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The interaction of urethane, an intravenous general anaesthetic, with phosphatidylcholine membranes

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The interaction of urethane ($C_2H_5OCONH_2$), a neutral, intravenous general anaesthetic, with model membranes of egg-yolk phosphatidylcholine has been examined by ^{2}H-NMR spectroscopy of deuterium-labelled lipids and anaesthetic as well as by ^{31}P-NMR spectroscopy. Urethane interacts at the membrane/water interface to increase the interlipid separation in the bilayer. The presence of cholesterol does not significantly alter this behaviour. However, cholesterol does act to displace urethane from a second weakly bound membrane site which is in fast exchange with urethane free in bulk aqueous solution.

Introduction

General anaesthesia is thought to occur by selective dysfunction of neural membranes. Although the molecular mechanism of anaesthesia is unclear, many of the mechanisms currently under investigation invoke interaction of the anaesthetic with the lipid moiety of the membrane [1,2]. Urethane is a neutral, intravenous general anaesthetic which affects both axonal and synaptic neural transmission [3]. It selectively inhibits axonal sodium conductance [4,5], antagonises the amino acid neurotransmitters acetylcholine, L-glutamate and carbachol [6] and potentiates the effects of the inhibitory neurotransmitter γ-aminobutyric acid. The anaesthetic effect of urethane is reversed at high pressures [7,8] and is additive with a wide range of other general anaesthetics, with the exception of intraval [7,9]. Therefore, urethane acts at a number of neural sites and its action may be mediated by a number of different mechanisms.

An ESR study of the action of urethane in a buffered aqueous dispersion of 64.3 mol% egg-yolk PC, 33 mol% cholesterol and 2.7 mol% egg-yolk phosphatidic acid indicated a small decrease in order reported by 1-acyl-2-[8-(4,4-dimethyloxazolidine-*N*-oxyl)palmitoylphosphatidylcholine, i.e., a 5% decrease in the order parameter at a urethane : lipid molar ratio of 5 : 1 [10]. However, this study examined only one position in the hydrocarbon region of the bilayer. In addition, there is some debate as to whether ESR spin probes are accurate in reporting perturbations of the bilayer region [11–14].

We present here ^{2}H-NMR studies of phospholipids deuterium-labelled at different positions which give comprehensive information on the urethane-induced perturbation of egg PC and egg PC-cholesterol model membranes. In addition, ^{2}H-NMR experiments using deuterium-labelled urethane give direct information on the environment(s) experienced by the anaesthetic in the system.

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; egg PC, egg-yolk phosphatidylcholine.

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Materials and Methods

The following deuterium-labelled phosphatidylcholines were prepared as described previously [15,16]: 1,2-di[9,10-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine (DOPC-9,10-d_4); 1,2-di[$^2H_{31}$]palmitoyl-*sn*-glycero-3-phosphocholine (DPPC-d_{62}); 1,2-di[2,2-$^2H_2$]palmitoyl-*sn*-glycero-3-phosphocholine (DPPC-2,2,2′,2′-d_4); 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine deuterium-labelled on the choline methyl groups (DPPC-d_9) and on both carbons of the choline methylene groups (DPPC-α,β-d_4) and cholesterol-3α-d_1. Egg-yolk phosphatidylcholine (egg PC) was prepared by the method of Singleton et al. [17]. Deuterium-labelled urethane ($C_2^2H_5OCONH_2$) was prepared from $C_2^2H_5O^2H$ (M.S.D. Isotopes) by the method of Guerci [18].

The lipid sample components were codissolved in chloroform and the solvent removed under high vacuum for 10–16 h. Urethane was added in the aqueous phase, in deuterium-depleted water (Aldrich Chemical Co.), and the lipid (450 mM) was dispersed by vortex mixing and repeated freeze-thaw cycles [19]. NMR spectra were recorded on a Nicolet 360 NB spectrometer operating at 55.4 MHz for ^2H and 146.2 MHz for ^{31}P. Deuterium spectra of urethane-d_5 were recorded either by a single pulse sequence of 60° pulses of approx. 35 μs duration, with a relaxation delay of 4 s, or by a quadrupolar echo sequence [20] with 60–80 μs spacing between 90° pulses (of 7 μs duration). Lipid deuterium spectra were recorded with the same quadrupolar echo sequence with the exception of the use of a 500 ms relaxation delay. Deuterium quadrupolar splittings were measured between the two most intense features of the powder pattern. Deuterium T_1 measurements were carried out using an inversion recovery pulse sequence, and T_2 measurements using a Hahn spin echo pulse sequence. Some T_2 values were verified using Carr-Purcell-Meiboom-Gill experiments.

Phosphorus spectra were recorded using a two-level broadband proton decoupling sequence using 25 μs 90° pulses, with decoupling power of 25 W during data acquisition and 5 W during the relaxation delay.

Results

In the samples prepared for ^2H-NMR spectroscopy, the deuterium-labelled lipid species is

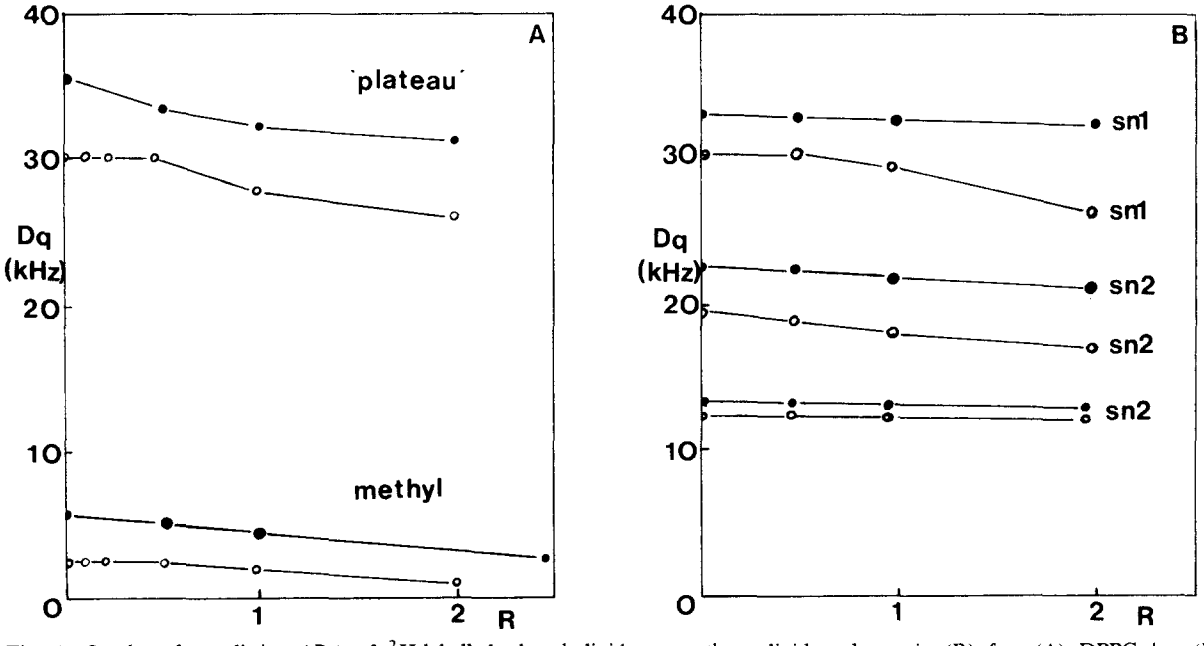

Fig. 1. Quadrupolar splitting (D_q) of ^2H-labelled phospholipid vs. urethane:lipid molar ratio (R) for: (A) DPPC-d_{62}; (B) DPPC-2,2,2′,2′-d_4. in (○) egg PC and (●) egg PC containing 20 mol% cholesterol at 22±2°C.

present at 5–10 mol% of a lamellar phase dispersion of egg PC or egg PC-cholesterol. Under these conditions the lipids are completely miscible [21]. As well, at 20°C, since the DPPCs are present as guests in the L_α phase of egg PC, they also are in the fluid L_α phase [15,16]. This fact is verified by their ^{2}H-NMR spectra (vide infra).

Acyl chain region

The effect of urethane on the acyl chain region of the model membranes was examined by ^{2}H-NMR of egg PC and egg PC-cholesterol dispersions containing DPPC-2,2,2′,2′-d_4 or DOPC-9,10-d_4.

Spectra from DPPC-d_{62} consisted of overlapping powder patterns indicative of the 'flexibility' or 'order' gradient along the chains in the L_α phase observed by others [22–24]. The innermost powder pattern assigned to the methyl deuterons of both chains had a quadrupolar splitting of approx. 3 kHz, as expected from a fluid lamellar L_α phase dispersion. In the absence of cholesterol the powder patterns from the 'plateau' region methylenes, which include those 3 to 10 carbons distant from the acyl linkage, occurred at the extreme edges of the spectra.

However, in the presence of cholesterol, the greatest intensity methylene powder patterns were bracketed by powder patterns of lower intensity, indicative of a change in the distribution of order parameters in this section of the chain. This effect has been examined previously by the use of selectively deuterium-labelled chains by Oldfield and co-workers [25].

The effect of addition of urethane to samples of egg PC and egg PC-cholesterol is illustrated in Fig. 1.

In the absence of cholesterol, urethane causes little detectable perturbation below a urethane: lipid molar ratio of 0.5 : 1. Above this concentration the splitting observed from all positions on the chain were reduced on increasing the urethane content; e.g., at a urethane : lipid molar ratio of 2.0 : 1 the splitting from the terminal methyl groups was reduced by 22%, and that from the 'plateau' methylenes by 11%.

In the presence of cholesterol, a similar reduction in splitting was observed, e.g., at a urethane : lipid molar ratio of 2.0 : 1 the splitting from the terminal methyl deuterons was reduced by 13% and that from the plateau methylenes by 8%.

The methylene groups adjacent to the acyl linkages of the two acyl chains are inequivalent [22,26]. The effect of urethane at this level of the bilayer was examined by ^{2}H-NMR of samples containing DPPC-2,2,2′,2′-d_4, the results of which are shown in Fig. 1B. In egg PC alone, the spectrum consisted of three superimposed powder patterns of splitting 29.5, 20.9 and 12.5 kHz, assigned to the two equivalent deuterons of the *sn*-1 chain and to the two inequivalent deuterons of the *sn*-2 chain [22,27].

The effect of urethane addition was to produce a small decrease in splitting from all three resonances, smaller than that observed from the plateau methylene deuterons of DPPC-d_{62}. For example, a urethane : lipid molar ratio of 2.0 : 1 the reductions in the splittings were 6, 8 and 3%, respectively. A similar difference in sensitivity to perturbation along the hydrocarbon chain has been noted for methochlorpromazine [15] but was not found for α-chloralose or chloralhydrate [16].

The effect of urethane in a mixture of egg PC and cholesterol was similar to that noted above; e.g., at a urethane : lipid molar ratio of 2.0 : 1 the

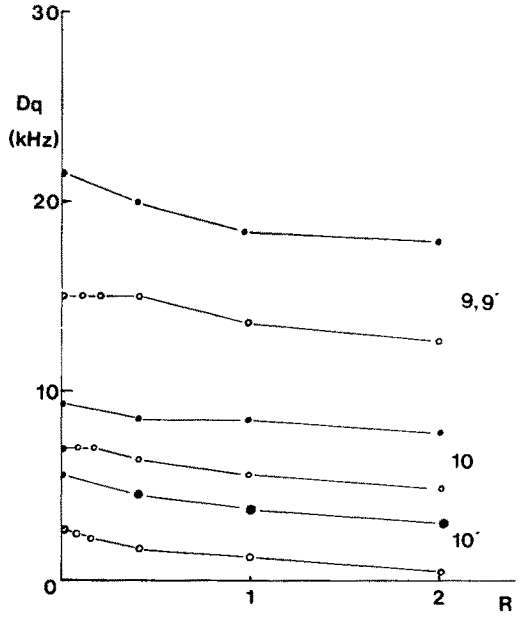

Fig. 2. Quadrupolar splitting (D_q) vs. urethane : lipid molar ratio (R) for DOPC-9,10-d_4 in (○) egg PC and (●) egg PC containing 33 mol% cholesterol at 22 ± 2°C.

splittings from DPPC-2,2,2′,2′-d_4 were reduced by 8, 6 and 2%, respectively, as shown in Fig. 1B.

Egg PC is a mixture of PCs containing saturated and unsaturated fatty acids. Therefore ^{2}H-NMR experiments were also performed on systems containing the unsaturated probe molecule, DOPC-9,10-d_4, the results of which are shown in Fig. 2.

In egg PC, the ^{2}H-NMR spectrum consisted of three overlapping powder patterns of 14.7 kHz, 6.7 kHz and 2.4 kHz splitting, assigned to the 9,9′-position deuterons, the 10-position deuterons and the 10′-position deuterons respectively [28].

In the presence of urethane, the splitting from the 9-position deuterons was unaffected until the urethane : lipid molar ratio exceeded 1 : 1, whereon the splitting decreased steadily with added urethane, e.g., at a urethane : lipid molar ratio of 2.0 : 1 the splitting was reduced by approx. 12%. The splitting from the 10-position deuterons decreased only above a urethane : lipid molar ratio of 0.5 : 1, e.g., at a urethane : lipid molar ratio of 2.0 : 1 the splitting was reduced by 20%. The 10′-position deuterons were the most sensitive to urethane addition: a detectable reduction in splitting occurred at urethane : lipid molar ratios of 0.1 : 1, and a ratio of 2.0 : 1 had further been reduced by approximately 75% (Fig. 2).

In the presence of cholesterol, the splittings from DOPC-9,10-d_4 also decreased in the presence of urethane, all positions experiencing some perturbation at a urethane : lipid molar ratio of 0.5 : 1. The 10′-position was again the most sensitive (Fig. 2).

The decrease in splittings observed in egg PC and in egg PC-cholesterol appeared to level-off above a urethane : lipid molar ratio of 2 : 1.

The headgroup region

The effect of urethane on motion in the headgroup region of the phospholipid component of these systems was examined by ^{2}H-NMR of DPPC-d_9 and DPPC-α,β-d_4, and by ^{31}P-NMR.

In both systems the presence of urethane gave rise to little detectable change in ^{31}P-NMR spectra, which were typical fluid lamellar L_α powder patterns with -45 ± 2 ppm chemical shift anisotropy. Similarly, the ^{2}H-NMR spectra from DPPC-d_9 were insensitive to urethane, consisting of a single powder patter of splitting 1.20 ± 0.05 kHz in egg PC and 1.18 ± 0.04 kHz in egg PC-cholesterol.

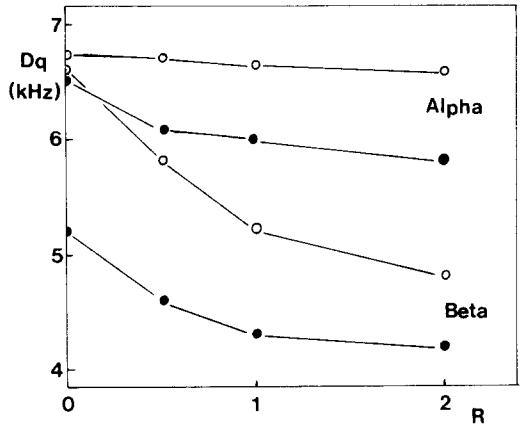

Fig. 3. Quadrupolar splitting (D_q) vs. urethane : lipid molar ratio (R) for DPPC-α,β-d_4 in samples of (○) egg PC and (●) egg PC containing 33 mol% cholesterol at 22 ± 2°C.

However, the addition of urethane did cause marked alterations in ^{2}H-NMR spectra from DPPC-α,β-d_4 in egg PC and egg PC-cholesterol membranes, as shown in Fig. 3.

In the cholesterol-free membrane, in the absence of urethane, the ^{2}H-NMR spectrum of DPPC-α,β-d_4 consisted of two overlapping powder patterns with splittings of 6.7 and 6.6 kHz. Urethane incorporation gave rise to a small decrease in the larger splitting and a marked decrease in the smaller splitting, as shown in Fig. 3. These resonances are assigned to the deuterons α and β to the phosphate group (i.e., *O*-methylene and *N*-methylene deuterons), respectively. The reduction in splitting appeared to level off above a urethane : lipid molar ratio of 2.0 : 1.

In the presence of cholesterol, in a urethane-free membrane, the ^{2}H-NMR spectrum of DPPC-α,β-d_4 consisted of two powder patterns of splitting 6.5 kHz and 5.2 kHz, assigned to the α- and β-methylene deuterons respectively [29]. Urethane addition again gave rise to a slight decrease in the α-position splitting and a more marked decrease in the β-position splitting.

Deuterium-labelled urethane

In aqueous solution, urethane-d_5 gave rise to two singlet peaks, of intensity ratio 3 : 2, separated by 2.87 ± 0.01 ppm, corresponding to the methyl and methylene deuterons, respectively.

In the presence of multilamellar liposomes urethane will be distributed between a membrane site(s) and aqueous solution since the partition coefficient (for a mixture of egg PC-cholesterol of molar ratio 2 : 1) is approximately unity [10]. Also, membrane-bound urethane will gain motional ordering with respect to the 'isotropic' urethane in bulk aqueous solution as a result of binding to the membrane. If urethane exchange between the bulk aqueous solution and a membrane site is slow on the NMR timescale then the observed ^{2}H-NMR spectrum will consist of separate signals for the bound and free anaesthetic. If exchange is fast then an averaged signal will be observed.

Fig. 4 shows a typical ^{2}H-NMR spectrum of urethane-d_5 in the presence of egg PC multilamellar liposomes. The two central peaks were centred at the isotropic resonance positions observed in aqueous solution. The powder pattern of smaller quadrupolar splitting was symmetrical about the methyl deuteron resonance frequency and that of larger splitting about the methylene resonance frequency. Addition of urethane gave rise to a linear decrease in both splittings, as shown in Fig. 5.

The singlet resonances in the presence of egg PC were broader than those in simple aqueous solution (linewidth at half height 55 ± 10 Hz compared to 10 Hz) and were unaffected by changes in urethane concentration or temperature. The spin-lattice relaxation times measured from these resonances lay in the range 0.40 ± 0.15 s, compared to 0.60 ± 0.05 s in aqueous solution.

The presence of cholesterol did not alter the magnitude or urethane-concentration dependence of the quadrupolar splittings from urethane-d_5. However, the linewidth of the central singlet resonances was markedly reduced on addition of cholesterol, as illustrated in Fig. 6. Linewidths

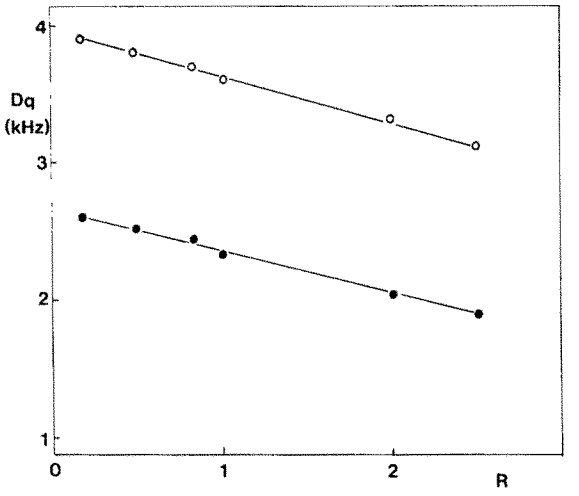

Fig. 5. Quadrupolar splitting (D_q) of urethane-d_5 in egg PC liposomes vs. urethane : lipid molar ratio (R); ○ methylene deuterons; ●, methyl deuterons.

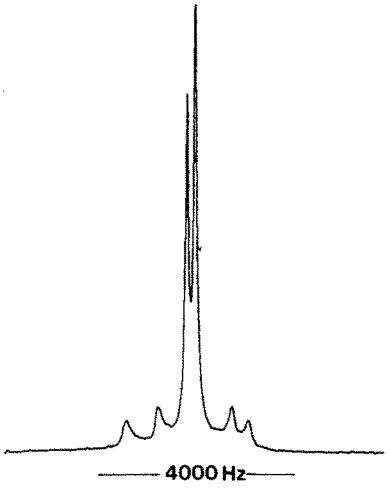

Fig. 4. ^{2}H-NMR spectrum of urethane-d_5 (C^2H_3-C^2H_2-OC-ONH_2) in egg PC at 22 ± 2°C at urethane : lipid molar ratio 1.0 : 1.

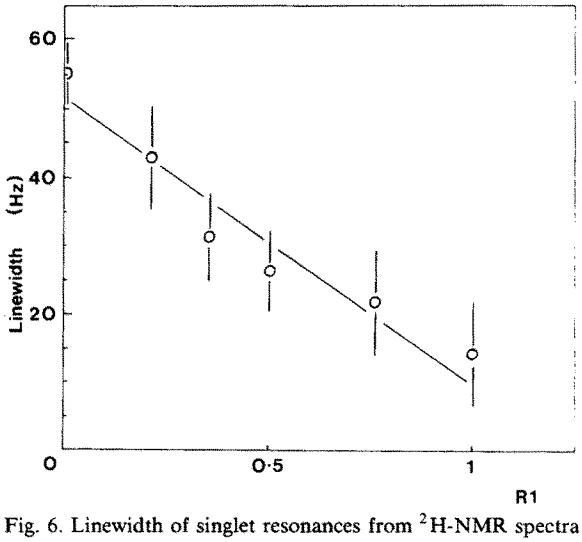

Fig. 6. Linewidth of singlet resonances from ^{2}H-NMR spectra of urethane-d_5 vs. cholesterol : egg PC molar ratio (R_1) at 22 ± 2°C.

calculated from spin-spin relaxation times showed the same trend. The linewidth was intensitive to changes in urethane concentration or temperature at each cholesterol concentration examined. In a sample equimolar in egg PC and cholesterol the linewidth approached that of a simple aqueous solution.

Discussion

The experimentally observed quadrupolar splitting, D_q, can be expressed by

$$D_q = 3/4(e^2qQ/h)S_{CD},$$

where (e^2qQ/h) is the deuterium quadrupolar coupling constant. The order parameter, S_{CD}, depends upon the average orientation of the C-^{2}H bond vector with respect to the axis of rotational symmetry, represented by

$$S_\alpha = \tfrac{1}{2}(3\cos^2\alpha - 1),$$

and the degree of orientational fluctuation around this average orientation, represented by S_γ [30], i.e.

$$S_{CD} = S_\alpha S_\gamma$$

Therefore, the quadrupolar splitting can be used to describe the 'fluidity' of a lipid in a bilayer only to the extent of defining the orientational flexibility of a given molecular segment, and has no simple relationship to the rates of motion of the segment.

It is clear (Figs. 1, 2 and 3) that different regions of the phosphatidylcholine molecule exhibit different degrees of sensitivity to the presence of urethane.

In the headgroup region of the bilayer, the β-methylene segment appears to be particularly sensitive to the presence of urethane, where the α-position methylene and trimethylammonium segments are relatively undisturbed by urethane addition, as is the phosphate group. This pattern of perturbation has been noted in the presence of chloroform [30], cholesterol (this study and Ref. 29), chloral hydrate [16] and as a result of increasing temperature in pure PC systems [29]. In all of these systems the effect is explained by an increase in intermolecular distance between the phospholipids in the bilayer, causing small changes in the time-average conformation of the headgroup and hence giving rise to the reduction in the β-methylene-deuteron quadrupolar splitting.

The urethane-induced disordering of the hydrocarbon region is apparent from the reduction in the observed quadrupolar splittings of phospholipids deuterium-labelled on the acyl chains. The disordering increases in segments further removed from the acyl linkage, indicated by the relatively small perturbation of the C2 methylenes (DPPC-2,2,2′,2′-d_4) compared to that of the terminal methyl group (DPPC-d_{62}). This pattern of disordering is consistent with an increase in intermolecular separation [31,32] as a result of urethane addition.

The presence of a cholesterol in the bilayer causes only minor deviations from the pattern of perturbation caused by urethane in egg PC bilayers, suggesting that the site for urethane binding responsible for disturbing the bilayer structure is not affected by cholesterol addition.

The experiments using deuterium-labelled urethane provide additional information on the nature of urethane binding in the bilayer.

The presence of quadrupolar powder patterns (Fig. 4) in the ^{2}H-NMR spectra of urethane-d_5 indicates that a population of ordered urethane exists at the bilayer in slow exchange with urethane elsewhere in the system. Since the presence of cholesterol has no effect on the splittings observed from urethane, it is most likely that this site represents that responsible for perturbations of the bilayer order.

In addition, the linewidth of the two central singlet resonances in the urethane-d_5 spectra decreases on addition of cholesterol. Therefore, in the absence of cholesterol the central resonances represent the signal from two populations of urethane in fast exchange on the NMR timescale [33], one of which is in the bulk aqueous solution and the other associated with the bilayer. A similar result has been observed in studies of tetracaine hydrochloride [32,34], where it was suggested that the latter site is in the electrical double layer of the membrane/water interface.

The reduction in linewidth on cholesterol addition indicates that urethane binding at this site is disfavoured by the presence of cholesterol. Since

cholesterol alters the intermolecular separation in the bilayer, and therefore alters the charge distribution and hydrogen bonding at the membrane surface, this may distabilise urethane weakly bound at the bilayer/water interface.

Therefore, there appear to be three sites for urethane in egg PC multilamellar dispersions: free in bulk aqueous solution; loosely bound to the membrane, in fast exchange with free urethane; and more firmly bound urethane, giving rise to the quadrupolar powder pattern, in slow exchange with the other urethane populations. Since cholesterol displaces the loosely bound urethane but does not significantly alter the pattern of urethane-induced membrane perturbation, the disordering effect arises from urethane at the third, firmly bound site.

The pattern of urethane-induced disordering is consistent with an increase in intermolecular separation of lipids in the bilayer and also the headgroup region is sensitive to low concentration of urethane. Therefore, it is highly probable that this third urethane site is the level of the headgroup or backbone region of the bilayer. Theoretical calculation of the electric field distribution across a simple model of a phospholipid bilayer indicate a maximum in the electric field intensity, where the interaction energy of a small dipolar molecule is greatest, in the headgroup-backbone region [35]. In addition, a number of workers have suggested evidence in support of headgroup-level interactions of other anaesthetics with phospholipid model membranes [36–39].

Therefore, the primary effect of urethane on bilayer membranes of egg PC and egg PC-cholesterol is to increase the interlipid separation via binding at the interfacial region and hence, to disorder the hydrocarbon interior of the membrane.

Acknowledgments

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