MOLECULAR PHARMACOLOGY, 12, 127-135

Site and Mechanism of Anesthetic Action

I. Effect of Anesthetics and Pressure on Fluidity of Spin-Labeled Lipid Vesicles

J. M. BOGGS,[1] T. YOONG,[2] AND J. C. HSIA[3]

Department of Pharmacology, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada M5S 1A8

(Received December 16, 1974)

SUMMARY

BOGGS, J. M., YOONG, T. & HSIA, J. C. (1976) Site and mechanism of anesthetic action. I. Effect of anesthetics and pressure on fluidity of spin-labeled lipid vesicles. *Mol. Pharmacol.*, 12, 000-000.

The lipid expansion hypothesis of anesthesia has been re-examined. We have measured the effect of anesthetics and pressure on the order parameter of phosphatidylcholine-cholesterol bilayer vesicles labeled with fatty acid spin labels. Concentrations of halothane, chloroform, diethyl ether, butanol, and benzyl alcohol which produce general anesthesia have no significant effect on the order of the bilayer structure, while 100 atm of helium have a constant small ordering effect with or without anesthetics. The lack of correlation between anesthetic and pressure effects on lipid model membranes fails to support the lipid fluidization hypothesis of anesthesia or to account for pressure reversal of general and local anesthesia.

INTRODUCTION

Anesthetics include a wide variety of simple, nonreactive substances which fit a strong correlation between anesthetic potency and lipid solubility. Current theories hold that they act at a hydrophobic site in nerve membranes by a physical mechanism. The nature of this hydrophobic site has been a subject of intensive investigation.

The effect of pressure may be a useful tool to determine the site and mechanism of anesthetic action, since 100-200 atm pressure can reverse (1-6), have no effect (5), or, as we shall show in the following paper, enhance (7-9) narcosis and nerve conduction block, depending on the anesthetic and organism studied. Since anesthetics have been shown to expand biological membranes (10), the critical volume hypothesis (3, 4) has been developed for the mechanism of anesthesia, based on the expansion of a hydrophobic region in nerve membranes with pressure counteracting this expansion and reversing the anesthetized state. Thus far no attempt has been made to account for other pressure effects on anesthesia.

The lipid bilayer has been suggested to be the site of this expansion via fluidization of the lipid fatty acid chains. Several lipid model studies have been performed to test the effect of anesthetics on lipid bilayer structure and the validity of this mechanism with regard to pressure reversal (11-15).

However, to the best of our knowledge,

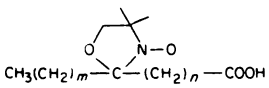
This investigation was supported by research grants from the Medical Research Council and the Defence Research Board of Canada.

[1] Recipient of a Medical Research Council predoctoral traineeship. Present address, Research Institute, Hospital for Sick Children, Toronto, Ontario, Canada.

[2] Recipient of a Department of Health and Welfare RODA summer scholarship.

[3] To whom requests for reprints should be addressed.

there has not been an adequate demonstration that clinical concentrations increase lipid fluidity to an extent which could account for expansion of membranes or suffice as a mechanism for anesthesia, particularly if the effects of pressure are also considered. There is no doubt that high concentrations of anesthetics can disturb bilayer structure, but it is crucial to demonstrate that this effect occurs at clinically significant concentrations. In the present work we have examined the effect of low concentrations of the neutral anesthetics benzyl alcohol, butanol, halothane, diethyl ether, and chloroform and of 100 atm pressure on the fluidity of lipid vesicles containing doxyl fatty acid spin labels [I(m,n)] in order to find the concentration



(I)

at which a detectable increase in fluidity occurs. The motional characteristics of fatty acid spin labels incorporated into lipid vesicles can give a measure of the fluidity of the lipid hydrocarbon chains, quantitatively expressed as an order parameter (16). The order parameter of these probes has been demonstrated to be sensitive to the ordering effects of cholesterol (17) and decrease in temperature (18). In a similar study Trudell *et al.* (11) found that high concentrations of anesthetics increased the fluidity of lipid bilayer vesicles in a linear fashion while pressure decreased the fluidity. However, the effects of anesthetic concentrations which produce general anesthesia [5 mmoles of anesthetic per liter of lipid (19)] were not examined.

The results presented in this paper show that anesthetics, at concentrations which cause narcosis and block nerve conduction, do not have a detectable effect on lipid fluidity. These results should permit a reevaluation of the fluidization theories. In the following paper (9) we present results showing that pressure enhances the effects of a spin label local anesthetic on nerve conduction and argue that a mechanism by which anesthetics directly induce protein conformation changes is required in order to account for the diverse effects of pressure on the anesthetized state.

METHODS

The fatty acid spin labels were prepared according to Hubbell and McConnell (16) and Waggoner *et al.* (20). 2,2,6,6-Tetramethylpiperidine-1-oxyl was prepared as described (21). Egg phosphatidylcholine was purchased from Pierce Chemical Company. Cholesterol was recrystallized in methanol (m.p. 140.5°). Halothane was obtained from Ayerst Laboratories (Montreal), and butanol, benzyl alcohol, chloroform, and diethyl ether, from Fisher Scientific Company (Toronto).

Preparation of lipid vesicles. A chloroform solution of the lipids, and fatty acid spin label when used, was evaporated, and a phosphate buffer, containing 0.15 M NaCl at pH 7.2 and the appropriate drug concentration, was added. For the TEMPO[4] partition coefficient measurements buffer containing 0.2 mM TEMPO was used. The lipid to doxyl fatty acid ratio was approximately 150:1. Lipid vesicles were prepared by hand dispersion of the lipid in buffer.

Drug effect. In order to measure the effect of the anesthetics on the order parameter, a stock solution of lipid vesicles was prepared containing 4.5 mg of lipid per 0.1 ml of buffer. Samples of 0.25 ml each (11.3 mg of lipid) were placed in disposable Pasteur pipettes with one end sealed. The samples were cooled in ice, the appropriate volume of drug was added, and the other end was sealed.

In the case of benzyl alcohol, the samples were titrated with 200 mM benzyl alcohol in the concentration range up to 36 mM, and with pure benzyl alcohol in the concentration range 50–300 mM. The concentrations were corrected for dilution. For measurement of the effect of benzyl alcohol on the TEMPO partition coefficient, the samples were titrated only with pure benzyl alcohol in the range 20–300 mM to minimize the effects of dilution. In the case of halothane the samples were

[4] The abbreviation used is: TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl.

titrated with 15 mM halothane in the range up to 3.6 mM, and with pure halothane in the range 7.5–30 mM. For chloroform the samples were titrated with 40 mM chloroform in the range up to 8 mM, and with pure chloroform in the range 20–60 mM. For ether the samples were titrated with 200 mM ether in the range up to 33 mM, and with pure ether in the range 20–735 mM. For butanol the samples were titrated with pure butanol in the entire concentration range. The drug solutions were made up 1–2 hr before use in 20-ml scintillation vials and placed in a sonicating bath to facilitate dispersal of the drug. For the titration 1-μl, 10-μl, and 50-μl Hamilton syringes were used.

The samples were then mixed on a Vortex mixer, allowed to equilibrate at room temperature overnight, and shaken to the small-diameter ends of the Pasteur pipettes, and the spectra were measured. Spectra measured after equilibration for 3 hr and overnight were identical in the case of halothane.

Effect of pressure. In order to examine the effects of pressure on the order parameter, the lipid vesicles were placed in a thick-walled quartz cell (outer diameter, 10 mm; inner diameter, 1.0 mm) connected to a pressure gauge and a helium tank (99.9%) containing 2200 psi. The helium tank was from Matheson of Canada, Ltd. The pipettes were opened and the samples quickly transferred to the pressure cell, and the spectra were immediately measured to avoid loss of drug by evaporation.

Measurement of ESR spectra. All spectra were recorded at room temperature on a Varian X-band E-6 ESR spectrometer. The temperature was controlled by a flow of air through the cavity at room temperature.

Analysis of data. Repetition of the experiments gave similar results, although the order parameter S for the entire curve could be shifted by approximately ± 0.04 for different vesicle preparations as a result of slight variations in the amount of cholesterol. Therefore the same stock solution of vesicles was used for each drug. Different control samples from this stock solution gave identical spectra. Although the error in measurement of S is ± 0.01, because of inaccuracy in manually measuring the hyperfine splitting as shown in Fig. 1, the spectra could be superimposed and examined to determine whether there was actually any change in hyperfine splitting. Thus, in the concentration regions of Figs. 2–6, where S is plotted as constant, the spectra were identical even though small variations in S were measured. No change in the spectra was observed by superimposition until the concentrations where S is plotted on the linear portions of the figures. Repeated measurement of the ESR spectrum on a single sample gave identical spectra.

The data were plotted on a linear dose-response curve and analyzed using a linear regression program with a Hewlett Packard 9810A calculator to fit to a straight line the points at which an increase in S was detected, by using as the initial point either (a) the control S at zero concentration or (b) the control S at the highest concentration at which no detectable change occurred. The value of S for the control was obtained by averaging the values in the concentration region where no change in the spectrum was detected. The standard deviations were 0.001–0.004. The standard deviations and correlation coefficients for the two methods of linearizing the data are given in Table 1. There was little difference in the goodness of fit between the two methods. Therefore the curves in Figs. 2–6 were drawn according to the second method and the lines were extrapolated back to zero and compared with the control values for S.

RESULTS

Figure 1 shows the resonance spectra of I(7,6) (8-doxylpalmitic acid) in phosphatidylcholine-cholesterol (molar ratio, 1:1) vesicles in the presence and absence of 300 mM benzyl alcohol. An empirical order parameter can be obtained from the hyperfine splittings $T'_{\parallel}$ and $T'_{\perp}$:

$$S = \frac{T'_{\parallel} - T'_{\perp}}{T_{zz} - T_{xx}}$$

where T_{zz} = 32 G and T_{xx} = 6 G are the nitroxide hyperfine tensors (16). S has a

TABLE 1

Comparison of methods of fitting data and significance of changes in order parameter S at low concentrations

Anesthetic and spin label	Control S	Extrapolated S[a]	Correlation coefficient	ΔS ($S_{extrap} - S_{control}$)	S from linear fit[b]	Correlation coefficient	ΔS predicted at local concentrations[c]
Benzyl alcohol							
5-Doxylpalmitate	0.737 ± 0.002	0.740 ± 0.009	−0.990	0.003 ± 0.011	0.737 ± 0.009	−0.992	0.01
8-Doxylpalmitate	0.675 ± 0.002	0.687 ± 0.004	−0.998	0.012 ± 0.006[d]	0.681 ± 0.006	−0.995	0.01
12-Doxylstearate	0.572 ± 0.003	0.574 ± 0.003	−0.964	0.002 ± 0.006	0.574 ± 0.003	−0.964	0.005
Halothane							
8-Doxylpalmitate	0.704 ± 0.001	0.700 ± 0.005	−0.938	0.004 ± 0.006	0.700 ± 0.004	−0.957	0.0004
12-Doxylstearate	0.551 ± 0.002	0.558 ± 0.005	−0.982	0.007 ± 0.007	0.552 ± 0.004	−0.990	0.001
Butanol							
8-Doxylpalmitate	0.696 ± 0.004	0.719 ± 0.006	−0.996	0.023 ± 0.010[d]	0.702 ± 0.008	−0.993	0.004
Chloroform							
8-Doxylpalmitate	0.687 ± 0.003	0.690 ± 0.009	−0.972	0.003 ± 0.012	0.687 ± 0.005	−0.991	0.004
Ether							
8-Doxylpalmitate	0.700 ± 0.004	0.704 ± 0.006	−0.995	0.004 ± 0.010	0.702 ± 0.006	−0.996	0.005

[a] Calculated by fitting data to a linear curve only up to point where S is constant, then extrapolating to zero concentration.

[b] Obtained by fitting data to a linear curve through zero concentration.

[c] Difference between control S at zero concentration and S at local concentrations, calculated from linear fit through zero.

[d] Significant difference.

maximum value of 1 for perfect order; as the fluidity increases S decreases.

By locating the nitroxide group at different positions along the fatty acid chain, different regions of the bilayer can be probed. In Fig. 2 the effects of benzyl alcohol on the order parameters of I(10,3) (5-doxylpalmitate), I(7,6) (8-doxylpalmitate), and I(5,10) (12-doxylstearate) are compared. Benzyl alcohol had no detectable effect on the order parameter until a concentration of 20–30 mM was reached. The concentration at which benzyl alcohol blocks nerve conduction for rat phrenic nerve is 4 mM, and for frog sciatic nerve, 12 mM (22). The concentration at which a decrease in S began was similar for all three spin labels. Thus it did not depend on the location of the probe within the hydrocarbon region of the bilayer. Phosphatidylcholine vesicles without cholesterol were also examined, but the changes in S were even smaller. The effect of pressure is shown for 8-doxylpalmitate. A pressure of 130 atm increased S by 0.02 ± 0.005 at all anesthetic concentrations and for the control vesicles.

The effect of benzyl alcohol and pressure on the partition coefficient of the water-soluble, lipophilic spin label TEMPO was also examined. Benzyl alcohol had no effect on the partition coefficient at concentrations below 100 mM for both phosphatidylcholine and phosphatidylcholine-cholesterol vesicles. Pressure decreased the distribution between membrane and aqueous phase by the same amount whether or not

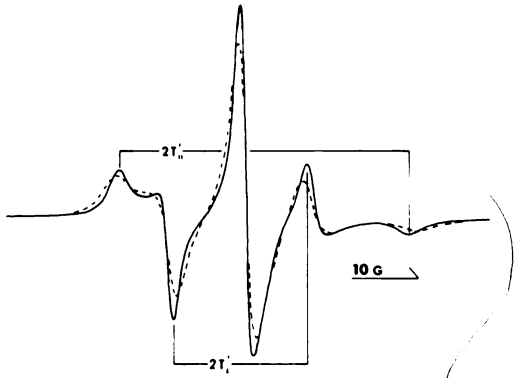


FIG. 1. *ESR spectra of 8-doxylpalmitate in phosphatidylcholine-cholesterol (molar ratio, 1:1) vesicles in the presence (——) and absence (- - -) of 300 mM benzyl alcohol*

$2T'_{\parallel}$ and $2T'_{\perp}$ were measured as indicated.

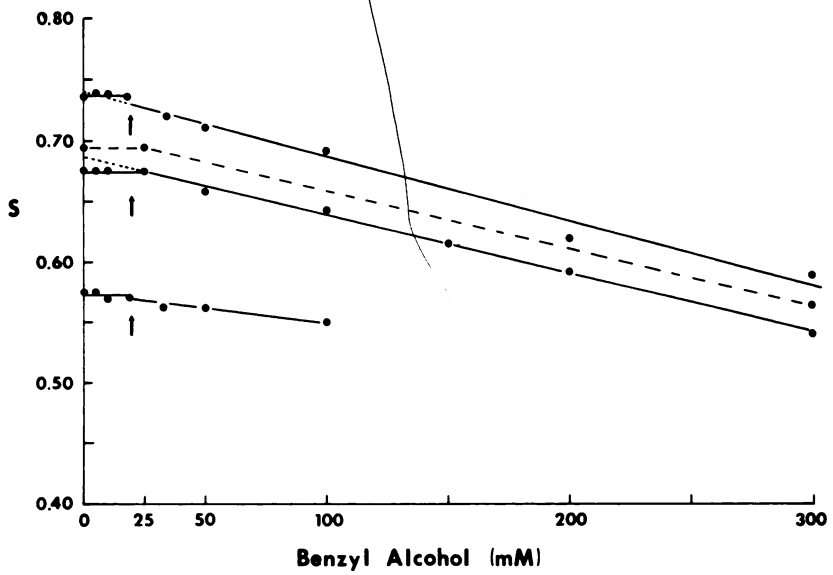


FIG. 2. *Effect of benzyl alcohol on order parameter S of 5-doxylpalmitate (upper), 8-doxylpalmitate (middle), and 12-doxylstearate (lower) in phosphatidylcholine-cholesterol vesicles*

The effect of 130 atm pressure on the order parameter of 8-doxylpalmitate is shown as the dashed line. The arrows indicate the concentrations which block frog sciatic nerve. As $T'_{\parallel}$ and $T'_{\perp}$ for 12-doxylstearate at high benzyl alcohol concentrations could not be resolved, S could not be calculated.

anesthetic was present.

Since the order parameter seemed to be more sensitive to the effect of anesthetic than the partition coefficient of TEMPO, only the effect of increasing drug concentration on the order parameter was examined for the other anesthetics. Figure 3 shows that halothane had no detectable effect on the order parameters of 8-doxylpalmitate and 12-doxylstearate at concentrations below 2.0–3.6 mM.

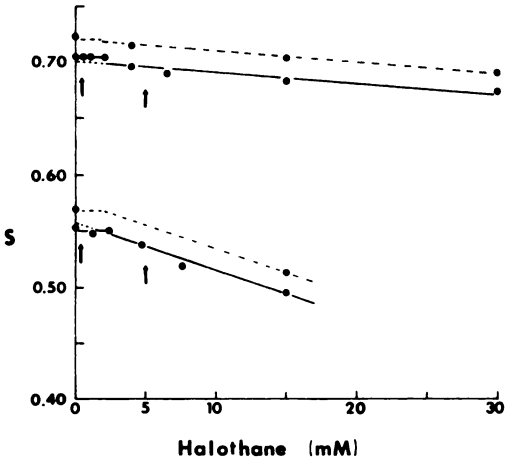


FIG. 3. *Effect of halothane at 1 atm (——) and 130 atm pressure (– – –) on order parameter S of 8-doxylpalmitate (upper curve) and 12-doxylstearate (lower curve) in phosphatidylcholine-cholesterol vesicles*

The arrows indicate the concentrations which produce general and local anesthesia.

Both probes were affected at the same concentration. The concentration required for general anesthesia is 0.4 mM (13), while that which blocks frog sciatic nerve conduction is 5 mM (19). Using the partition coefficient between lipid and aqueous phase, $P = 19 \pm 4$, determined by Trudell *et al.* (11), an aqueous concentration of 3.6 mM corresponds to 0.068 mole of halothane per liter of lipid. Thus the small decrease in S seen at 3.6 mM halothane is consistent with the decrease seen at 0.061 mole of halothane per liter of lipid by Trudell *et al.* (11, 12). As with benzyl alcohol, a pressure of 130 atm increased the order parameter by 0.02 ± 0.005 for both probes regardless of the halothane concentration. Benzyl alcohol had its greatest effect on the probes closer to the polar head group region, while the less polar halothane had a greater effect on the probe deeper in the hydrocarbon region. This may reflect the specific location of these anesthetic molecules dissolved in the bilayer.

Butanol had no effect on the order parameter of 8-doxylpalmitate at concentrations below 70–90 mM (Fig. 4). The concentration of butanol required for general anesthesia is 17 mM (13), while that required for nerve block is 68 nM (24). Ether and chloroform had no detectable effect on the order parameter until concentrations of 40 mM for ether (Fig. 5) and 8 mM for chloroform (Fig. 6). This is higher than the con-

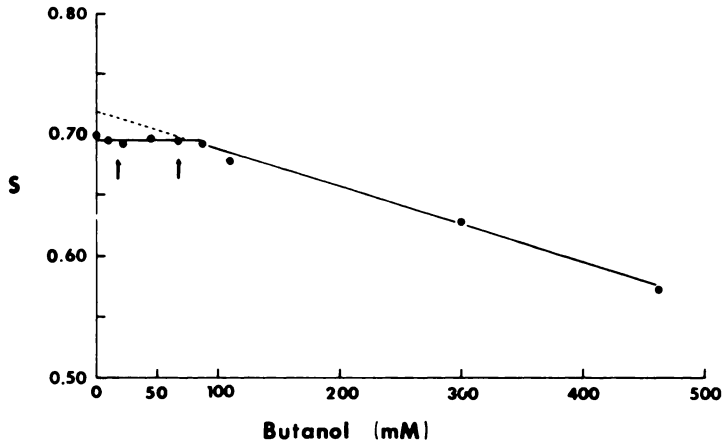


FIG. 4. *Effect of butanol on order parameter S of 8-doxylpalmitate in phosphatidylcholine-cholesterol vesicles*

The arrows indicate the concentrations which produce general and local anesthesia.

centrations required for general anesthesia and nerve conduction block by chloroform and for general anesthesia by ether (see Table 2).

As shown in Table 1, there is a significant difference between the extrapolated value for S and the control value only in the case of butanol and benzyl alcohol for the probe 8-doxylpalmitate. For the other anesthetics and spin labels, the data can be fitted as well or slightly better by a linear curve through zero concentration. Therefore the fluidity may increase linearly with increasing anesthetic concentration. Assuming a linear relationship between S and anesthetic concentration, the decrease in S predicted at the local anesthetic concentrations was calculated and is shown in Table 1. The decrease in S would be, for most anesthetics, less than 0.005. Such small changes would not be detectable by this technique.

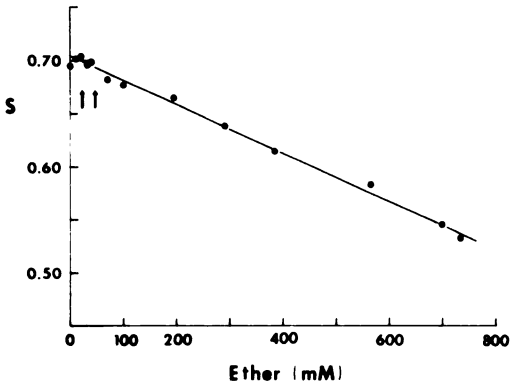


FIG. 5. *Effect of diethyl ether on order parameter S of 8-doxylpalmitate in phosphatidylcholine-cholesterol vesicles*

The arrows indicate the concentrations which produce general and local anesthesia.

DISCUSSION

The concentration of anesthetic at which a detectable increase in lipid fluidity occurred was much higher than that which produced general anesthesia, and in most cases was higher than that which blocks nerve conduction. Indeed, the concentrations at which significant increases in fluidity did occur correspond more closely to those causing lysis of erythrocyte ghosts (Table 2), in agreement with a previous ESR study of alcohols on lipid bilayers (14; reproduced in ref. 19). The mechanism of such a poisonous effect may well be different from that which causes anesthesia.

It is not possible to distinguish between the conclusion drawn from other studies of anesthetics in model systems, that there is a linear increase in fluidity with concentration, and the idea that there is a threshold below which no change occurs. This must be studied by a more sensitive technique than has been applied to anesthetic-membrane interactions. However, even if the effect is linear, the increase in fluidity produced by clinical concentrations would be extremely small. Such small increases in fluidity could not account for the large increases in volume of synaptosome and erythrocyte membranes produced by anes-

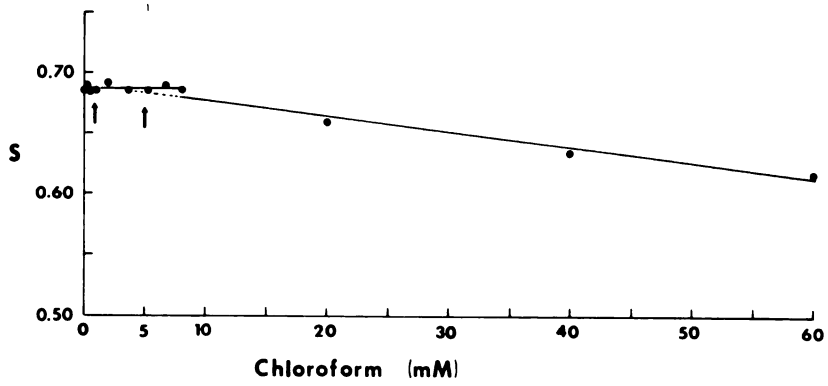


FIG. 6. *Effect of chloroform on order parameter S of 8-doxylpalmitate in phosphatidylcholine-cholesterol vesicles*

The arrows indicate the concentrations which produce general and local anesthesia.

TABLE 2

Comparison of drug concentrations which cause general and local anesthesia, cell lysis, and decrease in order parameter S

Anesthetic	50% loss of righting reflex in newt[a]	Complete block of frog sciatic nerve	Minimum decrease in S	Lysis of erythrocytes
	mM	*mM*	*mM*	*mM*
Benzyl alcohol		20[b]	20–30	85[c]
1-Butanol	17	68[d]	70–90	300[e]
Ether	25	50[b]	40–60	600[f]
Chloroform	0.90	5[b]	8–10	
Halothane	0.4	5[b]	2–4	14[f]

[a] Ref. 13. [b] Ref. 19. [c] Ref. 23. [d] Ref. 24. [e] Ref. 25. [f] Ref. 26.

thetics. This expansion is of the order of 0.5–0.6% at general anesthetic concentrations and 3–6% at local anesthetic concentrations (19). Anesthetic-induced phase transitions of a lipid at a temperature just below its transition temperature, as suggested by Trudell *et al.* (15), would amplify the effect of low anesthetic concentrations. However, even such a drastic change in lipid fluidity as the phase transition from a gel to liquid crystalline phase, which causes an abrupt decrease in S of 0.10 (16), is accompanied by an expansion in lipid volume of only 1.4% (27). The lack of fluidization found in this study is consistent with the finding of Seeman (28) that clinical concentrations of ethanol expand lipid bilayer vesicles by only 0.01% and 0.3% at general and local concentrations, respectively.

It is possible that the small increase in fluidity predicted at clinical anesthetic concentrations could be responsible for the small increases in permeability of phospholipid-cholesterol liposomes found by Johnson, Miller, and Bangham (13) at similar anesthetic concentrations. Alternatively, the permeability increase may be due to some action of the anesthetics at the polar head group region, which was not studied in the present work. Such an effect would be unlikely to result in significant volume changes.

The increase in order parameter produced by pressure is much larger than the decrease predicted to occur with clinical concentrations of anesthetics. Therefore pressures much less than 100 atm would be sufficient to counteract such small increases in fluidity. Furthermore, according to the critical volume hypothesis, the pressure required to remove the effects of an anesthetic should be directly proportional to the dose of the anesthetic agent. Yet similar pressures of 100–150 atm reverse both narcosis in animals and nerve conduction block (3, 4, 6), even though the concentration required to block nerve conduction is about 2–10 times greater for most neutral anesthetics (19). A mechanism for anesthesia by which fluidity increases linearly with concentration cannot accommodate these pressure results unless one postulates great differences in the partition coefficient of the drug in the membrane sites responsible for general and local anesthesia. However, for vesicles of phosphatidylcholine without cholesterol, in which the anesthetics would have a larger partition coefficient, the increase in fluidity produced was even less than that in phosphatidylcholine-cholesterol vesicles.

Since lipid bilayer fluidization is insignificant at clinical anesthetic concentrations and could not cause the observed expansion of membranes, while the pressure effects on fluidization do not correlate with the pressure effects on the anesthetized state, this simple mechanism is unlikely to be the mechanism of anesthesia. It is possible that the observed expansion of membranes is not relevant to the mechanism of anesthesia. However, the link between pressure counteraction of a volume increase at some site in the membrane and pressure reversal of anesthesia seems to be significant. Lipid bilayers of this sort may

not be an adequate model for the lipid region of nerve membranes. Complex lipids are present in these membranes which could conceivably undergo phase transitions to a nonlamellar phase upon interaction with drugs or ions, resulting in volume changes of the magnitude observed, and which could respond to pressure in various ways. However, no such behavior has yet been demonstrated, and no model system for the lipid region of membranes which can undergo such functional dynamic changes has been developed. Another mechanism which could account for the large volume changes as well as the diverse pressure effects is that of anesthetic-induced protein conformation change. A highly lipid-soluble molecule would also be able to interact hydrophobically with apolar sites on proteins. There is evidence that low concentrations of anesthetics and pressure can induce conformation changes in proteins. A discussion of the possibility of protein as the primary site of anesthetic action is presented in the following paper (9).

REFERENCES

1. Johnson, F. H. & Flagler, E. A. (1951) *Science*, **112**, 91–92.
2. Spyropoulos, C. S. (1957) *J. Gen. Physiol.*, **40**, 849–857.
3. Lever, M. J., Miller, K. W., Paton, W. D. M. & Smith, E. B. (1971) *Nature*, **231**, 368–371.
4. Miller, K. W., Paton, W. D. M., Smith, R. A. & Smith, E. B. (1973) *Mol. Pharmacol.*, **9**, 131–143.
5. Roth, S. H. (1975) in *Molecular Mechanisms of Anesthesia* (Fink, B. R., ed.), pp. 405–427, Raven Press, New York.
6. Roth, S. H., Smith, R. A. & Paton, W. D. M. (1972) in *Proceedings of the 5th International Symposium on Underwater Physiology* (Lambertsen, C. J., ed.), Academic Press, New York.
7. Youngson, A. F. & MacDonald, A. G. (1970) *Br. J. Anaesth.*, **42**, 801–802.
8. MacDonald, A. G. (1972) in *The Effects of Pressure on Organisms* (Sleigh, M. A. & MacDonald, A. G., eds.), pp. 209–231, Cambridge University Press, London.
9. Boggs, J. M., Roth, S. H., Yoong, T., Wong, E. & Hsia, J. C. (1976) *Mol. Pharmacol.*, **12**, 136–143.
10. Seeman, P. & Roth, S. H. (1972) *Biochim. Biophys. Acta*, **255**, 171–177.
11. Trudell, J. R., Hubbell, W. L. & Cohen, E. N. (1973) *Biochim. Biophys. Acta*, **291**, 321–327.
12. Trudell, J. R., Hubbell, W. L. & Cohen, E. N. (1973) *Biochim. Biophys. Acta*, **291**, 328–334.
13. Johnson, S. M., Miller, K. W. & Bangham, A. D. (1973) *Biochim. Biophys. Acta*, **307**, 42–57.
14. Paterson, S. J., Butler, K. W., Huang, P., Labelle, J., Smith, I. C. P. & Schneider, H. (1972) *Biochim. Biophys. Acta*, **266**, 597–602.
15. Trudell, J. R., Payan, D. G., Chin, J. H. & Cohen, E. N. (1975) *Proc. Natl. Acad. Sci. U. S. A.*, **72**, 210–213.
16. Hubbell, W. L. & McConnell, H. M. (1971) *J. Am. Chem. Soc.*, **93**, 314–326.
17. Schreier-Muccillo, Marsh, D., Dugas, H., Schneider, H. & Smith, I. C. P. (1973) *Chem. Phys. Lipids*, **10**, 11–27.
18. Jost, P., Libertini, L. J., Hebert, V. C. & Griffith, O. H. (1971) *J. Mol. Biol.*, **59**, 77–98.
19. Seeman, P. (1972) *Pharmacol. Rev.*, **24**, 583–655.
20. Waggoner, A. S., Kingzett, T. J., Rottschaefer, S., Griffith, O. H. & Keith, A. D. (1969) *Chem. Phys. Lipids*, **3**, 245–253.
21. Rozantzev, E. G. & Neiman, M. B. (1964) *Tetrahedron*, **20**, 131–137.
22. Staiman, A. & Seeman, P. (1974) *Can. J. Physiol. Pharmacol.*, **52**, 535–550.
23. Burgen, A. S. V. & Metcalfe, J. C. (1970) *J. Pharm. Pharmacol.*, **22**, 153–169.
24. Skou, J. C. (1958) *Biochim. Biophys. Acta*, **30**, 625–629.
25. Seeman, P. (1966) *Int. Rev. Neurobiol.*, **9**, 145–221.
26. Okumura, F., Yoshikawa, K., Ueda, I. & Koh, J. (1970) *Jap. J. Anesthesiol.*, **19**, 848–853.
27. Trauble, H. & Haynes, D. H. (1971) *Chem. Phys. Lipids*, **7**, 324–335.
28. Seeman, P. (1974) *Experientia*, **30**, 759–760.