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# THE PARTIAL MOLAR VOLUMES OF ANESTHETICS IN LIPID BILAYERS

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**The excess volumes of mixing of benzyl alcohol, halothane, and methoxyflurane in water and in suspensions of several lipid bilayers have been determined at 25°C using a novel excess volume dilatometer. The excess volumes of mixing in water were all found to be negative, whereas in lipid suspensions they were all more positive than those in water alone. From known partition coefficients the partial molar volumes of these three solutes in the lipid bilayers were calculated. These values were all close to the molar volumes of the pure anesthetics, as was a value determined for halothane in olive oil. Halothane was studied in dipalmitoylphosphatidylcholine below its phase transition, and was found to exhibit a much larger excess volume than in any other system we studied. The potency of these three anesthetics was determined in tadpoles. It was calculated that at equi-anesthetic doses these three agents caused an expansion in egg lecithin/cholesterol (2 : 1) bilayers of 0.21 ± 0.015%. This result is consistent with the hypothesis that general anesthetics act by expanding membranes.**

## Introduction

The potency of general anesthetics has long been known to correlate with lipid solubility [1]. More recently the fact that general anesthesia may be reversed by high pressure has led to the implication of volume changes in anesthetic action [2,3]. One model supposes that anesthesia occurs when the volume of a hydrophobic region is expanded beyond a certain critical volume by the absorption of molecules of an inert substance (the critical volume hypothesis). This expansion can be counteracted by elevated pressure thus reversing the anesthesia. The net fractional expansion, $E$, of the anesthetic site by a volatile agent is given by

$$E = \frac{\bar{V}_2 \cdot x_2 \cdot P_2}{V_m} - \beta(P_t - 1) \qquad (1)$$

where $\bar{V}_2$ is the partial molar volume of the anesthetic at its site of action, $x_2$ ($\ll 1$) is its mole fraction solubility at a partial pressure of one atmosphere and $P_2$ is the agent's partial pressure. $V_m$ is the molar volume of the site of action and $\beta$ is its isothermal compressibility with units of (pressure)$^{-1}$. $P_t$ is the total mechanical pressure.

The physiological location and molecular nature of the site of action of anesthetics is not known, but calculations show that simple liquids, such as olive oil, model the first term on the right side of Eqn. 1 quantitatively [2]. This leaves open the question of whether the hydrophobic site invoked in Eqn. 1 is in the lipid or the protein portion of some excitable membrane [3,4]. The excitable protein that might be involved in general anesthesia is not defined, and the effects of anesthetics on proteins are too variable [5] to enable a general model to suffice for an experimental test of a model of anesthetic action involving the hydrophobic region of proteins. On the other hand, the lipid region of membranes can be modeled to good approximation by lipid bilayers. Lipid bilayer partition coefficient data for gaseous and volatile anesthetics are available in the literature [6–9], but partial molar volume data are not. It was thus the object of this work to measure the partial

molar volumes of some anesthetics in lipid bilayers in order that the first term in Eqn. 1 can be evaluated. Our data show that anesthetics do expand lipid bilayers by an amount similar to that for olive oil. These data are thus consistent with the hypothesis that anesthetics act by expanding a lipid bilayer. They do not, of course, rule out alternative models, but they do contrast with the recent claims (based on thickness measurements) that anesthetics do not expand bilayers [10,11].

Since partial molar volumes of volatile solutes have not previously been measured in lipid bilayers it was necessary to devise a suitable technique. Partial molar volumes may be obtained either by density measurements on a series of individually prepared samples of known solute concentration, or by excess volume dilatometry. The latter method has several well known advantages, especially where volatile agents are employed [12,13]. The principle of the excess volume dilatometer is that known amounts of both components of the mixture are contained separately within the dilatometer. One can thus measure directly the excess volume change upon mixing known aliquots of the solute with the solvent. Our design was freely adapted from one of Pflug and Benson [14] with two primary differences. The first was to allow for mixing of successive aliquots of anesthetic without refilling the dilatometer. This was achieved by tilting the dilatometer. The second was to allow for the addition of small quantities of anesthetic to the solvent, because, in contrast to the mixtures commonly studied by physical chemists [12–14], ours displayed limited miscibility. A further limitation was that high concentrations of anesthetics would be unphysiological. The novel dilatometer which we designed is shown in Fig. 1 and its features and operation are described below.

## Materials and Methods

Egg lecithin was purchased from Lipid Products, Surrey, U.K. and used without further purification. Cholesterol (Sigma) was recrystallized two times in methanol. Dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidylcholine (DMPC) were from Calbiochem, San Diego, CA, and used without further purification. Halothane ($CF_3CHClBr$) was from Ayerst Laboratories Inc., New York, methoxyflurane ($CH_3 \cdot O \cdot CF_2CCl_2H$) from Abbott Laboratories, North Chicago, Il., and benzyl alcohol from Fisher Scientific, NJ. Unilamellar and multilamellar liposomes were prepared in distilled water by standard techniques as described previously [15]. Saturated lipids were vortexed 5–10°C above their transition temperatures. Although the use of distilled water is not physiological, it avoided uncertainties in salt concentration which might results from evaporation during degassing. The pH of the dispersions was 6.8. Final concentrations of lipid in suspensions were determined by dry weight with an uncertaintly of ±0.9% (standard deviation).

*Apparatus and procedure.* Dilatometric measurements were carried out using a specially designed relatively small excess volume dilatometer (Fig. 1). The size of the dilatometer was dictated by the availability and cost of lipids and is smaller than required for minimization of errors. The dilatometer was fabricated of pyrex glass by Ace Glass, Vineland, NJ. Capillaries, C, D and E (diameters of 0.04 to 0.062 cm) were made of precision bore tubing, with an inside diameter tolerance about ±0.001 cm. They were calibrated along their length by weighing mercury. The dilatometer is assembled using Apiezon T grease sparingly on all stop-cocks and joints. It is filled with mercury under vacuum (5–10 $\mu$mHg) until bulb B (~22 ml) is half full. Ice cold anesthetic was degassed by pumping and then introduced above the mercury in A (9.6 ml) through B and C. The anesthetics could not be added under vacuum because of their high vapor pressures. The amount of air dissolving during transfer to A can be minimized by adding the anesthetic in excess in B and withdrawing an aliquot into A quickly from the bottom. After vaporizing excess anesthetic from B, it is filled with excess degassed solvent (water or lipid suspension) and quickly closed by inserting H. The filled dilatometer was set in a double water bath. The outer bath was controlled at 24.8 ± 0.1°C, and the inner at 25.000 ± 0.003°C by a proportional temperature controller ((YSI 72) Yellow Spring Instruments Co., Yellow Springs, OH). The water surface of this bath was insulated with plastic spheres. After temperature equilibration, the mercury position corresponding to zero volume is read on calibrated capillaries D and E using a cathetometer (Precision Tool and Instrument Co., Surrey, U.K.). All levels were measured relative to a reference mark on each capillary tube. (By keeping the mercury

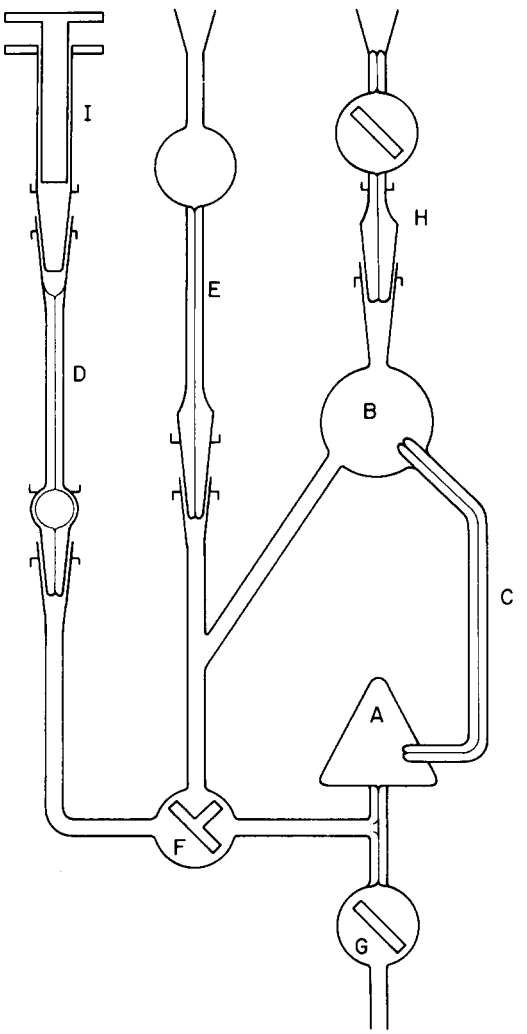


Fig. 1. The excess volume dilatometer. The solute and solvent were confined over mercury in A and B, respectively. Known aliquots of solute were transferred from A to B through calibrated capillary C ty tilting. The change in volume on mixing was monitored by the height of mercury in calibrated capillaries D and E. See text for details.

level in open capillary E relatively constant the hydrostatic pressure head in the dilatometer is maintained constant and no compressibility correction is then necessary.) By tilting the dilatometer, a slug of anesthetic is trapped between mercury in C. Its length is measured, and it is then pushed into B by operating stopcock F and syringe I. Mixing is done by a teflon-coated magnetic stir bar until the levels of mercury in capillaries D and E show no further change (usually from 15 min to 1.5 h). After mixing the volume change is read as the change in position of the mercury menisci in capillary tubes D and E. More solute is then added and the process repeated. The excess volume of mixing is evaluated from the slope of a plot of the moles of solute added versus the volume change.

The expected errors can be estimated from the uncertainties in temperature control, measurement of the position of mercury menisci and, in the case of lipid suspensions, dry weight determination. The temperature coefficient of the volume for the whole system when filled is 6.4 $\mu$l per degree Celsius. Temperature fluctuations during an experiment were never worse than ±0.003°C, and usually about ±0.002°C, yielding an uncertainty in the volume measurement of 19–12 nl. The standard deviation for measuring the position of a given meniscus with the cathetometer was determined to be ±0.0019 cm. Calculation shows that this would result in an uncertainty in the volume change (D+E) of ±9 nl. Combining errors for volume change and volume of halothane added yields an estimated error of 210 $\mu$l/mol in the final excess volume. This compares to experimentally determined standard deviations for the slope in plots such as Fig. 2 of 120–320 $\mu$l/mol for halothane in lipid suspensions. These errors in the excess volume appear to be far worse than the best obtainable in the literature for mixtures of two nonpolar liquids [12,13], but actually this originates largely from the restricted concentration range imposed on us and only to a lesser extent from the compromises made in the dilatometer design.

Densities of the pure anesthetics were determined in a Mettler/Paar (Graz, Austria) DMA 60 densitometer.

*Method of analysis.* If we consider a solution containing $n_A$ moles of A and $n_B$ moles of B, and assume the volume of this solution is so large that the additions of more A or B does not change the concentration to an appreciable extent, the partial molar volume, $\bar{V}_A$, of A in the solution at specified pressure, temperature and concentration is defined as the change in volume caused by adding one mole of A to this excess of solution. Thus

$$\bar{V}_A = \left(\frac{\partial V}{\partial n_A}\right)_{T,P,n_B} \tag{2}$$

One reason for introducing such a function is that the volume of a solution is not, in general, simply the sum of the volumes of the individual components. The volume, $V$, of a lipid suspension containing anesthetic is given by

$$V = n_1\bar{V}_1 + n_2\bar{V}_2 + n_3\bar{V}_3 + n_4\bar{V}_4 \quad (3)$$

where $n_i$ is number of moles and $\bar{V}_i$ is the partial molar volume of each component. Subscript $i = 1$ refers to the bulk water, 2 to lipid, 3 to anesthetic in water and 4 to anesthetic in lipid. $\bar{V}_i$ is an intensive property of the mixture and not dependent on the amount of each constituent. If a small extra aliquot of anesthetic is added to the suspension raising the moles of anesthetic in each phase to $n_3'$ and $n_4'$ and the total volume to $V'$, then

$$V' - V = \Delta V = (n_3' - n_3)\bar{V}_3 + (n_4' - n_4)\bar{V}_4 = \Delta n_3\bar{V}_3 + \Delta n_4\bar{V}_4 \quad (4)$$

In the excess volume dilatometer the volume of the unmixed, or pure, anesthetic remaining in bulb A has decreased by

$$\Delta V^\circ = (\Delta n_3 + \Delta n_4)\, V^\circ \quad (5)$$

where $V^\circ$ is the molar volume of anesthetic.
Thus the volume change, $V^E$, measured in the excess volume dilatometer is

$$V^E = \Delta V - \Delta V^\circ = \Delta n_3 V_3^E + \Delta n_4 V_4^E \quad (6)$$

where $V_i^E$ is the molar excess volume of anesthetic in the given solvent and

$$V_i^E = \bar{V}_i - V^\circ \quad (7)$$

In the absence of lipid Eqn. 6 reduces to

$$V^E = \Delta n_3\, V_3^E \quad (8)$$

so that the slope of the accumulative measured volume changes versus the accumulative moles of anesthetic added will yield the excess volume of the anesthetic in water.

In the presence of lipid, the total number of moles of anesthetic in the suspension, $n_t$, will be distributed between water and lipid with a lipid to water parition coefficient, $\lambda_{4/3}$, given by

$$\lambda_{4/3} = \frac{n_4}{\upsilon_4} \cdot \frac{\upsilon_3}{n_3} \quad (9)$$

where $\upsilon_i$ is the volume of the $i$th phase and

$$n_t = n_3 + n_4 \quad (10)$$

Rearranging Eqn. 6 and combining with Eqns. 9 and 10 yields

$$V_4^E = \frac{V^E}{\Delta n_t}(1 + A) - A V_3^E \quad (11)$$

where

$$A = \frac{\upsilon_3}{\upsilon_4} \cdot \frac{1}{\lambda_{4/3}} \quad (12)$$

Thus $V_4^E$ may be evaluated graphically in a manner similar to $V_3^E$ once the later has been determined independently, provided $\lambda_{4/3}$ is known.

*Determination of anesthetic potency*

The potency of the anesthetics was determined at 25 ± 1°C using the loss of righting reflex of tadpoles bathed in aqueous anesthetic solution as previously described [19]. All concentrations were checked by gas chromatography.

## Results

*Pure solvents*

According to Eqn. 8 a plot of the accumulative measured volume change, $V^E$, versus accumulative moles of benzyl alcohol added to water should be linear. Fig. 2 shows this to be the case at least up to 370 μmol. Thus the excess volume of benzyl alcohol in water, $V_3^E$, can be obtained from the slope by least squares fitting. Unconstrained fits always passed through the origin within the expected error. Quoted values are for fits constrained to pass through the origin. The value of $V_3^E$ (±S.D.) from three runs was −2.80 ± 0.099, −2.82 ± 0.070 and −2.67 ± 0.037 ml/mol, respectively. Mean value of these three runs is −2.76 ml/mol and the standard deviation between

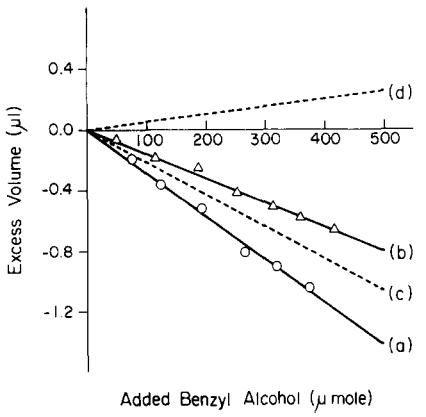


Fig. 2. Results of two experiments with benzyl alcohol. The accumulative moles of benzyl alcohol successively transferred from A to B (Fig. 1) are plotted against the respective total accumulated volume change. The solvents are (a) water, (b) an aqueous suspension of egg lecithin: cholesterol (2 : 1 mol ratio). The weight percent of lipid was 3.00 ± 0.01. The dashed lines show the relative contributions of benzyl alcohol in water (c) and in lipid (d) to the total volume change (b).

them is ±0.067 ml/mol. The partial molar volume of benzyl alcohol in water, $\bar{V}_3$, is, from Eqn. 7, 101.07 ± 0.067 ml/mol ($V^\circ$ = 103.83 ml/mol, Table I). This value was compared to the only available literature values obtained from density measurements using a pycnometer [16]. These values are 99.37 (1°C), 100.82 (20°C), 103.44 (40°C) and 105.20 (50°C) ml/mol. From them we can interpolate graphically a value of 101.2 ml/mol at 25°C which compares well with our own value.

The excess volume of halothane in water was obtained similarly and is −16.6 ± 0.56 ml/mol (three runs), which compares well with a value of −15.2 ml/mol obtained from the pressure dependence of halothane's solubility in water (Smith, R.A. and Miller, K.W., unpublished data). The excess volume of methoxyflurane in water was found to be −8.8 ± 0.20 ml/mol (two runs).

The excess volume of halothane was also determined in olive oil, which has traditionally been used as a model for the site of general anesthesia, and a value of −3.7 ± 0.12 ml/mol was obtained.

The limited solubility of hexane [17] prevented us from obtaining a value of its partial molar volume in water.

*Lipid dispersions*

Excess volumes were measured in three percent by weight lipid dispersions. Although higher lipid concentrations would have been advantageous, the increased turbidity and viscosity made it much more difficult to ensure complete degassing and thus to prevent artefacts due to inclusion of gas bubbles in the dilatometer, a common problem in densitometry [18].

When benzyl alcohol was added to an aqueous suspension of sonicated egg lecithin/cholesterol (2 : 1) vesicles linear plots were also obtained as shown for one experiment in Fig. 2. As before these plots passed through the origin within experimental error. The measured excess volume for the total system was less negative than in pure water, indicating that benzyl

TABLE I

MEAN PARTIAL MOLAR VOLUMES (ml/mol) OF ANESTHETICS IN PURE SOLVENTS AND IN LIPID BILAYERS AT 25°C

Numbers in parenthesis represent number of experiments. Errors are given as standard deviations between runs.

| Anesthetic<br>$V^\circ$ (ml/mol)<br>Solvent | Benzyl alcohol<br>103.83 | Halothane<br>106.35 | Methoxyflurane<br>116.24 |
|---|---|---|---|
| Water | 101.07 ± 0.067 (3) | 89.7 ± 0.56 (3) | 107.4 ± 0.20 (2) |
| Olive oil | – | 102.6 ± 0.12 (2) | – |
| Egg lecithin | – | 103.2 ± 0.13 (2) | 113.0 ± 0.20 (2) |
| DMPC | – | 106.41 ± 0.048 (2) | – |
| DPPC | – | 119 ± 1.4 (3) | – |
| Egg lecithin/cholesterol | 105.3 ± 0.26 (3) | 106.7 ± 0.50 (2) | 119.45 ± 0.016 (2) |

alcohol has a larger partial molar volume in lipid than in pure water. The actual excess volume of benzyl alcohol in lipid, $V_4^E$, can be obtained by using Eqn. 11 from the slope of (b) in Fig. 2, since $V_3^E$, has been determined independently and the partition coefficient $\lambda_{4/3}$ is known [8].

The calculated contributions of the water and of the lipid to the overall measured volume change are shown in the dotted lines (c) and (d), respectively, in Fig. 2. The mean excess volume of benzyl alcohol in egg lecithin/cholesterol (2:1) sonicated vesicles is 1.5 ± 0.26 ml/mol, yielding a partial molar volume, $\bar{V}_4$, of 105.3 ± 0.26 ml/mol (Table I). Since the partition coefficient of benzyl alcohol into egg lecithin alone is dependent on the concentration of benzyl alcohol [8], we did not attempt to measure the excess volume in this system.

The excess volumes of halothane in egg lecithin and in egg lecithin/cholesterol (2:1) are −1.9 ± 0.15 ml/mol and 2.6 ± 0.59, respectively, $\lambda_{4/3}$ used for calculation is from the data of Simon et al. [7]. The only other data on partition coefficients in these lipids at 25°C [9] yields lower excess volumes, values of −3.2 ± 0.13 and 0.3 ± 0.50 ml/mol, respectively. The latter values are used in Table I for consistency since the only partition coefficient data for methoxyflurane was determined by the same workers [9].

The excess volumes of methoxyflurane in egg lecithin and egg lecithin/cholesterol (2:1) are −3.2 ± 0.43 and 3.21 ± 0.016 ml/mol.

We also attempted to measure the excess volume of *n*-hexane in egg lecithin/cholesterol (2:1) vesicles. The limited solubility made it impossible to obtain good plots such as Fig. 2. However in two experiments small positive changes were observed suggesting an excess volume in the lipid of about 2–4 ml/mol.

To compare the partial molar volume of anesthetic in lipids in the gel and liquid crystalline states, we determined the excess volumes of halothane in DMPC and DPPC at 25°C. Values of 0.06 ± 0.048 and 13 ± 1.4 ml/mol, respectively, were obtained. We attempted to confirm this large difference by obtaining a value for DPPC at 42°C, but the high vapor pressure of halothane prevented us. (DPPC is the only lipid for which the partition coefficient has been determined in both the liquid crystalline and gel phases [7].) These experiments were done with vortexed lipid dispersions both because sonicated vesicles are not stable in the gel state and because sonication broadens the phase transition temperature [19]. Control experiments with egg lecithin/cholesterol (2:1) vortexed vesicles yielded a partial molar volume which was identical to that in sonicated vesicles of this lipid. Simon et al. [7] did not report data for the partition coefficient of halothane in DMPC, but his values at 25°C for DOPC, DLPC and egg lecithin are all equal within experimental error and $\lambda_{4/3}$ was therefore taken as 96.2 in DMPC.

The values of partial molar volumes in lipids in Table I are somewhat dependent on the partition coefficient. We give here the actual values of $\lambda_{4/3}$ used. Benzyl alcohol in egg lecithin/cholesterol 10.9 [8]; halothane in egg lecithin/cholesterol 85.8 [7] or 124 [9], in egg lecithin/cholesterol 52.5 [7] or 77.6 [9] and in DPPC 23.6 [7]; methoxyflurane in egg lecithin 135 and egg lecithin/cholesterol 80.0 [9]. Values from Ref. 9 were used for halothane in all tables. The sensitivity of $\bar{V}_3$ to $\lambda_{4/3}$ is not high as can be judged for the values for halothane cited in the text above.

*Anesthetic potency*

The aqueous concentrations which anesthetized half of a group of tadpoles were determined by analysis of dose response curves [20] to be: benzyl alcohol 2.0 ± 0.21 mM (±S.E.); halothane 0.23 ± 0.016 mM and methoxyflurane 0.21 ± 0.016 mM.

## Discussion

*Methodology*

We have determined the excess volumes of an alcohol, an ether, and a halogenated hydrocarbon in both water and in dilute aqueous suspensions of lipids using a novel excess volume dilatometer. These are the first direct measurements of excess volumes of mixing of simple solutes in lipid bilayers of which we are aware. The reliability of our dilatometer can thus only be assessed for the aqueous solutions where partial molar volumes of benzyl alcohol and halothane both agreed well with independent observations (see Results). The most serious problem we encountered when lipid suspensions were compared to water was

that of microbubbles. In turbid suspensions it was hard to ensure against them. If they were present in the upper bulb, then addition of a volatile solute tended to make them grow, resulting in an inflated value of the excess volume. In such cases the volume reading was often unstable with time. In practice this problem was usually avoided when sonicated suspensions of three, or less, weight percent of lipid were used. This had the unfortunate corollaries of limiting both the proportion of the volume change originating from the lipid and the total solubility in the suspensions (and hence the absolute magnitude of the volume change recorded) with some solutes (e.g., hexane). It also prevented a detailed investigation of the possible dependence of $V_3^E$ on lipid concentration. Nonetheless, reproducible excess volumes were obtained for these representative solutes and the method should be readily applicable to other liquid solutes as more partition coefficient data becomes available. The major limitation on solutes (apart from their physical state) is the possession of a high enough lipid/water partition coefficient to ensure an adequate contribution to the volume change from the lipid portion of the suspension.

*Excess volumes*

Benzyl alcohol, halothane and methoxyflurane all had negative excess volumes in water. Non-polar gases have excess volumes close to zero [12,21]. Thus, in our case solute-solvent interactions lead to a contraction in volume below that expected from simple summation. Hydrogen bonding and dipolar solutes would produce such changes by disrupting the open hydrogen bonded network of water [16]. The magnitude of the excess for halothane is particularly noteworthy.

In contrast to water, the excess volumes of all anesthetics in liquid crystalline lipid bilayers were small and exhibited little variation. This is also true in sodium dodecanoate micellar solutions where both *n*-alkanols [22] and *n*-alkanes [23] show excess volumes in the micelle that are scattered about zero, and their absolute magnitude is never more than a few percent of the solute's molar volume. In contrast, mixtures of non-polar liquids have excess volumes at infinite dilution that are usually positive and range from near zero to 15% of the solute's molar volume [21]. The partial molar volumes in bilayers and micelles thus tend to be smaller than those in non-polar solvents and are larger than those in water. Their magnitude is modelled, at least for halothane, by olive oil (Table I). Thus, the nature of the bilayer structure itself does not impose any exceptional value on the partial molar volume.

In egg lecithin/cholesterol positive excess volumes were obtained in three out of three cases, whereas negative excesses were observed in two out of two cases in egg lecithin alone. Furthermore, the excess volume of hexane in egg lecithin/cholesterol is also probably positive. Thus tentatively we conclude that the presence of cholesterol leads to an increase in partial molar volume of the anesthetic in the lipid by about 3.4–6.5 ml/mol. In the case of benzyl alcohol, NMR studies of the ring current shifts induced in the *N*-methyls of the choline head group suggest that the decrease in partition coefficient on addition of cholesterol results from an increase in the ratio of alcohol in the head group to that in the acyl chain region [8]. If this is generally true, then it follows from our results that the partial molar volume of solutes in the head group region must be larger than that in the acyl chain region.

The most dramatic difference in excess volume for halothane was obtained when DPPC and DMPC were compared at 25°C. The partial molar volume in the gel phase bilayer (DPPC) was nearly 13 ml/mol greater than that in the liquid crystalline bilayer. This effect might be due to the comparison of two different lipids, but when egg lecithin and DMPC are compared their partial molar volumes of halothane differ by only 2 ml/mol when self-consistent partition coefficients are used [7]. Thus the difference in the physical states of DMPC and DPPC must account for most of the large difference, and indeed this indication that the environment of halothane is different in the two phases is supported by $^{19}$F-NMR studies [24], which demonstrated a 0.2 ppm difference in chemical shift in the two phases of DPPC together with more complex changes revealed by proton decoupling and relaxation measurements. The implication that halothane interacts only with head group regions of the gel phase is also consistent with the NMR work with benzyl alcohol under similar circumstance [8]. Interaction with the acyl region occurs on melting. Curiously these changes are not associated with any dramatic changes in the enthalpy

and entropy of solution [7]. Thus the increasing partial molar volume on going from egg lecithin to egg lecithin/cholesterol (2:1) to gel phase lipid, according to the NMR evidence [8,23], may reflect an increasing displacement of solute from the acyl chain to the head group region.

*Anesthetic action*

It is possible to use our data to provide a partial test of the hypothesis that anesthetics act by expanding membranes [2–5,25]. The critical volume hypothesis states that general anesthesia occurs when the volume of some hydrophobic phase is expanded a constant amount by dissolution of any inert substance. The expansion required for anesthesia can be calculated from the first term in Eqn. 1 when volatile agents are considered [2]. Alternatively, when the anesthetic dose is expressed as an aqueous concentration, for example when aquatic animals are anesthetized, Eqn. 1 may be rewritten [26] at ambient pressure as

$$E^{50} = \frac{\bar{V}_2 \cdot \lambda_{4/3} \cdot C_3^{50}}{V_m} \qquad (13)$$

where $E^{50}$ is the expansion at a dose which anesthetizes 50% of a group of animals and $C_3^{50}$ is the aqueous concentration required to achieve this endpoint. $E^{50}$ should be constant for all anesthetics if the hypothesis is correct.

In Table II we present the results of such calculations using anesthetic data for tadpoles determined in this work, for goldfish [27] and for mice [5]. The partition coefficients used were from Ref. 9 for halothane and methoxyflurane (data from Ref. 7 were omitted for consistency) and from Ref. 8 for benzyl alcohol. We find that all the anesthetics expand these lipid bilayers. This expansion is less than one percent which is consistent with the predictions from studies of pressure reversal of anesthesia for newts [2] and mice [28]. Newts require very similar doses of anesthetic to tadpoles and an expansion of 0.2% is predicted to cause anesthesia [2]. In mice the equivalent figure is 0.4% [28]. Both these estimates are based on olive oil as a model of the site of action of general anesthesia. An indirect estimate of the expansion of red cells by volatile anesthetics at anesthetic concentrations is 0.4% [29], although higher estimates have been given for other agents [3].

Not only is the expansion of these lipid bilayers that is required for anesthesia of the correct magnitude, but it is reasonably constant when a given species and a given bilayer composition are considered (Table II). A wider range of anesthetics must be studied before this conclusion may be generalized, but it seems reasonably secure because the molar volumes of most anesthetics only cover a small range [2,3] and because the anesthetic potency of a wide range of anesthetics correlates well with their lipid solubility [9,30,31].

Neither of the lipid bilayer compositions used here is a significantly better model than the other. The egg lecithin model predicts a greater expansion in spite of the slightly larger partial molar volumes in the cholesterol containing bilayer. This is because the partition coefficients are consistently reduced by cholesterol [6–9,15].

TABLE II

CALCULATED BILAYER EXPANSION AT ANESTHETIC DOSES

Solvents: A, egg lecithin; B, egg lecithin/cholesterol.

| Anesthetic | Calculated expansion | | | | | |
|---|---|---|---|---|---|---|
| | Tadpoles | | Goldfish | | Mice | |
| | A | B | A | B | A | B |
| Benzyl alcohol | – | 0.22 | – | – | – | – |
| Halothane | 0.30 | 0.19 | 0.51 | 0.33 | 0.33 | 0.21 |
| Methoxyflurane | 0.34 | 0.21 | 0.60 | 0.37 | 0.56 | 0.37 |
| Mean ± S.D. | 0.32 ± 0.028 | 0.21 ± 0.015 | 0.56 ± 0.060 | 0.35 ± 0.028 | 0.45 ± 0.17 | 0.29 ± 0.11 |

Thus our data are consistent with the hypothesis that general anesthetics act by expanding a hydrophobic region which is well modeled by lipid bilayers. This conclusion is in contrast to X-ray and neutron diffraction studies in oriented lipid films [10,11], to magnetic resonance measurements in lipid vasicles [32] and to capacitance measurements in 'solvent-free' planar bilayers [33,34]. In all the latter studies no detectable increase in bilayer thickness was observed, indeed a decrease was possible in some studies [32,33]. However, anesthetics do increase the thickness of black lipid membranes [35], but this observation is not reproduced in 'solvent-free' bilayers [36] and therefore appears to be inapplicable to our system. Part of the failure to observe such an increase in thickness may arise from the small magnitude of the effect predicted by our results, but in many cases no changes were detected even when very high concentrations of anesthetics were employed [11].

One way of resolving this apparent discrepancy is to assume that the bilayer expands anisotropically [2,5,37,38]. Such an assumption may be justified by the observation that lipid bilayers have a positive coefficient of bulk thermal expansion but a negative coefficient of thickness thermal expansion [39,40]. Furthermore, general anesthetics do expand the area of lipid monolayers formed at the aqueous-gas interface [41]. If anisotropic expansion occurs when anesthetics dissolve in bilayers, their area must change by about the same percentage as their volume whilst the thickness remains essentially unchanged. In one study of monolayers [42] anesthetic concentrations expanded the area at constant pressure by 0.3% at 20°C. This is consistent with the idea of anisotropic expansion, but does not prove it.

One attraction of the idea that anesthetics expand bilayer area without altering thickness is that it may resolve the problem of why reducing bilayer volume by cooling does not reverse anesthesia in the same way that pressure does. Thus, after allowing for changes in bilayer partition coefficients with temperature, it is usually found that anesthetics tend to increase in potency as the temperature is lowered although the effect is not large [9,30,38]. It can be countered that temperature induced volume changes do differ from anesthetic induced ones in that the latter occur isothermally and thus may not be sensed by all parts of the system. In addition to this argument, we can now add that of anisotropic expansion. If anesthetics expand membranes without changing their thickness, but lowering temperature causes a net contraction with an increase in thickness, then the two changes would not be expected to compensate each other. The corollary of this is that pressure, since it reverses anethesia, must compress bilayers without causing much change in thickness. This prediction is testable by combining thickness measurements with bulk compressibility measurements. The latter measurement would also provide a severe test of the applicability of Eqn. 1 to the pressure reversal of anesthesia.

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