Magnetic Resonance Studies of Lipid-Protein Interfaces and Lipophilic Molecule Partitioning[a]

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INTRODUCTION

Lipophiles, including local and other general anesthetics, may partition into various locations of biological membranes, and the degree of partitioning depends upon various membrane properties including the electrostatics as well as the lipophile ionization state. Magnetic resonance methods such as spin-label electron spin resonance (ESR) and deuterium nuclear magnetic resonance (^{2}H-NMR) each show spectral properties that can be exploited to identify the various locations of lipophiles in biomembranes as well as determine directly the ionization state of a lipophile while in the bilayer.

In recent studies on nicotinic acetyl choline receptor (nAChR) rich membranes and protein-free model membranes, we suggest that:

1. the surface ionization state of lipid head groups and of a local anesthetic, tetracaine, can be directly measured on the membrane surface;
2. hexanol can partition into both the polar–apolar interface and the hydrocarbon core of lipid bilayers;
3. a series of general anesthetics (1-hexanol, urethane, ethanol, and diethyl ether) perturb lipids at the lipid-protein interface; and
4. the degree of perturbation of lipids at the nAChR interface is very different with similar anesthetic concentrations, but is similar for each anesthetic at a concentration that desensitizes 50% of the receptors.

METHODS

Deuterium NMR has been very successful in describing the order and dynamics of macromolecular complexes, in particular biomembranes.[1–5] The chemical labeling of membrane lipids and proteins with deuterons instead of protons at specific locations is now becoming achievable,[6] and in this way the experiment can be designed to yield information about any molecular location required, including the membrane surface. In an effort to gain an insight into membrane surface electrostat-

[a]The work described here was carried out by Sonia Louro, Vicki Abadji, David M. Fraser, Laszlo I. Horvath and Trevor W. Poile in collaboration with Keith W. Miller of the Harvard Medical School. This work was supported by studentships to D.M.F. (MRC) and T.W.P. (SERC), and by travel grants from SERC (GR/D/59070) to L.I.H., from the EPA Cephalosporin Fund, Royal Society and SERC (GR/D/69846) to A.W., and NIAAA (07040) to K.W.M.

ics, we have incorporated deuterons into the polar head groups of a wide range of phospholipids to study membrane surface architecture.[4,7-9]

The deuterium nmr spectrum from bilayers containing deuterons is a spherically averaged powder pattern from which two types of information can be gained. The separation of the spectral lines, the quadrupole splitting, $\Delta\nu_q$, gives an indication of the motional amplitude and/or orientation of the C^2H-bond.[1,3] The magnetic relaxation times, measured either from an inversion recovery experiment or line widths, yield dynamic information either of the fast (nsec) or slower (μsec) motions of the C^2H-group. In view of the rather large quadrupolar interaction ($\Delta E/h \sim 200$ kHz) of the deuteron ($m_1 = 1$) with the magnetic field, changes of local environment due to change of shielding of the nucleus, which normally lead to chemical shift changes ($\Delta E/h \sim 5$ kHz), are small and symmetrical in the two-line spectrum, and thus not readily detected.

The spin-label ESR method for studying biomembranes requires the incorporation of a probe nitroxide (at a ratio of 1 probe:100–200 endogenous lipids) into the membrane. An advantage over ^2H-NMR methods is that such lipid probes can be incorporated into natural biomembranes at low concentration ($\sim$nM of spin label in 30–50 μl sample) compared to ^2H-NMR (μM of ^2H in 0.5–1 ml). A further advantage with the spin-label ESR approach is that the averaging of the ^{14}N-electron hyperfine interactions occurs over a time range of 10^{-7}–10^{-9} s. Thus for fast ($\tau_c \sim 10^{-9}$ s) acyl chain rotational isomerizations, the order (given by an order parameter, S) of a membrane from determinations of the degree of anisotropy averaging, can be determined.[6,10-12]

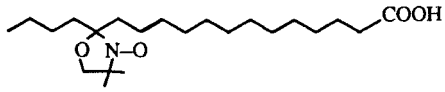
For the study of protein-lipid interactions, the rate of exchange for lipids onto and off the protein interface is found to be fast on the ^2H-NMR anisotropy averaging time scale [$\nu_{ex}^{-1} > \Delta\nu_q$ (max) $\sim$ 200 kHz] and slow on the ^{14}N-electron anisotropy averaging time scale [$\nu_{ex}^{-1} < (A_{90°} - A_{0°}) \sim 0.1$ GHz].[13] Therefore, in spin-label ESR experiments it has been possible to quantify the proportion of spin labels (and hence lipids) at the protein interface from the resultant two-component ESR spectrum recorded from protein-containing bilayers. In addition to this stoichiometry, which can be used to determine the size of the hydrophobic interface of an integral protein,[12] it has been possible to determine the selectivity of lipids for the protein interface, the exchange rates for lipids onto and off the protein interface, and the relative binding energies for lipids to a protein.[12,14]

No detailed description of the methods will be given here, but their use in studying membrane surface change, partitioning of lipophiles into membranes, will be described. The structures of the deuterated and spin-labeled lipids used in this study are shown in FIGURE 1.

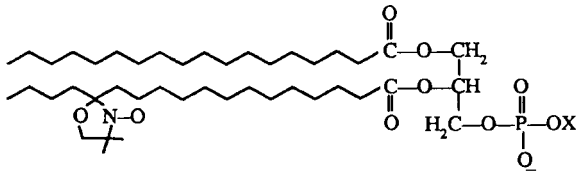
IONIZATION STATE OF BILAYER PHOSPHOLIPIDS AND PARTITIONED TETRACAINE

All naturally occurring phospholipids are either anionic or zwitterionic. The order and dynamics of these lipids in bilayers depend markedly on the ionization state of the polar head groups of the lipids.[10,15,16] One problem that exists in determining the ionization behavior of lipid polar groups when in the bilayer form, not least because of the shielding of the head groups that can occur with water, is that other lipids and ions exist and perturb the membrane surface electrostatics. By means of ^2H-NMR methods, such ionization behavior can be determined directly to give the true pK_a of shielded head groups.

In mixed bilayer dispersions of 1,2-dimyristoyl-*sn*-phosphatidyl-3-ethanolamine

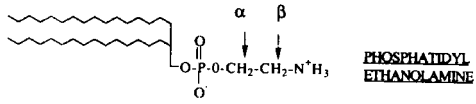


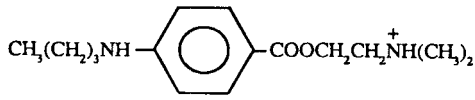
14-SASL



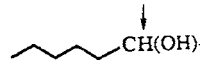
when X= —CH(OH)—CH(OH)—CH_2OH 14-PGSL

—CH_2—CH_2 —$N(CH_3)_3$ 14-PCSL





Tetracaine



hexanol

FIGURE 1. Formulae of the lipid spin labels (14-SASL, 14-PCSL, 14-PGSL), deuterated phospholipids (1,2 dimyristoyl-*sn*-phospho-3-ethanolamine-d_4, DMPE-d_4), and deuterated hexanol (α-C^2H_2-1-hexanol). Arrows denote sites of deuteration.

(DMPE), deuterons placed specifically in the head group (see FIGURE 1) are ideally placed at the bilayer polar–apolar interface to report on changes in surface properties at the molecular level. By titrating the bulk pH of such mixed DMPC/DMPE bilayers, the ^{2}H-NMR spectra (FIGURE 2) show that quadrupole splitting ($\Delta\nu_q$) is seen to change especially in the lower and higher pH ranges (FIGURE 3). The quadrupole splitting from the α-C^2H_2 group decreases at pH ~ 3, indicating that the phosphate group becomes protonated over this pH range; the β-C^2H_2 group does not respond to titration of the phosphate group, indicating a highly localized and sensitive orientational change at the bilayer surface. Similarly, at pH ~ 9–10, the β-C^2H_2 group of DMPE undergoes a pH-dependent change which can be fitted to a Henderson-Hasselbach formulation to give a pK_a of the amino group of DMPE of pH ~ 9.6.[17]

When tetracaine is incorporated into the bilayers, at a range of concentrations, a similar pH titration reveals a secondary change in the pH-dependent quadrupole splittings (FIGURE 3). Now both α-C^2H_2 and β-C^2H_2 groups respond to the same titration behavior, giving a pK_a for the secondary amino group of tetracaine at pH ~ 7.9 (from the α-C^2H_2) and 8.1 (from the β-C^2H_2 group). This titration behavior is in the range (pH 6–9) of that reported for tetracaine in aqueous dispersions, but here the molecule is titrated while in the membrane itself. In addition, the membrane ionization is monitored in a nonperturbing way. The changes in the quadrupole

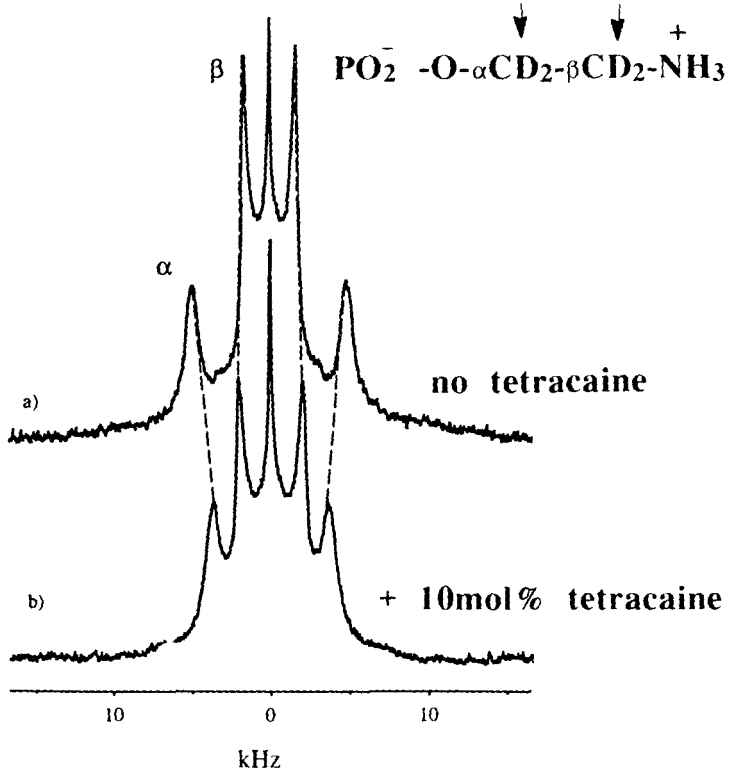


FIGURE 2. ^{2}H-NMR spectra for DMPC/DMPE-d_4 bilayers (1:1; mol:mol ratio) at 57°C with and without tetracaine at pH 5.5.

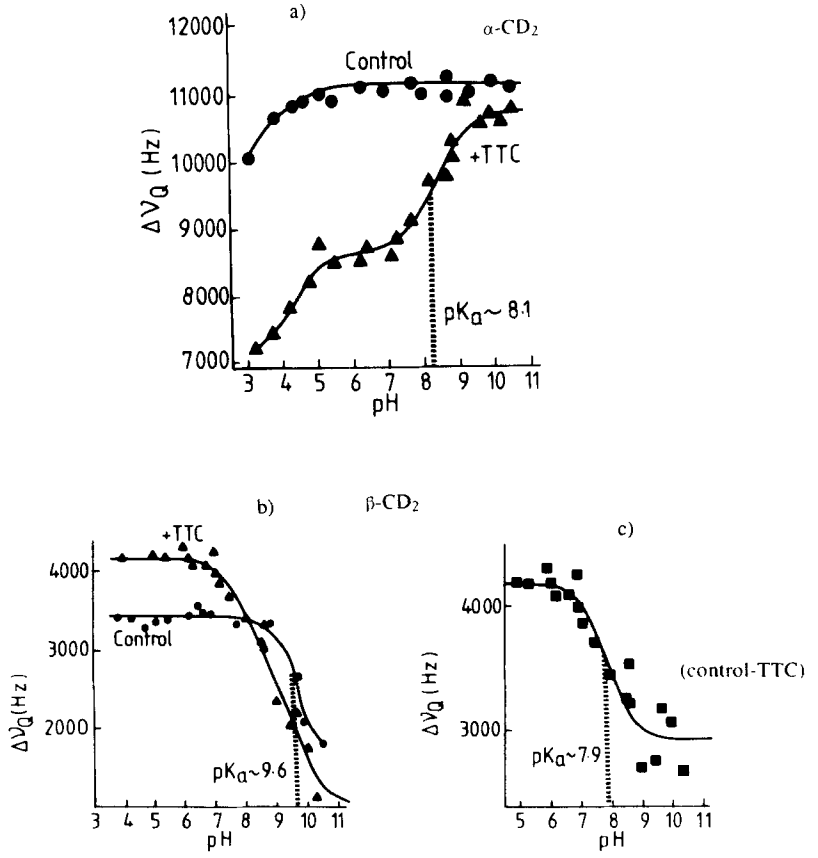


FIGURE 3. Deuterium NMR quadrupole splittings ($\Delta\nu_q$) measured from spectra similar to those in FIGURE 2 for DMPE-d_4 in DMPC bilayers (DMPC:DMPE; 1:1; mol:mol) at 57°C with varying pH (control, circles) and with added tetracaine (10 mol%) (TTC, triangles). The titration behavior for tetracaine (squares) sensed by the β-C^2H_2 was deduced by subtraction of the quadrupole splittings for the control and TTC (adapted from Watts and Poile).[17] All data were fitted using a standard Henderson–Hasselbach relationship.

splittings reflect only changes in the amplitude and/or orientation of the individual lipid C^2H_2 group at the bilayer surface. Such alterations may well be due to hydrogen bond formation for the deprotonated phosphate and protonated amino group. The perturbation of the two head group methylenes by tetracaine shows that the local anesthetic exchanges quickly ($\nu_{ex} > 10$ kHz) between the aqueous and bilayer phase and interacts with all positions in the polar head group.

PARTITIONING OF HEXANOL INTO MEMBRANES

Conventional methods for determining the partition behavior of lipophiles suffer from serious disadvantages. In particular, fast exchange (with respect to the experimental time scale) of lipophiles out of the aqueous phase and into the membrane

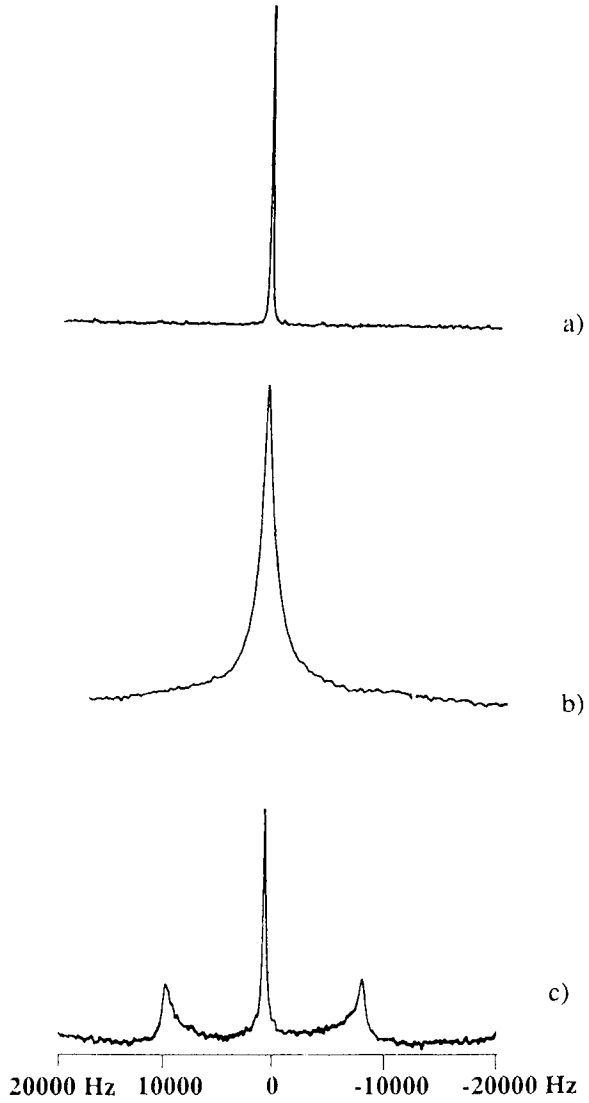


FIGURE 4. Deuterium NMR spectra (61.3 MHz) of α-C^2H_2-1-hexanol in water (a), a solubilized (hexanol:asolectin; 4:1 mole ratio) asolectin dispersion (b), and a DMPC bilayer dispersion (c) (1:10; hexanol:DMPC mole ratio).

bilayer core, can significantly distort a measured partition coefficient when determined by centrifugation or filtration methods.

The ^{2}H-NMR spectrum of lipophiles is very different for monomers (or small micelles) of the lipophile in solution when compared with the lipophile in a relatively ordered membrane bilayer core.[19] We have exploited this spectral difference between isotropically and anisotropically moving lipophiles for 1-hexanol, which was deuter-

ated in the α-C^2H_2 position[18] (FIGURE 1). In free solution, ^{2}H-1-hexanol gives rise to an isotropic single line spectrum (width, $\Delta \sim 20$ Hz) (FIGURE 4). When added to a phospholipid dispersion (at less than 4:1 hexanol:lipid mole ratio) such as asolectin or DMPC, a new spectral component is recorded (FIGURE 4) with a well-resolved quadrupole splitting of ~17 kHz together with an isotropic line (width, $\Delta \sim 20$ Hz) that is characteristic of the spectrum from hexanol in water. The exchange of ^{2}H-1-hexanol into bilayers from the isotropic phase is slow on the quadrupolar anisotropy averaging time scale; $\nu_{ex} < 17$ kHz, the width of the broad spherically averaged spectrum.

At high hexanol:lipid mole ratios of greater than ~4:1, a single isotropic line shape was recorded and the dispersion clarified as micelles were formed. The ^{31}P-NMR spectra (not shown) confirm that at these high hexanol concentrations, the bilayers are solubilized. In addition, the rotational rate for the isotropic hexanol in such micelles is fast ($\tau_R^{-1} < 200$ Hz), implying that the molecular complex has a

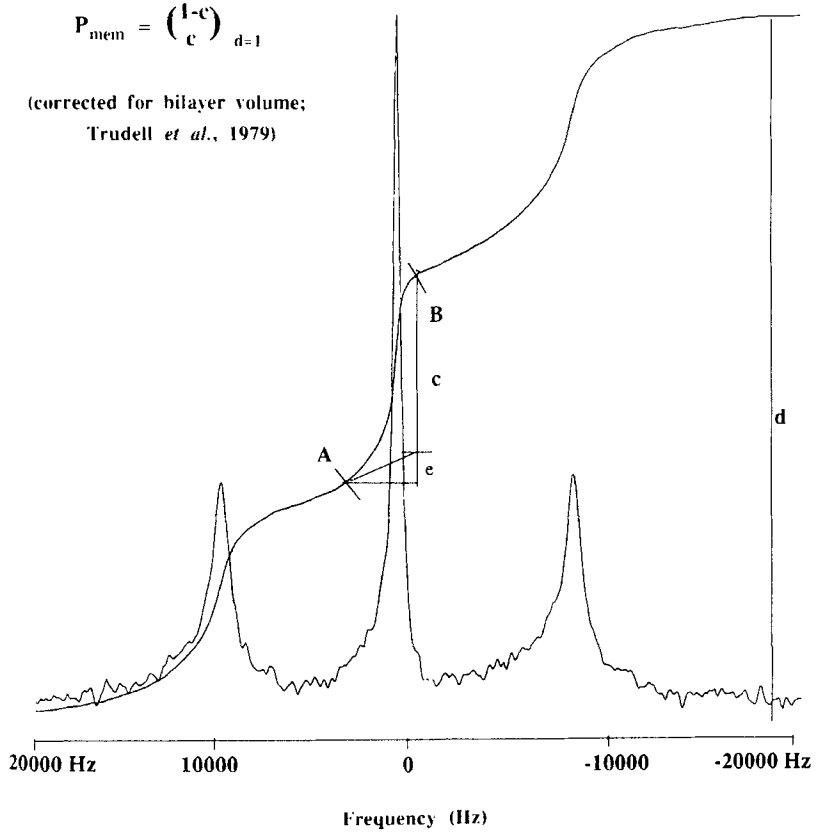


FIGURE 5. Deuterium NMR spectra (61.3 MHz) for α-C^2H_2-1-hexanol in DMPC bilayers as in FIGURE 4, showing the integration method used to quantitate the proportions of hexanol in the aqueous phase (c) and in the bilayer environment ($1 - c$), where the total integrated intensity (d) is normalized. The partition coefficient P_{mem} is then the ratio of free to bound hexanol, $(1 - c/c)_{d=1}$ (From Fraser.[19] Reprinted by permission.)

small (<50 nm) radius and can readily and quickly orientate through 90° in the magnetic field.

Quantitation of the two spectral components allows calculation of a partition coefficient, P_{mem}, since the concentration of hexanol in any one environment is proportional to the integrated spectrum for each component. Thus:

$$P_{mem} = \frac{[\text{hexanol}]_b}{[\text{hexanol}]_f} = \left(\frac{1 - \Sigma c}{\Sigma c}\right)_{\Sigma d = 1}$$

where $[\text{hexanol}]_b$ and $[\text{hexanol}]_f$ are the concentrations of hexanol in the bilayer and fluid phases, respectively, and Σc is the integrated intensity of the narrow NMR spectral component if Σd is the normalized total integrated spectral intensity as shown in FIGURE 5. A correction is then made to P_{mem} for the bilayer and aqueous volumes.[20]

The values of P_{mem} for 1-hexanol partitioning into DMPC bilayers is shown in FIGURE 6. At low hexanol concentrations (hexanol:DMPC < 0.5:1 mol ratio), there is a concentration-dependent partition coefficient until, at higher hexanol content, the dependence of P_{mem} on hexanol:DMPC ratio is less marked, giving a partition coefficient of ~3. The increase of hexanol in DMPC bilayers does not lead to a change in the order of the environment for hexanol in the bilayers, since the quadrupole splitting for the α-C^2H_2 hexanol is relatively unchanged at $\Delta\nu_q \sim 17$ kHz; this rather high value is similar to the order ($S \sim 0.1$) of the lipid bilayer for acyl chain carbon atoms around C12–C14.[1] Hexanol in the more disordered environment and which gives rise to the isotropic but broad spectrum may be located randomly at the bilayer interface or possibly in micelles of hexanol and lipid. However, since the proportion of isotropic component in the ^{31}P-NMR spectra (not shown) does not increase proportionately with increasing hexanol concentration, it is not likely that the hexanol is in an isotropically moving particle containing phospholipids.

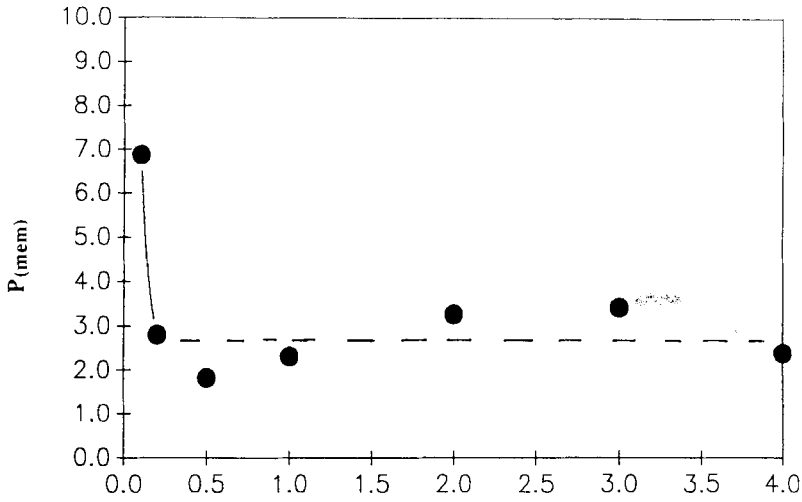


FIGURE 6. The variation of P_{mem} (defined and measured as in FIGURE 5) as a function of hexanol concentration in DMPC bilayers up to the solubilizing concentration of 4:1, hexanol: DMPC mole ratio. (From Fraser.[19] Reprinted by permission.)

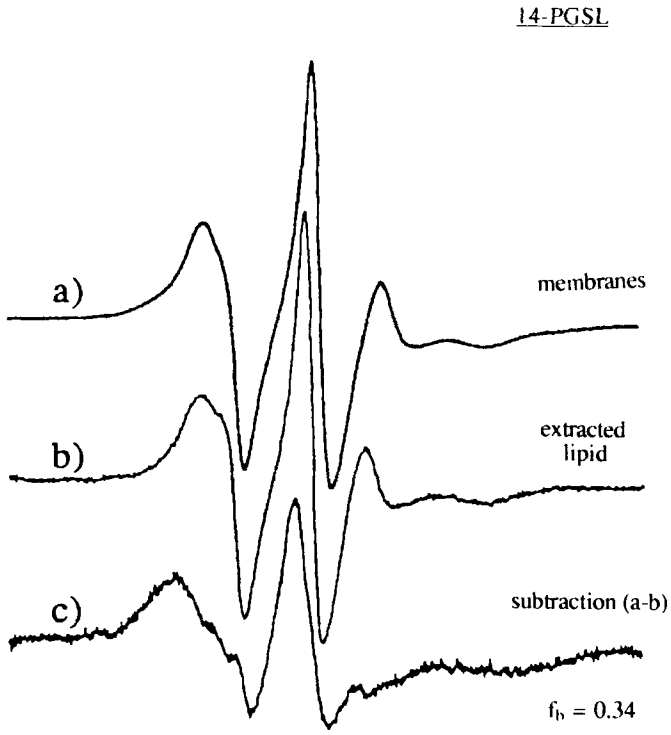


FIGURE 7. ESR spectra for 14-PGSL (1 label:150 endogenous lipids) in nAChR-rich membranes from *T. nobliana* at 273 K (upper spectrum). Spectral subtraction methods were used to give the narrow lipid-like component (middle spectrum; $1 - f_b \sim 0.58$) and the motionally restricted component (lower spectrum; $f_b \sim 0.42$). The two lower spectra were then computer simulated to give spectra that were added and compensated in line broadening to give estimates for molecular exchange rates between the two motionally distinct environments. (From Fraser *et al.*[23] Reprinted by permission.)

Thus, ^{2}H-NMR methods may be usefully applied to determine equilibrium partition coefficients of deuterated lipophiles in biomembranes in a nonperturbing way.

PARTITIONING OF GENERAL ANESTHETICS INTO THE LIPID INTERFACE OF THE NICOTINIC ACETYL CHOLINE RECEPTOR IN MEMBRANES

The slow exchange of spin-labeled phospholipids between the motionally restricted interface of integral proteins and the bulk, protein-free bilayer has been successfully examined with the nicotinic acetyl choline receptor (nAChR) in both enriched natural membranes[21] and reconstituted membranes.[22] In this study, our original experiments[21] on enriched nAChR membranes (but from *T. nobliana*) have been repeated, but the alteration in the fraction of lipid labels at the protein

interface (f_b) caused after the addition of a range of lipophilic general anesthetics has been studied.[23]

The lipid spin-labeled analogues of stearic acid, phosphatidylcholine and phosphatidyl glycerol, with the nitroxide at the C14 position of the *sn*-2 chain (FIGURE 1), have been incorporated into nAChR-rich membranes at probe concentrations (1 spin label per 150 endogenous lipids). The ESR spectra recorded over a range of temperatures for all labels are two-component in nature (FIGURE 7), one component characteristic of slowly ($\tau_R < 10^{-7}$ s) moving labels at the protein interfacial boundary and the other component characteristic of acyl chain labels probing the bulk bilayer and with fast ($\tau_R > 10^{-8}$ s) molecular motion. Quantitation of the two slowly exchanging components ($\nu_{ex} < 10^7$ Hz) by both spectral subtraction and computer simulation methods has confirmed previous work[12,22] that charged fatty acid and phosphatidyl glycerol labels preferentially interact with the protein interface when compared to phosphatidyl choline label (FIGURE 8). This selectivity is reflected in the proportion of lipids at the protein interface (14-SASL, $f_b \sim 0.44$; 14-PGSL, $f_b \sim 0.42$; and 14-PCSL, $f_b \sim 0.33$) and in the more restricted motion of the charged labels at the protein interface when compared with the 14-PCSL as reflected in the narrower line widths of the motionally restricted components for the charged spin labels and off-rate from the protein interface estimated from simulations (TABLE 1).

Addition of urethane, ethanol, hexanol, and diethylether to nAChR-rich membranes reduces the proportion of motionally restricted labels (f_b) at the protein interface (TABLE 1). Although the fraction of each label displaced from the interface ($\Delta f_b/f_b$) is different at the same concentration for each lipophile, a similar fractional

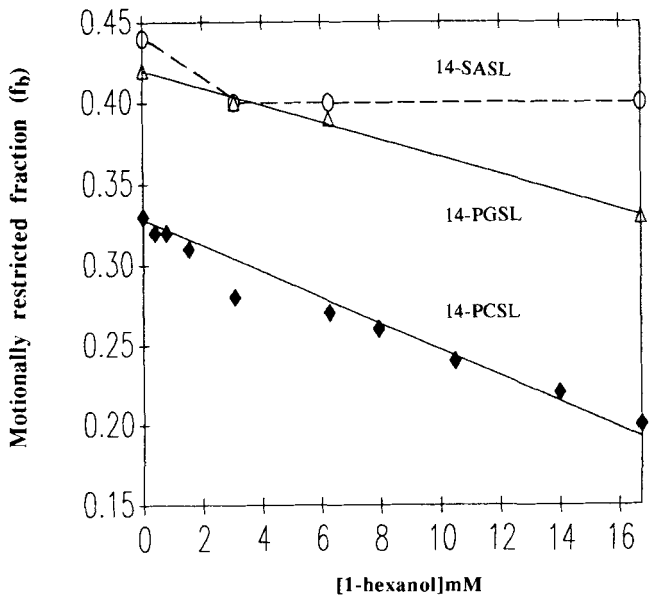


FIGURE 8. Variation in the fraction of motionally restricted lipids ($1 - f_b/f_b$) in nAChr-rich membranes at 273 K for 14-SASL, 14-PCSL and 14-PGSL as a function of membrane hexanol. (From Fraser *et al.*[23] Reprinted by permission.)

TABLE 1. Summary of Spin-Label Results on nAChR-Rich Membranes[a]

Spin Label	$(\Delta f_b/f_b \times c)$ $[M^{-1}]$	$\Delta f_b/f_b$	τ_b^{-1} $[10^6\ s^{-1}]$
14-SASL			
Control		0	8
Hexanol	−1.40	0	8
14-PGSL			
Control		0	12
Hexanol	−12.80	−0.09	15
14-PCSL			
Control		0	18
Hexanol	−23.60	−0.15	27
Urethane	−0.69	−0.09	25
Diethylether	−2.11	−0.14	26
Ethanol	−0.09	−0.03	22
	Structure		Dynamics
	Concentration dependence	Functional dependence	

[a]The fraction of lipids in the protein boundary is (f_b) and the change in f_b with added lipophile (Δf_b). The concentration dependence ($\Delta f_b/f_b \times c$) and fractional change at a concentration of lipophile that desensitizes 50% of the receptors ($\Delta f_b/f_b$) is also given with the off rates (τ_b^{-1}) for labels from the protein-lipid interface. (Adapted from Fraser *et al.*[23])

displacement ($\Delta f_b/f_b$) is observed (within a factor of 5) for concentrations of the lipophile, which desensitizes 50% of the receptors in functional studies.

In addition to the perturbation of the lipid-protein interface by the general anesthetics tested, the order of the bulk lipid phase is also perturbed by the lipophiles in agreement with previous observations.[24] However, the quantitation of this perturbation through apparent order parameter measurements (FIGURE 9) intitially requires the subtraction of the broad component to give the bulk more mobile spectral component. It is then this narrower component that can be used to calculate an apparent order parameter (S_{app}) from the ratio of the observed anisotropy ($A_{/\!/} - 2A_\perp$) to the maximum possible anisotropy [$A_{zz} - \frac{1}{2}(A_{xx} + A_{yy})$] and corrected for the polarity of the spin-label environment.[25] The changes in the order parameter (S_{app}) are similar for 14-SASL and 14-PCSL, but rather less marked for 14-PGSL. The reduction in order on partitioning of general anesthetics has been observed previously,[26] but in this present study the contribution to line-position changes attributable to spectral overlap from the component due, in turn, to labels interacting with the protein interface has been accounted for. In addition to disordering of the bulk fluid phase by the general anesthetics, the mobility of the interfacial lipids is also changed. The off rate (τ_b^{-1}) of the lipids from the receptor interface are increased by addition of the general anesthetics (TABLE 1) and the line widths of the motionally restricted components become larger, indicating increased local motion at the protein interface.

From this work it appears that the protein-lipid interface does indeed become perturbed by general anesthetics as does the bulk lipid bilayer. However, one uncertainty in this work on the natural membrane is whether the total number of lipids (spin labels, endogenous lipids, and lipophiles) at the interface decreases when anesthetic is added or whether the relative association of the label for the protein interface ($K_R = K_{PC} - K_L$ where PC is the PC spin label, L is the lipophile, and K_R the relative association constants) decreases. Only by carrying out reconstitution experi-

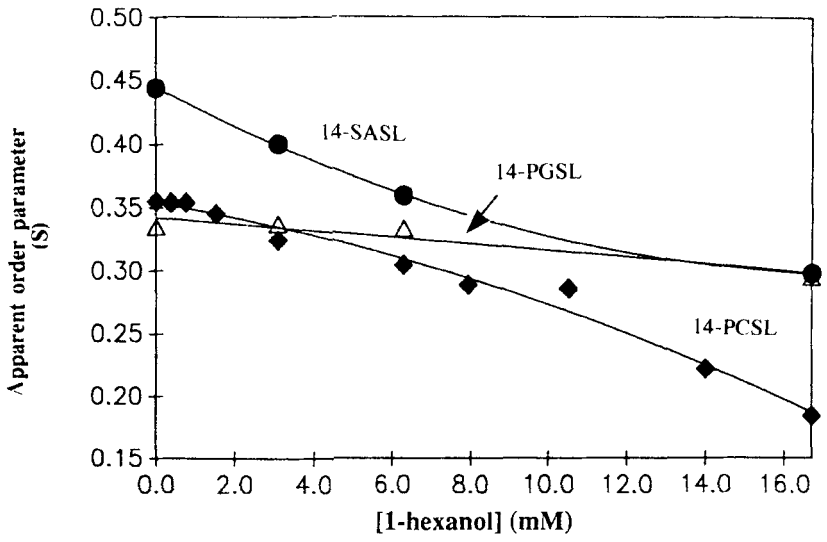


FIGURE 9. Apparent order parameter for the bulk fluid ESR spin-label spectrum for 14-SASL, 14-PGSL, and 14-PCSL in nAChR-rich membranes after subtraction of the motionally restricted component.

ments in which the lipid:protein ratio (n_t) of the complex is varied can an attempt be made to answer this question. Since $n_t = n_f + n_b$ (where n_t is the number of free lipids and not at the protein interface and n_b is the number of lipids in the protein boundary), then n_f/n_b (or $1 - f_b/f_b$) plotted with n_t gives the number of lipids at the protein interface (N_1) at $n_f/n_b = 0$, and $(K_R)^{-1}$ is given by $n_t = 0$. Now the relative binding energy ($\Delta G_{PC} - \Delta G_L = RT \ln K_R$) for a lipophile compared to the phosphati-

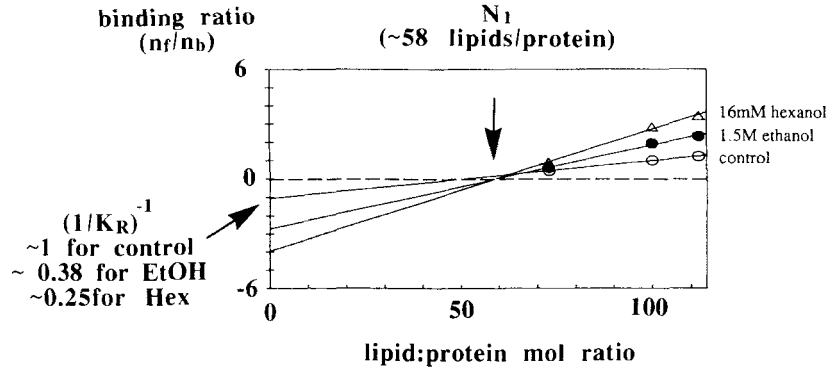


FIGURE 10. Titration with lipid-protein mole ratio (n_t) of the fraction of 14-PCSL spectrum motionally restricted in reconstituted nAChR-DOPC complexes with no added lipophile, with 16 mM hexanol, and 1.5 M ethanol. The number of lipids at the nAChR-lipid interface is 48–58 (at $n_t = 0$) and the relative association constant for 14-PCSLs over DOPC is given by $1/K_R$ at $(1 - f_b/f_b) = 0$. (From Fraser.[19] Reprinted by permission.)

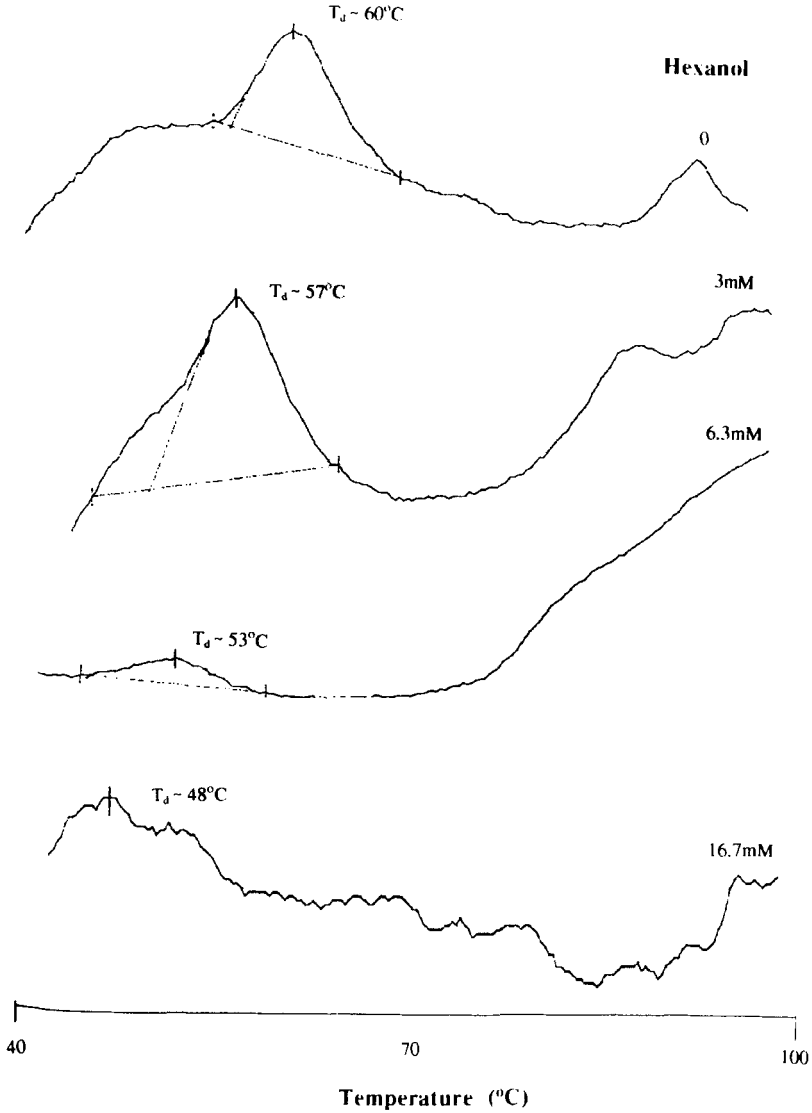


FIGURE 11. DSC thermograms showing the denaturation temperatures of nAChr in enriched membranes at increasing hexanol concentrations. (From Fraser *et al.*[23] Reprinted by permission.)

dyl choline spin label can be estimated, as performed to determine lipid selectivity in a wide range of membranes reconstituted with integral proteins.[12,14]

Thus, to gain further insight into lipophile action, experiments have been performed with reconstituted bilayers containing 1,2-dioleoyl-*sn*-phospho-3-choline (DOPC) and the nAChR at a range (73, 101, 112 lipids/protein) of protein-lipid ratios and temperatures (273, 280, and 293 K). In common with the experiments on

nAChR-rich membranes from *T. nobliana,* the fraction of motionally restricted spin labels decreased with separately added ethanol (1.5 M) and hexanol (16 mM) (FIGURE 10) at all temperatures studied.[18] By analyzing the binding ratio for motionally restricted lipids with chemically determined lipid:protein mol ratio, it is possible to deduce the number of lipids at the protein interface, N_l. A value for N_l of 48–58 molecules of DOPC around the protein can be estimated from FIGURE 10, apparently independently of the anesthetic concentration. If the number of lipids that can interface with the protein is unchanged, then the relative association constant, K_R, given by $(1 - f_b/f_b)^{-1}$ at $n_t = 0$, must change. The intercept at $n_t = 0$ for the control spin-labeled sample with no anesthetic, shows that the 14-PCSL shows no added affinity ($K_R = 1$) for the nAChR over DOPC. However, at the ethanol and hexanol concentrations tested (1.5 M ethanol and 16 mM hexanol), K_R is 0.38 and 0.25, respectively. This gives a value of $(\Delta G_L - \Delta G_{PC})$ of 0.2 kJ· mol^{-1} and 0.3 kJ· mol^{-1} for the nAChR interface induced by the presence of the anesthetics. Such energies are relatively small but not insignificant when compared with RT ~ 2.2 kJ· mol^{-1} at 273 K, especially when this energy is averaged over all protein-lipid interfacial sites. Thus, the change in the association of lipids with the protein interface by anesthetics probably reflects a predominantly hydrophobic rather than electrostatic perturbation of the lipid-protein interface induced by the anesthetics. In addition, even though the absolute concentration of each anesthetic in the membrane is very different, the degree of displacement is similar in energetic terms. In an attempt to monitor the stability of the nAChR in the presence of hexanol, the thermal denaturation of the protein in membranes has been studied (FIGURE 11). At hexanol concentrations up to 16.4 mM, the protein denatures at lower temperatures, suggesting that the protein is stabilized by lipid-bilayer protein interactions and that these can be perturbed by hexanol.

In no way do these experiments prove that general anesthetics selectively partition to a protein-lipid interface[27,28] or the bulk bilayer, but the results suggest that some general effects may be involved in the interaction of lipophiles with functional proteins. The acyl chain length cutoff and pressure-reversal phenomena should now be attempted with this method.

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