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An anaesthetic-induced phosphatidylcholine hexagonal phase

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The interaction of trichloroethylene, an inhalational general anaesthetic, with phosphatidylcholine model membranes has been studied by ^{31}P- and ^{2}H-nuclear magnetic resonance spectroscopy. The incorporation of increasing amounts of trichloroethylene induces a phase transition from a multilamellar bilayer to a hexagonal phase. The effect is enhanced by increasing temperature. No indication of an intermediate cubic (C_{II}) phase was discovered.

Introduction

The mechanisms of general anaesthesia, although not well understood on a molecular level, are membrane related [1–3]. Previously it has been reported that anaesthetics modulate the formation of an inverted hexagonal phase in aqueous media [4,5]. In some of these experiments the lipid component contained significant fractions of phospholipids such as phosphatidylethanolamines or cardiolipins which are known to form a hexagonal phase readily in aqueous dispersion [6–11].

We wish to report that at 20°C trichloroethylene induces a hexagonal phase in aqueous egg yolk phosphatidylcholine dispersions at an anaesthetic: lipid molar ratio of approximately 2 : 1. Above this concentration increasing temperature further favours the hexagonal phase.

The effects are of particular interest in light of the postulated role of phosphatidylcholines as bilayer stabilising components of natural membranes [11].

Materials and Methods

The following were prepared and incorporated, in small amounts (5–10 mole percent), in multilamellar liposomes of 1,2-diacyl-*sn*-glycero-3-phosphocholine, extracted from hens' egg yolk, as described previously [12]: 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, deuterium labelled at the 9 and 10 positions of both olefinic chains (1,2-di[9,10-$^{2}H_{2}$]oleoyl-*sn*-glycero-3-phosphocholine or DOPC-d_4); and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, deuterium labelled on the choline methyl groups (DPPC-d_9), on both carbons of the choline methylene groups (DPPC-$\alpha,\beta d_4$), on the methylene group adjacent to the ester groups of both palmitoyl chains (1,2-di[2-$^{2}H_{2}$]palmitoyl-*sn*-glycero-3-phosphocholine or [2,2'-$^{2}H_{4}$]DPPC and on all positions of both palmitoyl chains, 1,2-di[$^{2}H_{31}$]palmitoyl-*sn*-glycero-3-phosphocholine ('perdeuterated'-DPPC or DPPC-d_{62}). The total lipid concentration was maintained at 450 mM.

Deuterium labelled trichloroethylene ($^{2}HCCl:CCl_2$) was prepared from 1,1,2,2-tetrachloro[1,2-$^{2}H_{2}$]ethane (MSD Isotopes) by the action of sodium hydroxide [13] and purified by

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distillation. The product was verified by ^{2}H and ^{13}C nuclear magnetic resonance. Protonated trichloroethylene was distilled prior to use.

Nuclear magnetic resonance spectra were determined on a Nicolet 360 NB spectrometer operating at 55.43 MHz for deuterium, using a quadrupolar echo sequence [14] with a 60–80 μs spacing between the 90° pulses (of 7 μs duration) and a 0.35 s recycle time. ^{31}P-NMR spectra were recorded at 146.14 MHz with broadband proton decoupling using a two-level decoupling sequence in which the 90° pulse was 25 μs and the decoupling power was 25 watt during data acquisition and 5 watt during the magnetisation recovery time.

Deuterium quadrupolar splittings were measured between the two most intense peaks of the powder pattern and are reproducible to less than two percent.

Results

The effect of adding trichloroethylene to an aqueous dispersion of 1,2-diacyl-*sn*-glycero-3-phosphocholine from egg yolk (egg PC), monitored by ^{31}P-NMR is illustrated in Fig. 1. In the absence of the anaesthetic the spectrum consisted of a broad asymmetric resonance characteristic of a multilamellar bilayer (L_α) structure [15,16] with a chemical shift anisotropy of approximately −45 ppm. Addition of trichloroethylene, over a range of trichloroethylene : egg PC molar ratios 0.0 : 1 to 2.05 : 1, caused little change in the ^{31}P-NMR spectra. At a trichloroethylene : egg PC molar ratio of 2.3 : 1 the ^{31}P-NMR spectrum indicated the presence of a second powder pattern of much smaller intensity than the first. The chemical shift anisotropy of this new powder pattern was opposite in sign to, and of approximately half the magnitude of, that of a pure egg PC dispersion. Increasing the concentration of trichloroethylene caused an increase in the relative intensity of this second powder pattern. At a trichloroethylene : egg PC molar ratio of 5.2 : 1 the spectrum consisted of one powder pattern of chemical shift anisotropy of +18 ppm and a second powder pattern of opposite anisotropy and much smaller intensity, arising from a small fraction of the lipid remaining in a bilayer phase.

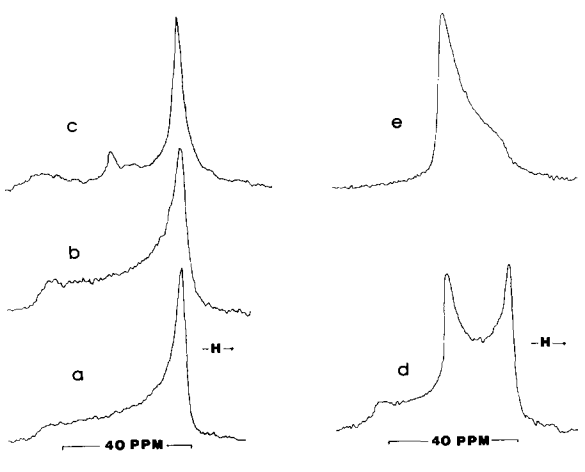

Fig. 1. ^{31}P-NMR spectra of aqueous dispersions of egg yolk phosphatidylcholine in the presence of trichloroethylene at 20°C. Trichloroethylene : egg yolk PC molar ratio: (a) 0 : 1, (b) 2.0 : 1, (c) 2.3 : 1, (d) 4.1 : 1, (e) 5.2 : 1.

Increasing the temperature from 20°C to 55°C was analogous to increasing the anaesthetic concentration. A sample of trichloroethylene : egg PC molar ratio 2.3 : 1 gave rise to a ^{31}P-NMR spectrum indicating the presence of a small fraction of lipid in a hexagonal phase (Fig. 1c). Increasing the temperature of this sample led to an increase in the intensity of the hexagonal phase signal: at 37°C the intensities of the hexagonal and bilayer signals were approximately equal. Further heating to 55°C results in virtually complete conversion of the sample to the hexagonal phase.

This behaviour is indicative of a phase transition from a bilayer (L_α) to a hexagonal phase in which lipid molecules cluster to form long cylinders surrounding an aqueous channel [17]. It is not possible to distinguish between an H_I and an H_{II} phase on the basis of ^{31}P-NMR data [17]. However, the hydrocarbon chain region of the two phases have different packing and order profiles and it is possible to use these differences to obtain evidence for the presence of a given phase (vide infra). An alternative explanation of the ^{31}P powder pattern (of chemical shift anisotropy +18 ppm) has been suggested [18], in which the lipid maintains a bilayer structure. The change in ^{31}P-NMR spectra is suggested to arise from an alteration of the headgroup conformation.

If this were the cause of the observations above one would require two populations of phospholi-

pid in the bilayer, of different headgroup conformations, which do not interconvert rapidly on the NMR timescale. In order to determine that this was not the case, experiments were performed using deuterium-labelled phosphatidylcholines incorporated into the egg yolk phosphatidylcholine dispersions.

The magnitudes of the quadrupolar splittings measured from these experiments are dependent upon the order parameter of the deuterium-labelled segment of the phospholipid molecule and also upon the motions of the molecule as a whole. If the lipids form a hexagonal phase then diffusion of the lipid molecules around the circular cross-section of the lipid cylinders provides additional averaging of the quadrupolar coupling tensor, resulting in halving of the previously observed quadrupolar splitting [6,19]. Deviation from a 'halved' value indicates a difference between the 'internal' or segmental order parameter of the deuterium-labelled segment in each phase, and from this data one may distinguish between an H_I and an H_{II} phase.

The effects of trichloroethylene addition on samples containing DPPC-d_{62} are shown in Fig. 2. Trichloroethylene: PC molar ratios to 2.0 : 1 produced little discernible change in the order profile of the palmitoyl hydrocarbon chains. A trichloroethylene: PC molar ratio of 4.2 : 1 produced two superposed spectra, of which the quadrupolar splittings of one were approximately half the values of the other, the smaller being assigned to the perdeuterated chains of DPPC in a hexagonal phase. The larger spectrum, identified by the quadrupolar splittings of the terminal methyl group and the 'plateau' region methylene deuterons, was similar to that of samples containing no trichloroethylene. This suggests that the remaining multilamellar bilayer structure is little affected by the presence of the hexagonal phase.

In the hexagonal phase (formed at a trichloroethylene: PC molar ratio of 5.2 : 1) the largest quadrupolar splitting observed in the DPPC-d_{62} spectrum was approximately 13 kHz, very close to half the value of the plateau region deuterons of the bilayer phase. The central powder pattern collapsed to a broad singlet, (approx. 1 kHz wide), which, on cooling the sample to 5°C, was resolved into a powder pattern of quadrupolar splitting 0.6

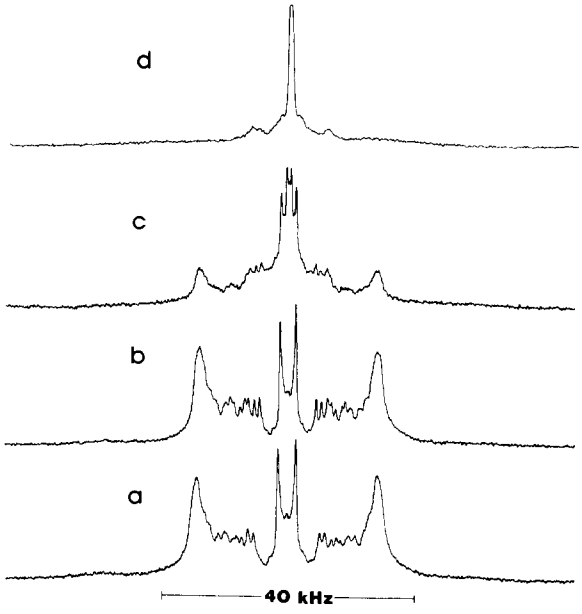

Fig. 2. ^{2}H-NMR spectra of egg PC samples containing approx. 5% perdeuterated dipalmitoylphosphatidylcholine in the presence of trichloroethylene at 20°C. Trichloroethylene: phosphatidylcholine molar ratio: (a) 0 : 1, (b) 1.7 : 1, (c) 4.2 : 1, (d) 5.2 : 1.

kHz, a value significantly less than half the value found for the terminal methyl group deuterons in the lamellar phase. At this temperature the ^{31}P-NMR spectrum was a typical hexagonal phase powder pattern (of chemical shift anisotropy +18 ppm).

The spectra from samples containing other phospholipids having deuterons in the hydrocarbon region of the lipid parallel those of DPPC-d_{62}. The ^{2}H-NMR spectrum of DOPC-d_4 in egg yolk phosphatidylcholine, in the absence of trichloroethylene, consisted of three superposed powder patterns of quadrupolar splittings 15.0, 6.8 and 2.7 kHz. These resonances have been assigned to deuterons at the 9-position of both chains, the 10-position of the *sn*-1 chain and the 10-position of the *sn*-2 chain, respectively [21]. These values fell to 7.4, 2.9 and 0.8 kHz on addition of trichloroethylene (trichloroethylene: PC, 4 : 1) consistent with the formation of a hexagonal phase [22,19].

The quadrupolar splittings from the hexagonal phase lipid were less than half the corresponding bilayer phase value, suggesting that either the segmental order parameter is different in the two

phases or that the axis of segmental rotation (which averages the quadrupolar interaction) is tilted, with respect to the molecular axis, at a different angle from that in the bilayer phase.

A similar reduction in ^{2}H-NMR quadrupolar splitting was apparent in the terminal methyl group of DPPC-d_{62} (this work) and has also been noted (for C_{11}-labelled oleate incorporated into DOPC) by other workers [20].

The spectrum of [2,2′-$^2H_4$]DPPC in egg yolk phosphatidylcholine, in the absence of trichloroethylene, consisted of three powder patterns of quadrupolar splitting 29.5, 19.4 and 12.5 kHz, assigned to the deuterons of the methylene adjacent to the ester linkage of the *sn*1 chain and to the nonequivalent deuterons at this position on the *sn*2 chain [23].

As is clear from Table I, there is little discernible effect on these splittings on addition of trichloroethylene to a trichloroethylene: PC molar ratio of 1.8 : 1. However, at a trichloroethylene: PC molar ratio of 3.5 : 1 one observes four distinct powder patterns of quadrupolar splittings 27.7, 18.9, 12.8 and 6.5 kHz. We suggest that this is a result of the superposition of spectra from hexagonal and bilayer phases of the lipid in which the quadrupolar splittings of the hexagonal phase lipid deuterons are half the value of those from the bilayer phase.

To complete the analysis of this phase behavior experiments were also carried out using the headgroup labelled phosphatidylcholines DPPC-d_9 and DPPC-$\alpha,\beta d_4$, the results of which are shown

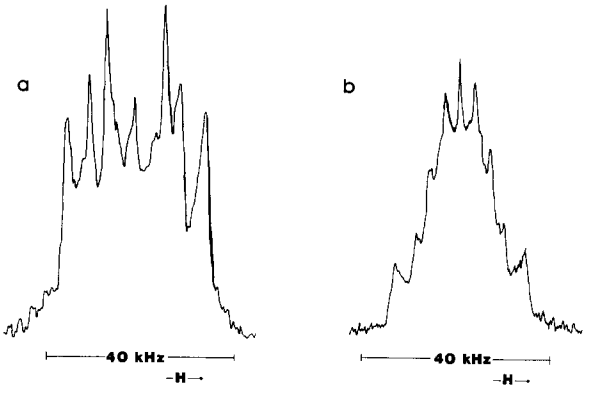

Fig. 3. ^{2}H-NMR spectra of egg phosphatidylcholine samples containing approx. 5% [2,2′-$^2H_4$]DPPC in the presence of trichloroethylene at 20°C. Trichloroethylene: phosphatidylcholine molar ratio: (a) 0 : 1, (b) 3.5 : 1.

in Table I. Addition of trichloroethylene caused little perturbation of the observed quadrupolar splitting until a trichloroethylene: PC molar ratio of 2 : 1 was exceeded. At trichloroethylene: PC molar ratios of 4 : 1 the values of the quadrupolar splittings from the DPPC-$\alpha,\beta d_4$ spectrum were halved, from 6.5 and 4.5 kHz at low trichloroethylene: PC molar ratios to 3.0 and 2.1 kHz. Similarly the quadrupolar splitting from DPPC-d_9 spectra fell from 1.1 kHz to 0.48 kHz.

These ^{2}H-NMR results confirm the formation of a hexagonal phase. We now turn our attention to the trichloroethylene itself within the lamellar and hexagonal phases.

The interaction of trichloroethylene with aque-

TABLE I

QUADRUPOLAR SPLITTINGS (kHz) OF DEUTERIUM-LABELLED PHOSPHATIDYLCHOLINE SPECIES INCORPORATED INTO EGG PHOSPHATIDYLCHOLINE DISPERSIONS CONTAINING TRICHLOROETHYLENE

Spectra recorded at 22 ± 2°C.

Trichloroethylene: egg PC (molar ratio)	DOPC-d_4			[2,2′-$^2H_4$]DPPC			DPPC-d_9	DPPC-$\alpha,\beta d_4$	
	9,9′,	10,	10′	*sn*1	*sn*2	*sn*2			
0.25 : 1	14.6	7.1	2.6	29.5	19.4	12.5	1.12	6.5	4.8
1.00 : 1	16.5	6.8	2.7	27.0	18.7	13.0	1.02	6.5	4.2
1.80 : 1	16.5	7.0	2.7	27.1	18.3	13.0	1.08	6.6	4.5
3.5 : 1	7.4	2.9	0.8	27.7	18.9	12.8 (6.5)	–	6.0	4.2
4.2 : 1	–	–	–	–	–	–	0.48	3.0	2.1

TABLE II

QUADRUPOLAR SPLITTINGS OBSERVED FROM DEUTERIUM LABELLED TRICHLOROETHYLENE IN AQUEOUS DISPERSIONS OF EGG PC AND CHEMICAL SHIFT ANISOTROPIES FROM THE CORRESPONDING ^{31}P SPECTRA

Spectra recorded at 22 ± 2°C.

Trichloroethylene : egg phosphatidylcholine (molar ratio)	Quadrupolar splitting (kHz)	Chemical shift anisotropy (ppm)
0.54 : 1	8.0	−45.2
1.04 : 1	7.3	−44.8
2.0 : 1	5.7	−44.3
2.1 : 1	6.3	−44.0
	4.9	
4.3 : 1	5 kHz [a]	−43.8
	& Central peak [b]	+18
5.2 : 1	Single peak [c]	+18

[a] The intensity of the powder pattern was considerably smaller than that of the broad central peak.
[b] Width at half-height approx. 1.5 kHz.
[c] Width at half-height 1.2 kHz.

ous dispersions of egg yolk lecithin was also monitored by ^{2}H-NMR spectroscopy of the deuterated anaesthetic, trichloroethylene-d_1. The results of these experiments are collated in Table II and Fig. 4. At a low concentration of trichloroethylene (trichloroethylene : PC, 0.54 : 1) the ^{2}H-NMR spectrum consisted of a single powder pattern of quadrupolar splitting 8 kHz (Fig. 4a). Increasing the trichloroethylene : PC molar ratio to 1.04 : 1 gave rise to a spectrum consisting of a single powder pattern of 7.3 kHz quadrupolar splitting. At trichloroethylene concentrations approaching those required for the phase transition, viz. trichloroethylene : PC 2.0 : 1 and 2.1 : 1, the spectra indicate the presence of two distinct powder patterns (indicated in Figs. 4b and 4c), the phosphorus spectra of which indicate the presence of a bilayer phase only. Increasing the trichloroethylene : PC molar ratio further to 4.3 : 1 produced a ^{31}P-NMR spectrum indicative of an approximately equimolar mixture of hexagonal and bilayer phase phospholipids. The ^{2}H-NMR spectrum of this sample (Fig. 4d) consisted of a broad central peak (width at half-height approx. 1.5 kHz) of much greater intensity than two 'satellite' peaks approx. 5 kHz apart. Further increasing the trichloroethylene : PC

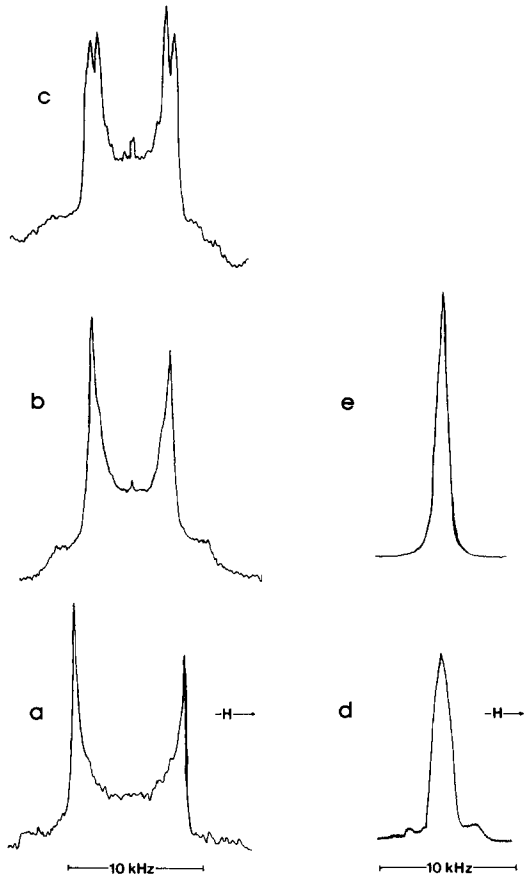

Fig. 4. ^{2}H-NMR spectra of egg phosphatidylcholine samples in the presence of deuterium-labelled trichloroethylene at 20°C. Trichloroethylene : phosphatidylcholine molar ratio: (a) 0.54 : 1, (b) 2.0 : 1, (c) 2.1 : 1, (d) 4.3 : 1, (e) 5.2 : 1.

molar ratio (to 5.2 : 1) gave rise to a ^{2}H-NMR spectrum in which the 'outer' powder pattern has entirely disappeared. The ^{31}P-NMR spectrum of this sample indicated the presence of hexagonal phase only.

Discussion

The formation of an inverted hexagonal phase in aqueous dispersions of phospholipids has previously been shown to occur in the presence of anaesthetics [4,5] although it has not always been recognized as such [5]. The results presented above indicate that trichloroethylene causes a phase transition of this type in systems of unsaturated phosphatidylcholines. The phosphorus spectra indicate

that a detectable hexagonal phase is formed at trichloroethylene: PC molar ratios above 2:1. Thayer and Kohler [18] have shown that the presence of a powder pattern of chemical shift anisotropy +18 ppm may arise from a bilayer lipid phase in which the phosphate headgroup is distorted. The results from ^{2}H-NMR experiments using [2,2'-$^2H_4$]DPPC, DPPC-d_{62} and DOPC-d_4, however, clearly show that the hydrocarbon region also experiences much greater motional freedom at trichloroethylene: PC molar ratios greater than 2:1. The degree of this additional freedom of movement is concomitant with that provided by rotation of the lipid around the aqueous channel of a hexagonal phase structure [19].

The use of ^{31}P-NMR to identify L_α-H_I or L_α-H_{II} phase transitions is an equivocal technique and X-ray evidence is required to distinguish between H_I and an H_{II} phase with absolute certainty.

However, the hydrocarbon chains of lipids in H_I and H_{II} phases are in significantly different environments. One might reasonably expect that in a normal (H_I) hexagonal phase the headgroup region, and hydrocarbon chain segments near to the headgroup, would have a similar or slightly reduced order parameter compared to that in the bilayer, whilst chain segments nearer to the terminal methyl group would experience a greater order because of the increased packing density of the hydrocarbon chains. This effect has been noted in deuterium-labelled phosphatidylcholines in hexagonal (H_I) phases induced by sodium cholate [24].

Similarly, one would expect the headgroup region and proximal methylene groups to experience a similar or slightly greater order in the inverted hexagonal phase (H_{II}) than in the bilayer phase, and the hydrocarbon chain segments nearer to the terminal methyl group to experience a reduced order.

The results presented above show that the quadrupolar splittings from DPPC-d_9, DPPC-$\alpha,\beta d_4$ and [2,2-$^2H_4$]DPPC in hexagonal phase lipids are very close to half their value in the bilayer phase, and those from DOPC-d_4 and the terminal methyl group of DPPC-d_{62} are significantly less than half their value in the bilayer phase. A similar observation has also been made by other workers [20] on mixtures of dioleoylphosphatidylcholine and dioleoylphosphatidylethanolamine, a system thought to produce an H_{II} phase.

In light of these results it is reasonable that, although the hexagonal phase was not identified by X-ray diffraction, the ^{2}H-NMR data indicate that it is an inverted hexagonal phase (H_{II}) which is formed.

It appears that the phase transition caused by trichloroethylene does not involve the formation of lipidic particles or intermediate cubic phases. There was no indication of an isotropic component, in any of the spectra, of the type associated with these intermediate structures [25,26]. The mechanism of the phase transition is therefore similar to the 'direct' interconversion of L_α to H_{II} phases described by Hui et al. [27] in which 'troughs' in adjacent bilayer surfaces form linear 'fault lines' or connections between bilayers, converting the layers into the cylinders of the hexagonal phase.

In view of the results from the deuterium-labelled trichloroethylene experiments it is probable that this mechanism is initiated as follows. The trichloroethylene, at low concentrations, is sequestered deep into the centre of the bilayer. The high olive oil/water partition coefficient and high fat solubility of trichloroethylene [28,29] support the hypothesis of trichloroethylene interacting predominantly with the hydrocarbon interior of the phospholipid bilayer. In this region the trichloroethylene is ordered, giving rise to the quadrupolar splitting of 8 kHz observed at a trichloroethylene: PC molar ratio of 0.54:1. Because of the fluid nature of this region the trichloroethylene does not significantly disturb the order of the hydrocarbon chains, as was observed in the experiments using DPPC-d_{62} (Fig. 2). Increasing the trichloroethylene concentration leads to further sequestering of the trichloroethylene in the bilayer, with concomitant reduction of the ordering of the trichloroethylene molecule. At the limit of bilayer stability, which we estimate to occur between trichloroethylene: PC molar ratios of 2.0:1 and 2.3:1, the trichloroethylene disrupts the hydrocarbon-hydrocarbon boundary inside the bilayer, leading to 'troughs' of the type described by Hui et al. [27], and ultimately to formation of a hexagonal phase. In the hexagonal phase the trichloroethylene has a significantly reduced order due to a greater motional freedom in the hydrocarbon-hy-

drocarbon interface region, giving rise to the broad central component (unresolved quadrupole splitting) of the ^{2}H-NMR spectra of deuterotrichloroethylene samples.

In the concentration range immediately preceding hexagonal phase formation the ^{2}H-NMR spectra of deuterotrichloroethylene indicate two distinct environments for the anaesthetic in the bilayer phase. Since egg yolk PC is a mixture of a variety of phosphatidylcholines [30] it is possible that this result is due to lateral phase separation [31], the trichloroethylene associating more readily with unsaturated phosphatidylcholines. The 'dynamic shape' models of lipid phase transitions [10,32,33] suggest that unsaturated lipids more readily form hexagonal phases. Thus the trichloroethylene-rich, unsaturated lipid-rich regions act as 'fault lines' for formation of the trough structure intermediate between the L_α and H_{II} phases. Thus in the ^{2}H-NMR spectra of the deuterium-labelled trichloroethylene samples the powder pattern of smaller quadrupolar splitting is from trichloroethylene in this 'fault' zone or early hexagonal phase, the other from trichloroethylene in the bilayer, saturated lipid-rich region.

In this connection it is of interest to note that the ^{2}H-NMR spectrum of a sample containing DOPC-d_4 shows little sign of a bilayer component at a trichloroethylene: PC molar ratio of 3.5 : 1, whereas that from a sample containing [2,2'-$^2H_4$]DPPC indicates both bilayer and hexagonal phase components.

It should also be noted that the ^{2}H-NMR spectra of the headgroup labelled DPPC-d_9 and DPPC-$\alpha,\beta d_4$ exhibit a significant degree of line-broadening on formation of the hexagonal phase. The cause of this is presumably dynamic rather than conformational since the quadrupolar splittings in the hexagonal phase are almost half the value of their equivalent in the L_α phase.

Conclusion

The presence of the general anaesthetic trichloroethylene in aqueous dispersions of egg yolk phosphatidylcholines induces a bilayer (L_α) to a hexagonal phase transition at a trichloroethylene: egg yolk phosphatidylcholine molar ratio of 2.3 : 1. This transition appears to be direct, i.e. there is no evidence to suggest an intermediate phase. However, at trichloroethylene : phosphatidylcholine molar ratios between 2.0 : 1 and 2.3 : 1 there are two distinct environments for trichloroethylene in a bilayer (L_α) phase which may be the result of lateral phase separation of the phosphatidylcholine components. At trichloroethylene concentrations above the phase transition threshold the effect of increasing temperature is to favour the hexagonal phase.

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