NUCLEAR MAGNETIC RESONANCE PARTITIONING STUDIES OF SOLUTE ACTION IN LIPID MEMBRANES

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INTRODUCTION: Lipid theories of anesthesia implicate perturbation of membrane lipids as the locus for acute anesthetic action. [1] Chronic exposure to alcohols and anesthetics induces an adaptive response in membrane phospholipids that confers resistance to many of the acute actions of alcohols and anesthetics. [2]

We have proposed a colligative thermodynamic reformulation of the Meyer-Overton hypothesis for anesthetic action. [3,4] This reformulation implicates configurational entropy (S_{cf}), the entropy imparted by a solute upon a membrane structure in the partitioning process, as the driving force of solute action on cooperative membrane equilibria. Solute potency is determined by the competing contributions of configurational and thermal entropy (ΔS_t). Equilibria most susceptible to solute action (where dilute concentrations of solutes induce a perturbation equivalent to a large change in temperature) involve large changes in configurational entropy and small changes in thermal entropy according to the following relation. [3]

$$\Delta T / T_m = \Delta S_{cf} / \Delta S_t \qquad (1)$$

ΔT is the perturbation of the midpoint temperature, T_m, from its value in the absence of solute. The thermal entropy of an equilibrium is deduced from calorimetry and is approximately constant for solute levels of biological relevance. The remaining unknowns are the configurational entropy, which is determined from the partitioning of the solute, and the perturbation of the equilibrium midpoint.

The colligative thermodynamic framework implicates solute partitioning as the energetic force that drives perturbations of cooperative membrane equilibria by altering the relative free energies of membrane states. Tests of the framework require simultaneous measures of solute partitioning and membrane structure over a range of solute concentrations and temperatures.

Spin label partitioning protocols have often been used in ESR studies of membrane structure. [5] Such studies are designed so that the spin label partitioning probe is a nonperturbing reporter of membrane structure. To study solute action on membranes, however, a partitioning probe should serve the multifarious role of membrane perturbant, reporter of perturbations, and reporter of solute partitioning. Since NMR methods are not limited to dilute solute levels, such flexibility is offered. Furthermore, complementary structural information is available from simultaneous wideline ^1H [6], ^2H [7], or ^{31}P [8] studies.

PARTITIONING APPROACH TO SOLUTE ACTION: In this abstract, we describe a ^1H NMR partitioning approach based on the uncharged local anesthetic alcohol, benzyl alcohol. Benzyl alcohol is a clinically used topical bacteriostatic agent. A variety of commercial pharmaceutical agents prepared for injection contain benzyl alcohol for its preservative properties and for pain relief.

The partitioning approach is based on (*i*) the sensitivity of the ring proton chemical shift to the polarity of its environment and (*ii*) the sensitivity of the ring proton linewidth to membrane binding. The chemical shift of the ring resonances in a hydrophobic environment are shielded and resolved from the ring resonances of the aqueous alcohol. The sensitivity of the ring proton resonance to its environment provides a means of discriminating the aqueous alcohol resonance from the partitioned alcohol resonance. The dependence of the three chemically distinct ring proton chemical shifts on their environment is shown in Table 1 for benzyl alcohol (5 mole fraction %) in a variety of bulk solvents at 22°C. The resonance exhibits a diamagnetic shift in hydrophobic solvents. A modest correlation between the chemical shift and Hildebrandt's solubility parameter (δ^*) for the solvent is evident.

TABLE 1

SOLVENT	δ^*	δ (ppm from TMS)			δ (ppm) avg
Water	23.4	7.41	7.41	7.41	7.41
Methanol	14.5	7.32	7.32	7.23	7.29
1-Propanol	11.9	7.29	7.25	7.17	7.24
1-Butanol	11.4	7.29	7.24	7.16	7.23
1-Octanol	10.3	7.27	7.21	7.12	7.20
1-Decanol	-	7.27	7.21	7.12	7.20
Acetone	9.9	7.34	7.30	7.21	7.28
Methylene Chloride	9.8	7.29	7.29	7.29	7.29
Chloroform-d_1	9.2	7.32	7.32	7.32	7.32
Carbon Tetrachloride	8.6	7.19	7.16	7.16	7.17
Hexane	7.3	7.20	7.20	7.13	7.18

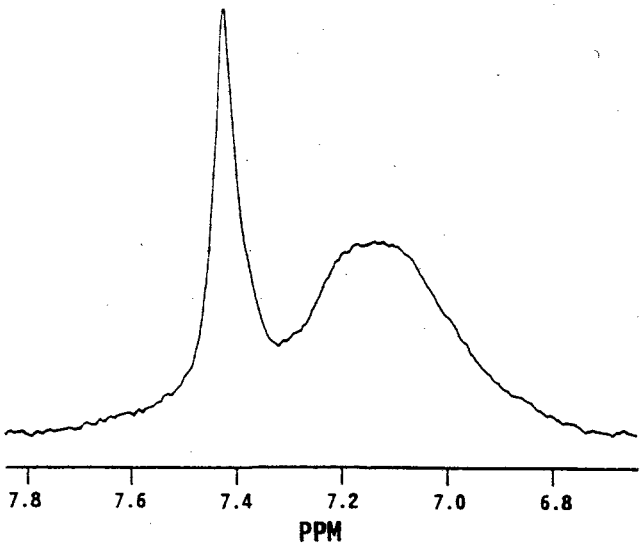

Figure 1: The ring proton resonances of benzyl alcohol are shifted upfield and broadened upon binding to lecithin membranes.

Further discrimination stems from the motional restrictions imparted by the membrane environment that is reflected in the spin-spin relaxation. The ring resonances corresponding to the free and bound drug are shown in Figure 1 for a lecithin model membrane in the L_α state. The resonance of the bound agent is broadened due to immobilization in the membrane. The T_2 of the bound agent is approximately 6 msec, while the T_2 of the free agent is more than three orders of magnitude greater (11 sec). The different relaxation properties allow for spectral editing based on T_2 using spin echoes.

The partitioning of benzyl alcohol into membranes is modest. Consequently, the lipid to water ratios of the sample must be large to obtain accurate simulations of the broad bound resonance, while a sample size and geometry consistent with high Zeeman field homogeneity must be maintained. In practice, to reduce sample demands, an internal acetate standard was used to determine the aqueous alcohol concentration in a dilute membrane suspension. Since the lipid concentration is known, the intramembrane concentration is obtained by difference to yield the partition coefficient. To ensure that the integrated aqueous resonance is not contaminated by the broad bound resonance, a CPMG sequence is used to delay acquisition by 25 msec in order to filter the broad bound-drug resonance. This filtering method also removes the dipolar broadened lipid resonance to improve baseline definition.

Since the colligative thermodynamic framework equates the action of solute and temperature through entropy, precise temperature regulation is required. The aqueous benzyl alcohol resonance exhibits a temperature dependent chemical shift. In order to maintain a consistently reproducible temperature, we have taken advantage of this temperature dependence. The chemical shift differences between the HOD and free benzyl alcohol resonances as a function of temperature is shown in Figure 2.

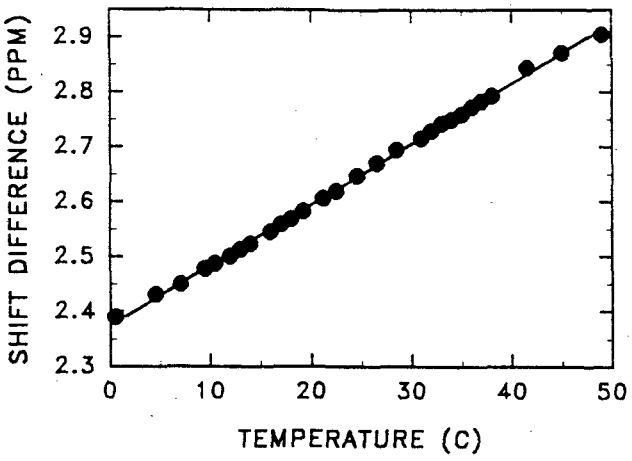

Figure 2: The chemical shift difference between the benzyl alcohol ring proton resonance and the HOD resonance shows a temperature dependence.

ANALYSIS OF PARTITIONING: The degree of anesthetic partitioning into a membrane system is sensitive to and characteristic of the state of the lipid assembly. The equilibrium constant, K_{eq}, is deduced from the partitioning changes[4] characteristic of the interchange between membrane states. The temperature dependence of the partitioning exhibits the following functional form for a state change between two membrane structures. [3]

$$Kp = \frac{Kp^{\alpha} + Kp^{\beta} \exp C(T-T_m)}{1 + \exp C(T-T_m)} \quad (2)$$

$$C = \frac{\Delta H_{vH}}{RTT_m} \simeq \frac{\Delta H_{vH}}{RT_m^2} \quad (3)$$

The partition coefficient for the membrane states α and β are Kp^{α} and Kp^{β}, respectively. These partition coefficients are not necessarily constant and may be altered to include a temperature dependence. The total partition coefficient is Kp. The midpoint temperature is Tm. A fit of the experimental data to this function yields partition coefficients for each phase, the midpoint temperature, and the van't Hoff enthalpy (ΔH_{vH}), a measure of the cooperativity of the equilibrium.

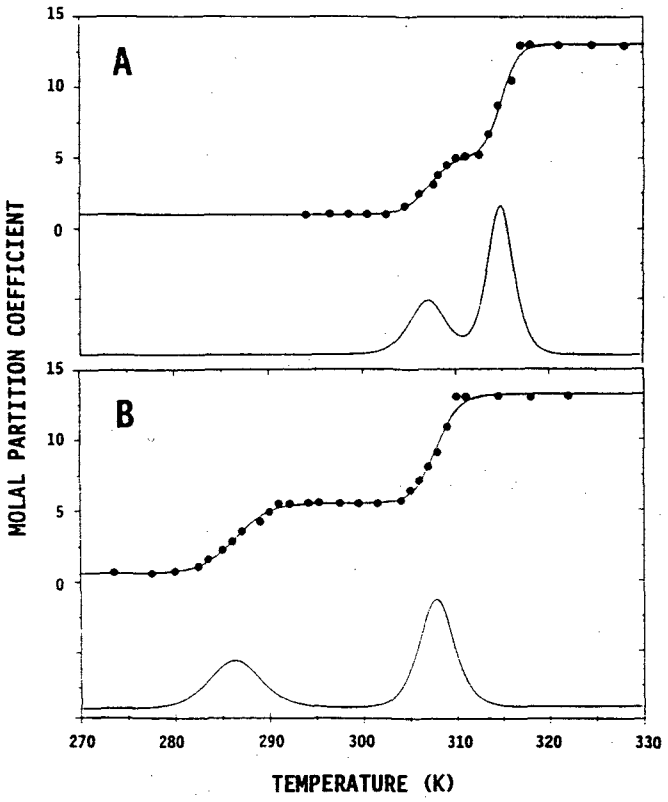

Figure 3: The molal partition coefficient of benzyl alcohol into multilamellar DPPC membranes is shown as a function of temperature for two concentrations of benzyl alcohol. The fit corresponds to the theoretical multiparameter least-squares analysis described in the text. The derivative of the fit to the data is shown offset below. The percent mole fraction intramembrane benzyl alcohol concentrations at the $L_{\beta'} \rightarrow P_{\beta'}$ equilibrium midpoint are as follows: Panel A: $L_{\beta'}$ = 0.23%, $P_{\beta'}$ = 1.1%; Panel B: 2.2%, 16.8%; The mole fraction benzyl alcohol concentrations at the $P_{\beta'} \rightarrow L_{\alpha}$ equilibrium midpoint are as follows: Panel A: $P_{\beta'}$ = 0.84%, L_{α}= 2.1%; Panel B: 11.7%, 24.2%; For comparative purposes, general anesthetic intramembrane concentrations are considered less than 5 mole fraction percent.

The analytical framework presented is not specific to the partitioning analysis. It is broadly applicable to any technique in which the observable is characteristic of each state.

ALCOHOL ACTION IN MODEL MEMBRANES: The lecithin membrane, DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine), adopts three well-studied structures or phases, a gel-structure ($L_{\beta'}$), a ripple-structure ($P_{\beta'}$), and a fluid bilayer-structure (L_{α}). [9] Since the interchange between these three membrane structures is driven by

entropy, changes in solute, temperature and pressure alter the energetic balance to favor a given structure. The $L_{\beta'} \rightarrow P_{\beta'}$ equilibrium (pretransition) exhibits an equilibrium midpoint temperature determined by calorimetry as 34.8°C. [9] This change in state is accompanied by a small change in thermal entropy (12.5 J mol^{-1} K^{-1}). The $P_{\beta'} \rightarrow L_{\alpha}$ (main transition) exhibits an equilibrium midpoint temperature determined by calorimetry as 41.0°C. This change in state is accompanied by a relatively large change in thermal entropy (85.6 J mol^{-1} K^{-1}). [9] The large difference between the thermal entropy changes associated with these two equilibria provides a simple system in which to test the predictions of the colligative thermodynamic framework, that solute action occurs through entropy and that equilibria characterized by a small thermal entropy change should be most susceptible to perturbation.

The temperature dependence of benzyl alcohol partitioning at two substantially different alcohol concentrations is shown in Figure 3. Figure 3A corresponds to benzyl alcohol concentrations below that required for general anesthesia; whereas, the concentration in Figure 3B is near that required for local anesthesia. The partition coefficients obtained by the NMR method are in excellent agreement with direct radiolabel measures. [10] Two discontinuities correspond to the two membrane equilibria. It is these *changes* in partitioning that provide the configurational entropy by which solutes perturb equilibria. The low entropy $L_{\beta'} \rightarrow P_{\beta'}$ equilibrium exhibits greater sensitivity to the alcohol than the high entropy $P_{\beta'} \rightarrow L_{\alpha}$, as qualitatively predicted by the thermodynamic model.

The quantitative test for the model is shown in Figure 4. The partitioning method provides intramembrane solute concentrations, which, in turn, provide the magnitude of the configurational entropy imparted to each membrane structure. This contribution lowers the free energy of each state according to the magnitude of the partitioning, and thereby alters the difference in free energy and shifts the equilibrium. The experimental points are in good agreement with the theoretical predictions (represented by the lines) at dilute alcohol concentrations for which the thermodynamic treatment is derived and which corresponds to pharmacological levels

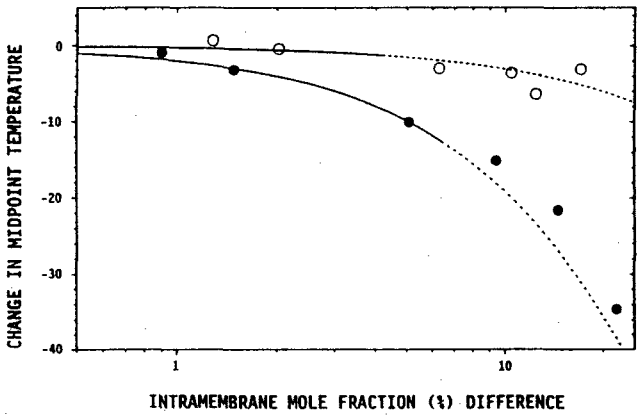

Figure 4: The dependence of the equilibrium midpoint temperature of DPPC on the presence of benzyl alcohol. The benzyl alcohol intramembrane concentration difference between the initial and final states at the equilibrium midpoint is shown. Data are presented for the low entropy $L_{\beta'} \rightarrow P_{\beta'}$ (pretransition; filled circles) and the high entropy $P_{\beta'} \rightarrow L_{\alpha}$ (main transition; open circles) equilibria. The colligative thermodynamic predictions (eq. 1) are represented by the lines. The solid portions of the lines designate the average intramembrane concentrations at the midpoint which correspond to the range of pharmacological relevance (< 5 m.f.%).

for general anesthesia.

Particularly striking is the dramatic contrast in benzyl alcohol sensitivity exhibited by these two equilibria. At average intramembrane concentrations of 5 m.f.%, the low entropy equilibrium is perturbed by approximately 12°C, whereas the high entropy equilibrium is perturbed by approximately 1°C. Not only does this observation support the predictions of the thermodynamic model, but it demonstrates that remarkably low intramembrane concentrations of nonspecific solutes can precipitate quite substantial effects upon membrane structure.

ALCOHOL ACTION IN LIPOSOMES MADE FROM RATS CHRONICALLY EXPOSED TO ANESTHETICS: Rat liver microsomes obtained from rats exposed to nitrous oxide or fed ethanol were isolated and liposomes formed from the extracted phospholipids. Shown in Figure 5 are representative benzyl alcohol partitioning traces for the liposomes prepared from the ethanol-fed and control animals. The partition coefficient into

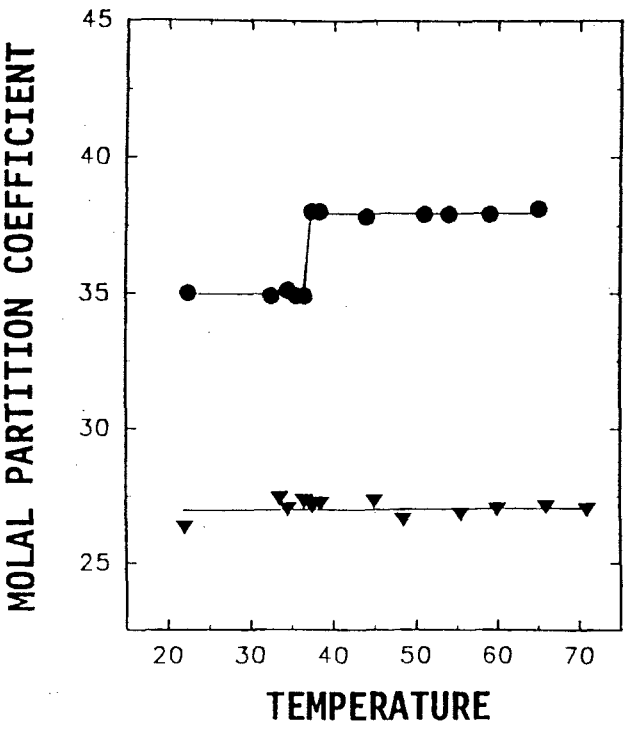

Figure 5: Benzyl alcohol partitioning traces are shown for liposomes made from rat-liver microsomal-phospholipids. The samples represented in the lower trace (filled triangles) were prepared from chronically ethanol-fed rats. The samples represented in the upper trace (filled circles) were prepared from their pair-fed littermates.

the control samples is larger than the treated samples. This difference in partitioning is characteristic of 'membrane tolerance'. [2] A structural equilibrium is apparent in the control samples near 37°C that is lacking in the samples obtained from treated animals. Similar results are obtained from the chronic nitrous oxide paradigm. These results evidence an adaptive response to the chronic presence of anesthetic agents that results in altered domain structure in the reconstituted system. Similarly, structural lipid domains are predicted at the anesthetic locus in our thermodynamic reformulation of the Meyer-Overton hypothesis.

CONCLUSIONS: Alcohols and anesthetics act through the entropy imparted by partitioning to modify membrane architecture. Analysis of anesthetic action requires simultaneous measures of solute partitioning and membrane structure over a wide range of solute concentrations. NMR partitioning methods offer unique advantages in such inquiry since the solute can serve the multifaceted role of perturbant, reporter of membrane perturbations, and reporter of solute partitioning.

METHODS: Spectra were obtained on a Bruker 8.5T AM spectrometer operating at 360 MHz with deuterium lock. Lipids were dried under N_2, evacuated (<5 mTorr) for a minimum of 3 hours (natural lipids) or overnight (synthetic lipids) and hydrated in a cut-off 5 mm tube with a tris-KCl-D_2O buffer containing benzyl alcohol and the acetate internal standard. The sample tube containing the multilamellar vesicles was placed coaxially in a 10 mm tube and centered within the limits of the decoupling coil for quantitative detection. Spectra were obtained from a Bloch decay or from a spin echo CPMG sequence to delay acquisition 25 msec in order to filter broad resonances. Typically the CPMG sequence was used for fluid phase lipids, and whenever the baseline was ill-defined due to the bound agent. The Bloch decay was typically used for gel-state lipids. ^{31}P spectra were obtained periodically to ensure the absence of small vesicular structures.

The chronic animal models and the membrane preparations have been described elsewhere. [11]

The 'molal' partition coefficients reported here are mixed unit values ([moles alcohol / kg lipid] / [moles alcohol / liter deuterated buffer]) in order to reference the heavy water buffer partition coefficient to literature values for normal buffer. The molal partition coefficients are converted to mole fraction partition coefficients for all thermodynamic calculations.

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