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Solvent Effects on Halothane: ^{19}F Nuclear Magnetic Resonance in Solvents and Artificial Membranes

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SUMMARY

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This study of solvent effects on halothane ^{19}F nuclear magnetic resonance chemical shifts was undertaken in order to elucidate environmental factors contributing to observed halothane ^{19}F NMR shifts in the presence of phospholipid suspensions in aqueous media. Halothane ^{19}F NMR chemical shifts are correlated by Hildebrand's δ function or refractive index for non-hydrogen-bonding solvents. No significant correlation was observed for other solvent properties, such as dielectric constant. Water and alcohols show positive deviations from the correlation with δ. Halothane molecules associated with both synthetic and natural phospholipid multilayer vesicles show significant line broadening of the fluorine signal and appear to be in an environment which may be characterized by a δ value of 9.3 ± 0.3 $(cal/cm^3)^{1/2}$ by comparison with the observed correlation of chemical shift with δ in isotropic solvent systems.

INTRODUCTION

Integral with theories of the mechanism of action of general anesthetics have been assumptions or explicit statements regarding the molecular nature of the primary site of anesthetic action. The high lipid solubility of anesthetic agents (1, 2), which otherwise seem to exhibit few common properties, and the known expansion of certain cell membranes in the presence of anesthetics have led research in anesthesia mechanisms to focus on the cell membrane as the site of anesthetic action. Subsequent to the crucial interaction of the anesthetic agent and its site of action in membrane, secondary events such as perturbation (3, 4) of the lipid matrix of the membrane and/or induction of conformational changes in membrane-associated proteins (5-7) are presumed to lead to anesthesia. The intent of much recent work on the mechanism of anesthetic action has been characterization of the nature of the anesthetic site, or anesthetic interaction (8). However, assessment of the microenvironment(s) of an anesthetic molecule in the heterogeneous, anisotropic milieu con-

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stituted by a membrane in an aqueous system is a formidable task, and more readily studied bulk properties may not necessarily accurately reflect the microenvironment of the anesthetic.

Mullins (9) emphasized the potential importance of solution theory (10, 11) as a means of estimating some properties of the site of anesthetic action and of predicting certain requirements of effective anesthetic molecules. Mullins suggested that the membrane interaction site for anesthetic molecules can be characterized by a measure of solvent cohesiveness, δ (12), of approximately 10 $(cal/cm^3)^{1/2}$. Lever *et al.* (13) and Miller *et al.* (14, 15), by examining deviations of a series of anesthetic gases from Meyer-Overton (1, 2) or Mullins correlations (16) in a series of solvents whose δ values varied from 6 to 10 $(cal/cm^3)^{1/2}$, concluded that the biological site of action of these anesthetic molecules has solvent properties which may be characterized by a δ value of 9.2–10.0. In contrast, Bennett *et al.* (17), utilizing the correlation reported by Koski *et al.* (18) between anesthetic potency and the van der Waals *a* constant, estimated a value of 4.3 $(cal/cm^3)^{1/2}$ for the primary site of anesthetic action. These methods of estimating the solvent properties of the site of anesthetic action are all necessarily indirect attempts to characterize the solvent nature of the membrane site of anesthetic action.

Nuclear magnetic resonance and electron spin resonance techniques have been particularly useful for evaluation of the membrane organization-related effects of anesthetics and other molecules as well as structural and dynamic processes within membranes (3, 19, 20). Such utility arises from sensitivity of the nuclei or electron to both the complex sum of environmental forces and the motional characteristics of the system.

Thus, under favorable circumstances, it should be feasible to assess directly the microenvironment experienced by the anesthetic molecule while interacting with the membrane, by employing nonperturbing measurements such as nuclear magnetic resonance. We report here the results of ^{19}F NMR measurements intended to assess the feasibility of establishing a nonperturbing measure of the microenvironment experienced by halothane (2-bromo,2-chloro,1,1,1-trifluoroethane) in a variety of solvents and in natural and artificial phospholipid bilayer membranes. Because it contains a trifluoromethyl group, halothane is readily amenable to studies employing NMR methodology (21) without necessity for the introduction of extrinsic labels either to the halothane molecule itself or to the membrane environment.

MATERIALS AND METHODS

N-Methylformamide (99%) and octane (Gold Label) were obtained from Aldrich Chemical Company. Prior to use, dioxane was passed through an alumina column (Brockman activity grade 1), after which the dioxane was mixed with deionized water. All other organic solvents and inorganic salts employed were of reagent grade and used without further purification. Long-chain alkanes (n = 9–16) were a gift of Dr. Mahendra K. Jain. Halothane without preservative was supplied by Ayerst Laboratories.

NMR tubes (10 mm) were obtained from Wilmad Glass Company, and prior to each use were cleaned in chromic acid cleaning solution, rinsed with deionized water, soaked in aqueous disodium EDTA, and rinsed again in deionized water. All ^{19}F chemical shifts are reported relative to external standards of 1% hexafluoroacetone in deuterium oxide sealed in glass capillary tubes, which were cleaned prior to each use in the same way as NMR tubes.

Except where noted, the ^{19}F NMR spectra were obtained on a Varian XL-100 Fourier transform NMR spectrometer equipped with multinuclear capacity, using a field of 94.1 MHz and typically a sweep width of 1000 Hz. Other measurements were made on a JEOL FX 60 NMR spectrometer, employing an external hexafluoroacetone reference run separately. Chemical shifts have not been corrected for bulk diamagnetic susceptibility effects, which are expected to be very small (see DISCUSSION).

To each NMR tube containing solvent

were added 10 μl of halothane, which on a volume basis would be 47.1 mM. The external standard capillary tube was introduced into the sample tube, the tube was immediately tightly capped, and the spectra of both were recorded simultaneously. A ^{19}F NMR spectrum was obtained for each phospholipid- or solvent-halothane mixture at 25°.

Crystalline, synthetic grade 1 β,γ-dipalmitoyl-DL-α-phosphatidylcholine (99%) was purchased from Sigma Chemical Company. The lipid was applied to an alumina column (activity grade 1), 50 g of alumina per gram of lipid, in alcohol-free chloroform. The column was washed, the lipid was eluted with a 30% methanol–70% chloroform mixture, and the solvent was rapidly removed from the lipid under vacuum. Purity of the phospholipid was verified by thin-layer chromatography. Phospholipid concentration was confirmed by the phosphorus determination method of Lowry and Tinsley (22). Bovine heart lecithin (lot T1196), egg lecithin (lot T1236), and bovine phosphatidylserine (lot T1323C) were obtained from Analabs and were used without further purification.

The buffer employed for liposome preparation was 2-(*N*-morpholino)ethanesulfonic acid (Sigma). The 5 mM 2-(*N*-morpholino)ethanesulfonic acid buffer solution was prepared with deionized water, and the pH was adjusted to 6.4 by addition of 1 N hydrochloric acid. Sodium chloride was added to the buffer to make the final buffer solution 0.1 M in sodium chloride.

Liposomes were prepared by adding an appropriate amount of buffer solution to the purified, dried phospholipid. Glass beads were added to this mixture, followed by rotation at 50° for 3 hr. This preparation yields a stable suspension of concentric multilayer vesicles of somewhat varying size.

The synthetic phosphatidylcholine-vesicle suspension contained 250 mg of phospholipid per milliliter (53.6 mM). The suspension of vesicles composed of a mixture of bovine or egg lecithin and phosphatidylserine contained 25 mg of phospholipid per milliliter.

The partition coefficient for the partitioning of halothane between aqueous buffer and synthetic dipalmitoylphosphatidylcholine multilayer vesicles was estimated employing integrated ^{19}F NMR spectra of a settled liposome-halothane mixture (0.145 M halothane and 0.34 M phospholipid in 21.5 ml), from which appropriate aliquots were withdrawn. At equilibrium at 5°, P, as defined by Seeman *et al.* (23), is 9. This result is consistent with the data of Katz and Diamond (24) for the partitioning of nonelectrolytes between dimyristoyllecithin and water.

RESULTS

^{19}F NMR chemical shifts for halothane in a variety of solvents (including a series of dioxane-water mixtures) are tabulated in Table 1. The solvent dielectric constant varied from 1.84 (pentane) to 182.4 (*N*-methylformamide).

^{19}F NMR spectra were obtained on samples of halothane added to a suspension of synthetic dipalmitoylphosphatidylcholine under conditions favoring substantial partitioning of the anesthetic into the phospholipid. For example, for 7 μl of halothane per 250 mg of dipalmitoylphosphatidylcholine in a 1-ml volume it can be calculated that at equilibrium at least 80% of the halothane is in the phospholipid phase. ^{19}F NMR spectra obtained after addition of 5 μl of halothane to 50 mg of dipalmitoylphosphatidylcholine or 7 μl of halothane to 250 mg of dipalmitoylphosphatidylcholine in 1-ml volumes revealed broad, unresolved resonances (94 Hz in the latter case) at approximately 6.5 and 6.4 ppm, respectively, relative to the reference signal. This position is upfield relative to the chemical shift of halothane in buffer alone.

Halothane added to bovine phosphatidylserine–bovine lecithin (1:1) vesicles in aqueous buffer suspension (3.75 μl of halothane per 25 mg of phospholipid) resulted in a single, unresolved signal at 6.49 ppm. Using a partition coefficient of 9, it can be calculated that 25% of the halothane in this system is partitioned into the phospholipid.

Experiments employing the JEOL FX 60 NMR spectrometer yielded similar results, although the absolute values of the chemical shifts were slightly different as a result of the separately run external stan-

TABLE 1

Halothane ^{19}F chemical shifts

Solvent	^{19}F chemical shift[a]	δ[b]	$(E - 1)/(2E + 1)$[c]
	ppm	*(cal/cm^3)$^{1/2}$*	
Methanol	4.667	14.5	0.48
Pentane	4.688	7.1	0.18
Diethyl ether	4.912	7.4	0.34
Heptane	4.980	7.4	0.19
Acetone	5.060	9.9	0.46
Cyclohexane	5.114	8.2	0.20
Octane	5.246	7.5	0.19
Nonane	5.251		
Decane	5.328	6.6	0.20
Dodecane	5.494	7.9	0.20
1-Butanol	5.558	11.4	0.46
Tetradecane	5.602		
Toluene	5.640	8.9	0.24
N-Methylformamide	5.640	16.1	0.5
Hexadecane	5.670	8.0	
Dioxane	5.732	10.0	0.21
Benzene	5.819	9.2	0.23
Dimethylformamide	5.918	12.1	0.48
Dioxane (80%, w/w)	5.934		0.43
Dioxane (60%, w/w)	6.167		0.47
Methylene chloride	6.211	9.8	0.42
Dioxane (50%, w/w)	6.272		0.48
Propylene glycol	6.396	12.6	0.48
Dioxane (30%, w/w)	6.464		0.49
1,2-dichloroethane	6.482	9.9	0.43
Dioxane (10%, w/w)	6.561		0.49
Aqueous MES[d] buffer (5 mM)	6.625		
Chloroform	6.635	9.2	0.36
Carbon tetrachloride	6.681	8.6	0.23
Water	6.700	23.4	0.49
Carbon disulfide	7.275	10.0	0.26
Ethylene bromide	8.061	10.2	0.36
Bromoform	8.898	10.5	
Methylene iodide	10.870	11.8	0.37
Aqueous DPC,[e] 25°C	6.9		
Aqueous DPC,[e] 50°C	7.4		
Aqueous PC:PS,[f] 25°C	6.5		

[a] Shifts are reported for the downfield peak of the halothane doublet.

[b] Values were taken from refs. 12, 25, and 26.

[c] Solvent dielectric constants E were obtained from ref. 27.

[d] 2-(*N*-Morpholino)ethanesulfonic acid.

[e] Synthetic dipalmitoylphosphatidylcholine multilayer vesicles. Shifts were obtained with a JEOL FX 60 NMR spectrometer (5 μl of halothane per 250 mg of phospholipid in a 1-ml volume).

[f] Bovine phosphatidylserine–bovine lecithin (1:1) vesicles in aqueous buffer (3.75 μl of halothane per 25 mg of phospholipid).

dard employed. These experiments are summarized as follows. At 30° the addition of 5 μl of halothane to 1 ml of synthetic dipalmitoylphosphatidylcholine vesicles (250 mg/ml) resulted in a broad signal (70 Hz) centered at 6.9 ppm from the external standard. At 50° the same sample showed a shift of 7.4 ppm (44 Hz). In aqueous buffer over this temperature range no alteration in chemical shift or spectrum morphology of the halothane ^{19}F signal was observed.

DISCUSSION

In Fig. 1 halothane ^{19}F NMR chemical shifts are plotted as a function of δ for the non-hydrogen-bonding solvents in Table 1. The positive correlation, determined by a linear least-squares fit to the data, is characterized by a slope of 0.76.

A δ value of 7.7 can be estimated for halothane using the reported latent heat of vaporization at its boiling point and the method of calculation described by Hildebrand *et al.* (12). The observed ^{19}F chemical shift of neat halothane, 5.31 ppm, indicates that, although it was not plotted, halothane itself falls on the plot in Fig. 1.

From Table 1 it is apparent that the observed fluorine chemical shifts of halothane in a variety of solvents reveal no significant correlation with solvent dielectric constant or Kirkwood's dielectric function (28, 29). The fluorine chemical shift of halothane in a series of *n*-alkanes (C_5 to C_{16}) reveals a distinct downfield shift, from 4.69 ppm ($n = 5$) to 5.70 ppm ($n = 16$), with increasing carbon chain length (Fig. 1).

The observed solvent shielding of the fluorine nuclei of halothane in principle contains contributions due to bulk magnetic susceptibility, van der Waals interactions, solvent magnetic anisotropy, effects of electric fields, and specific solute-solvent interactions. These potential solvent effects are considered below.

Bulk susceptibility corrections of ^{19}F shifts in solvent systems of the sort employed in this study are typically very small (30, 31). From the data of Abraham and Wileman (32) it is possible to calculate that for halothane, in the solvent systems employed in this study, errors of less than 10% in the most unfavorable cases (comparing *n*-pentane and carbon tetrachloride) result from ignoring bulk volume susceptibility corrections. Furthermore, the observed halothane ^{19}F NMR chemical shifts relative to external hexafluoroacetone for the six solvents which showed the smallest deviations from the line in Fig. 1 do not correlate with solvent diamagnetic susceptibilities.

Several anisotropic solvents, such as benzene, toluene, and carbon disulfide (33), show no significant deviations from the correlation shown in Fig. 1, although aromatic compounds (but not carbon disulfide) fall substantially above the line in a plot of solvent refractive index (27) vs. observed ^{19}F shift. From these observations and the determined solvent anisotropic contribution for a series of fluorine-containing molecules, we can conclude that solvent magnetic anisotropy contributes little to the observed solvent shift in the studies reported here. The contribution of solvent anisotropy to proton chemical shifts is also reported to be small (34).

The effect of an electric field on nuclear shielding is expected to be most important

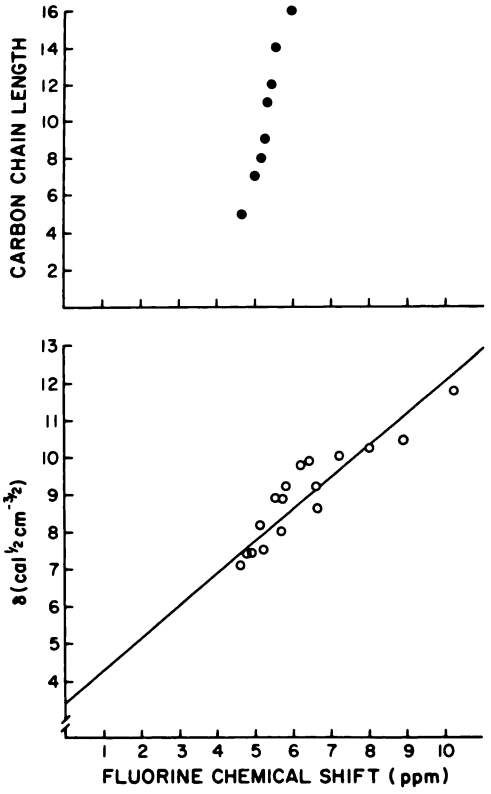

FIG. 1. *Halothane ^{19}F solvent chemical shifts*

Top: plot of carbon chain length of a series of n-alkanes vs. fluorine chemical shift (parts per million) of halothane (10 μl of halothane per 2 ml of solvent) relative to an external standard of 1% hexafluoroacetone in D_2O at 25°

Bottom: plot of Hildebrand's δ values for a series of non-hydrogen-bonding solvents (see Table 1) vs. fluorine chemical shift of halothane (10 μl of halothane per 2 ml of solvent) relative to an external standard of 1% hexafluoroacetone in D_2O at 25°.

for highly polar molecules in solvents of high dielectric constant and thus is likely to be relatively small in the solvent systems employed here. Evaluation of the contribution of this term to the observed solvent shifts is a matter of controversy with regard to the fluorine nucleus (33–35).

Terms involving van der Waals interactions and specific solvent-solute interactions are the most probable contributors to the fluorine chemical shifts reported here (36). In nonpolar solvents the observed correlation of fluorine chemical shifts with solvent polarizability (37) and solvent refractive index (38) strongly suggests a predominant role for London forces in the generation of the observed fluorine chemical shifts. Furthermore, the correlation of halothane ^{19}F chemical shifts with δ in non-hydrogen-bonding solvents suggests a dominant role of solvent-solvent forces in determining the observed shifts (12). Although van der Waals interactions are perhaps the most important contributors to fluorine solvent shifts, Abraham and Wileman (32) pointed out that the van der Waals term can be expressed as a product function of solute and solvent terms, which is consistent with the use of solvent parameters such as δ.

In addition to an increased electric field contribution to the observed fluorine chemical shifts in polar solvents (33), specific interactions between the solvent and the fluorine atoms of halothane are clearly important.

The summation of van der Waals, solute-solute, electrostatic solute-solute, and solute-solvent interactions characterizes the energy required to form a solvent cavity, E_{cav}, of sufficient size to contain the solute (39, 40) and interactions between the solute in its cavity with an average solvent entironment. E_{cav} can be estimated from bulk solvent properties via the solvent cohesive energy density function proposed by Hildebrand and Scott (10–12):

$$\delta = \left(\frac{-E}{V}\right)^{1/2} \quad (1)$$

where E is approximately the molal energy of vaporization of the gas phase at zero pressure and V is the molal volume of the liquid. Since δ is related to the $a/\bar{v}^2$ term in van der Waals' equation (41), and thus to dispersion properties such as refractive index, the observed correlations of halothane ^{19}F chemical shifts with δ and refractive index in non-hydrogen-bonding solvents suggest that the fluorine nucleus in this system is sensitive to such solvent-solvent interactions (42).

Use of δ explicitly assumes that no positive solute-solvent interactions are involved in the determination of fluorine chemical shifts (43). Such interactions are clearly present in many of the solvents we have investigated here (30, 44). Hydrogen-bonding interactions between solvent and halothane are strikingly reflected in Table 1.

Water and alcohols show positive deviations (not plotted) from the correlation shown in Fig. 1. *N*-Methylformamide and dimethylformamide also show positive deviations, which may be due to direct halothane-solvent associations (45). The effect of hydrogen bonding is to produce an upfield shift of the fluorine resonance, presumably as a result of deshielding of the fluorine nucleus because of interactions with the hydrogen-bonding proton. Therefore hydrogen bonding by solvent molecules must be ruled out in some manner before ^{19}F chemical shifts become useful diagnostics of membrane solvent properties.

The immobilization of anesthetics and related compounds has been noted in several NMR and ESR investigations of small molecule interactions with organized lipid states such as membranes and some membrane models (46, 47). The observed ^{19}F NMR chemical shift in the phosphatidylcholine bilayer is significantly downfield from that observed for halothane in isotropic hexadecane (see Fig. 1), consistent with the predicted organization of the hydrocarbon chains and head groups of the phospholipid (48).

Suspensions of synthetic dipalmitoylphosphatidylcholine in aqueous buffer undergo a gel-to-liquid crystalline phase transition at about 41°. The mixtures of natural phospholipids employed here are

considerably above their phase transition temperatures at 25°. The distribution of halothane in the bilayers reflects these intrinsic properties of lipid aggregate states and exerts an effect on them. These topics will be the subject of a subsequent communication; however, the arguments below can be considered to represent average properties of the halothane-phospholipid system.

Since the halothane in the aqueous phospholipid suspensions described here is exchanging between aqueous and membrane environments, the resulting chemical shifts will represent a weighted average of the shifts in the two environments. The results reported for halothane and synthetic dipalmitoylphosphatidylcholine represent a substantial contribution from membrane-bound anesthetic, while the signal arising from the bulk aqueous phase is of substantial importance in the results obtained employing natural lipids. However, since the partition coefficient for halothane between buffer and the natural phospholipids employed here was not determined, accurate distributions of halothane between buffer and lipid at the concentrations of lipid and halothane used here cannot be estimated. Since the equilibrium distribution of small molecules in membrane systems is a function of the aggregate state of the membrane and the concentration of the small molecule, it is not unlikely that the partition coefficient for halothane into the natural lipids will be higher than for halothane into synthetic dipalmitoylphosphatidylcholine.

The results of Diamond and Katz (49) suggest that nonelectrolytes (whose octanol/water partition coefficients are no greater than 13) partition into dimyristoyllecithin into sites whose solvent properties on the average resemble isoamyl alcohol. Halothane, whose octanol/water partition coefficient is 65, may penetrate even more deeply into the membrane interior. An apparent δ of 9.1 $(cal/cm^3)^{1/2}$ can be estimated from Fig. 1 to characterize the average membrane environment experienced by halothane at low halothane-to-phospholipid mole ratios. This value is presumably representative of halothane partitioned into the gel phase (see RESULTS). The apparent δ value estimated here for the membrane environment of halothane is substantially lower than that expected in highly ordered polymeric structures (26). At temperatures above 41°, the apparent δ value appears to increase by approximately 0.5 $(cal/cm^3)^{1/2}$. The chemical shift of halothane in all the lipids investigated was consistent with a δ value of 9.3 ± 0.3 $(cal/cm^3)^{1/2}$, even though the nature of the lipid aggregate state varied from the gel to liquid crystalline.

Several useful generalizations have emerged recently as a result of magnetic resonance studies on phospholipids (50). Considerable differences in the mobility of the terminal portion of the methylene chains of the phospholipids are observed in synthetic dipalmitoylphosphatidylcholine upon transition to the liquid crystalline state. Furthermore, comparison between fully saturated lecithins and the variably unsaturated, naturally occurring lecithins reveals greater sequential mobility differences, which are substantially smaller in the methylene carbons near the polar head groups than in the region near the terminal methyl groups. The constancy of the chemical shifts of halothane in the lipids reported here suggests that the halothane molecules may be largely distributed in the methylene carbons closer to the polar head groups than to the terminal methyl groups.

Recently reported studies of Trudell and Hubbell (51) involving the 19F NMR spectra of halothane in sonicated egg lecithin show a sharp doublet, whose chemical shift in the presence of phospholipid is somewhat farther upfield from the signal of halothane in water than is reported here. Relative to the chemical shift of halothane in water, we can estimate from the data of Trudell and Hubbell (51) that the apparent δ value of halothane in sonicated egg lecithin in aqueous suspension is slightly but significantly smaller than in unsonicated lipid systems. The looser organization of the phospholipid in the sonicated vesicles, as opposed to the more structured phospholipid of the unsonicated, multiple bilayer vesicles used in the

experiments reported here, may account for differences in linewidth. However, it is striking that although the chemical shift of halothane is a sensitive function of solvent intermolecular interactions, at the mole ratios of halothane to phospholipid employed in these experiments 19F chemical shifts do not appear to reflect significant structural transformations in the lipid multilayer assembly, such as the gel-to-liquid crystalline transition, the effect of introducing unsaturation, or the effects of sonication (resulting in single bilayer structures). These results suggest that these halothane molecules in the lipid are perturbant to the lipid packing in some manner which results in a local environment for halothane that is not significantly different in the various lipid states considered here (52). As a result, the microenvironment of halothane does not effectively change even when longer-range lipid associations are altered as a result of temperature, sonication, or chemical structure.

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