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On the Coupling between Anesthetic Induced Membrane Fluidization and Cation Permeability in Lipid Vesicles

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SUMMARY

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The effect of anesthetics on the rubidium-86 ion efflux from phospholipid vesicles was studied in the presence of the ionophores gramicidin A and valinomycin and in the absence of ionophores. Anesthetics of known lipid/buffer partition coefficient were used. Pentobarbital, halothane and butanol all increased the three types of ion efflux. The effects were linearly dependent on each anesthetics' membrane concentration over a range of anesthetic mole fractions in lipid from 0.02 to 0.3, and concentrations known to produce anesthesia produced clearly significant increases in each case. The effect of a given membrane concentration of all the anesthetics on each of the three modes of ion efflux was similar, suggesting that a single perturbation of the lipids is involved in every case. The effects of anesthetics on cation permeability correlated better with their perturbation of lipid bilayers (reported by a freely rotating hydrophobic fluorescent probe [1,6-diphenyl 1,3,5-hexatriene]) than with those reported by spin-labeled lipid probes undergoing anisotropic motion. Coupling between the permeability increases and the perturbation of the bilayer structure was strong, the functional changes being about an order of magnitude larger than the structural changes. The lipid perturbation hypothesis of anesthetic action has been criticized because the structural perturbations observed at anesthetic concentrations are small, but such strong coupling between structural and functional changes may resolve this problem.

INTRODUCTION

The site of action of anesthetics has received considerable attention recently. Because the physiological site (or sites) of action within the central nervous system remains undetermined, much of this work follows the classical physicochemical approach of correlating anesthetic potency with the physical properties of anesthetics. Advances in basic membrane science have provided new models and techniques that have considerably extended the scope of such an approach. The possibility that anesthetics interact directly with hydrophobic regions of membrane proteins remains untested for lack of a suitable model, while considerable progress has been made in understanding interactions between anesthetics and lipid bilayers, which present a readily available, well defined model.

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Changes in bilayer fluidity and permeability provide successful models for the major features of anesthetic potency (1–3) and its reversal by pressure (4–6) but a number of problems remain unresolved. First, there is some question whether fluidity changes do in fact occur at low concentrations of anesthetic (7). Even though these doubts have themselves been questioned by more recent work on halothane (8), the size of the perturbations measured are at least comparable to the errors involved in spectroscopic studies of structural perturbations. But then, the volume changes predicted by theoretical studies of pressure reversal are also small (9). Second, the problem of how a perturbation of the state of the lipid bilayer may be coupled to and disrupt the function of some excitable protein remains largely unstudied. A small change in the state of lipids may cause a large change in a protein's functional capacity. If so this would resolve the difficulty associated with the small changes observed in lipid bilayer structure. A third problem, that of specificity (i.e., how does a generalized lipid perturbation have fairly specific effects on membrane function), may be related to specificity either in the primary anesthetic-lipid interaction (10) or in the coupling of the lipid perturbation to protein function. A recent study that examined the effect of benzyl alcohol in the lipids of a calcium-magnesium ATPase from sarcoplasmic reticulum sheds some light on this complex problem but high concentrations of this agent were required (11), and electrophysiological evidence suggests excitable ionophores would be more suitable models of anesthetic action (12, 13).

One approach to the problem of lipid-protein coupling is to study in well defined lipid bilayers the function of simple ionophores of known structure and defined mechanism, for example certain antibiotics. Since these systems are often more sensitive to anesthetic perturbations than are spectroscopic probes of the state of lipid bilayers, they provide incidently an approach to the determination of the size and linearity of the lipid perturbation at low anesthetic concentration.

Previously it has been shown that the ion carrier, valinomycin, provides a useful model for examining anesthetic (1) and pressure (4, 5) induced changes in the lipid bilayer. However, excitable fluxes in nerves are carried by ion channels, and the observation that the life time of gramicidin A channels in black lipid membranes is related to membrane thickness (14) suggested that it might be a more suitable model. Recently several authors have elaborated this possibility in some detail (15–17). We chose to study gramicidin A in lipid vesicles both because the primary anesthetic-lipid interaction has been well characterized in them and because black lipid membranes, which would offer the opportunity of resolving single channel events, are saturated with solvents such as hexadecane. We argued these would influence the subtle anesthetic-lipid interaction and it seemed wiser, at least initially, to characterize the effects of anesthetics in the absence of such potentially confounding variables.

We present here a comparison of the effects of anesthetics on ion fluxes mediated by the carrier, valinomycin, and the channel former, gramicidin A, in phospholipid bilayers, using a new method of higher precision than previously reported. Experiments were carried out under conditions for which the membrane concentration of the anesthetic can be accurately estimated. The magnitude of the observed effects can, thus, be readily related to the anesthetic concentration.

MATERIALS AND METHODS

Valinomycin (A grade) was obtained from Calbiochem, Los Angeles, Cal., gramicidin A from Nutritional Biochemicals Corp., Cleveland, Ohio, egg phosphatidylcholine and phosphatidic acid (Grade 1) from Lipid Products, Surrey, England, and cholesterol (chromatographic grade) from Sigma Chemical Company. All were used as supplied, except cholesterol which was recrystallized from methanol. Rubidium-86 and calcium-45 were from New England Nuclear, Boston, Mass. 1,6-Diphenyl-1,3,5-hexatriene (DPH) and tetrahydrofuran were from Aldrich, Milwaukee, Wis. Spin-labeled 8-doxylstearic acid was synthesized by Dr. M. J. Pringle in this laboratory. All

other reagents were analytical grade.

Phospholipid vesicles were prepared by first drying down 90 μmoles of phospholipid from stock solutions in chloroform containing 96 mole % phosphatidylcholine and 4 mole % phosphatidic acid. Five milliliters of buffer (0.12 M KCl, 0.03 M RbCl (containing 0.25 mCi of ^{86}Rb), Tris-HCl 10 mM at pH 7.0 or 8.1) was added and the suspension sonicated in a stoppered conical tube in a bath sonicator (Heat Systems, model 5 × 5) at 25° under nitrogen. The clear suspension was passed through a Sephadex G-50 (coarse) column, preequilibrated with nonradioactive buffer, the vesicles collected in the void volume, and diluted to 0.2–0.4 mM phospholipid in the above buffer without radiolabel and containing in some cases anesthetic. After 15–30 min equilibration in a water bath at 25°, valinomycin, or gramicidin A, was added in ethanol to give, usually, a final concentration of about 1–10 nM respectively. This was sufficient to ensure a flux rate 6–10 times higher than the background due to passive permeability. The final ethanol concentration, 0.01% v/v, was shown in control experiments to have a negligible effect.

Ion leak rate from the vesicles was determined by ultrafiltration. At intervals of 10–20 min 2 ml. aliquots were tranfered to 10 ml filtration cells (Amicon, Lexington, Mass., model 12) and filtered through an XM-50 ultrafilter (Amicon) under 4 atm of nitrogen. The first 0.3 ml of filtrate was discarded and the remainder (usually 1.2 ml) was collected for scintillation counting in aquafluor (New England Nuclear, Boston, Mass.). A few samples were analyzed by Cherenkov radiation. Filtration time varied from 1½ to not more than 5 min. We have previously shown that no vesicles appear in the filtrate (19). The incubation mixture was also assayed for phosphorous (20) and total radioactivity.

In some experiments both rubidium and calcium fluxes were determined simultaneously and the buffers then contained 0.09 M RbCl and 0.03 M $CaCl_2$ in 10 mM Tris-HCl at pH 7.

Because of its volatility halothane incubations were performed in a 30 ml glass syringe capped by a two-way valve through which appropriate amounts of saturated halothane solution were added. Final halothane concentrations were determined by gas chromatography at the end of each experiment. Even with these precautions concentrations were often 5–20% below those expected.

Permeability was treated as a first order process (1, 5) given by

$$\frac{dn}{dt} = \frac{PA}{V}(N - n) \quad (1)$$

where n is the number of counts per minute at time t which have leaked from liposomes of surface area A and internal volume V, containing N counts per minute. P is the permeability coefficient. Plots of ln $(N - n)$ versus t were found to be linear over 300 to 480 minutes, depending on the concentration of ionophores used, and the slope is thus proportional to P.

Samples for fluorescence depolarization were prepared by the method of Lenz et al. (21). Phosphatidylcholine suspensions were sonicated with the microtip of a probe sonifier (Heat Systems, model W185) at 0°. Metal debris and large vesicles were precipitated at 100,000 × g for 30 min. Pentobarbital was mixed with lipids before sonication, while halothane was added after centrifugation, using the technique described above. Four and a half milliliters of liposome suspension (about 0.7 mM) was mixed with 1 μl of 2 mM DPH in tetrahydrofuran and vigorously stirred for 1 hr. Fluorescence depolarization was determined at 25° in a subnanosecond spectrofluorimeter (S.L.M. Series 400, Urbana, Ill.). The sample was excited at 360 nm and recorded at 430 nm. Microviscosity was calculated from the fluorescence depolarization by the method of Shinitzky and Barenholz (22). The depolarizations reported at 10, and at 30 MHz modulation were not significantly different.

Samples of phospholipid vesicles with anesthetics for spin label studies were prepared according to the method described previously (23).

RESULTS

The diffusion of $^{86}Rb^+$ ion through the lipid bilayer was treated as described above

(equation 1 and figure 1). If values for the internal volume and surface area are assumed the permeability coefficient, P, may be calculated. The internal volume of a 4% phosphatidic acid-96% phosphatidylcholine liposome has been shown to be 6.7×10^{-18} cm^3 and its external surface area to be 8.6×10^{-12} cm^2 (24). Hence, for $^{86}Rb^+$ passive diffusion at 25° we calculated $P = 4.8 \times 10^{-13}$ cm/sec compared to a value for $^{42}K^+$ of 7.3×10^{-13} cm/sec. at 37° in a similar preparation (1) and of 1.3×10^{-14} cm/sec at 4° in egg phosphatidylcholine vesicles using slightly different numbers for external surface area and internal volume (25).

The carrier mechanism of valinomycin transportation has been well established both in black lipid membranes and in liposomes (26–30). Although the evidence that gramicidin A dimerizes to form ion conducting channels in black lipid membranes is strong, its effects in vesicles have not been characterized (29, 31). Indeed the inability to record single channel events in vesicles makes a full characterization difficult. Nonetheless, the presence of hydrocarbon solvent in black lipid membranes renders them needlessly complex for anesthetic studies. We therefore characterized the gramicidin A mediated cation flux in our vesicles as follows. First, incorporation of gramicidin A into vesicles caused the same increase in efflux when the antibiotic was added to the suspension or when it was dried down with the lipid before the vesicles were prepared. This reduced the probability that the increase in efflux resulted from some nonspecific interaction. Secondly, gramicidin selected rubidium over calcium (Fig. 1) just as it does in black lipid membranes (29). Valinomycin showed the same selectivity. We were unable to demonstrate a dependence of permeability on the square of the gramicidin concentration because the range of permeabilities that can be studied by our technique is too narrow. However, permeability increased with gramicidin concentration over the limited range studied. In a typical experiment the mean number of gramicidins per liposome was 0.4. Assuming that all gramicidin is present as dimers (31), up to 20% of the liposomes

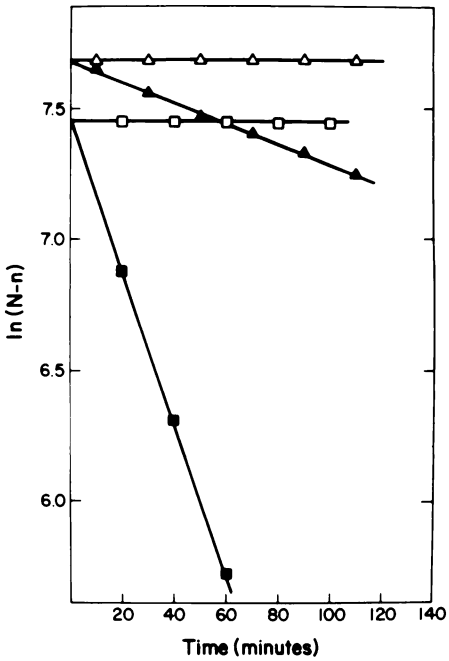

FIG. 1. *The efflux of $^{86}Rb^+$ (solid symbols) and $^{45}Ca^{2+}$ (unfilled symbols) from 96% egg phosphatidylcholine-4% egg phosphatidic acid sonicated vesicles*

Triangles, in the presence of 0.1 μM gramicidin A and 0.59 mM phospholipid; squares in the presence of 1 nM valinomycin and 0.57 mM phospholipid. N is the total counts and n the filtrate counts determined at time t. The slopes of the lines through the points for rubidium and calcium efflux are, respectively, $-3.95 \pm 0.13 \times 10^{-3}$ (SD) and $-1.7 \pm 2.2 \times 10^{-5}$ in the gramicidin experiment, and $-2.89 \pm 0.036 \times 10^{-2}$ and $-9.5 \pm 2.8 \times 10^{-5}$ in the valinomycin experiment and $-2.3 \pm 0.12 \times 10^{-4}$ and $-0.5 \pm 5.8 \times 10^{-6}$ in the unmodified liposomes (not shown).

contain a channel at any given time. Under these conditions the measured cation flux is 2×10^{-3}/min and remains linear for at least 300 min. This suggests that gramicidin, like valinomycin, must be in dynamic equilibrium with the liposomes on the time scale of this experiment. Studies in planar bilayers suggest a single channel might empty a vesicle in much less than one second, but no evidence for a rapid efflux phase from those vesicles initially containing two gramicidins is seen at 0.1 μM (Fig. 1), or at 1 μM gramicidin (not shown). Possibly the kinetics of channel formation is strongly affected by vesicle curvature, but in any event the rate-limiting process in the fluxes

measured here remains uncertain.

All three anesthetics increased all the ionic fluxes examined in a concentration dependent manner. Control experiments showed that pentobarbital at high pH had little effect on the valinomycin mediated flux. The effect increased at lower pH showing the uncharged molecule to be the primary affector. The pH dependence of pentobarbital partitioning has been determined in egg phosphatidylcholine (19). It is thus possible to calculate the dependence of the permeability increase upon the bilayer concentration of pentobarbital. Figure 2 shows the data of several experiments for each of the cation fluxes. The effect of the anesthetic on all three types of permeability is remarkably consistent and independent of pH when the membrane concentration is adjusted appropriately. Halothane yielded similar results over the same concentration range (not shown) when a partition coefficient of 152 moles halothane per ml lipid/ moles halothane per ml buffer (Porter & Miller, unpublished data) was used to calculate the bilayer concentration. For butanol a partition coefficient of 3.2 was assumed. This value is for dimyristylphosphatidylcholine (32), but comparison of results obtained for benzyl alcohol and for butane, both in saturated and unsaturated lipids, suggests that the value used will not be in error by more than 30% (33, 34). We thus calculate that the valinomycin mediated cation flux in the presence of butanol was linear down to a mole fraction of 0.02 in the lipid (Fig. 3). The slopes of these graphs quantitate the dependence of the increase in permeability on membrane concentration. The results of a linear regression through the origin in each case are given in Table 1. When the best fit was not constrained to pass through the origin the predicted intercept varied randomly above and below ln $(P/P_0) = 0$. The mean of all intercepts determined was −0.025, and in no case was the intercept more than two standard deviations from the origin. (In five of seven cases it was within one standard deviation of the origin.) The mean correlation coefficient was 0.983. The linearity of these plots is thus established within quite

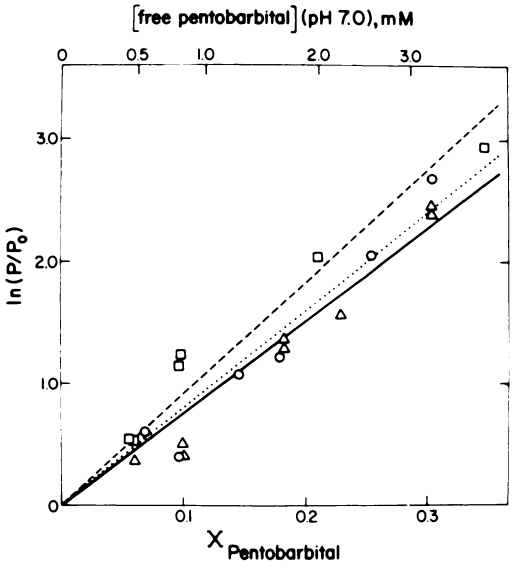

FIG. 2. *The effect of pentobarbital on rubidium efflux in the absence of ionophore,* ○, *and in the presence of valinomycin,* □, *and gramicidin A,* △

The membrane concentration was calculated as described in the text. The ratio of anesthetic permeability, P, to control permeability, P_o, is determined from the slopes of plots such as those in Figure 1. Least squares fitted lines, through the origin for no ionophore (dotted); valinomycin (dashed); and gramicidin A (solid).

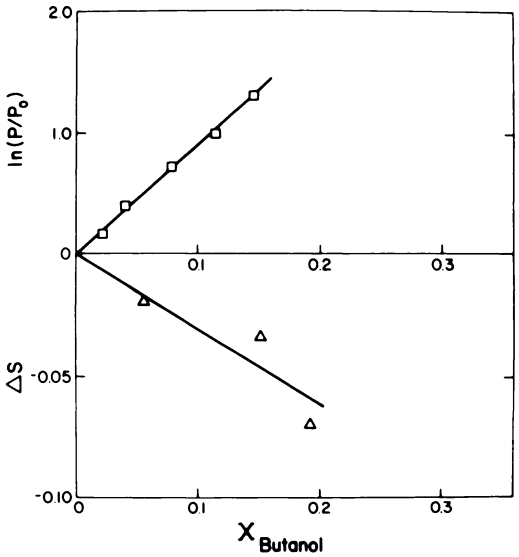

FIG. 3. *The effect of n-butanol on valinomycin mediated rubidium permeability* □, *and on the change in order parameter,* ΔS, *of 8-doxylstearic acid* ▽

close limits. Comparison of our data for valinomycin with a previous determination using a dialysis technique (5) shows good agreement for pentobarbital and butanol, although our errors are considerably smaller. Our value for halothane is larger than the previous one, emphasizing the need to directly monitor the concentration of this volatile agent.

Table 1 shows that when these anesthetics are examined at constant membrane concentration, the effect of each of them on a given type of cation flux is remarkably similar.

Figure 3 also shows the dependence of the order parameter of 8-doxylstearic acid in phospholipid vesicles on the membrane concentration of butanol. Order decreases in an approximately linear fashion, but the errors in determining ΔS are relatively larger at a given membrane concentration than those of the permeability study.

The fluorescence depolarization data for DPH showed that both halothane and pentobarbital (pH 7.0) fluidized the bilayer. The calculated microviscosity of the control bilayers was 0.96 poise, in agreement with Shinitzky and Barenholz (22). At mole fractions of 0.05 pentobarbital and 0.38 halothane the microviscosity was 0.87 and 0.63 poise respectively. Under these conditions fluorescence life times detected by phase and modulation methods were homogeneous, but we were unable to study higher concentrations of pentobarbital. Control experiments showed that pentobarbital at a mole fraction of 0.5 reduced the quantum yield of steady state DPH fluorescence, and in the presence of vesicles it showed different fluorescence life times by phase and modulation detection methods. Recent studies have suggested that DPH does not rotate freely in bilayers of saturated lipids and that the assumptions inherent in microviscosity calculations may be invalid (35, 36). However, 20–30° above the phase transition there is free rotation (36), so that our use of the microviscosity formalism in egg lethicin bilayers is probably a good approximation.

TABLE 1

The increase in $^{86}Rb^+$ efflux in 96% egg phosphatidylcholine:4% phosphatidic acid liposomes in the presence of anesthetics

The figures are the slopes of plots such as Figure 2. The units are ln (P/P_0) at unit mole fraction of anesthetic in lipid ± S.D.

Ionophore	Anesthetic		
	Pentobarbital	Halothane	*n*-Butanol
None	8.0 ± 0.43	10.4 ± 0.45	—
Valinomycin	9.2 ± 0.48	9.0 ± 0.29	9.1 ± 0.12
Gramicidin A	7.5 ± 0.32	6.8 ± 0.30	(5.7)[a]

[a] Single determination.

DISCUSSION

Our results establish with high accuracy the relationship between the concentration of the anesthetic in the bilayer and the ion permeability through it. They enable us to estimate the size of the change at physiologically relevant concentrations and to examine the coupling between the lipid perturbation and the function of carrier and pore forming ionophores. These experiments were not designed to directly evaluate the mechanisms involved; nonetheless it is possible to relate these results to more mechanistic studies and to correlate them with structural changes induced in the bilayer.

The dependence of the anesthetic induced increase in ion flux upon membrane concentration was in each case highly linear. No deviations were noted even though the anesthetic mole fraction was varied over more than an order of magnitude. Two conclusions relevant to anesthetic studies may be inferred. First, in these permeability studies it is valid to extrapolate from high ($\times \sim 0.35$) to low anesthetic concentrations, which suggests that the same will be true of measurements, often made with less sensitive techniques, of the underlying lipid perturbation. (This might not be so in other types of permeability if the coupling between lipid perturbation and ionophore is nonlinear). Second, the suggestion that there is a nonlinear relationship between anesthetic concentration and membrane fluidization, particularly at low concentrations (7) appears now to be highly improbable. Our finding in this respect confirms earlier permeability studies in a wide range

of lipids (5) and extends a recent spin label study with halothane in cholesterol containing bilayers (8). The previous results (7) might be in error through failure to adequately control the volatile anesthetics studied or to the inherently large errors associated with electron spin resonance work. The latter point is underscored by the comparison of methods presented in Fig. 3.

The membrane concentrations corresponding to anesthesia may be calculated. Using the buffer concentrations summarized in a previous paper for general anesthesia in newts and goldfish (5), together with a recent figure for pentobarbital in tadpoles (37), the corresponding mole fractions in our bilayer are 0.044, 0.041 and 0.019 for halothane, butanol and pentobarbital, respectively. Values given for nerve block by Seeman (12) are higher than for general anesthesia and yield corresponding mole fractions of 0.37, 0.14 and 0.17. Using the valinomycin data, the changes of permeability at general anesthetic concentrations are respectively 1.5, 1.5 and 1.2 times, and at nerve block 28, 3.7 and 5.0 times, control values. Thus quite clearly measurable changes in permeability are observed in each case, while those related to nerve blocking concentrations are substantial. There remains little doubt that permeability, as well as order parameter (8) changes in bilayers are detectable at physiological concentrations, although in the case of general anesthesia these changes are small.

Table 1 shows that at equal membrane concentrations all the anesthetics produced very similar effects on each mode of ion transport including that with no ionophore present. The underlying perturbation, thus, probably occurs within the lipids and does not represent an ionophore-anesthetic interaction. This perturbation is not markedly dependent on the anesthetics' structure but is related to their lipid solubility. The coupling between this apparently uniform perturbation and all the ion fluxes is very similar, although somewhat less effective with gramicidin A than valinomycin.

The question of coupling between lipid perturbation and membrane function is now central to development of the fluidized (or expanded) lipid hypothesis of anesthetic action. Our fluorescence data, when plotted against membrane concentration, yields a straight line. Taking a mole fraction of 0.02 to 0.04 as the anesthetic concentration the change in microviscosity is 2½–5%. Similar considerations at nerve blocking concentrations yield changes of 16–42%. For halothane a value of the partial molar volume in egg phosphatidylcholine is available (Bennett & Miller, unpublished data) and the calculated expansion is 1% for anesthesia and 8% for nerve block in these bilayers. Our spin-label study with butanol yields an order parameter change of 2% at general anesthetic concentrations and 7% at nerve block (Fig. 3). In corroboration of the order of magnitude of these changes an order parameter change of about 1% at an anesthetic dose of halothane was reported in a careful study of egg phosphatidylcholine: cholesterol liposomes (8). Thus at general anesthetic concentrations a 2–5% change in structural parameters produces a 20–50% change in ion permeability. The functional change is about an order of magnitude larger than the associated structural perturbation in the lipids.

Are such changes as these sufficient to account for anesthesia? Such a question is hard to answer on the present information. First, the ionophores studied here are very simple; more complex ones, such as alamethicin with its potential dependent "gate," might couple to the lipid perturbation much more strongly. Second, the boundary lipid close to physiological ionophores, such as rhodopsin (38) or Ca^{2+}-Mg^{2+} ATPase (11), might be more or less perturbed by anesthetics. On the other hand, the anesthetic induced change in time constants of the decay phase of miniature end-plate currents at the neuromuscular junction is only about a factor of two at blocking doses (39). Thus, the observed magnitude of the changes in simple bilayer systems does not currently provide any evidence for rejecting the lipid perturbation model. Unequivocal support for the model can, of course, only come from work on physiological systems, but studies on more complex model ionophores could provide a

further test.

We now consider the nature of the lipid perturbation that influences the permeability by considering spectroscopic evidence on anesthetic-lipid interactions. These three anesthetics were chosen to reveal different aspects of the interaction. Halothane is relatively nonpolar, is evenly distributed through the bilayer (40), decreases the order parameter of spin probes at different depths in the bilayer (6, 7) and decreases the microviscosity of the hydrocarbon interior. Butanol is similar but on average probably distributes preferentially into the bilayer's interface because of its hydrogen bonding capacity (34). Pentobarbital is probably also amphiphatic, and most significantly, it is the only anesthetic studied here which increases the order parameter reported by spin-labeled lipid probes (10, 23, 41). This ordering effect is stronger at the fifth than at the eighth acyl carbon. Spin-labels deeper in the bilayer yield little information because of the high fluidity, but DPH fluorescence depolarization reveals a decrease in microviscosity. Thus the observed changes in permeability correlate best with the changes in microviscosity derived from the fluorescence depolarization of DPH for these anesthetics. This strongly suggests that the underlying lipid perturbation resides in the interior of the bilayer in each case. The lack of correlation between ion permeability and order parameter in lipid vesicles has also been noted for a number of steroids (42, 43).

The correlation of the anesthetic effects with hydrocarbon fluidity recalls the established effects of increasing acyl unsaturation and decreasing cholesterol content in increasing valinomycin mediated cation fluxes (27, 28, 44). It has also been observed that the opposite effects of anesthetics and pressure on valinomycin mediated cation permeability correlated with expected changes in bilayer density (5). Further work may show the density and microviscosity descriptions to be equivalent to some degree. The advantage of the density description is that it is a thermodynamic quantity whereas the spectral parameter is more difficult to interpret precisely. The correlation of these structural parameters with anesthetic action on ion fluxes does not imply that they are directly related to the mechanism of the anesthetic effect. Other properties which are co-variant, such as dielectric constant, may well be more relevant. More detailed studies will be required to answer such questions.

It has recently been shown that anesthetics decrease the overall conductance observed in black lipid membranes in the presence of a gramicidin derivative by affecting the number of channels conducting, with unchanged unit conductance, at a given time (18). These effects have been related to changes in bilayer thickness and tension. We found the effects of anesthetics on the effects of gramicidin mediated cation flux in liposomes to be in the opposite direction. If the rate limiting step in our work is the transfer of gramicidin between vesicles, these observations are not contradictory. However, if channel duration is rate limiting, the discrepancy between the liposome and planar bilayer work might be resolved by the recent observation that the thickness of black lipid membranes is increased by anesthetics while that of liposomes is changed little (45) or, as many authors have predicted, decreases slightly (46, 47). Studies in "solvent-free" planar bilayers may yet resolve these uncertainties and provide more detailed insights of the mechanisms involved (48, 49).

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