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Partitioning of four modern volatile general anesthetics into solvents that model buried amino acid side-chains

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Abstract

Partitioning of four modern inhalational anesthetics (halothane, isoflurane, enflurane, and sevoflurane) between the gas phase and nine organic solvents that model different amino acid side-chains and lipid membrane domains was performed in an effort to define which microenvironments present in proteins and lipid bilayers might be favored. Compared to a purely aliphatic environment (hexane), the presence of an aromatic-, alcohol-, thiol- or sulfide group on the solvent improved anesthetic partitioning, by factors of 1.3–5.2 for halothane, 1.7–5.6 for isoflurane, 1.7–7.6 for enflurane, and 1.5–7.3 for sevoflurane. The most favorable solvent for halothane partitioning was ethyl methyl sulfide, a model for methionine. Enflurane and isoflurane partitioned most extensively into methanol, a model for serine, and sevoflurane into ethanol, a model for threonine. Isoflurane also partitioned favorably into ethyl methyl sulfide. The results suggest that volatile general anesthetics interact better with partly polar groups, which are present on amino acids frequently found buried in the hydrophobic core of proteins, compared to purely aliphatic side-chains. Furthermore, if an anesthetic molecule was located in a saturated region of a phospholipid bilayer membrane, there would be an energetically favorable driving force for it to move into several higher dielectric microenvironments present on membrane proteins. The results provide evidence that proteins rather than lipids are the likely targets of volatile general anesthetics in biological membranes.

Keywords: Solvation; General anesthetic; Amino acids; Protein; Lipid

Abbreviations: HPLC, high performance liquid chromatography; $\Delta\Delta G$, change in the transfer free energy

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1. Introduction

The site of action of the volatile general anesthetics remains undefined, despite extensive studies over several decades [1]. The current lack of understanding in this field follows from (i) the relative weakness of the interactions between the volatile general anesthetics and macromolecules (with dissociation constants in the millimolar range); and (ii) the fact that techniques to *directly* study the binding of volatile anesthetics to proteins have only recently been introduced [2–4]. Nevertheless, current consensus favors membrane proteins that function as ion channels and neurotransmitter receptors as the targets for the volatile general anesthetics [5], despite an almost complete lack of direct evidence for this. Indirect support comes from studies showing that volatile general anesthetics alter the *activity* of a number of membrane proteins such as voltage-gated [6] and ligand-gated ion channels [7]. However, studies that address the effect of anesthetics on the function of membrane proteins are unable to distinguish between direct effects on proteins vs. indirect effects resulting from changes in lipid bilayer properties. Support for direct effects has been provided by recent photoaffinity labeling studies indicating that halothane interacts directly with the nicotinic acetylcholine receptor from *Torpedo nobiliana* [8], and the Ca^{2+}-ATPase from skeletal muscle sarcoplasmic reticulum [9].

What factors determine the binding of general anesthetics to a macromolecular target? This question has been addressed by linear solvation analysis correlating the potency of 32 general anesthetics in mice with their octanol–water partition coefficients and the presence or absence of polar hydrogen atoms [10]. A polar hydrogen atom is attached to a carbon atom that is covalently bound to an electronegative oxygen or halogen. Molecules with polar hydrogens are approximately 80 times more potent as anesthetics than those lacking this function. These authors concluded that there is an important polar component to the site of anesthetic action.

Examining principally the *n*-alkanes and *n*-alcohols and correlating animal anesthetic potency with hydrogen-bonding ability, dipole moment, and molecular volume, it was found that molecular volume (a measure of the hydrophobic effect) was most important for anesthetic potency [11]. In addition, the ability to accept a hydrogen bond decreased potency. Lecithin was shown to be a better model of the anesthetic site than *n*-octanol, when the modern clinical anesthetics (halothane, isoflurane, enflurane, desflurane, and sevoflurane) along with cyclopropane and fluoroxene were examined [12].

In order to better understand the energetics and molecular interactions that underlie protein–anesthetic complexation, the partitioning of four volatile general anesthetics (Fig. 1) between the gas phase and nine solvents that model amino acid side-chains that are generally

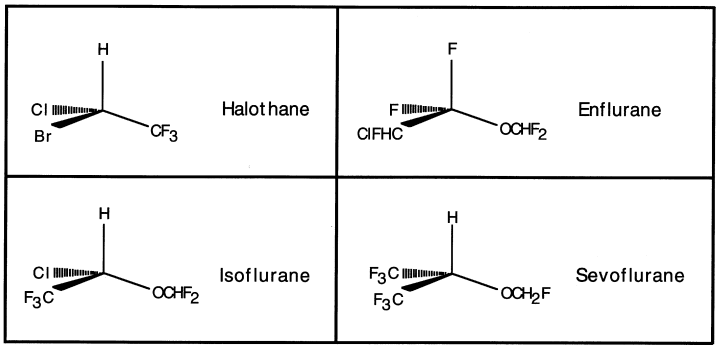


Fig. 1. Chemical structures of the four volatile general anesthetics examined. Halothane is a halogenated alkane. Isoflurane, enflurane and sevoflurane are halogenated ethers. Halothane and isoflurane are represented as being composed of a single optical isomer, although a 50:50 mixture of the D and L enantiomers was used.

found buried in proteins was examined. It is clear that using isotropic solvents to model anisotropic structures such as proteins and lipid membranes may only provide approximate interaction energies. Nevertheless, the use of octanol as a model for biological membranes has seen widespread use in the arena of medicinal chemistry, and has provided a framework for detailed quantitative structure-activity relationships (QSARs) [13].

2. Materials and methods

2.1. Materials

Halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) was purchased from Halocarbon Laboratories (Hackensack, NJ, USA). The thymol preservative in the commercial halothane was removed with an aluminum oxide column [14]. Isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether) was purchased from Ohmeda PPD Inc (Liberty Corner, NJ, USA). Enflurane (2-chloro-1,1,2-trifluoroethyl difluoromethyl ether) was obtained from Anaquest (Madison, WI, USA), and sevoflurane (fluoromethyl 2,2,2-trifluoro-1-(trifluoromethyl) ethyl ether) from Abbott Laboratories (North Chicago, IL, USA). Toluene (99.8%, HPLC grade) and *n*-hexane (99 + %, capillary GC grade) were from Sigma Chemical Co (St Louis, MO, USA). Benzene (99.8%), 1-hexene (99 + %), pyrrole (98%), 1-propanethiol (99%), and ethyl methyl sulfide (99%) were from Aldrich Chemical Co (Milwaukee, WI, USA). Methanol (99.9 + %, HPLC grade) was from Fisher Scientific (Fairlane, NJ, USA), and ethanol (dehydrated, 200 proof) was from Pharmco Products, Inc (Bayonne, NJ, USA).

2.2. Determination of solvent / gas partition coefficients and transfer free energies

Partition coefficients were determined essentially as described [15] using a 228-ml round-bottom flask that had three openings sealed with aluminum foil-covered corks. Aliquots (5–25 μl) of liquid anesthetic were introduced and the system resealed. The flask was placed in a constant temperature incubator (Model 12-140, Quincy Labs, Chicago, IL, USA) at 25°C. After anesthetic vaporization, a sample of gas (1 ml) was withdrawn and analyzed by gas chromatography using an HP 6890 Series instrument (Hewlett Packard, Wilmington, DE, USA), with a 30-m 5% phenyl substituted methylpolysiloxane column (internal diameter of 0.32 mm). The oven temperature was 80°C, with the electron capture detector operating at 350°C. Organic solvent was added and the system allowed to equilibrate with continuous stirring for 30–40 min. A second gas sample was then withdrawn and analyzed by gas chromatography. The partition coefficient was calculated using a standard conservation of matter relationship:

$$C_{g1} \cdot V_{g1} = C_{g2} \cdot V_{g2} + C_s \cdot V_s, \quad (1)$$

where C_{g1} and C_{g2} are the measured gas phase anesthetic concentrations before and after solvent addition, respectively, V_{g1} and V_{g2} are the initial and final gas phase volumes, V_s is the volume of added organic solvent, and C_s is the calculated concentration of anesthetic in the solvent at equilibrium. The anesthetic concentrations correspond to the densities in the gas and solvent phases. This approach assumes that there is minimal loss of anesthetic from the system during the equilibration period. To verify this, HPLC experiments to directly calculate C_s for selected solvents (methanol, ethanol and hexane) were carried out for halothane, which has a measurable extinction coefficient at 240 nm because of its C—Br bond. Loss of anesthetic from the flask was on the order of 1–2% per hour, and was therefore considered to be within the experimental error of the technique.

The transfer free energies of the anesthetics between the gas (g) and solvent (s) phases was calculated as

$$\Delta G_{g \rightarrow s} = -RT \ln(C_s / C_{g2}), \quad (2)$$

where R is the gas constant (1.987 cal/mol K), T is the temperature in kelvin, and C_s and C_{g2} are as defined above. The ratio C_s/C_{g2} represents the solvent/gas partition coefficient. Partition coefficients were determined between three and 10

times for each individual anesthetic–solvent combination. Data are expressed as mean ± S.D.

2.3. Selection of organic solvents

Methanol, ethanol, toluene, and ethyl methyl sulfide are the accepted models for the side-chains of serine, threonine, phenylalanine, and methionine, respectively [16]. The remaining solvents represent compromises, necessitated by the requirement for a liquid phase at room temperature. Thus, 1-propanethiol is the solvent selected to model cysteine because methylthiol is a gas at room temperature. Similarly, *n*-hexane was selected as an overall model of the aliphatic amino acids alanine, valine, leucine and isoleucine, because methane, propane, 2-methylpropane, and butane are gases under ambient conditions. Because methylindole and *p*-cresole are solids at room temperature, it was necessary to select alternative molecules as models for the tryptophan and tyrosine side-chains. Therefore the interactions of the anesthetics with the tryptophan and tyrosine side-chains were examined using benzene, toluene and pyrrole. Fig. 2 shows the solvent structures and the amino acid side-chains that they represent.

3. Results

The partition coefficients for the four volatile general anesthetics halothane, isoflurane, enflurane, and sevoflurane between the gas phase and the nine organic solvents are given in Table 1. For all four anesthetics, partitioning was found to be more favorable into the solvents that have either aromatic character, or contain an alcohol-, thiol-, or sulfide group, compared with the purely aliphatic solvent *n*-hexane.

The unsaturated solvent 1-hexene also provided a more favorable environment compared to *n*-hexane for all four anesthetics studied. The ether-containing anesthetics (isoflurane, enflu-

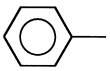
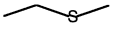
Solvent	Chemical structure	Microenvironment modeled
n-Hexane		Aliphatic - A, V, L, I
1-Hexene		Unsaturated acyl chain
Benzene		Aromatic - F, Y, W
Toluene		Aromatic - F
Pyrrole		Aromatic - W, Heme
Methanol		Serine
Ethanol		Threonine
Ethyl methyl sulfide		Methionine
1-Propanethiol		Cysteine

Fig. 2. Chemical structures of the solvents used and the biological membrane microenvironments that they model. Amino acids are identified using the standard single letter abbreviations.

Table 1
Solvent/gas partition coefficients for the four volatile general anesthetics for nine different solvents[a]

Solvent	Halothane	Isoflurane	Enflurane	Sevoflurane
n-Hexane	316 ± 22 ($n = 3$)	149 ± 4 ($n = 3$)	168 ± 6 ($n = 5$)	106 ± 6 ($n = 5$)
1-Hexene	618 ± 28 ($n = 5$)	258 ± 10 ($n = 5$)	329 ± 12 ($n = 6$)	156 ± 5 ($n = 5$)
Benzene	632 ± 202 ($n = 8$)	445 ± 55 ($n = 3$)	692 ± 18 ($n = 5$)	307 ± 7 ($n = 5$)
Toluene	870 ± 18 ($n = 4$)	408 ± 40 ($n = 5$)	646 ± 16 ($n = 5$)	311 ± 8 ($n = 6$)
Pyrrole	527 ± 19 ($n = 5$)	302 ± 4 ($n = 5$)	541 ± 12 ($n = 5$)	296 ± 4 ($n = 6$)
Methanol	897 ± 43 ($n = 3$)	814 ± 33 ($n = 6$)	1275 ± 37 ($n = 6$)	657 ± 16 ($n = 6$)
Ethanol	629 ± 75 ($n = 7$)	409 ± 47 ($n = 6$)	556 ± 27 ($n = 6$)	775 ± 12 ($n = 5$)
1-Propanethiol	423 ± 77 ($n = 6$)	384 ± 21 ($n = 5$)	504 ± 3 ($n = 5$)	248 ± 25 ($n = 5$)
Ethyl methyl sulfide	1656 ± 35 ($n = 6$)	835 ± 52 ($n = 5$)	863 ± 20 ($n = 5$)	453 ± 13 ($n = 5$)

[a] For each case, the partition coefficient is given followed by *n,* the number of experiments. The errors are S.D.

rane, and sevoflurane) interacted better with the solvents having either aromatic character, or containing an alcohol-, thiol-, or sulfide group, than with 1-hexene. This was also generally true in the case of halothane, with the exceptions of partitioning into pyrrole and 1-propanethiol.

Compared to a purely aliphatic environment (*n*-hexane), the presence of an aromatic-, alcohol-, thiol- or sulfide group improved anesthetic partitioning, by factors of 1.3–5.2 for halothane, 1.7–5.6 for isoflurane, 1.7–7.6 for enflurane, and 1.5–7.3 for sevoflurane. As shown in Fig. 3, the most favorable environment for halothane was ethyl methyl sulfide, a model for methionine. Fig. 4 shows that isoflurane partitioned to the greatest extent into methanol, a model for serine, and also into ethyl methyl sulfide, a model for methionine. Enflurane also partitioned best into methanol, a model for serine, as shown in Fig. 5, followed by ethyl methyl sulfide, a model for methionine. Fig. 6 shows that sevoflurane partitioned most extensively into methanol and ethanol, models for serine and threonine, respectively, followed by ethyl methyl sulfide, a model for methionine. Compared to both *n*-hexane and 1-hexene, isoflurane, enflurane, and sevoflurane partitioned better into solvents with either aromatic character, or into those containing an alcohol-, thiol-, or sulfide group.

In comparison with *n*-hexane the results suggest that volatile general anesthetics interact more favorably with somewhat polar groups, which are present on amino acids generally found buried in the hydrophobic core of proteins, such as phenylalanine, tyrosine, tryptophan, cysteine and methionine [17]. In addition, the results reveal that volatile general anesthetics are predicted to interact quite favorably with serine and threonine

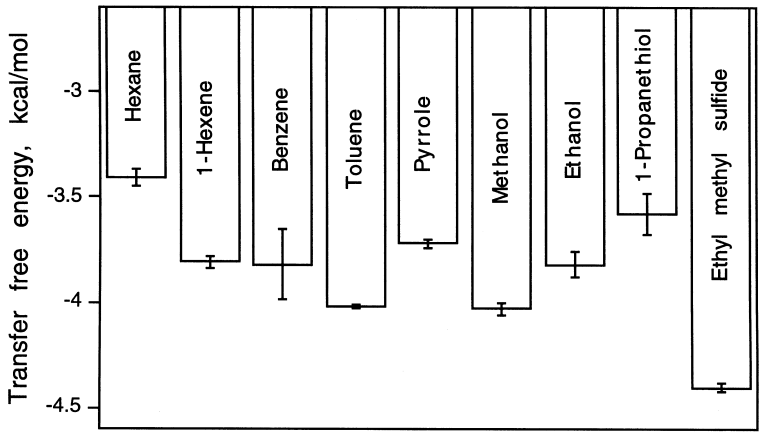


Fig. 3. Transfer free energies for halothane between the gas and nine solvent phases that model various microenvironments present in biological membranes. Error bars are S.D.

side-chains which may be found either buried in the hydrophobic cores of proteins, or exposed to the aqueous environment [17].

4. Discussion

Because the site of action of the volatile general anesthetics is unknown, investigations into the molecular features of the in vivo targets have been confined to correlations of whole animal potency data with anesthetic partitioning into model solvents. The solvents that have been examined include olive oil, *n*-octanol, hexadecane and lecithin. The solvents that best approximate the whole animal site of action are *n*-octanol [10,18] and lecithin [12]. The conclusion to be drawn from these studies is that the in vivo site is hydrophobic in character but also has an important integral polar component. This polar feature has been interpreted in terms of the hydrogen bond donor and acceptor potential of the anesthetics, attributed to the presence of 'acidic' hydrogens (as donors) and ether oxygens (as acceptors). According to this view, anesthetics act by competing for hydrogen bonds with native protein

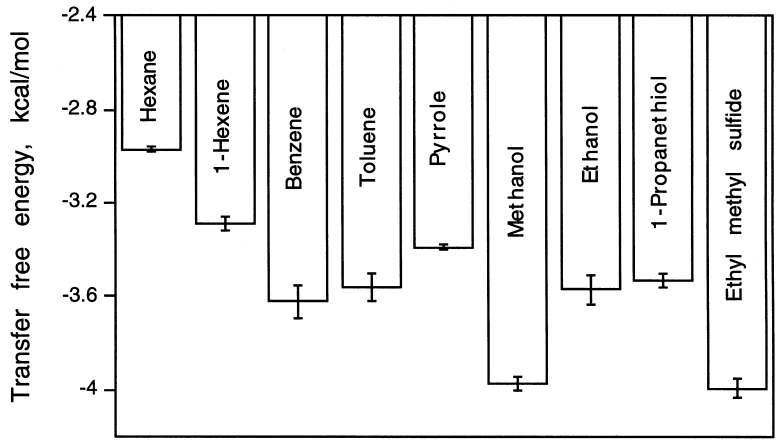


Fig. 4. Transfer free energies for isoflurane between the gas and nine solvent phases that model various microenvironments present in biological membranes. Error bars are S.D.

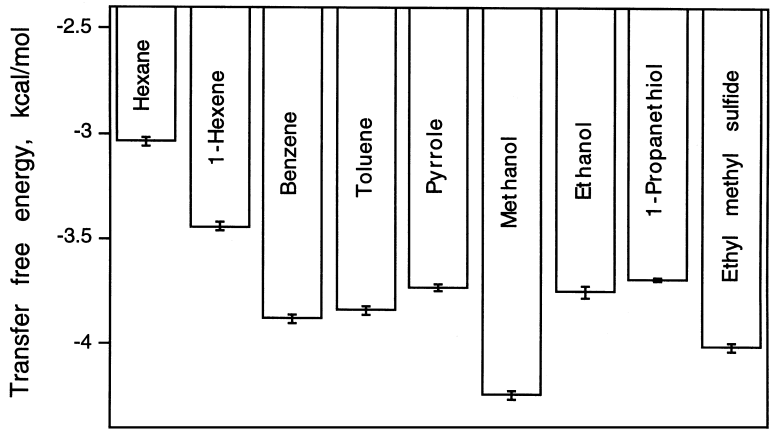


Fig. 5. Transfer free energies for enflurane between the gas and nine solvent phases that model various microenvironments present in biological membranes. Error bars are S.D.

hydrogen bond partners, and thereby alter protein function.

The current study explores the interaction energies between four modern volatile general anesthetics and nine bulk solvents that model the side-chains of amino acids generally found buried in the interior of proteins. The goal was to define how different microenvironments present in biological membranes interact energetically with the volatile general anesthetics. This is in contrast to the earlier solvation studies which sought to define which single solvent best correlated with whole animal anesthetic potency data [10,12,18]. The overall finding is that there are a number of amino acid side-chains including the aromatic (phenylalanine, tyrosine, and tryptophan), alcohols (serine and threonine), and the sulfur-containing residues (cysteine and methionine) that are predicted to provide more favorable interaction energies with the four volatile general anesthetics as compared to the aliphatic amino acid residues (alanine, valine, leucine, and isoleucine).

In the present study, *n*-hexane was used as an example of a neutral apolar environment. This solvent serves as an overall model for the aliphatic amino acid side-chains of alanine, valine, leucine,

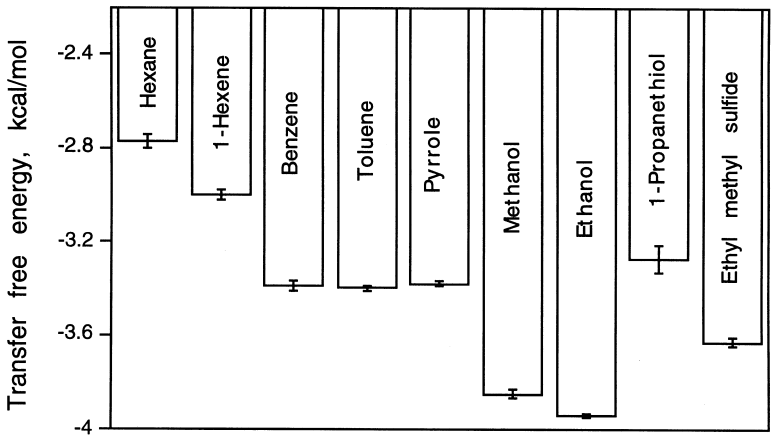


Fig. 6. Transfer free energies for sevoflurane between the gas and nine solvent phases that model various microenvironments present in biological membranes. Error bars are S.D.

and isoleucine, and also for the saturated domains of the phospholipid acyl chains. The solvent/gas partition coefficient determined for halothane is comparable to that reported for hexadecane [11], and somewhat greater than that reported for a saturated bilayer composed of dilaurylphosphatidylcholine [19]. Partitioning into lipid bilayers is predicted to be less extensive as compared to isotropic solvents because of the entropic penalty that limits anesthetic mobility in the bilayer. The results with *n*-hexane are in agreement with many prior studies indicating that a hydrophobic environment is the primary feature determining volatile general anesthetic interactions with biological systems [10,11]. This follows from the present results because the free energy of transfer from the gas phase to water is close to zero for all four volatile general anesthetics examined [20]. Therefore, approximately 3 kcal/mol of binding energy can be attributed to the hydrophobic effect, when a volatile general anesthetic (halothane, isoflurane, enflurane, or sevoflurane) binds to a target molecule, corresponding to a K_d of 6.3 mM for a single binding site, at 25°C (where $\Delta G = RT \ln K_d$).

In general, the anesthetics that contain an ether group (isoflurane, enflurane, and sevoflurane) are found to partition well into the solvents containing the alcohol groups methanol and ethanol, models for serine and threonine, respectively. This suggests that these anesthetics are able to form hydrogen bonds as acceptors. There is also the potential that they may be forming hydrogen bonds as donors, since they contain two (isoflurane and enflurane) to three (sevoflurane) acidic (or polar) hydrogens (Fig. 1). In addition, the chlorine atom present on isoflurane and enflurane is predicted to be able to act as a hydrogen bond acceptor [21]. In this context, it is of interest that serine residues in the transmembrane domains of the $GABA_A$-receptor α_1 subunit have been suggested to be crucial for enflurane binding based upon site-directed mutagenesis experiments [7]. In addition, all three of the ether-containing anesthetics partitioned well into ethyl methyl sulfide.

The halogenated alkane halothane partitions most favorably into ethyl methyl sulfide which serves as a model for methionine. This is in good agreement with the work showing that binding of halothane to the hydrophobic core of a four-α-helix bundle scaffold is improved by a factor of 3.5 when a leucine residue in the vicinity of the binding pocket is replaced by a methionine [22]. The sulfur atom of methionine is postulated to be a hydrogen bond acceptor [23], but may also serve as an electron acceptor [24]. The partition coefficients for halothane between methanol and ethanol and the gas phase are, however, comparable to those for the ether anesthetics, indicating that halothane is able to form weak hydrogen bonding interactions with the alcohol groups, either as an acceptor or as a donor.

The aromatic solvents benzene, toluene, and pyrrole provide better environments for all four general anesthetics as compared to the aliphatic *n*-hexane. The values of the solvent/gas partition coefficients for halothane, isoflurane, enflurane, and sevoflurane are comparable to those reported for benzene at 37°C previously [12]. This suggests that aromatic residues such as phenylalanine, tyrosine, and tryptophan are likely to engage in favorable interactions with volatile general anesthetics. This is in accord with experimental work on proteins such as bovine serum albumin [4,25,26], haloalkane dehalogenase [27], dichloromethane dehalogenase [28], and synthetic four-α-helix bundle proteins [29–31] that reveals that halogenated alkane anesthetics localize in the vicinity of aromatic residues. Crystal structures for haloalkane dehalogenase, dichloromethane dehalogenase, and hemoglobin indicate that both chlorine and bromine can act as hydrogen bond acceptors with aromatic ring hydrogens [27,28,32]. In addition, the relatively polarizable chlorine and bromine atoms may act as electron acceptors in combination with oxygen and nitrogen atoms, and with aromatic rings [33]. The finding that pyrrole provides a more favorable environment than *n*-hexane also suggests that heme groups would interact favorably with volatile general anesthetics, as previously shown for the inhalational anesthetic xenon [34].

Cysteine is predicted to interact favorably with all four volatile general anesthetics based upon the partitioning data using 1-propanethiol. The

ether anesthetics (isoflurane, enflurane, and sevoflurane) interact most favorably with 1-propanethiol, compared to *n*-hexane, with $\Delta\Delta G = -0.5$–0.6 kcal/mol. However, halothane also partitioned to a greater extent into 1-propanethiol compared with *n*-hexane, by a $\Delta\Delta G = -0.17$ kcal/mol. Reduced cysteine residues are common in buried hydrophobic regions of proteins [17] and can act as hydrogen bond donors and acceptors [35,36]. It is predicted that cysteine residues would interact favorably with the halogens, ether oxygens, and acidic hydrogens on the anesthetic molecules. Perhaps the more favorable interaction with the ether anesthetics compared to halothane is due to the oxygen acting as a hydrogen-bond acceptor.

The unsaturated solvent 1-hexene allowed for greater partitioning of all four volatile general anesthetics as compared to *n*-hexane. This suggests that unsaturated regions of lipid membranes will provide more suitable environments for these compounds than do the saturated portions. Biological membranes are typically highly unsaturated, a feature that is known to influence properties such as the phase transition temperature and the overall bilayer thickness [37–39]. Nevertheless, for all four anesthetics, the solvents that model somewhat polar amino acid side-chains were able to accommodate anesthetics in an energetically more favorable manner than either *n*-hexane or 1-hexene. The only exceptions to this general finding were for halothane partitioning into pyrrole and 1-propanethiol, which were both somewhat less favorable than for 1-hexene by 0.1 and 0.2 kcal/mol, respectively.

It is concluded that there are a number of amino acid side-chains including the aromatic-, alcohol-, and the sulfur-containing residues that provide more favorable interaction energies with the four volatile general anesthetics as compared to the aliphatic amino acid residues. In addition, compared to partitioning into *n*-hexane and 1-hexene, the results with the remaining more polar solvents suggest that from a purely energetic standpoint, modern clinical volatile general anesthetics prefer to interact with protein rather than with lipid. These findings lend support to the argument that proteins [18,40] rather than lipids are the likely targets of volatile general anesthetics in vivo.

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