Alcohol Binding to Liposomes by ^2H NMR and Radiolabel Binding Assays: Does Partitioning Describe Binding?

Anil K. Dubey, Vladimir A. Eryomin, Theodore F. Taraschi, and Nathan Janes
Department of Pathology, Anatomy, and Cell Biology, Medical College of Thomas Jefferson University, 1020 Locust Street, Philadelphia, Pennsylvania 19107

ABSTRACT Implicit within the concept of membrane-buffer partition coefficients of solutes is a nonspecific solvation mechanism of solute binding. However, ^2H NMR studies of the binding of $^2H_6$-ethanol and [1-$^2H_2$] n-hexanol to phosphatidylcholine vesicles have been interpreted as evidence for two distinct alcohol binding modes. One binding mode was reported to be at the membrane surface. The second mode was reported to be within the bilayer interior. An examination of the ^2H NMR binding studies, together with direct radiolabel binding assays, shows that other interpretations of the data are more plausible. The results are entirely consistent with partitioning (nonspecific binding) as the sole mode of alcohol binding to liposomes, in accord with our previous thermodynamic interpretation of alcohol action in phosphatidylcholine liposomes [Janes et al., (1992) *Biochemistry* 31, 9467–9472].

INTRODUCTION

The binding of lipophilic solutes to membranes is traditionally described in terms of an agent's "lipid solubility," implying a nonspecific dissolution mechanism of binding, and it is expressed in those terms as a "partition coefficient" (Seeman, 1972; Janoff et al., 1981; Taheri et al., 1991). This view has been challenged by studies that implicate distinct surface and core binding modalities for ethanol (Kreishman et al., 1985) and hexanol (Fraser et al., 1991). This paper critically examines the evidence presented for the two-site surface/core binding model, and asks whether the partition coefficient, an inherent measure of nonspecific solute solvation, remains an appropriate formulation.

The term "lipid solubility" was developed by early workers to describe the pharmacological action of nonspecific lipophilic solutes (Meyer, 1899; Overton, 1901). These workers used olive oil as a model for cellular lipids and correlated solute action with oil solubility using terminology appropriate for bulk substances. Subsequent studies have shown that the partitioning of solutes into selected-bulk, three-dimensional solvents correlates well with the binding of solutes to two-dimensional membrane solvents (Seeman, 1972; Jain and Wray, 1978; Hill, 1975; Janoff et al., 1981) and yields similar correlations with biological endpoints (Seeman, 1972; McCreery and Hunt, 1978; Lyon et al., 1981; Janoff et al., 1981; Janoff and Miller, 1982; Taheri et al., 1991).

Classical thermodynamic treatments developed for solute/solvent interactions in bulk solutions have been applied widely to solute/membrane interactions using a single nonspecific solvation mechanism as the energetic source of solute action on phase equilibria (Hill, 1975; Rowe, 1981; Kamaya et al., 1981; Rowe, 1982). Subsequently, the use of colligative thermodynamics of bulk substances was validated in membranes by simultaneous determination of alcohol binding and action (Janes et al., 1992; Ma et al., 1992; Janes, 1995). Similarly, the effects of pressure and solutes on membranes have been modeled using thermodynamics developed for bulk substances (Tamura et al., 1991; Kaminoh et al., 1991). These studies indicate that the activity of alcohol within the membrane is homogeneous. From this standpoint, adsorptive binding to interfacial sites must be small, as all of the bound alcohol acts energetically like solubilized or partitioned alcohol.

Yet, membranes differ dramatically from bulk fluid oils (Smith and Oldfield, 1984). They are structurally and dynamically anisotropic, with a character intermediate between isotropic fluid and anisotropic solid bulk phases. Their polarity as solvents is depth dependent. They possess well-defined polar interfaces. Solutes are not distributed uniformly throughout the membrane, but exhibit a concentration profile dependent on the chemical makeup of the solute, according to the maxim "likes dissolve likes." Alkanes prefer the hydrocarbon interior of the bilayer (Simon et al., 1977; White et al., 1981); normal alcohols are tethered in the polar headgroup region by their hydroxyl group, whereas their chains intercalate within the lipid acyl chains with anisotropic motional restrictions similar to that of the acyl chains (Thewalt et al., 1986; Westerman et al., 1988); and charged lipophiles tend to situate their charges among the lipid charges in the headgroup region and their hydro-

Received for publication 4 December 1995 and in final form 5 February 1996.

Address reprint requests to Dr. Nathan Janes, Department of Pathology, Anatomy and Cell Biology, Medical College of Thomas Jefferson University, 271 Jeff Hall, 1020 Locust Street, Philadelphia, PA 19107. Tel.: 215-955-1174; Fax: 215-923-2218; E-mail: janesn@jeflin.tju.edu.

Dr. Eryomin's permanent address is: A.N. Bakh Institute of Biochemistry, Russian Academy of Sciences, Leninsky Prospekt 33, Moscow, 117071 Russia

Abbreviations: Pam_2PtdCho: 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; Myr_2PtdCho: 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; MLV: multilamellar vesicles; SUV: small unilamellar vesicles; NMR: nuclear magnetic resonance; mf %: mole fraction percent; Kp: partition coefficient.

0006-3495/96/05/2307/09 $2.00

phobic portions within the chain region (Herbette et al., 1985; Young et al., 1992).

As a result, regular solution theory—which describes the energetics of bulk solutions—requires modification for proper employment in membrane semisolids (Simon et al., 1979; Katz, 1988). Similarly, comparisons of the enthalpy and entropy of solute transfer (the energetic basis of partitioning) indicate differences between two and three-dimensional solutions (e.g., Seelig and Ganz, 1991; Wimley and White, 1993). Despite these differences, the energetics were analyzed in terms of a single nonspecific solute binding modality for both bulk fluids and membranes.

Statistical models treat the membrane as a composite solvent wherein a depth-dependent and surface-density-dependent summation of microscopic solvent character best describes the solubilization process (e.g., Marqusee and Dill, 1986). This model, however, does predict distinct core and surface binding modalities for some solutes. The concentration dependence of solute binding in gel-state membranes that was tentatively interpreted as supportive of an interfacial mechanism of binding (De Young and Dill, 1988), however, probably arises from the appearance of the ripple state, which is greatly stabilized at higher solute levels (Janes et al., 1992; Ma et al., 1992; Wang et al., 1993).

Given the parallels and the discrepancies between the treatment of solute binding with two-dimensional and three-dimensional substances, it was of interest to further explore those cases of solute binding to membranes where both interfacial and core modalities have been reported. Ethanol and hexanol were reported to exhibit distinct surface and core binding sites in lipid membranes based on ^2H NMR spectroscopic studies of the binding of the deuterated alcohols (Kreishman et al., 1985; Kreishman et al., 1986; Fraser et al., 1991). This paper critically examines this interpretation.

MATERIALS AND METHODS

Synthesis of [1-$^2H_2$] n-hexanol

Hexanol, specifically deuterated in the α-position, was prepared from hexanoic acid by reduction (Brown and Subba Rao, 1956). Anhydrous diethyl ether (125 ml; Fisher, Pittsburgh, PA) containing 2.4 g (57 mM) lithium aluminum deuteride (Aldrich, Milwaukee, WI) was placed in a two-neck, round-bottom flask with a reflux condenser, dropping funnel, and drying tubes. An ether solution (70 ml) containing 9.5 g (81 mM) hexanoic acid (Aldrich, Milwaukee, WI) was slowly added with magnetic stirring and cooling to produce a gentle reflux. The reaction was stopped after 6 h with slow addition of water, followed by addition of 150 ml of 10% sulfuric acid solution. The contents were transferred to a separation funnel and sequentially extracted with ether, neutralized with saturated aqueous sodium bicarbonate, reextracted with ether, and distilled at ambient pressure with collection of the fraction boiling at 156.5°C for a yield of ~50%. The product purity was assessed by high-resolution ^1H NMR. Protonation at the α-position was estimated at 1.2%, and unreacted hexanoic acid was less than 0.5%.

Preparation of liposomes

Phospholipids, stored in chloroform (Avanti Polar Lipids, Alabaster, AL) and assayed for purity by thin layer chromatography (>99%), were dried to a thin film under dry N_2, evacuated overnight (< 5 mTorr), and hydrated by vigorous vortexing. For the high-resolution studies of hexanol-d_2, liposomes were hydrated with Tris-buffered saline (150 mM NaCl, 10 mM Tris, pH 7.4). The buffer contained 3 mM sodium acetate-d_3 as an internal standard and [1-$^2H_2$] n-hexanol. Samples were incubated for >3 h at 30°C to ensure solute equilibration. The supernatant was isolated from the multilamellar suspension by centrifugation (Sorvall RC-5B centrifuge) at 30°C for 1 h at 1900 g. For the wide-line study of hexanol-d_2, liposomes (500 mg/ml) were hydrated using ^2H-depleted water containing 150 mM KCl and hexanol-d_2 (30 mf %).

Sonicated vesicles were prepared by sonication of the multilamellar vesicles to optical clarity at ~30°C using a MedSonic W-225 sonicator (Misonix, Farmingdale, NY) operating at 20 kHz and a power setting of 3 with a 3.2-mm microtip for 3 min intervals followed by 3–4 min pauses to prevent undue heating. Metal particles and any remaining multilamellar vesicles were removed by high speed centrifugation. The concentration of the vesicle suspension was determined from the total phosphorus according to Bartlett (1959).

Liposomes for the experiments with ethanol were prepared in a similar manner as those for the hexanol experiments with the following exceptions. The buffer contained 100 mM sodium acetate-d_3 and ethanol-d_6 (Cambridge Isotope Laboratories, Andover, MA). Samples were equilibrated for >3 h at 46°C, excepting gel-state ($L_{\beta'}$) samples that were allowed to equilibrate at 4°C overnight. The samples were prepared in constricted 5-mm NMR tubes and flame-sealed to prevent the evaporation of ethanol, which was observed in conventionally capped buffer blanks during equilibration.

Nuclear magnetic resonance

Fully relaxed spectra were obtained on a Bruker (Billerica, MA) widebore 8.5 T spectrometer operating at 55.3 MHz using the ^2H channel of a 5-mm high-resolution probe without sample rotation. High-resolution experiments with hexanol were obtained as Bloch decays under the following typical conditions: 30° flip angle, 1.7-s interpulse spacing, 600-Hz spectral window, 2-K data points zero filled to 8 K, 2-Hz exponential filter, and 34,000 transients. The linewidths reported are corrected for the broadening due to the exponential filter. Wideline spectra with hexanol were obtained as Bloch decays under the following conditions: 4° flip angle, 100-ms interpulse spacing, 83-kHz spectral window, 16-K data points, and 200-Hz exponential filter.

Experiments with ethanol and membranes were obtained as Bloch decays with a 3° flip angle, an 83-kHz spectral window, 0.6-s interpulse spacing, 32-K data points zero filled to 128 K, a 5-Hz exponential filter, and 10,000 transients. The flame-sealed 5-mm tubes were 8 cm in length, and the sample was centered within the limits of the detection coil without the use of a spinner. Experiments with ethanol and castor oil were obtained with a 30° flip angle, a 5-kHz spectral window, 0.82-s interpulse spacing, 8-K data points zero-filled to 16 K.

Partition coefficients

Alcohol binding was determined using a dual radiolabel centrifugal approach modified from Katz and Diamond (1974a) as described in Janes et al. (1992). Intramembrane alcohol concentrations are expressed in mole fraction units, [mol drug bound/(mol phospholipid + mol drug bound)]. The partition coefficients (Kps) are expressed in molal units as [(mol drug bound/kg memb)/(mol drug free/kg buffer)]. The membrane mass is defined as inclusive of intramembrane alcohol. The membrane mass is not corrected for the water of hydration, nor for any nonsolvent water corrections in the dual radiolabel binding studies (Katz and Diamond, 1974b).

RESULTS

^{2}H NMR of ethanol-d_6 binding

The high-resolution ^{2}H NMR spectrum of 200 mM ethanol-d_6 in solution is shown in Fig. 1A. The four-line spectrum exhibits a downfield HOD resonance (which includes the exchanged 200 mM ethanol-OD as well as the natural abundance contribution of 16.5 mM), the ethanol methylene-d_2, an internal 100 mM sodium acetate-d_3 standard, and the ethanol methyl-d_3, respectively. Shown in Fig. 1, *B-E* are the spectral changes induced by increasing concentrations of dipalmitoylphosphatidylcholine ($Pam_2PtdCho$) multilamellar vesicles in the liquid-crystalline state (L_α-state, 46°C). Spectral intensities are normalized using the acetate-d_3 resonance. The methylene-d_2 resonance is significantly broadened and diminished in intensity as the concentration of the membrane is increased. At the highest concentration of membranes used (1E; 350 mg/ml), this resonance is broadened beyond detection. No narrow aqueous resonance is observed superimposed above the broadened methylene-d_2, as would be expected if the free and bound populations were in slow exchange. The progressive broadening observed is characteristic of a fast exchange process expressed as a weighted average of narrow free and broad membrane-bound resonances. Similar, but less pronounced, changes are observed for the methyl-d_3 and HOD resonances. The latter is a consequence of fast exchange between free and membrane-bound water (Salsbury et al., 1972). By contrast, the acetate methyl-d_3 resonance is nearly unaffected as expected, because acetate interacts with membranes to a lesser degree than does ethanol (Katz and Diamond, 1974c). No other broad signals were observed over the 80-kHz window that would correspond to immobilized ethanol.

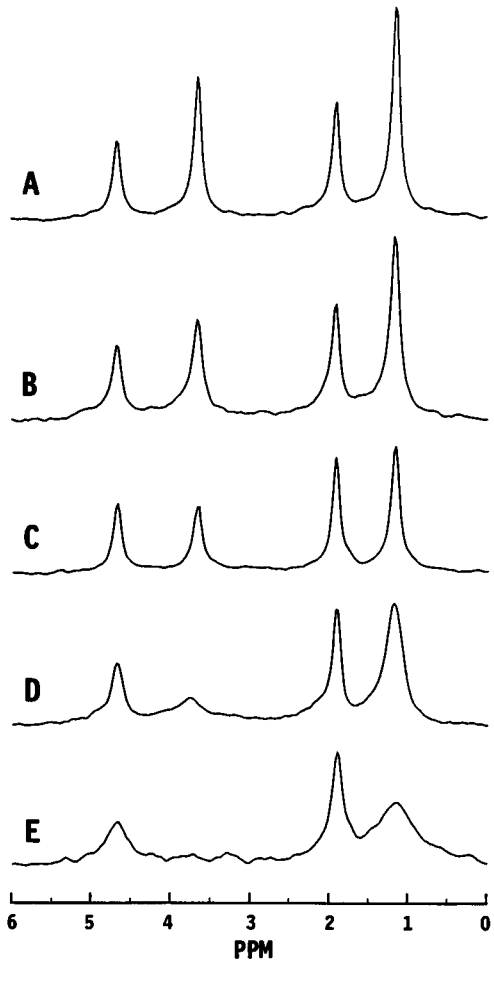

FIGURE 1 ^{2}H NMR spectra of the binding of ethanol-d_6 to $Pam_2PtdCho$ membranes at 46°C. (*A*) Aqueous blank 200 mM ethanol, 100 mM acetate-d_3; (*B*) $Pam_2PtdCho$ 35 mg/ml; (*C*) 70 mg/ml; (*D*) 100 mg/ml; (*E*) 350 mg/ml.

Are differences in the line broadening of the methylene-d_2 and methyl-d_3 consistent with partitioning as the sole mechanism of ethanol binding, or should a second interfacial site be invoked? To address this issue, membranes were examined in the gel state ($L_{\beta'}$). Gel-state membranes exhibit condensed or "frozen" hydrocarbon chains, but retain the lipid/water interface. The condensation of the hydrocarbon chains is thought to prevent appreciable binding of most solutes within the membrane core (Janes et al., 1992; Ma et al., 1992; Luxnat and Galla, 1986); consequently, gel-state membranes offer the potential for the direct observation of the putative interfacial component of ethanol binding without interference from core binding.

Shown in Fig. 2A is the aqueous ethanol-d_6 spectrum in the absence of membrane. When this buffer is used to hydrate $Pam_2PtdCho$ (100 mg lipid/ml buffer) and the dispersion is equilibrated overnight in the gel state (25°C), the spectral features are not appreciably altered, as shown in Fig. 2 *B*. Induction of the fluid L_α-state in the same sample (Fig. 2 *C*, 46°C) causes the substantial decreases in intensity and broadening of the ethanol resonances that were demonstrated in Fig. 1 (intensities are normalized using the acetate signal).

The similarities between the buffer blank and the gel-state spectra are not consistent with substantive interfacial binding in the gel-state membrane. It is possible that the receptivity of the interface to ethanol is also reduced in the gel-state membrane. An alternative explanation, that is consistent with the observed spectra, implicates a nonspecific mechanism of ethanol partitioning.

As a model for the core effects upon the spectral features of the ethanol resonances, spectra of ethanol-d_6 in a viscous bulk oil solvent, castor oil, are shown as a function of temperature in Fig. 2, *D-F* (intensities are normalized with the spectrometer's internal reference). Castor oil is a triacylglycerol composed of 87% ricinoleic acid (12-hydroxyoleic acid). Fig. 2 *D* shows the spectrum of 200 mM ethanol-d_6 in castor oil at 46°C. Both the methylene-d_2 and the methyl-d_3 resonances are narrow and well resolved. (The ethanol hydroxyl-d_1 resonance has exchanged with the oil hydroxyl and is presumably broadened beyond detection).

Fig. 2 *E*, obtained at 20°C, shows the consequences of increasing the viscosity. The methylene-d_2 resonance is

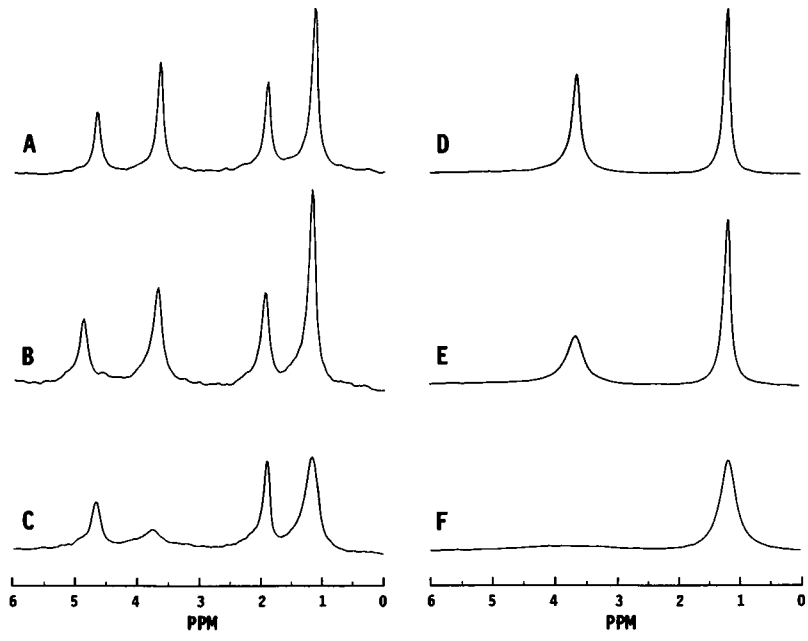

FIGURE 2 ^{2}H NMR spectra of ethanol-d_6 as a function of membrane structure and solvent viscosity. (*A*) Aqueous blank 200 mM ethanol-d_6, 100 mM acetate-d_3; (*B*) with gel-state $Pam_2PtdCho$ 100 mg/ml at 25°C; (*C*) with fluid-bilayer $Pam_2PtdCho$ 100 mg/ml at 46°C. 200 mM ethanol-d_6 in castor oil at (*D*) 46°C, (*E*) 20°C, (*F*) -8°C.

broadened, whereas the methyl-d_3 remains narrow. Fig. 2 *F* is obtained at -8°C, where the oil is still fluid, but quite viscous. The methylene-d_2 resonance is broadened almost beyond detection. The methyl-d_3 resonance is also broadened, but to a lesser extent. The spectral series obtained in this bulk solvent bears a strong resemblance to that obtained in the presence of membranes. Similar results were obtained in hexadecane (not shown). Thus, differential effects upon the methylene-d_2 and methyl-d_3 resonances are obtained from a viscous bulk solvent.

Direct measures of ethanol binding

Partition coefficients obtained for ethanol in fluid $Myr_2PtdCho$ membranes (37°C) as a function of ethanol concentration using a direct dual radiolabel centrifugal approach are shown in Fig. 3. The partition coefficient obtained at trace ethanol levels (0.21) is in good agreement with the literature value (Katz and Diamond, 1974c). The binding of ethanol is unchanged at ethanol concentrations of 5 μM and 400 mM, as expected for an ideal solvation process at dilute concentrations. No evidence for cooperative, anticooperative, or saturable ethanol binding is present.

^{2}H NMR of [1-$^2H_2$] n-hexanol binding

The high-resolution ^{2}H NMR spectrum of 20 mM [1-$^2H_2$] n-hexanol in solution is shown in Fig. 4 *A*. The three line spectrum includes a downfield peak from natural abundance HOD, the 1-hexanol-d_2 resonance, and an upfield resonance from the internal standard, 3 mM acetate-d_3. Addition of this solution to fluid L_α $Myr_2PtdCho$ multilamellar vesicles (36 mM, 30°C) induces a dramatic loss in the intensity of the hexanol resonance and a modest broadening of its linewidth from 2.4 to 8.8 $\pm$ 2.0 Hz (n = 3) (Fig. 4 *B*). The other resonances are unaffected. The intensity remaining corresponds to a hexanol concentration of 7.5 $\pm$ 0.4 mM (n = 3).

Are both the loss in intensity and the line broadening consistent with partitioning as the sole mechanism of hexanol binding, or must a second interfacial binding site be invoked? To investigate whether the broadening of the isotropic resonance is derived from surface adsorption, the multilamellar vesicles and the supernatant were separated by low speed centrifugation at 30°C and analyzed. The spectrum of the supernatant (Fig. 4 *C*) reproduced that of the lipid dispersion, indicating that the broadening was due to changes within the supernatant caused by the presence of lipids. A phosphorus assay confirmed that the supernatant contained a small amount of phospholipid (110 $\pm$ 30 μM; n = 3), presumably as small vesicular structures. Similar results were obtained when the membranes were hydrated in

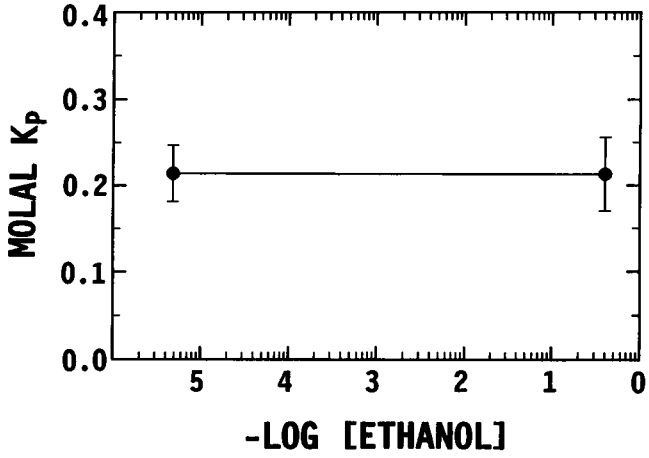

FIGURE 3 Ethanol binding in $Myr_2PtdCho$ membranes determined by a direct dual radiolabel binding assay.

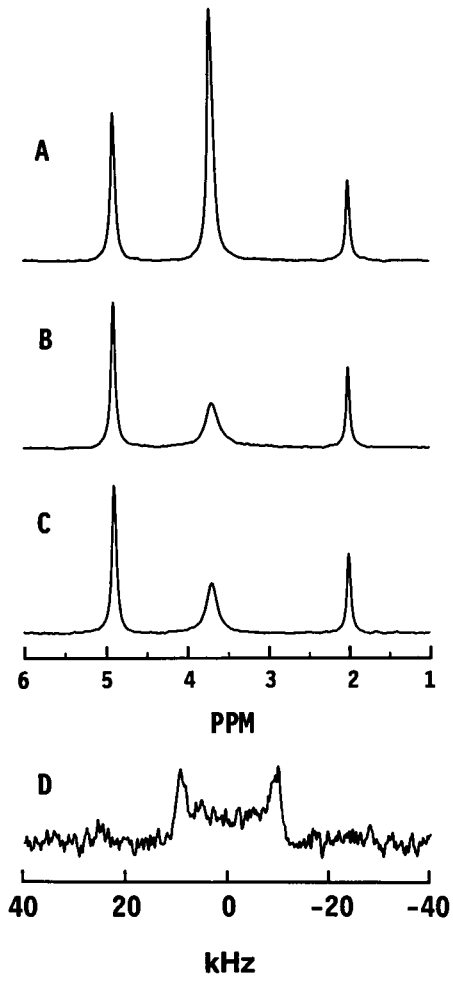

FIGURE 4 Deuterium NMR spectra of [1-$^2H_2$] n-hexanol binding to $Myr_2PtdCho$ membranes at 30°C. (*A*) Buffer solution containing 20 mM 1-hexanol-d_2, 9 mM acetate-d_3. (*B*) Buffer added to 36 mM $Myr_2PtdCho$ membranes. (*C*) Supernatant of *B* after centrifugal separation of multilamellar structures. (*D*) Concentrated membrane dispersion of $Myr_2PtdCho$ (500 mg/ml) with 1-hexanol-d_2 (0.3 mol fraction) in ^2H-depleted water.

the absence of hexanol, and hexanol-d_2 was added to the supernatant after centrifugation (except that at high hexanol concentrations, 45 mM and 60 mM, the resonance narrowed considerably, presumably due to changes in the lipid structure). Thus, the broadening of the free hexanol resonance stems from an interaction with small lipid structures and not from a second site within the multilamellar vesicles.

Most (60%) of the hexanol-d_2 intensity was absent from the high-resolution spectrum. Hexanol-d_2 is thought to intercalate within the acyl chains of the membrane and yield a characteristic Pake doublet with a quadrupole splitting of 19.5–20 kHz (Westerman et al., 1988). Concentrated membrane dispersions (500 mg/ml) were examined for the resonance of hexanol-d_2 bound to the multilamellar structures. The powder pattern, shown in Fig. 4 *D*, exhibits a quadrupole splitting of 19.4 kHz. No significant high-resolution signals are visible, indicating that nearly all of the hexanol is bound at these high membrane concentrations (the solution was made with ^2H-depleted water, and lacked an internal ^2H standard). The absence of any significant visible isotropic hexanol signal reinforces the conclusion that the broadening observed in less concentrated dispersions does not derive from a second site on the multilamellar vesicles.

To verify that the lipid remaining in the supernatant caused the broadening of the free hexanol resonance, sonicated unilamellar vesicles (SUVs) were prepared in the same hexanol/buffer system. The ^2H NMR spectra shown in Fig. 5 illustrate the broadening of the hexanol-d_2 resonance, which is induced by increasing SUV concentration.

The correlation between SUV concentration and hexanol-d_2 linewidth is shown in Fig. 6. The broadening correlated linearly with the vesicle concentration. The broadening observed for the supernatant of the multilamellar dispersion was in accord with the broadening observed for sonicated vesicles of similar concentration. Experiments performed at lower hexanol concentrations (8 mM; not shown) yielded similar results, consistent with the linear dependence observed.

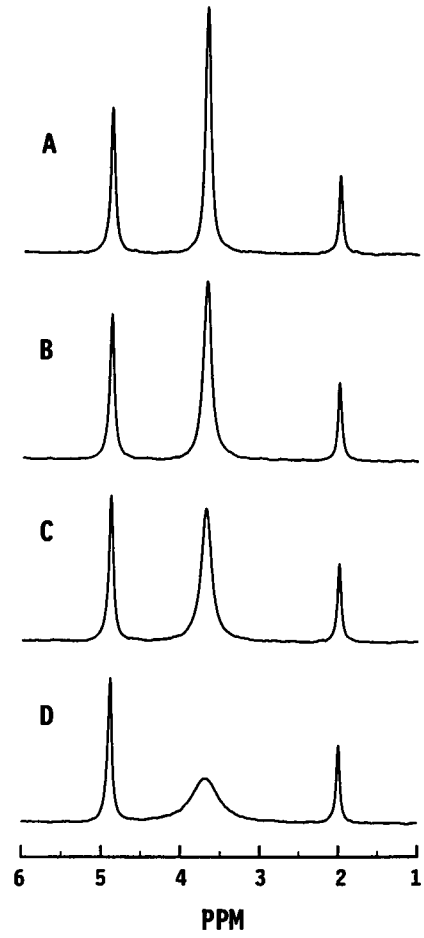

FIGURE 5 Deuterium NMR spectra of [1-$^2H_2$] n-hexanol binding to $Myr_2PtdCho$ SUVs at 30°C as a function of SUV concentration: (*A*) 0 μM, (*B*) 47 μM, (*C*) 98 μM, (*D*) 400 μM.

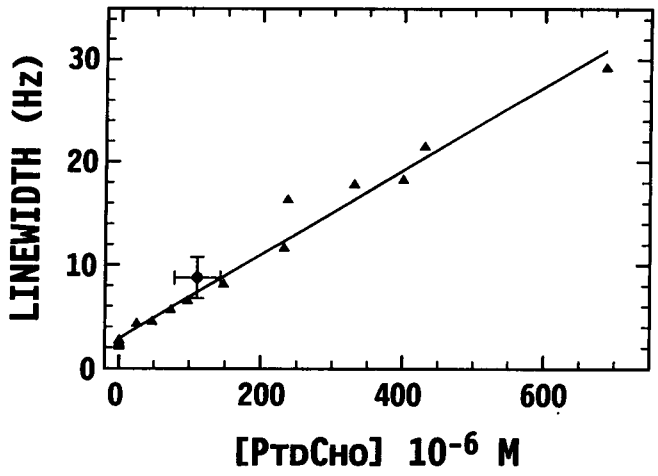

FIGURE 6 Correlation of ^2H linewidths-at-half-height of the [1-$^2H_2$] n-hexanol resonance with Myr_2PtdCho vesicle concentration in SUVs (8; least squares linear fit) and multilamellar dispersions (●; n = 3).

Direct measures of hexanol binding

The concentration dependence of hexanol binding to Myr_2PtdCho membranes in their liquid-crystalline state has been obtained previously in this laboratory using a direct radiolabel centrifugation technique, and the results are shown in Fig. 7 (Janes et al., 1992). At trace hexanol levels (37 μM free; 0.23 mf % bound) the molal partition coefficient is 91.7. The binding is slightly anticooperative and falls to 73.3 at the highest levels examined by the centrifugal method (2.55 mM free; 11.3 mf % bound). These results can be compared to those obtained by integration of the ^2H spectra of hexanol-d_2 in the presence and absence of lipid (Fig. 4). A partition coefficient of 63 ± 3 molal units at 7.5 ± 0.4 mM free hexanol (25.3 mf % bound) is obtained, in agreement with the radiolabel results.

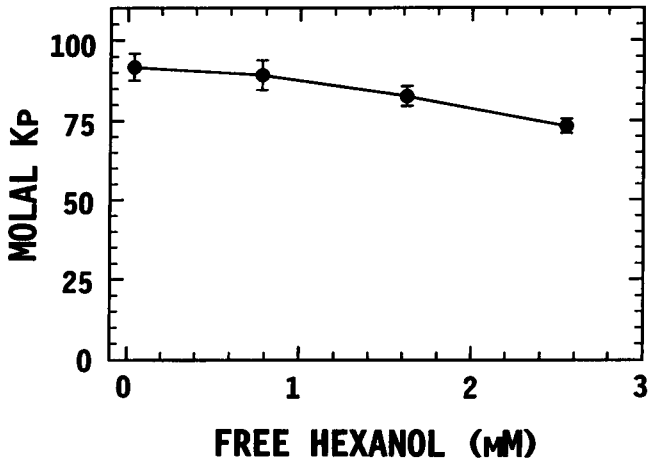

FIGURE 7 The dependence of hexanol binding on hexanol concentration as determined by a dual radiolabel centrifugal approach. Data from Janes et al. (1992).

DISCUSSION

In the previous section, we showed that the ^2H NMR and the direct radiolabel binding assay are consistent with a partitioning mechanism for the interaction of ethanol with model PtdCho membranes, in accord with our earlier thermodynamic analysis of solute action in membranes. In this section, we critically review the basis of the dual site surface/core interpretation of the ^2H NMR results.

Ethanol binding to liposomes

At the heart of the evidence for the two-site model of ethanol binding were the differential visibilities of the methylene-d_2 and methylene-d_3 resonances relative to the HOD resonance in the ^2H NMR spectrum (Kreishman et al., 1985, 1986). The NMR-invisible methylene-d_2 intensity was postulated as representing immobilization in both surface and core sites, whereas the NMR-invisible methyl-d_3 resonance was postulated to represent immobilization only at surface sites.

Our results confirm the experimental observation, but our interpretation differs. The spectral changes are reproduced in a viscous bulk oil solvent where ethanol binds through nonspecific solubilization. Presumably, the spectra reflect the differential rotational mobilities of the -CD_2OH and -CD_3 rotors in a viscous hydrocarbon environment (Woessner et al., 1969). In the membrane dispersion, fast exchange of the differentially broadened bound-ethanol population with the free ethanol population imparts differential broadening upon the resultant resonances. By contrast, the two-site model assumed slow exchange between both bound methylene-d_2 sites and the free methylene-d_2 in solution. The broadening that we show for the methylene-d_2 resonance provides no indication for a narrow free resonance line superimposed upon the broadened resonance which would be indicative of the slow exchange predicted in the two-site model.

The applicability of the two-site formalism can be further assessed by considering the predictions that were made within this interpretive framework. 1) Approximately two-thirds of the total binding at 200 mM ethanol was adsorbed at the surface. 2) The surface component of binding was highly cooperative. 3) The total binding greatly exceeded commonly cited values.

Because two-thirds of the total binding was found at the surface within the two-site analysis, we anticipated that we should observe spectral changes due to surface binding in the gel state, if the model were correct. The spectra that we obtained with gel-state lipids were quite similar to the buffer blank and markedly different from those we obtained with fluid-phase lipids. In the gel state, the core sites are "frozen" and (with rare exceptions) solutes are expelled from the acyl-chain region. Surface sites, albeit with reduced surface area, remain. Yet, we find no evidence for significant surface binding in the gel state.

The two-site analysis posits highly cooperative surface binding, whereas core binding remains constant. The total ethanol binding was found to rise by six-fold (i.e., 8.7 molal units at 35 mM and 56 molal units at 350 mM). By contrast, the total ethanol binding, determined here by a direct dual radiolabel method, exhibits no change between trace (5 μM) and 400 mM ethanol.

Published binding constants for ethanol in fluid PtdCho membranes are 1–2 orders of magnitude smaller than reported using the two-site interpretation of the ^{2}H NMR. The following values in molal units have been determined by indirect means: 0.105 (Hill, 1975), 0.07 (Rowe, 1981), 0.062 (Kamaya et al., 1981), 0.15 (Rowe, 1982), and 0.14 (Wang et al., 1993). Extrapolation from the binding behavior of longer chain alcohols yields 0.68 molal units (Jain and Wray, 1978). Direct measures using the radiolabel centrifugal technique and ^{3}H-water as a solvent marker yield 0.22 molal units (Katz and Diamond, 1974c). Our values, 0.21 molal units, support the latter direct measure and are in qualitative agreement with indirect determinations and extrapolations. Binding deduced using the two-site analytical framework is not.

Hexanol binding to liposomes

The spectral data on which the two-site hypothesis for hexanol binding was based differs from that obtained here in several key aspects (Fraser et al., 1991). 1) The broadened isotropic resonance of [1-$^2H_2$] n-hexanol, assigned to the adsorbed species, was reported shifted downfield by 2.7 ppm from the aqueous or free resonance. 2) The linewidth reportedly increased from 50 Hz for the free resonance to 100 Hz for the putative adsorbed resonance. 3) Complete equilibration of the hexanol/membrane dispersion required 3 days. 4) A dramatic anticooperativity in the total binding of hexanol was noted.

The 2.7-ppm shift reported for the adsorbed species represents a shift of approximately one-quarter of the usual deuterium/proton chemical shift range. Chemical shift changes arising from changes in the alcohol environment generally are more modest and often difficult to resolve. We have previously found that the aromatic ring protons of benzyl alcohol are shifted upfield by ~0.25 ppm upon binding to PtdCho membranes (Ma et al., 1992). Upfield shifts of similar magnitude were also observed in bulk nonpolar solvents. By contrast, any shift changes that might be attributed to the benzyl alcohol methylene protons were smaller and unresolved. ^{13}C possesses a 10-fold greater chemical shift range than deuterium, but the 1-^{13}C-butanol resonance was not shifted, only broadened (Rowe et al., 1987). No shifts in any of the isotropic resonances are observed here. No new resonances are apparent.

The putative adsorbed hexanol resonance was reported to be broadened by 50 Hz (~1 ppm) at half-height (50 Hz free, 100 Hz adsorbed). The broadening that we observe here is almost an order of magnitude smaller (2.4 Hz in the blank and 8.8 Hz in the presence of liposomes).

The putative adsorbed hexanol resonance required 3 days for complete equilibration. This time significantly exceeds that reported elsewhere in similar PtdCho/alcohol systems where partition coefficients are reported: 33 min for a series of alcohols, including hexanol, in PtdCho liquid-crystalline and gel states (Jain and Wray, 1978); 3 h for hexanol in the liquid-crystalline state, and 16 h for hexanol in the gel state of PtdCho, (Janes et al., 1992). Numerous other studies of alcohol/PtdCho studies report no special precautions for equilibration (Kamaya et al, 1981; Rowe, 1982; Rowe, 1985; Hill, 1974; Hill, 1975; Rowe et al., 1987). By contrast, we observe no spectral changes after 3 h.

The total binding derived from the two-site model was reported to be precipitously anticooperative (Fraser et al., 1991). As the total drug/lipid ratio increased from 0.1, 0.25, and 0.5, the molal Kp was reported to drop to 138, 56, and 38, respectively–a decrease of 72%. For comparison with Figs. 4 and 7, the lowest drug/lipid ratio (0.1) corresponded to a calculated free-hexanol level of 0.9 mM. By contrast, the anticooperativity that we observe is much more modest. Based on the radiolabel approach, the molal Kp drops from 91.7 (37 μM free; 0.23 mf % bound) to 73.3 (2.55 mM free; 11.3 mf % bound), and from the ^{2}H NMR to 63 (7.5 mM free; 25.3 mf % bound)–a decrease of 31% over a much larger concentration range. In accord with this weak anticooperativity, no nonlinear effects were reported in the effects of hexanol on the main phase transition temperature of PtdCho (Hill, 1974; Kamaya et al., 1981). Thus, the two-site model overestimated the anticooperativity of hexanol binding.

Within the partitioning formalism, alcohol-dependent, alcohol binding, whether cooperative or anticooperative, simply indicates that the membrane can no longer be considered as a lipid membrane, but is a lipid/alcohol membrane mixture. At 25 mf %, there are only three PtdCho per alcohol so the receptivity for any added solute may change, as would be expected, for instance, if the membrane contained 25 mf % cholesterol instead of hexanol.

The main difference between our results and those used to formulate the two-site model, is that our studies used the vertical sample geometry in 5-mm tubes common to high-resolution NMR, whereas the previous workers used a horizontal geometry in 10-mm tubes. Optimizing the Zeeman field homogeneity is much more difficult in a horizontal geometry, especially with samples of large diameter (linewidths tend to be much greater). Furthermore, inhomogeneities in the diamagnetic susceptibility of the sample as the phospholipid settles may lead to chemical-shift changes and slow equilibration times.

In summary, what appears to be two binding sites in the ^{2}H NMR spectra of hexanol-d_2 is actually a single site derived from different membrane morphologies. Bound to multilamellar structures, hexanol exhibits a quadrupolar powder pattern. Bound to very low levels of rapidly tumbling small vesicular structures, hexanol is in fast exchange and a modest broadening is imparted to the free resonance.

This interpretation is consistent with nonspecific partitioning as the sole mechanism of hexanol binding.

Implications for interfacial theories of alcohol action

Our thermodynamic analysis of alcohol action in membranes demonstrated that partitioned alcohols perturb the properties of the entire membrane, both surface and core, by altering the energetic balance among membrane structures (Janes et al., 1992; Ma et al., 1992; Wang et al., 1993). Thus, no specific mechanism of surface adsorption need be invoked to account for alcohol-induced alterations at the membrane surface.

CONCLUSIONS

Our experiments and analysis have led us to the following conclusions: 1) Ethanol binding to phosphatidylcholine membranes is consistent with a single nonspecific mechanism of partitioning. 2) The ^{2}H NMR results are consistent with different mobilities of the ethanol methylene-d_2 and methyl-d_3 rotors in a viscous environment. 3) The molal partition coefficient for ethanol in phosphatidylcholine liposomes is consistent with values obtained from indirect determinations, and extrapolations from the long-chain alcohols. 4) Ethanol binding to phosphatidylcholine membranes is concentration independent up to at least 400 mM. 5) Hexanol binding to phosphatidylcholine membranes is consistent with a single, nonspecific mechanism of partitioning. 6) The ^{2}H NMR results are consistent with hexanol binding to two populations of vesicles–large multilamellar structures and trace levels of small vesicular structures. 7) The equilibration of hexanol with liquid-crystalline phosphatidylcholine liposomes is complete within 3 h. 8) Hexanol binding is weakly anticooperative.

This work was supported by US Public Health Service grants AA00163, AA07186, AA07215.

REFERENCES

Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* 234:466–468.

Brown, H. C., and B. C. Subba Rao. 1956. A new powerful reducing agent-sodium borohydride in the presence of aluminum chloride and other polyvalent metal halides. *J. Am. Chem. Soc.* 78:2582–2588.

De Young, L. R., and K. A. Dill. 1988. Solute partitioning into lipid bilayer membranes. *Biochemistry.* 27:5281–5289.

Fraser, D. M., L. C. M. Van Gorkom, and A. Watts. 1991. Partitioning behaviour of 1-hexanol into lipid membranes as studied by deuterium NMR spectroscopy. *Biochim. Biophys. Acta.* 1069:53–60.

Herbette, L., C. A. Napolitano, F. C. Messineo, and A. M. Katz. 1985. Interaction of amphiphilic molecules with biological membranes. A model for nonspecific and specific drug effects with membranes. *In* Advances in Myocardiology, Vol. 5. P. Harris and P. A. Poole-Wilson, editors. 333–346.

Hill, M. W. 1974. The effect of anaesthetic-like molecules on the phase transition in smectic mesophases of dipalmitoyllecithin I. The normal alcohol up to C = 9 and three inhalation anaesthetics. *Biochim. Biophys. Acta.* 356:117–124.

Hill, M. W. 1975. Partition coefficients of some anaesthetic-like molecules between water and smectic mesophases of dipalmitoyl phosphatidylcholine. *Biochem. Soc. Trans.* 3:149–152.

Jain, M. K., and L. V. Wray. 1978. Partition coefficients of alkanols in lipid bilayer/water. *Biochem. Pharmacol.* 27:1294–1295.

Janes, N., J. W. Hsu, E. Rubin, and T. F. Taraschi. 1992. Nature of alcohol and anesthetic action on cooperative membrane equilibria. *Biochemistry.* 31:9467–9472.

Janes, N. 1995. The nature of alcohol and anesthetic action in membranes. A thermodynamic description of Meyer's "solution reaction". *Prog. Anesth. Mech.* 3:10–15.

Janoff, A. S., M. J. Pringle, and K. W. Miller. 1981. Correlation of general anesthetic potency with solubility in membranes. *Biochim. Biophys. Acta.* 649:125–128.

Janoff, A. S., and K. W. Miller. 1982. A critical assessment of the lipid theories of general anaesthetic action. *In* Biological Membranes, Vol. 4. D. Chapman, editor. Academic Press, New York. 417–476.

Kamaya, H., S. Kaneshina, and I. Ueda. 1981. Partition equilibrium of inhalation anesthetics and alcohols between water and membranes of phospholipids with varying acyl chain-lengths. *Biochim. Biophys. Acta.* 646:135–142.

Kaminoh, Y., T. Tatara, P. R. Krishna, H. Kamaya, and I. Ueda. 1991. Effects of pressure on anesthetic potency and thermodynamic activity theory of anesthesia. *Pharmacol. (Life Sci. Adv.)* 10:75–86.

Katz, Y., and J. M. Diamond. 1974a. A method for measuring nonelectrolyte partition coefficients between liposomes and water. *J. Membr. Biol.* 17:69–86.

Katz, Y., and J. M. Diamond. 1974b. Nonsolvent water in liposomes. *J. Membr. Biol.* 17:87–100.

Katz, Y., and J. M. Diamond. 1974c. Thermodynamic constants for nonelectrolyte partition between dimyristoyl lecithin and water. *J. Membr. Biol.* 17:101–120.

Katz, Y. 1988. On the use of regular solution theory as a theoretical frame for the analysis of membrane phenomena. *Biochim. Biophys. Acta.* 939:19–28.

Kreishman, G. P., C. Graham-Brittain, and R. J. Hitzemann. 1985. Determination of ethanol partition coefficients to the interior and the surface of dipalmityl-phosphatidylcholine liposomes using deuterium nuclear magnetic resonance spectroscopy. *Biochem. Biophys. Res. Comm.* 130: 301–305.

Kreishman, G. P., C. Graham-Brittain, H. Schueler, and R. Hitzemann. 1986. On the use of nuclear magnetic resonance spectroscopy to study the mechanisms of ethanol action. *Alcohol Drug Res.* 6:1–13.

Luxnat, M., and H.-J. Galla. 1986. Partition of chlorpromazine into lipid bilayer membranes: the effect of membrane structure and composition. *Biochim. Biophys. Acta.* 856:274–282.

Lyon, R. C., J. A. McComb, J. Schreurs, and D. B. Goldstein. 1981. A relationship between alcohol intoxication and the disordering of brain membranes by a series of short-chain alcohols. *J. Pharm. Exp. Ther.* 218, 669–675.

Ma, L., T. F. Taraschi, and N. Janes. 1992. Nuclear magnetic resonance partitioning studies of solute action in lipid membranes. *Bull. Magn. Reson.* 14:293–298.

Marqusee, J. A., and K. A. Dill. 1986. Solute partitioning into chain molecule interphases: monolayers, bilayer membranes, and micelles. *J. Chem. Phys.* 85:434–444.

McCreery, M. J., and W. A. Hunt. 1978. Physico-chemical correlates of alcohol intoxication. *Neuropharmacol.* 17:451–461.

Meyer, H. 1899. On the theory of alcohol narcosis. What property of anesthetics produces their anesthetic action? *Arch. Exper. Path. U. (Naunyn-Schmiedeberg)* 42:109–118.

Overton, C. E. 1901. Studien uber die Narkose, zugleich ein Beitrag zur allgemeiner Pharmakologie. G. Fischer, Jena; 1991. Studies of Narcosis. R. L. Lipnick, editor. Chapman and Hall, New York.

Rowe, E. S. 1981. Membrane buffer partition coefficient for ethanol in dimyristoylphosphatidylcholine. *Alcohol. Clin. Exp. Res.* 5:259–263.

Rowe, E. S. 1982. The effects of ethanol on the thermotropic properties of dipalmitoylphosphatidylcholine. *Mol. Pharmacol.* 22:133–139.

Rowe, E. S. 1985. Thermodynamic reversibility of phase transitions. Specific effects of alcohols on phosphatidylcholines. *Biochim. Biophys. Acta.* 813:321–330.

Rowe, E. S., A. Fernandes, and R. G. Khalifah. 1987. Alcohol interactions with lipids: a carbon-13 nmr study using butanol labeled at C-1. *Biochim. Biophys. Acta.* 905:151–161.

Salsbury, N. J., A. Darke, and D. Chapman. 1972. Deuteron magnetic resonance studies of water associated with phospholipids. *Chem. Phys. Lipids.* 8:142–151.

Seelig, J., and P. Ganz. 1991. Nonclassical hydrophobic effect in membrane binding equilibria. *Biochemistry.* 30:9354–9359.

Simon, S. A., W. L. Stone, and P. Busto-Latorre. 1977. A thermodynamic study of the partition of n-hexane into phosphatidylcholine and phosphatidylcholine-cholesterol bilayers. *Biochim. Biophys. Acta.* 468: 378–388.

Simon, S. A., W. L. Stone, and P. B. Bennett. 1979. Can regular solution theory be applied to lipid bilayer membranes? *Biochim. Biophys. Acta.* 550: 38–47.

Smith, R. L., and E. Oldfield. 1984. Dynamic structure of membranes by deuterium NMR. *Science.* 225:280–288.

Seeman, P. 1972. The membrane actions of anesthetics and tranquilizers. *Pharmacol. Rev.* 24:583–655.

Taheri, S., M. J. Halsey, J. Liu, E. I. Eger II, D. D. Koblin, and M. J. Laster. 1991. What solvent best represents the site of action of inhaled anesthetics in humans, rats, and dogs? *Anesth. Analg.* 72:627–634.

Tamura, K., Y. Kaminoh, H. Kamaya, and I. Ueda. 1991. High pressure antagonism of alcohol effects on the main phase-transition temperature of phospholipid membranes: biphasic response. *Biochim. Biophys. Acta.* 1066:219–224.

Thewalt, J. L., A. P. Tulloch, and R. J. Cushley. 1986. A deuterium NMR study on labelled n-alkanol anesthetics in a model membrane. *Chem. Phys. Lipids.* 39:93–107.

Wang, D.-C., T. F. Taraschi, E. Rubin, and N. Janes. 1993. Configurational entropy is the driving force of ethanol action on membrane architecture. *Biochim. Biophys. Acta.* 1145:141–148.

Westerman, P. W., J. M. Pope, N. Phonphok, J. W. Doane, and D. W. Dubro. 1988. The interaction of n-alkanols with lipid bilayer membranes: a 2H-NMR study. *Biochim. Biophys. Acta.* 939:64–78.

White, S. H., G. I. King, and J. E. Cain. 1981. Location of hexane in lipid bilayers determined by neutron diffraction. *Nature.* 290:161–163.

Wimley, W. C., and S. H. White. 1993. Membrane partitioning: distinguishing bilayer effects from the hydrophobic effect. *Biochemistry.* 32:6307–6312.

Woessner, D. E., B. S. Snowden Jr., and G. H. Meyer. 1969. Calculation of NMR free induction signals for nuclei of molecules in a highly viscous medium or a solid-liquid system. *J. Chem. Phys.* 51:2968–2976.

Young, H. S., V. Skita, R. P. Mason, and L. G. Herbette. 1992. Molecular basis for the inhibition of 1,4-dihydropyridine calcium channel drugs binding to their receptors by a nonspecific site interaction mechanism. *Biophys. J.* 61:1244–1255.