pressure there is an additional decrease of the action potential to 14% of its original control height. Thus pressure appears to enhance the nerve-blocking effect of TEMPO. Upon release of pressure, the action potential returns to its anesthetized value; thus the effect is readily reversible. A pressure of 100 atm of helium is sufficient to reverse the nerve-blocking action of gaseous anesthetics (2, 10). This amount of pressure alone has no effect on either amplitude or duration of the evoked action potential (1, 10) or on the resting potential (1). Figure 2 shows the shift in dose-response curve to the left by pressure. The ED_{50} for frog sciatic nerve under 100 atm pressure is 8.5 mM.

In order to determine whether the enhanced activity of TEMPO under pressure was due to a change in binding of TEMPO to the membrane, the effect of pressure on the ESR spectrum was monitored. An analysis of the spectrum of TEMPO in lipid model membranes and in nerve membranes has been reported (17). A typical resonance spectrum of TEMPO in lipid model membranes and in nerve membranes at 1 atm and under 150 atm pressure is shown in Fig. 3. Peak *a* is due to TEMPO dissolved in the apolar region of the membrane, while peak *b* is due to free TEMPO in the aqueous phase. TEMPO

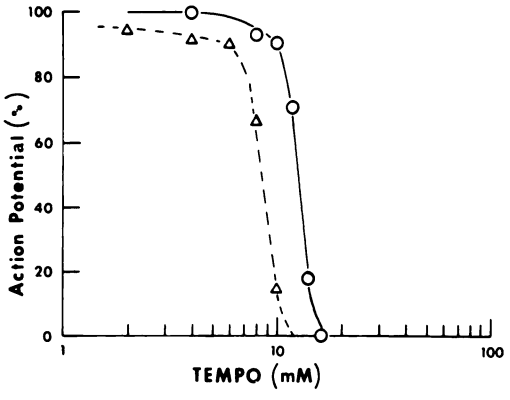


FIG. 2. *Comparison of log dose-response curves for TEMPO on frog sciatic nerve at atmospheric* (○) *and 100 atm* (△) *pressure*

Each point for the curve under pressure was obtained on a different nerve.

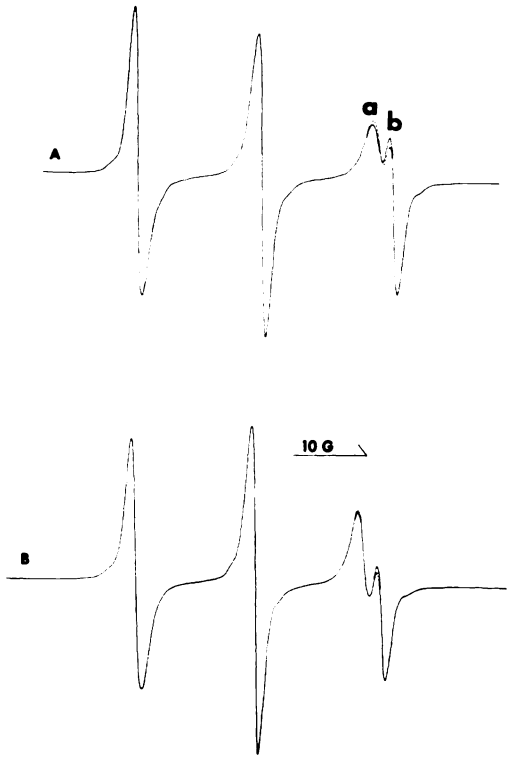


FIG. 3. *ESR spectra of TEMPO in phosphatidylcholine-cholesterol vesicles (A) and frog sciatic nerve (B) at 1 atm (- - -) and 150 atm (——) pressure.*

experiences an environment in the nerve membrane similar to that in phosphatidylcholine-cholesterol bilayers.

Pressure does not affect the relative intensities of peaks *a* and *b* significantly (Fig. 3B), indicating that pressure causes only a small decrease in the partition coefficient of TEMPO in nerve membranes, similar to its effect on lipid bilayers (6, 17) (Fig. 3A). No change in polarity or mobility of the binding site could be seen, contrary to our earlier report (17). It is possible that most of the TEMPO is bound to nonspecific sites in the membrane and that any small changes in binding to specific sites are obscured. However, the spectra obtained at low concentrations of TEMPO (0.1 mM) were similar to those at nerve-blocking concentrations (6 mM).

TEMPO is reduced by the nerve membrane to its diamagnetic hydroxylamine derivative, TEMPHO. The activity of TEMPHO was measured by reducing

TEMPO with ascorbate and protecting the nerve membrane with catalase (16). The dose-response curve for TEMPHO on frog sciatic nerve is shown in Fig. 4. TEMPHO is more active than TEMPO, with an ED_{50} of 7.3 mM, and its activity is also enhanced by pressure, although to a lesser extent than for TEMPO.

This raised the possibility that the observed reversible pressure enhancement was due to an enhanced rate of reduction of TEMPO under pressure and its corresponding reoxidation when pressure was released. However, as shown in Fig. 5, the rate of reduction, although time-dependent, was independent of pressure. Reduction continued at the same rate when pressure was released: no reoxidation occurred.

Similar pressure enhancement of nerve block was observed when pressure was applied to a partially blocked nerve from which the surrounding TEMPO solution had been removed. The significance of this experiment will become apparent from the discussion below. During measurement of the blocking effect of TEMPO the nerve is normally suspended in 1 ml of TEMPO solution. Since TEMPO in solution can exchange with any TEMPHO produced by the nerve, the amount of TEMPHO present during measurement of the dose-response curves is probably insignificant. Furthermore, TEMPHO in the aqueous phase is reoxidized to TEMPO by oxygen

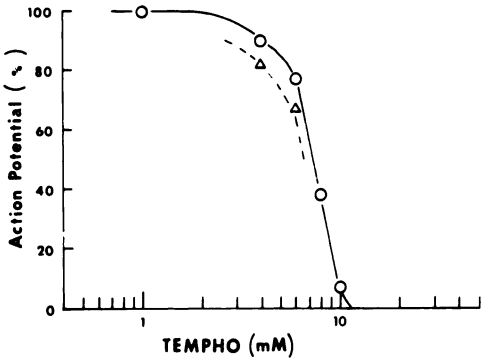


FIG. 4. *Log dose-response curves for TEMPHO on frog sciatic nerve at atmospheric* (○) *and 100 atm* (△) *pressure*

The points under pressure were obtained on separate nerves.

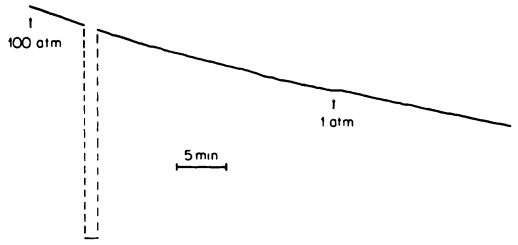


FIG. 5. *Rate of reduction of TEMPO by frog sciatic nerve, measured as decrease in height of membrane peak a under 100 atm and 1 atm pressure with time*

The dashed line indicates the baseline-zero signal.

in solution. However, if after partial block has occurred the TEMPO solution surrounding the nerve is removed, the concentration of TEMPHO in the nerve will build up. The height of the action potential was monitored over a period of time (1 hr) during which significant reduction of TEMPO had occurred (50% reduced) and the action potential remained constant. Thus TEMPHO has approximately the same activity as TEMPO at equal membrane concentrations, and the differences in ED_{50} may be due to different partition coefficients. These results indicate that the pressure enhancement observed in this experiment when the surrounding TEMPO solution had been removed was not due to reduction of TEMPO.

Combinations of lidocaine with TEMPO and benzyl alcohol with TEMPO were studied to give some information on the effect of TEMPO on the nerve response to other anesthetics and pressure. Pressure was found to have no effect on the nerve-blocking activity of lidocaine and benzyl alcohol. Figure 6 shows that the effects of lidocaine and TEMPO and benzyl alcohol and TEMPO are roughly additive at atmospheric pressure. One-half the concentration of TEMPO producing 38% block, plus one-half the concentration of lidocaine producing 34% block, produced 37% block. In the case of benzyl alcohol, one-half the TEMPO concentration producing 50% block and one-half the benzyl alcohol concentration producing 46% block resulted in 60% block. The effect was observed repeatedly. Because the dose-response curves are not exactly parallel, it is difficult to con-

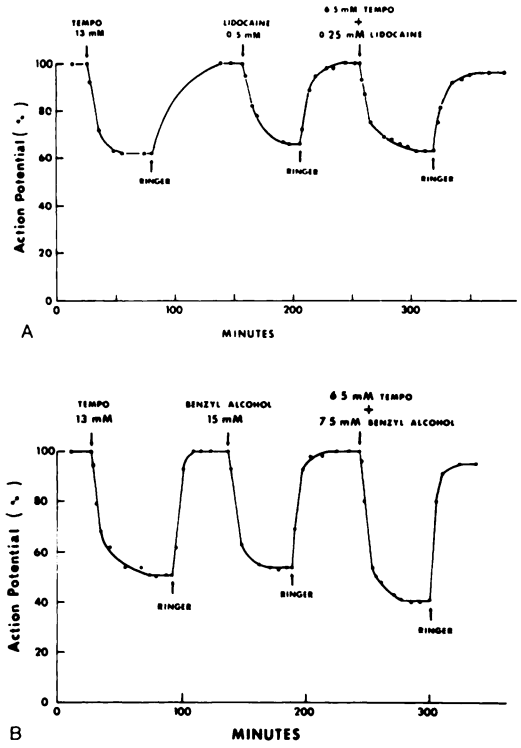


FIG. 6. *Depression of compound action potential of frog sciatic nerve by approximately equieffective concentrations of TEMPO and lidocaine (A) or benzyl alcohol (B), and by combinations of one-half of each of these concentrations*

clude anything from this apparent small potentiation with benzyl alcohol. In any case there is not any great interaction. The combination of lidocaine and benzyl alcohol has been shown to be additive (18).

The effect of pressure on a combination of lidocaine and 4 mM TEMPO is shown in Fig. 7A. There is a slight enhancement of nerve block, from 62% to 66%. This is the amount of enhanced activity that would be expected from TEMPO alone, as can be seen from Fig. 2. The effect of pressure on the block produced by a combination of benzyl alcohol with 2 mM TEMPO is shown in Fig. 7B. There was enhancement of nerve block, from 44% to 56%. This is much greater than the enhanced effect expected from 2 mM TEMPO alone, but not as great as if the 44% block were produced solely by TEMPO.

These results indicate that the amount of enhancement observed does not depend on the extent of total nerve block or disruption, since similar block by TEMPO alone would result in 100% block under pressure (see Fig. 2). It appears that TEMPO acts at a different site and by a different mechanism than either lidocaine or benzyl alcohol, which themselves may act at different sites. These anesthetics cannot substitute for TEMPO or displace it from its site of action, since they do not affect the pressure response to TEMPO significantly. Furthermore, the presence of TEMPO does not affect the nerve response to lidocaine or benzyl alcohol under pressure.

DISCUSSION

Pressure has been found to enhance the nerve conduction-blocking activity of TEMPO. A study of the mechanism of pres-

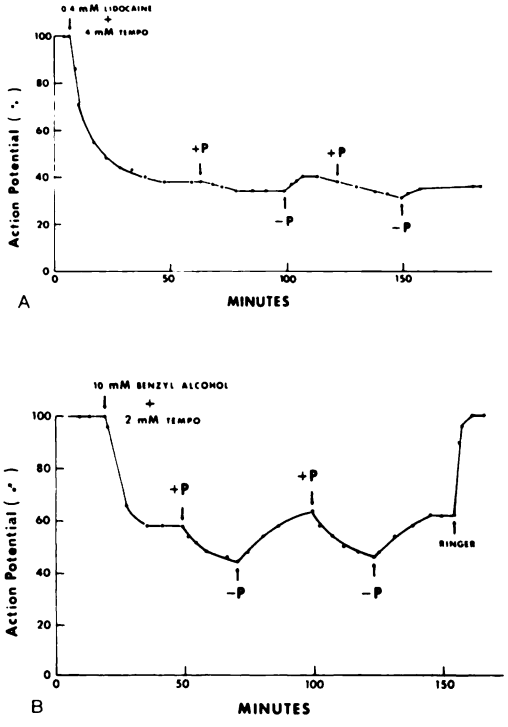


FIG. 7. *Effect of application and release of 100 atm pressure on action potential of frog sciatic nerve partially blocked with a combination of 0.4 mM lidocaine and a low concentration of TEMPO (4 mM) (A) and a combination of 10 mM benzyl alcohol and 2 mM TEMPO (B)*

A 2 mM concentration of TEMPO has no effect by itself. ±P indicates application or release of pressure at the time indicated by an arrow.

sure enhancement showed that pressure causes no significant change in the membrane concentration of TEMPO. No significant change in binding site or in the environment of TEMPO under pressure could be detected. This enhancement does not appear to be due to any unique property of TEMPO and must be considered to be one of several pressure effects on anesthesia. In view of the diverse pressure effects on the activity of various anesthetics, it is probable that different anesthetics act on different sites which do not respond in the same way to pressure.

At present those hypotheses which propose that anesthetics act on some membrane site by a physical mechanism are the most satisfactory. Since clinical concentrations of anesthetics do not have any detectable effect on lipid bilayer fluidity, lipid bilayer fluidization is unlikely to be the mechanism of action (8). No results are yet available to prove that nerve membrane protein is the primary site of anesthetic action. However, a hypothesis based on anesthetic perturbation of protein structure can accommodate both pressure reversal and enhancement.

According to Le Chatelier's principle, an increase in pressure will favor any process tending to reduce the volume of the system and will suppress any change tending to increase the volume (19). Expansion of a protein by anesthetics and reversal by pressure have been observed in the case of the enzyme luciferase (20). Clinical concentrations of anesthetics inhibit luminescence in bacteria and fireflies (20, 21) by expanding the enzyme luciferase, while pressure reverses the inhibition by favoring the lower-volume, active state of the enzyme (22). Formation of apolar bonds with proteins could induce a conformation change. In the case of luciferase, the conformation change is believed to result in neutralization of charged groups, resulting in release of bound water and expansion of the system (22).

Pressure and anesthetics can also cause volume decreases through their interaction with protein. The depolymerization of microtubules by pressure results in a volume decrease which has been suggested to be due to formation of shells of bound water around the binding site of the polymer subunits (23, 24). Anesthetics also cause depolymerization of microtubules (23), and an additive interaction between subeffective doses of anesthetics and pressure on cell division has been reported (25).

The decrease in entropy resulting from a volume decrease would tend to make such a conformation change unfavorable. However, a polar, amphiphilic anesthetic such as TEMPO might stabilize such a conformation change by binding to exposed hydrophobic sites and interacting with water through hydrophilic bonds. Although other studies have shown that TEMPO binds to extracted membrane proteins to a much lower extent than to the membrane lipids (26), TEMPO binds to a hydrophobic myelin protein.[6] Furthermore, only a small amount of TEMPO bound to a specific protein may be necessary to stabilize a protein conformation which results in nerve conduction block.

The diverse pressure effects on anesthesia can therefore be rationalized, based on the sign and net volume change of the anesthetic-produced membrane protein conformation changes. Thus pressure may cause reversal or enhancement of anesthesia simply by counteracting an anesthetic-induced protein conformation change, which results in a volume increase, or by favoring a conformation change, which results in a volume decrease. The activity of anesthetics which do not cause any change in volume would not be altered by pressure.

It is clear that anesthetics can affect a variety of processes in a pressure-sensitive way. Although it is not known which proteins in nerve membranes bind anesthetics, resulting in nerve conduction block or loss of consciousness, either pressure reversal or enhancement could result, depending on how the anesthetic and pressure disrupt or stabilize the molecular forces holding the protein in its native conformation. The mechanism by which anesthetic binding to protein results in nerve conduction block and narcosis must be

[6] J. M. Boggs, unpublished observations.

studied on a molecular level. This will require the development of model and reconstituted systems to elucidate the diverse effects of anesthetics and pressure.

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