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Chain-length dependence of *n*-alkane solubility in phosphatidylcholine bilayers: a ^{2}H-NMR study

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Phosphatidylcholine bilayer membranes containing ^{2}H-labelled *n*-alkanes have been studied by ^{2}H-NMR as a model system for the investigation of molecular theories of general anesthesia. The solubilities of *n*-alkanes in lipid bilayers have been determined by measurement of the relative intensities of a powder pattern signal arising from orientationally ordered, membrane-soluble alkane and a sharp signal in the ^{2}H-NMR spectrum resulting from isotropically reorienting alkane. The ordering profiles for the ordered *n*-alkane as determined from the quadrupole splittings for different segments along the chain are similar to those described earlier for *n*-hexane, *n*-octane and *n*-dodecane, suggesting that the restricted motions undergone by the *n*-alkanes of chain length from 6 to 19 are basically similar. For this homologous series of *n*-alkanes, it was found that membrane solubility dropped sharply at an alkane chain length which depended on lipid chain length, degree of unsaturation, cholesterol concentration in the bilayer, and temperature. The results show that the incorporation of *n*-alkanes in lipid bilayers is a complex function of lipid composition. The implications of these results in relationship to the observed 'cut-off' in anesthetic potency in the *n*-alkane homologous series are discussed.

Introduction

The nature of the interaction of *n*-alkanes with phospholipid bilayer membranes is of interest for a number of reasons. Firstly, alkanes are important in the preparation of planar black lipid membranes which have been used for many years in the study of electrolyte and non-electrolyte transport across membranes [1]. Secondly, a knowledge of the manner in which alkanes are arranged in bilayers should further our understanding of how to treat the bilayer as a solvent for other molecules [2]. Finally, alkanes have several pharmacological effects [3], which are believed to result from their interactions with cellular membranes.

One pharmacological effect of *n*-alkanes, especially the shorter chain analogs, is the ability to act as general anesthetics. Haydon et al. [4] postulated that this ability was due to interactions with the bilayer. The detailed mechanism by which this occurs, whether the alkane binds initially to a lipid site or directly to a membrane protein, remains unknown and has been the subject of much discussion [5–7]. It has been known for many years that a sudden drop in the potency of an alkane as an anesthetic occurs at a well-defined chain length of between C_6 and C_{10} depending on the species [8,9]. For example, Mullins showed that mice were anesthetized by *n*-nonane, but were unaffected by *n*-decane [10]. It has been postulated that this cut-off in anesthetic potency results from either a sudden drop in solubility of *n*-alkanes as one ascends the homologous series [11] or from decreased binding of *n*-alkanes at a hydrophobic site of defined size on an unknown target protein in the central nervous system [12,13].

It is apparent that, if the lipid-based unitary theories of general anesthesia are to be consistent with the observed cut-off in anesthetic potency in the *n*-alkane series, it is essential to determine their solubilities in bilayer membranes. BLM experiments by White [14] and Fettiplace et al. [15] with phosphatidylcholine bilayers showed that *n*-alkane solubility indeed decreased with increasing chain-length, but that the cut-off in solubility occurred at tetradecane. Similarly, Coster and

Abbreviations: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitcylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; egg-PC, egg-yolk Phosphatidylcholine; BLM, black lipid membranes.

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Laver [16] found a decrease in the incorporation of *n*-alkanes into egg-PC bilayers as the chain length increased. A further set of BLM experiments by Haydon's group [4] in which cholesterol was mixed with egg-PC yielded capacitance measurements which showed that the cut-off in solubility occurred at *n*-nonane, in better agreement with the capacities of the *n*-alkanes to act as anesthetics.

Previous ^{2}H-NMR studies of *n*-alkane-containing phospholipid multibilayers, by Pope et al. [17,18] and Jacobs and White [19] have focussed on measuring orientational ordering of both the *n*-alkane and lipid components of the membrane rather than on measuring the solubilities of *n*-alkanes in the bilayer. The form of the order parameter profiles for the *n*-alkane chains is consistent with a model in which the chains are intercalated between the lipid hydrocarbon chains. A model in which the alkane molecules were 'sandwiched' between monolayers at the bilayer center could therefore be ruled out. The former model based on ^{2}H-NMR results is in agreement with both X-ray [17,20] and neutron diffraction [21] data.

In the course of these earlier ^{2}H-NMR studies [17,18] a number of spectra were recorded which indicated that the *n*-alkane was contained not only in the bilayer but also in a separate phase which was in slow exchange (on the NMR time-scale) with the ordered or 'dissolved' component. In such cases a sharp central peak appeared in the spectrum which indicated that alkane molecules were reorienting isotropically. The component represented alkane in a disordered environment which was insoluble in the bilayer. The present work has extended and quantified these results. It is an attempt to determine the dependence of the solubility of *n*-alkane in bilayers, upon *n*-alkane chain length, lipid acyl chain length and unsaturation, cholesterol concentration, and temperature.

Experimental Procedures

Materials

Unlabelled DMPC, DPPC, DSPC and egg-PC were obtained from Avanti Polar Lipids, Inc., Birmingham, AL. Perdeuterated *n*-alkanes, ^2H_2O and deuterium-depleted water were purchased from MSD Isotopes, Montreal, Canada and Sigma Chemical Co., St. Louis, MO. Perdeuterated *n*-nonane was prepared by catalytic exchange at high temperature and pressure, of *n*-decanoic acid with ^2H_2O in a pressure vessel [22]. If more severe conditions than those described in this reference were employed, approximately 15% of the decarboxylated product, perdeuterated *n*-nonane, was obtained in addition to the major product, perdeuterated *n*-decanoic acid. The desired product was isolated by ether extraction from the basic reaction mixture. The alkane was purified by fractional distillation to give material with physical properties consistent with published data.

Sample preparation and data acquisition

Lipid-bilayer samples of known composition were made by addition of appropriate quantities by microsyringe of perdeuterated *n*-alkane and ^{2}H-depleted water to a 5 mm o.d. glass tube containing a known amount of pure dry phospholipid. Depending on the volatility of the *n*-alkane, cooling with ice, solid CO_2 or liquid nitrogen was employed during the additions. The molar ratios of alkane/lipid/water were either 0.1 : 1 : 25 or 0.5 : 1 : 25. A number of bilayer samples containing 25 mol% cholesterol (molar ratio of lipid/cholesterol = 3 : 1) were also prepared. In these instances the PC and cholesterol were co-dissolved in chloroform and the solvent removed with a stream of dry N_2 gas followed by drying under high vacuum for 12–24 h.

The above NMR samples tubes were then flame sealed, and the contents thoroughly mixed by equilibration at a temperature above the main gel to liquid-crystalline phase transition (T_m) of the lipid, as well as by repeated centrifugation. ^{2}H-NMR spectra were recorded at 14–15 K above T_m on a Bruker CXP-300 spectrometer operating at 46.063 MHz using a quadrupole echo pulse sequence. Typically, spectral widths were in the range 25–40 kHz. Integrals were obtained digitally. Further details about data acquisition and processing, the spectrometer, and its sample temperature control system are described in previous publications [17,18,23,24].

Interpretation of NMR spectral parameters

The theory of ^{2}H-NMR as applied to bilayer membranes is well established and a full discussion can be found elsewhere [25,26]. Of the several parameters that can be measured in the ^{2}H NMR spectrum, relative signal intensities and time-averaged quadrupole couplings (ν_Q) have been utilized in this study. In measuring the distribution of ^{2}H-labelled *n*-alkanes between bulk phases it has been assumed that relative signal intensities accurately reflect relative populations of ^{2}H-nuclei in the different bulk phases. In the case of fast anisotropic motions in systems with a random or spherical distribution of director axes, such as aqueous multilamellar dispersions of lipids, the latter parameter, ν_Q, is directly proportional to the quadrupole splitting ($\Delta\nu_i$) between the 90° edge singularities of the uniaxial spectral powder pattern (i.e. the Pake doublet). This quantity is given by:

$$\Delta\nu_i = (3/4)\nu_Q\langle(3/2)\cos^2\sigma_i - (1/2)\rangle$$

where ν_Q is the solid-state quadrupole coupling constant associated with a given C-^{2}H, bond and $\langle(3/2)\cos^2\sigma_i -$

$(1/2)\rangle$, or the order parameter S_{C-^2H}, is the time-average of the quantity in brackets, taken over all angles (σ_i) that the C-^{2}H$_i$ bond direction assumes with respect to the time averaged principal axis of the electric-field-gradient tensor during the time-course of the NMR measurement. A zero value for the order parameter can result from a ^{2}H-labelled site in a molecule being in a phase where it undergoes rapid isotropic motion or it can result from a situation where the time-averaged orientation of the C-^{2}H$_i$ bond with respect to the most ordered or principal molecular axis, is at the 'magic' angle ($\cos^{-1}1\sqrt{3}$). Earlier ^{2}H-NMR studies of *n*-alkane-containing phospholipid bilayers suggest that this is unlikely, so that any sharp spectral signals can probably be assigned totally to solute undergoing rapid isotropic motion. The NMR technique is unable, however, to determine unambiguously whether that component exists as microlenses between the two monolayers of the bilayer, or as micelle-like aggregates at the water-lipid interface.

Results and Discussion

Representative 46.063 MHz ^{2}H-NMR spectra of 9 mol% solutions of perdeuterated *n*-dodecane and *n*-hexadecane in bilayers of DMPC are shown in Figs. 1(a) and (b), respectively. The most prominent features of the spectrum in Fig. 1(a) are two broadened powder patterns. The pattern with maxima separated by approx. 8 kHz, consists of several unresolved Pake doublets, which on the basis of published data [17,18] were assigned to the ten methylene sites in *n*-dodecane. The central powder pattern with splitting width of approx. 2 kHz was assigned to the two methyl groups. The spectrum in Fig. 1(a) differs somewhat from those reported earlier [17,18] for the same system, in two features; namely, the resolution is poorer and a small sharp peak in the center is evident, reflecting the presence of an isotropic component in the sample. This peak accounts for about 1% of the total signal. The poorer resolution results from the higher hydrations used in this study (lipid/H_2O = 1 : 25), it being noted earlier that better resolved spectra were obtained at lower hydration (lipid/H_2O = 1 : 9). This study was undertaken at the higher hydration so as to more closely approximate a biological situation. Another problem associated with higher hydrations is that the time taken for the relative intensity of the central peak compared to that of the ordered components to reach a constant value (i.e., equilibration time) becomes inordinately long. In the case of the longer chain alkanes this may be several weeks, even at the water concentration used here. In general it is found that the percentage of ordered material increases with time. In addition, at higher hydrations, the separation of the ordered and isotropic components in the spectra are difficult particularly in the case of the short chain *n*-alkanes and at higher alkane concentrations. The problem here is that the isotropic peak becomes broad (presumably either due to a shorter T_2, or to exchange of alkane between isotropic and ordered environments occurring at a rate which is intermediate on the NMR time-scale) and is difficult to separate from the C^{2}H$_3$ peak of the ordered component. The above factors are largely responsible for the scatter observed in measurements of the relative amounts of ordered and disordered components.

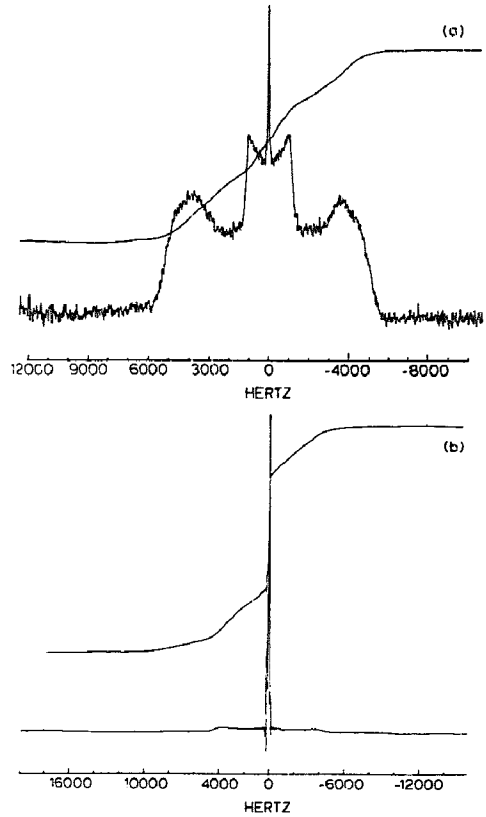

Fig. 1. Representative ^{2}H-NMR spectra of perdeuterated *n*-alkanes in lipid bilayers formed from DMPC/H_2O/*n*-alkane (1 : 25 : 0.1): (a) *n*-dodecane, T = 310 K; (b) *n*-hexadecane, T = 310 K.

The corresponding data for *n*-hexadecane (Fig. 1(b)) show a large isotropic signal which accounts for 50% of the total spectral intensity. The origin of the minor isotropic peak slightly down field of the major isotropic signal is unknown. Spectra of *n*-hexadecane-d_{34} mixed with ^{2}H-depleted water indicate that this peak does not result from a different chemical shift associated with any of the segments in hexadecane. One possibility is that one isotropic peak arises from *n*-hexadecane microlenses between the monolayers of the bilayer and the other peak from micelle-like aggregates of hydrocarbon

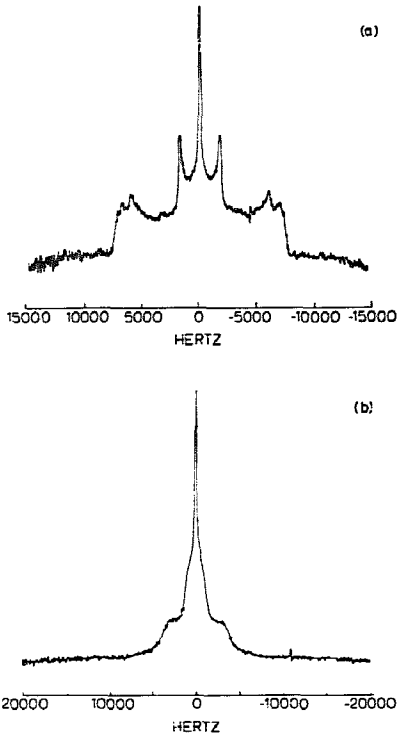

Fig. 2. ^{2}H-NMR spectra recorded at 310 K of (a) perdeuterated *n*-nonane in lipid bilayers of molar composition DMPC/cholesterol/H_2O/*n*-nonane (1:0.33:25:0.1), and (b) *n*-hexane in bilayers of composition DMPC/cholesterol/H_2O/*n*-hexane (1:0.33:25:0.5).

at the lipid/water interface, although it would not be possible on NMR evidence alone, to make assignments. The spectra for each of the *n*-alkanes of chain length greater than ten show the same features as those described above. In the case of *n*-hexane a further isotropic peak was observed which was attributed to solute vapor above the bulk sample in the NMR tube.

The ordered component in Fig. 1(b) on expansion shows a spectrum similar to that for *n*-dodecane (Fig. 1(a)). This is also true for the other *n*-alkanes examined in this study. In some instances individual methylene singularities in the broad methylene signal could be resolved (Fig. 2a). The similarity in the spectra for various *n*-alkanes infers that they all undergo similar types of motions in the bilayer. These motions, which have been described earlier by us [17,18] and by Jacobs and White [19], include a shuffling between the two monolayers in a direction normal to the bilayer surface, as well as rotation about this normal axis and *gauche-trans* isomerization.

Although the poor spectral resolution in most cases of the signals in the ordered component precludes a detailed comparison of quadrupole splittings at analogous sites in the homologous series, the following trends were observed. At constant alkane concentration in the bilayer and at comparable reduced temperatures the methylene and methyl splittings for a given alkane tend to decrease with increasing phospholipid acyl chain length. The effect is more pronounced for long chain alkanes (nonadecane) than for shorter chain analogs (e.g., octane). Generally lower splittings were observed for the *n*-alkanes in egg-PC than in any of the PC with saturated chains. These results probably reflect the greater volume in the center of the bilayer in the case of egg-PC. Thus, molecules of *n*-alkane intercalated in egg-PC bilayers would be expected to experience greater motional freedom and hence be more disordered. The addition of cholesterol to the lipid bilayer increases the methylene splittings for any given alkane. At a lipid to cholesterol ratio of 3 to 1, typically the increase is 2–3 kHz. These observations are consistent with the well-known ordering effect of cholesterol [27]. Increasing the concentration of an *n*-alkane in the bilayer is found to decrease the methyl and methylene splittings of the solute. Since the ordering of the solute reflects that of the lipid acyl chains, this result shows the disordering effect *n*-alkanes have on bilayer structure. A similar effect is observed with increasing temperature in a given phospholipid/*n*-alkane system.

The parameter of more direct concern in this study, however, is not the quadrupole splitting but the relative signal intensity of the isotropic and ordered components in the ^{2}H-NMR spectrum, which gives a measure of the solubility of an alkane in the lipid bilayer. Figs. 1(a) and (b) show integral plots which can be used to determine the relative contributions to the overall signal of the isotropic and ordered components. In determining the contribution of the ordered component, allowance was always made for the signals in the 'wings'; that is, the part of the powder pattern between the 0° and 90° edge singularities. Separation of the isotropic and ordered components by this method is straightforward for the longer chain *n*-alkanes and low solute concentrations. It becomes difficult, however, for octane and hexane particularly at increased solute concentrations, since the splittings for the ordered component decrease with increasing alkane concentrations, while the linewidth of the isotropic component becomes broader in the shorter chain alkanes, reflecting a reduction in spin-spin relaxation time T_2. (This is illustrated in Fig. 2b for the case of *n*-hexane in a DMPC/cholesterol (3:1) bilayer for an alkane/lipid ratio of 0.5.)

The solubilities of *n*-alkanes in egg-PC and egg-PC/cholesterol bilayers, determined as described above, are shown in Fig. 3 as a function of *n*-alkane chain length. Under our experimental conditions for a molar ratio of alkane/lipid/H_2O of 0.1:1:25, at 37°C, there is a

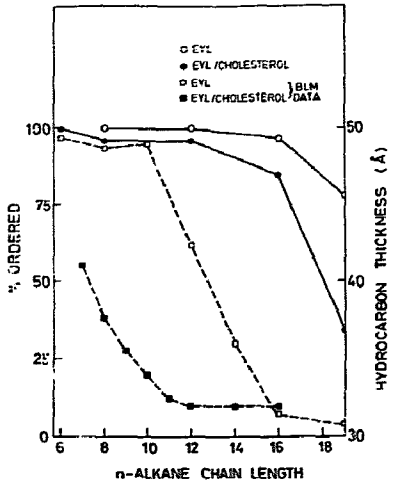

Fig. 3. Plot of percentage of ordered spectral component versus *n*-alkane chain length for bilayers of molar composition, egg-PC/*n*-alkane/H_2O (1:0.1:25) and egg-PC/cholesterol/*n*-alkane/H_2O (1:0.33:0.1:25). Dashed lines are plots of hydrocarbon layer thickness (right-hand scale) of BLM formed with different *n*-alkanes, versus *n*-alkane chain length. The latter data are from Haydon et al. [4]. EYL, egg-PC.

trend of reduced solubility of the alkanes with increasing alkane chain length. However, it is not particularly marked, with *n*-nonadecane still being 80% soluble in the bilayer. The addition of cholesterol, so that the molar ratio of egg-PC/cholesterol is 3:1, results in a reduction in alkane solubilities which is most pronounced for the longer-chain alkanes. The solubility of *n*-nonadecane drops to about 35%.

In the same figure is also plotted the hydrocarbon layer thickness of black lipid membranes (BLM) formed with different *n*-alkanes, as determined by Haydon et al. [4]. The point of sudden decline in bilayer thickness in both the egg-PC and egg-PC/cholesterol BLM, which can be attributed to decreased absorption of *n*-alkane [4], differs considerably from that found by NMR solubility measurements. It would be difficult to duplicate the conditions of these BLM experiments in our NMR observations, partly because the exact amount of *n*-alkane absorbed in the BLM bilayer is unknown. In a steady-state situation, the alkane in the bilayer is in thermodynamic equilibrium with bulk lipid-alkane solution in the surrounding Plateau-Gibbs border (annulus) and with *n*-alkane in the aqueous solution. NMR measurements of the concentration dependence of alkane solubility (vide infra), show it to be reduced at higher concentrations, the effect being enhanced by the presence of cholesterol. In addition our measurements for egg-PC bilayers were made at 37 °C in contrast to the BLM experiments which were performed at 20 °C. NMR measurements of *n*-alkane solubility in DMPC and egg-PC ilayers, with and without cholesterol show that *n*-alkane solubility increases with increasing temperature (vide infra). Hence, lower overall solubility would be expected for data collected at 20 °C. Thus, the two sets of data in Fig. 3 may not be comparable.

n-Alkane solubility in DMPC as well as egg-PC bilayers was examined, and the results are shown in Fig.

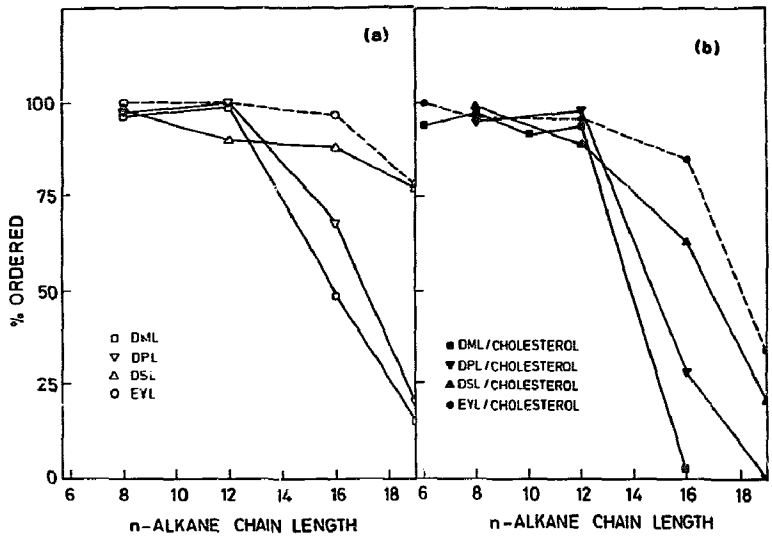

Fig. 4. Plot of percentage of ordered spectral component versus *n*-alkane chain-length for bilayers of molar composition (a) diacylphosphatidylcholine/*n*-alkane/H_2O (1:0.1:25) and (b) diacylphosphatidylcholine/cholesterol/*n*-alkane/H_2O (1:0.33:0.1:25). Spectra for DMPC, DPPC and DSPC containing bilayers were recorded at 310, 329 and 345 K, respectively (T_m + 14 K). EYL, egg-PC; DML, DMPC; DPL, DPPC; DSL, DSPC.

4(a). Clearly, the longer chain *n*-alkanes were far less soluble in DMPC than in egg-PC bilayers. To investigate the dependence on *n*-alkane solubility as a function of lipid acyl chain length, these experiments were repeated for DPPC and DSPC bilayer dispersions. Measurements in all cases were made 14–15 K above the main thermal transition of the phospholipid (DPPC, $T = 329$ K; DSPC, $T = 345$ K). *n*-Alkanes with a chain length of 12 carbons or fewer were almost completely soluble in all the bilayers examined. However, the longer chain *n*-alkanes showed increasing solubility as the chain length of the lipid acyl chains increased. For example, hexadecane is 50% dissolved in DMPC, 68% dissolved in DPPC and 88% dissolved in DSPC. Both *n*-hexadecane and *n*-nonadecane were more soluble in egg-PC bilayers than in dispersions of DSPC which has saturated acyl chains of comparable length to those in egg-PC. A similar pattern emerged for bilayer dispersions containing 25 mol% cholesterol (Fig. 4(b)). While solubilities of the *n*-alkanes up to and including *n*-dodecane remained high, the solubilities of the longer chain *n*-alkanes were reduced most by the presence of cholesterol. For example, *n*-hexadecane which is 50% dissolved in DMPC bilayers is almost completely insoluble in DMPC/cholesterol bilayers. The solubility difference between bilayers not containing cholesterol and those that do, decreases as the length of the lipid acyl chain increases. For instance *n*-hexadecane is 88% dissolved in DSPC bilayers but only 63% dissolved in DSPC/cholesterol bilayers. Thus, cholesterol, reduces the solubility of *n*-alkanes in lipid bilayers, the size of the effect being dependent upon the chain length of both the *n*-alkane and phosphatidylcholine.

It is informative to consider how these data relate to theoretical predictions of the state of the hydrophobic interior of a bilayer. The molecular mean-field calculations of Gruen and Haydon [28] have predicted the order-parameter profile for the acyl chains of DPPC. The theoretical profile agrees well with the experimental values measured by ^{2}H-NMR [25]. The profile shows a 'plateau' region extending from carbons 2 to 10, which is entropically less favorable to the inclusion of *n*-alkane molecules. The more disordered region near the bilayer midplane is predicted to be the favored location of hydrophobic solutes. Shorter chain alkanes might be expected to fit easily into this region. As the chain-length of the *n*-alkane increases, a point is reached at which the entire *n*-alkane chain can no longer be accommodated in the disordered region but must extend partially into the more ordered region. Alkanes of this chain length and longer would tend to be less soluble in the bilayer. In this framework, the almost complete solubility of *n*-alkanes up to *n*-dodecane in all bilayers, both in the absence and presence of cholesterol, would imply that the disordered region is large enough to easily accommodate *n*-dodecane. *n*-Hexadecane, however, must extend partially into the ordered region of the bilayer and would consequently tend to be less soluble. The higher solubility of *n*-hexadecane in egg-PC and DSPC bilayers implies that the disordered region is larger for lipids with longer acyl chains.

It has been shown [25] that the order-parameter

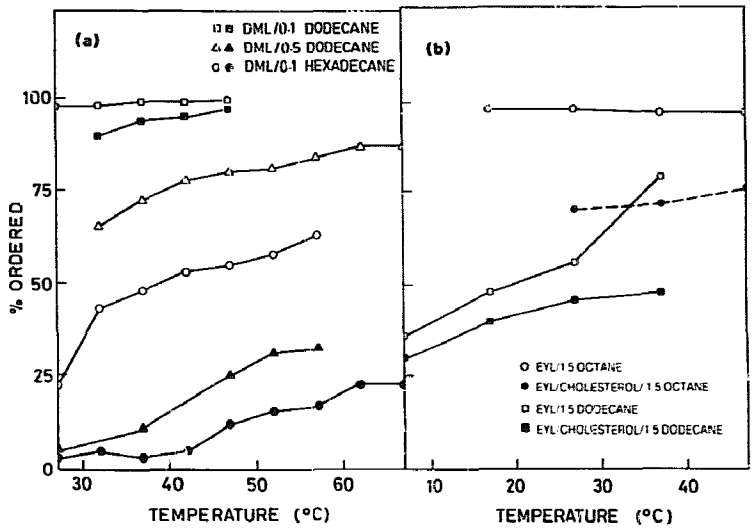

Fig. 5. Plot of percentage of ordered spectral component versus temperature for bilayers of various composition in the absence of cholesterol (open symbols) and for a PC/cholesterol ratio of 3:1 (full symbols). Results in (a) are for DMPC (DML) bilayers of various composition, while (b) refers to egg-PC (EYL) bilayers with a high concentration of *n*-alkane (alkane/lipid ratio of 1.5).

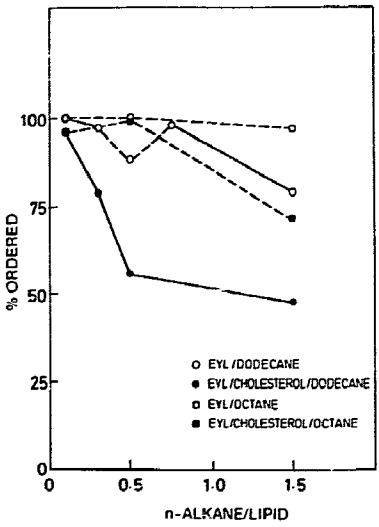

Fig. 6. Plot of percentage of ordered spectral component versus *n*-alkane/lipid ratio. For bilayers of molar composition, egg-PC/*n*-alkane/H_2O (1 : *x* : 25) and EYL/cholesterol/*n*-alkane/H_2O (1 : 0.33 : *x* : 25). Spectra were recorded at 310 K. EYL, egg-PC.

profile flattens as the temperature increases, and at the same time the plateau region disappears. In the context of the model described, we predict that *n*-alkane solubility in the bilayer would increase. The plot of percentage of ordered component versus temperature for DMPC, egg-PC and egg-PC/cholesterol bilayers containing *n*-alkanes (Fig. 5(a) and (b)) shows this to be the case. This effect is more pronounced for the longer chain *n*-alkanes.

The addition of cholesterol to a bilayer is known [27] to increase the order of lipid chain segments in contact with the rigid portion of the molecule which is 'anchored' by the hydroxyl group near the water/lipid interfacial region. Thus, the order-parameter plateau region of the lipid acyl chains should become more ordered in the presence of cholesterol. This would in turn be expected to reduce the solubility of *n*-alkanes which extend into this region. Our data show that the addition of 25 mol% cholesterol to the bilayer has little effect on the solubilities of *n*-alkanes up to *n*-dodecane but substantial reductions in solubility were observed for *n*-hexadecane and *n*-nonadecane.

Finally the effect of increasing the ratio of *n*-alkane to lipid on the percentage of ordered component in the spectrum, was investigated. The results (Fig. 6) show a decrease in ordered component with increasing *n*-alkane/egg-PC. The effect is more marked for *n*-dodecane than for *n*-octane. The addition of cholesterol to the bilayer accentuates the difference between the effects of *n*-dodecane and *n*-octane on decreasing the percentage of ordered component. The results suggest that at high *n*-alkane concentrations, *n*-octane can still be entirely accommodated in the disordered region of the bilayer, whereas some *n*-dodecane molecules have been forced either out of the bilayer (disordered component) or into the ordered region. Cholesterol, by filling up the void spaces in the disordered region of the bilayer exaggerates these differences between *n*-dodecane and *n*-octane.

In conclusion, our data confirm that the solubility of *n*-alkanes in multibilayer dispersions is chain-length dependent. Solubility is also dependent upon the chain length of the acyl chain of the lipid, the presence of cholesterol in the bilayer, temperature and concentration of the *n*-alkane. All of these trends are consistent with a model in which the *n*-alkane 'dissolves' in the disordered region of the bilayer at its center. The implication of these data in regard to the molecular mechanism of general anesthesia is that the 'cut-off' in anesthetic potency in the *n*-alkane series may be the result of a drop in solubility in the bilayer with increasing chain-length. To more firmly establish this, it would be necessary to show that the drop in solubility, of alkanes in membranes of identical lipid composition to that of the anesthetic site, occurs at the same chain length to that for which the drop in anesthetic potency takes place in a given species. Since the site of general anesthesia remains unknown, the lipid composition at that site cannot as yet be determined. Consequently it is still not possible to decide unambiguously between a lipid based unitary theory or direct protein binding, but the present results suggest that the former cannot be ruled out.

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