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Interaction of Halothane with Lipid Bilayers

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SUMMARY

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The bilayer-saline partition coefficient of halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) was directly measured as a function of temperature in phospholipids of various hydrocarbon chain lengths and degrees of saturation, both with and without cholesterol. The major conclusions of this research can be summarized as follows: 1) The bilayer-saline partition coefficient as a function of temperature is similar for the lipids dilauroylphosphatidylcholine, dioleoylphosphatidylcholine and egg phosphatidylcholine over a temperature range from 10 to 60°, where each lipid is in the liquid-crystalline state. Over this range the partition coefficient versus temperature curve exhibits a change from positive to negative slope for all three lipids. 2) The bilayer-saline partition coefficient increased with increasing temperature by a factor of 4 at the phase transition (40°) of dipalmitoylphosphatidylcholine. At this temperature, the partition coefficients of all four lipids mentioned were similar. 3) The addition of cholesterol to egg phosphatidylcholine reduces the partition coefficient by a factor of 2 at a 2:1 mole ratio and 2.7 at a 1:1 mole ratio at 25°. In these bilayers the partition coefficient was independent of temperature. 4) From (3) and other data we suggest that the reduced partial pressure necessary to produce anesthesia at lower temperatures for halothane is not due to an increase in the bilayer-saline partition coefficient.

INTRODUCTION

The membrane-saline partition coefficient is an important parameter to know in order to differentiate the mechanisms of general anesthesia. As all mammalian membranes contain a variety of phospholipids with varying amounts of cholesterol (1), we thought it was important to measure directly the partition coefficient of the anesthetic gas halothane in a variety of phospholipid and phospholipid-cholesterol bilayers. Halothane was of particular interest since there has been controversy in the literature regarding the value of the halothane partition coefficient, as has been recently pointed out by Mastrangelo *et al.* (2).

It is well known that the gas pressure necessary to cause anesthesia depends on temperature (3). Therefore, we have also examined the temperature dependence of the partition coefficient of halothane into lipid bilayers to see if this effect could be explained in terms of a change in solubility of the gas in the membrane. In this context, it is of interest to investigate if Overton's Rule applies at all temperatures.

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MATERIALS AND METHODS

The lipids used in these experiments were DPL[1] (Sigma Chemical Co.), DOL (Supelco, Inc.), DLL (Supelco, Inc.), EPC (Nuthill), and cholesterol (Supelco). Olive oil was purchased from Sigma. All lipids were used without further purification. Halothane-1-^{14}C (1,1,1-trifluoro-2-bromo-2-chloroethane-1-^{14}C) was purchased from New England Nuclear (Lot No. 1033-029); as purchased its specific activity was 2.6 mCi/mmole. Its purity as given by New England Nuclear was 99%. It was not checked further. However, it is noteworthy that the value of the partition coefficient was independent of the age of the sample over a period of six months. It was subsequently diluted with twice distilled Fluothane (Ayerst) to remove the 0.01% thymol and to obtain a specific activity more amenable to our experimental conditions. The water was twice distilled in an all quartz still and the sodium chloride used was washed with chloroform to remove nonpolar impurities.

The procedures for measuring the partition coefficients are described elsewhere (4). Basically, a sample of radioactive halothane is injected into a closed chamber that is connected through a gas phase with four other chambers. Two of the chambers contain vortexed lipid-dispersions at a concentration of 10 mg/ml. The other two chambers contain 0.1 M NaCl, which is hereafter referred to as the saline phase. The temperature of this apparatus was controlled by a regulated water jacket. The temperature could be measured to a precision of ±0.2°. After injection of a fixed amount of halothane in the gas phase, it was necessary to wait one hour to obtain equilibrium conditions. This was ascertained by measuring the counts in each phase as a function of time. Once equilibrium was obtained, the temperature was either increased when the experiment commenced at low temperatures or decreased when the experiment was commenced at high temperatures. Experimentally similar results were obtained under these conditions.

The units used in this paper are unitary (5) or mole fraction units unless otherwise stated. This means that the membrane-saline partition coefficient, K, is defined as follows:

$$K = \frac{n}{N_1 + n} \Big/ \frac{n}{N_2 + n}$$

where n = moles of halothane
N_1 = moles of solvent
N_2 = moles of water

The partial molal energy of transfer is obtained from the equation:

$$\overline{\Delta G}^\circ = -RT \ln K \qquad (1)$$

The partial molal entropy, $\Delta\bar{S}$, and enthalpy, $\Delta\bar{H}$, of transfer are obtained from equation (1) via:

$$\overline{\Delta H}^\circ = \frac{\partial(\overline{\Delta G}^\circ/T)}{\partial(1/T)} = -\frac{\partial(R \ln K)}{\partial(1/T)} \qquad (2)$$

$$\overline{\Delta S}^\circ = \frac{\overline{\Delta H}^\circ - \overline{\Delta G}^\circ}{T} \qquad (3)$$

RESULTS

The partition coefficient of halothane was first measured as a function of concentration of halothane in the aqueous phase, in egg lecithin bilayers at 25°. We found that the partition coefficient was independent of aqueous concentrations of halothane from 1.1 μM to 940 μM. The value of the partition coefficient is $4.08 \pm 0.23 \times 10^3$ over this concentration range. This latter concentration corresponds to about a 1.3% partial pressure of halothane (2) in the gaseous phase, which is considerably greater than the pressure necessary to abolish the righting reflex in mice (0.77%) (6) or to depress synaptic conduction in the dentate gyrus (0.3–0.4%) (7). Once the fact that the partition coefficient is independent of pressure had been established, we commenced to measure the partition coefficient using very dilute concentrations in the aqueous phase (usually about 5 μM). This was done so that we could use the "law of ideality of dilute solutions" (8) and assume that the activity coefficient is unity (5). Regardless

[1] The abbreviations used are: DPL, dipalmitoylphosphatidylcholine; DOL, dioleoylphosphatidylcholine; DLL, dilaurylphosphatidylcholine; EPC, egg phosphatidylcholine.

of the value of the activity coefficient, the fact that the partition coefficient is independent of the aqueous concentration (hence partial pressure) means that we are operating in the Henrys' Law region. Hence the unitary free energy of transfer $\Delta G°$ of halothane from water into EPC at 25° is −4.92 kcal/mole. Since the other phospholipids exhibited similar behavior in regard to their partition coefficients we assume that their partition coefficients are also independent of the partial pressure over the stated range.

Figure 1 shows the temperature dependence of the partition coefficient of halothane from water to bilayers of DLL (●) and EPC (×) over the temperature range 10–60° for the former and 20–55° for the latter. The partition coefficient of halothane into DOL is experimentally indistinguishable from that of DLL over the temperature range 20–55°. It is omitted from the figure for purposes of clarity. It is seen from Fig. 1 that the partition coefficient undergoes a change of slope over the stated temperature range. This behavior is rather typical of hydrophobic interactions (5, 9). The position of the maximum occurs where the partial molal enthalpy of transfer, $\Delta H°$, is zero (9). Due to the resolution of these measurements we are not able to ascertain precisely where this maximum is. Table 1 lists the thermodynamic transfer parameters of halothane into these dispersions at 25° and 37°.

Figure 2 shows how the halothane partition coefficient varies with increasing temperature in a bilayer comprised of DPL (●). The abrupt break in the curve is indicative of the well characterized phase transition of DPL at 41° (11). There is a depression of the transition temperature due to the incorporation of halothane into the dispersion (12). We minimized this effect by keeping the concentration in the aqueous phase low (~500 μM). At this aqueous concentration of halothane the depression of the transition temperature would be about 3° (12). In Fig. 2 it is seen that the partition coefficient decreased by a factor of four from above to below the transition.

Below the phase transition the partition coefficient is virtually independent of temperature, whereas above the transition it decreases with temperature. It is noteworthy that at 40° (where each of the lipids is in the liquid-crystalline state) the partition coefficient is about the same in all four lipids tested. This implies that the region of the bilayer where the halothane partitions is similar for all of these lipids. Also shown in Fig. 2, for comparative purposes, is the value of the partition coefficient (×) of halothane obtained by Hill (12) by measuring the freezing point depression of DPL caused by halothane. It is obvious that the freezing point depression method gives comparable results to the directly measured values.

Figure 3 shows the effect of increasing the cholesterol concentrations in EPC-cholesterol dispersions. It is clear from this figure that cholesterol decreases the partition coefficient in a manner that is dependent on its concentration in the bilayer (Fig. 4). This behavior has been demonstrated in other systems using other molecules as probes (1, 4, 13). One striking difference between the curves in Figs. 1 and 3 is the

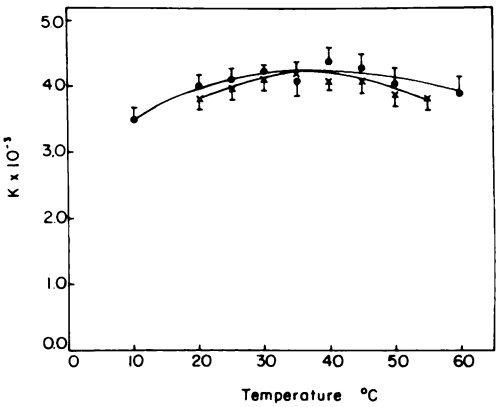

FIG. 1. *The bilayer-saline (0.1 M NaCl) partition coefficient of halothane as a function of temperature for dilaurylphosphatidylcholine* (●) *and egg phosphatidylcholine* (×)

Not shown in this figure is the partition coefficient for dioleoylphosphatidylcholine which is experimentally indistinguishable from dilaurylphosphatidylcholine. Error bars (representing the standard deviation) are only shown in one direction for clarity. The egg phosphatidylcholine data represent the average of eight experiments whereas the others were for four experiments. The curves are hand drawn.

TABLE 1

Thermodynamic transfer parameters of halothane from saline into lipid bilayers in unitary units

Lipid	Temperature	$-\overline{\Delta G}^{oa}$	$\overline{\Delta H}^{o}$	$\overline{\Delta S}^{od}$
		kcal/mole	*kcal/mole*	*cal/mole/deg*
Egg phosphatidylcholine	25	4.92 ± 0.03	1.0 ± 0.4	19.9
	37	5.12 ± 0.03	0.0 ± 0.5	16.5
Dilaurylphosphatidylcholine	25	4.91 ± 0.03	1.2 ± 0.5	20.5
	37	5.12 ± 0.03	0.0 ± 0.5	16.5
Dioleoylphosphatidylcholine	25	4.92 ± 0.04	0.8 ± 0.4	19.2
	37	5.14 ± 0.05	0.0 ± 0.5	16.6
Dipalmitoylphosphatidylcholine	25	4.12 + 0.08 −0.03	0.0 ± 0.2	13.8
	40	5.16 ± 0.03	−0.5 ± 0.4	14.9
Egg phosphatidylcholine-cholesterol (2:1)	25	4.52 ± 0.04	0.0 ± 0.3	15.2
	37	4.71 + 0.04 −0.06	0.0 ± 0.3	15.2
Egg phosphatidylcholine-cholesterol (1:1)	25	4.33 + 0.10 −0.08	0.0 ± 0.3	14.5
	37	4.50 + 0.08 −0.09	0.0 ± 0.3	14.5
Octanol	25	4.42[b]		
Olive oil	24	5.29 ± 0.21[c]		

[a] Calculated from the mean value in Figs. 1 and 2.

[b] From reference 10 taking the volume of octanol to be 157 cm^3/mol.

[c] Assuming the molar volume to be 980 cm^3/mol (25).

[d] The digit after the decimal point is not significant and is included to make Eq. (2) consistent.

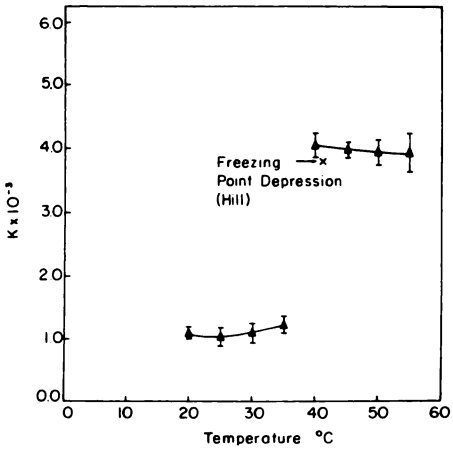

FIG. 2. *The bilayer-saline (0.1 M NaCl) partition coefficient of halothane into dipalmitoylphosphatidylcholine (▲) as a function of temperature*

The data were obtained from four experiments. Also shown for comparative reasons are the data obtained by Hill (12) from measuring the freezing point depression of dipalmitoylphosphatidylcholine produced by halothane (×).

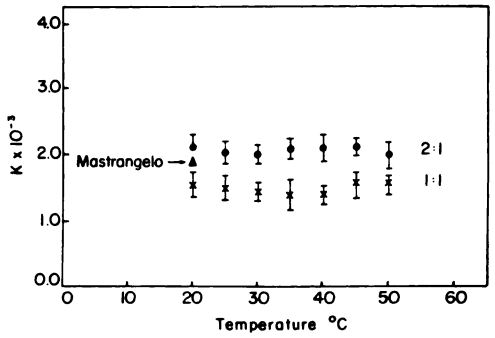

FIG. 3. *The bilayer-saline (0.1 M NaCl) partition coefficient for halothane in egg phosphatidylcholine-cholesterol (2:1, (●)) and egg phosphatidylcholine-cholesterol (1:1, (×)) as a function of temperature*

The results are the average of four experiments each. Also shown is the partition coefficient of halothane obtained by Mastrangelo *et al.* (2) (▲) for an egg phosphatidylcholine-cholesterol (2:1) bilayer at 20°.

noticeable lack of curvature in the partition coefficient versus temperature curves for the cholesterol-containing bilayers. From Eq. (2) it is clear that the partial molal enthalpy of transfer is experimentally zero over the entire temperature range.

The partition coefficient of halothane has also been measured in the isotropic liquid olive oil. The free energy of transfer was

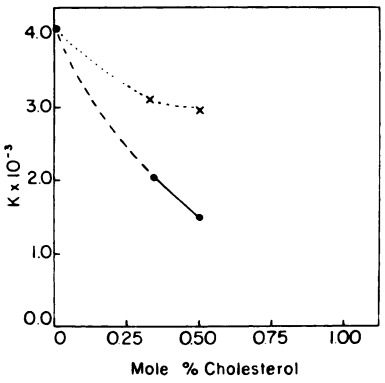

FIG. 4. *A plot of the bilayer-saline (0.1 M NaCl) partition coefficient of halothane into egg phosphatidylcholine bilayers as a function of cholesterol concentration at 25°* (●)

These data were obtained from the data in Fig. 3. The symbols designated by (×) denote the partition coefficient recalculated assuming that the halothane does not partition into the cholesterol in the bilayer. To obtain these numbers, we experimented under conditions where $n \ll N_1$. Then we assumed that the partition coefficient into cholesterol molecules was zero and that halothane only partitioned into EPC. The partition coefficient was then recalculated under these conditions using as N_1 the number of moles of solvent that halothane partitions into, that is, the number of EPC molecules. The differences between the recalculated partition coefficients (×) and the zero mole percent cholesterol point reflect the modification of EPC by cholesterol.

found to be -5.29 ± 0.21 kcal/mole. (Table 1) which is in agreement with previously published data (14).

DISCUSSION

In this paper we have measured the partition coefficient of the anesthetic gas, halothane (2-bromo-2 chloro-1,1,1 trifluoroethane), into a variety of lipid bilayers, both with and without cholesterol, as well as in the isotropic liquid olive oil. Our conclusions may be summarized as follows:

1) The partition coefficient is independent of the chain length of the lipid from C_{12}–C_{18} (DLL-DOL). EPC has a large variation in chain lengths. In this vein the partition coefficient is experimentally insensitive to the presence of double bonds in the hydrophobic region as DOL has two double bonds per molecule whereas DLL has none. The partition coefficient of halothane into DLL, DPL, EPC, and DOL are similar at comparable temperatures when all the lipids are in their liquid-crystalline state.

2) In lipid bilayers without cholesterol which do not undergo a phase transition in this temperature range (DLL, DOL, EPC) the partition coefficient versus temperature curves exhibit a change in curvature over the temperature range investigated.

3) The partition coefficient increases by a factor of four when DPL goes through its phase transition temperature.

4) Cholesterol decreases the partition coefficient of halothane by a factor of 2 into a 2:1 (EPC-cholesterol) and 2.7 into a 1:1 mole ratio dispersion at 25°. Our results at 2:1 EPC-cholesterol are comparable to those obtained by Mastrangelo *et al.* (2) who measured the partition coefficient into dispersions at much higher lipid concentrations.

5) With cholesterol in EPC, the halothane partition coefficient is virtually temperature independent.

As these measurements represent thermodynamic quantities that measure the work done in transferring halothane from an aqueous phase to bilayers, they are naturally incapable of answering questions as to where the halothane is located and how these molecules may cause synaptic and axonal blockade. However, with other preexisting data some light may be shed on these questions.

From partition coefficient, nuclear magnetic resonance (15), and electron spin resonance measurements (16), it is thought that halothane is located in the hydrophobic region for cholesterol-free lipid bilayers above their transition temperature. For pure DPL bilayers below the transition we found a significant decrease in the free energy and entropy of transfer and an increase (becomes less positive) in the enthalpy of transfer (Table 1) as compared to those parameters above the transition temperature. The reduction in $\Delta S°$ in the gel state is due to the tighter packing of the acyl chains which will restrict the movement of halothane.

The reduction in the partition coefficient of halothane that is observed when choles-

terol is incorporated into egg phosphatidylcholine bilayers results primarily from a significant decrease in the partial molal entropy of transfer (at 25°), (Table 1), as the reduction in the partial molal enthalpy of transfer (less positive) would favor an increased partition coefficient. This reduction in entropy of transfer cannot simply be explained as a decrease in the volume available for partition, since we have previously seen the partition coefficient of halothane into cholesterol-free bilayers is practically insensitive to the length of the acyl chain. This phenomenon was also found in other bilayer systems (17, 18). The reduction in the entropy of transfer caused by cholesterol is most likely due to two effects: first, the well known "ordering effect" that cholesterol has on phospholipids above their phase transition temperature (1), and second, the fact that halothane appears to have a much lower partition coefficient into cholesterol than into phospholipid. Considering the first effect, it is known from spectroscopic techniques (19) that upon the addition of cholesterol the flexibility of the acyl chains in contact with the steroid nucleus decreases. In fact, the order parameters of the methylene groups in contact with the steroid nucleus are not very different than those for DPL below the phase transition temperature. X-ray diffraction (20, 21) studies show that a large portion of the methylene groups of the phospholipids of the bilayer are in contact with the steroid nuclei. Thus, should halothane partition into this region of the bilayer it would be expected to have a smaller entropy of transfer. We note that Koehler *et al.* (15) have indicated that halothane, at least below the phase transition temperature, is located near the interfacial region of the bilayer, where the cholesterol steroid nucleus is located when cholesterol is added to bilayers (20, 21). Consistent with the notion that the ordering effect of cholesterol on the phospholipid chains causes the decrease in entropy of transfer, is the observation that the entropy of transfer is lower for DPL below its transition than above (Table 1). Note that for both DPL in the gel state and EPC:cholesterol bilayers the enthalpy of transfer is zero. The second consideration in the reduction of the partition coefficient caused by cholesterol is that the partition coefficient of halothane into cholesterol is smaller than it is into phospholipid. That halothane has a lower partition in cholesterol than EPC is indicated by the fact that the curve of Fig. 4 extrapolates approximately to zero partition coefficient at 100% cholesterol. We note that Miller and Yu (13) obtained a similar dependence for the partition coefficient of pentobarbitone into phosphatidylcholine bilayers containing various amounts of cholesterol. Thus, the higher the molar ratio of cholesterol in the bilayer, the lower the partition coefficient of halothane into the bilayer. To get some idea of the relative magnitude of these two effects, we have made calculations correcting for the number of "zero partition coefficient" cholesterol molecules in the bilayer. This was done by simply assuming that all the halothane partitions into the EPC and none into the cholesterol and therefore assuming the bilayer to be comprised entirely of EPC molecules (see Fig. 4). It was found that the partition coefficient for 2:1 EPC: cholesterol bilayers is lower by about 25% than expected for bilayers composed of unperturbed EPC molecules (Fig. 4). This calculation was done assuming a zero partition coefficient for halothane into cholesterol. We note an assumption of a larger partition coefficient would result in an even larger difference than 25%. Although there are many assumptions involved in this calculation, it does show that the entire reduction in partition coefficient is not caused by the small partition coefficient of the cholesterol molecule itself. Therefore, the "ordering effect" of cholesterol on the phospholipids must play a major role in reducing the partition coefficient of halothane into EPC: cholesterol bilayers, at least at 2:1 molar ratios. As more cholesterol is added, the second effect of cholesterol having a lower partition coefficient than EPC becomes more important. This can be seen in Fig. 4 for the case of 1:1 molar ratio EPC:cholesterol. Thus, the reduction in both the entropy of transfer and partition coefficient of halothane into bilayers caused by cholesterol can be explained in terms of the ordering effect of cholesterol on phospholip-

ids and the reduced partition coefficient of the cholesterol molecule itself. However, the reduction in enthalpy of transfer caused by cholesterol is not understood at present.

At higher temperatures, the thermodynamic transfer parameters in cholesterol-free and cholesterol-containing bilayers can be explained entirely in entropic terms (Table 1). This implies, on the average, that a halothane molecule in a cholesterol-containing bilayer is somewhat more restricted in its motion than it is in a cholesterol-free bilayer. This, of course, is just one possible interpretation. It is well known that partial molal quantities reflect the system behavior and not simply the probe, which in this case is halothane.

Thus, the amount of cholesterol in the bilayer significantly moderates the partition coefficient. This observation was also found by others (4, 13). As the cholesterol content of nerve membranes, myelin, and some red blood cells all differ (1), one does not expect them to have the same concentration of anesthetic molecules at a given partial pressure of halothane. This may be one reason why some cells are more sensitive to anesthetics than others.

The effect of temperature on anesthetic potency has been examined in many systems (3). It is found that the lower the temperature of the given preparation, the lower the partial pressure of gas that is necessary to produce general anesthesia. One possible explanation of this phenomenon would be to assume that the membrane water partition coefficient increases as the temperature decreases over a wide temperature range. Such an effect has indeed been observed for n-hexane (4). However, for halothane in the physiological ranges of temperature (37° and below) the partition coefficient either decreases with decreasing temperature (Fig. 1) or remains unchanged for bilayers of EPC containing 2:1 or 1:1 mole ratios of cholesterol (Fig. 3). Thus the effects of temperature on anesthetic potency for halothane cannot easily be explained by an increase in solubility of the anesthetic in the lipid components of a membrane. It is known that temperature, by itself, has profound physiological and anesthetic effects as many of the processes involved in excitable membranes exhibit very large Q_{10}'s (22, 23). Thus the effects of temperature on anesthetic potency are more likely to involve a protein or lipoprotein component of the membrane than the lipid region (14, 24).

The free energy of transfer of halothane into the isotropic liquid, olive oil, is greater than it is in the lipid bilayers and is considerably higher than it is in octanol (Table 1). However, these differences can be made more transparent if we compare the partition coefficients in molal units rather than unitary units. In these units the partition coefficient for octanol, olive oil at 25°, and EPC are 200, 140 and 114, respectively. The fact that the bilayer has a lower partition coefficient than the isotropic liquids is not surprising in view of its more ordered structure (4).

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