THE EFFECTS OF ANAESTHETICS ON LIPID POLYMORPHISM

by

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ABSTRACT

The effects of representative anaesthetics on the polymorphic phase behavior of phospholipids, particularly phosphatidylethanolamine, is studied. The series of normal alcohols and alkanes destabilize the bilayer structure of hydrated phosphatidylethanolamine causing the lipid to adopt hexagonal (H_{II}) structures at lower temperatures than controls. By exception, ethanol and butanol stabilize the bilayer configuration. Chloroform also initiates bilayer destabilization, producing this effect at clinically relevant concentrations of anaesthetic. The charged anaesthetics tested show the ability to stabilize the bilayer configuration of phosphatidylethanolamine to higher temperatures than controls; this occurs in a single or mixed lipid system (ie. phosphatidylethanolamine / phosphatidylserine). In all cases the ability of an anaesthetic to alter the polymorphic phase behavior of phosphatidylethanolamine is a function of its individual potency. The observed effects of anaesthetic agents on lipid polymorphism can be interpreted in relation to the molecular shapes of the lipids and anaesthetics involved.

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CHAPTER 1

INTRODUCTION

Anaesthetic molecules provide a particularly useful, yet seldomly applied, tool for the study of membrane structure. It is surprising to many that very little is known about how anaesthetics work. The term anaesthetic applies to a diverse range of molecular structures which have a single common property - they are invariably lipid soluble. Since these agents interact with cell membranes and have a large effect on cell function (most noticeably in nerve cells) they provide a means for gaining much information on how membranes are structured and how this structure relates to function. It is commonly accepted that both general and local anaesthetics act in a general manner with the lipid or lipid-protein component of the cell membrane rather than a specific interaction with a membrane receptor (1). This hypothesis is based on a large variety of documented studies from which a number of generalizations can be drawn, for example: 1.) greater lipid solubility constitutes a more potent anaesthetic, 2.) a vast variety of chemically disparate drugs are able to produce nerve block, and 3.) action potentials in artificial lipid bilayers are blocked by anaesthetics such a cocaine or chlorpromazine (see Ref.1).

The general rule of Meyer and Overton applies for most anaesthetic studies, it states that, "narcosis commences when any chemically indifferent substance has attained a certain molar concentration in the lipoids of the cell" (2 and 3). In other words, anaesthesia is produced when a critical volume is occupied by an anaesthetic in the membrane:

under conditions of local anaesthesia this average volume occupation is 0.3% of the membrane volume. Similarly, high membrane concentrations of anaesthetics, which correspond to around one anaesthetic molecule per five phospholipid molecules, are invariably associated with the threshold of membrane lysis (1).

By definition, anaesthesia constitutes action potential blockade without a measurable affect on the resting membrane potential; anaesthetics are not, however, selective between nerve and other cell types. Action potential initiation and propagation requires influx of sodium ions across the cell membrane through a proposed sodium channel; subsequently, an efflux of potassium ions occurs, the result being a depolarization of the cell membrane. Anaestheics presumably block this process by interfering with, or in some way disrupting, the sodium channel. Seeman (1) has stated that "for any particular hypothesis to be termed a 'theory of anaesthesia', it is necessary for that hypothesis to explain the manner in which the sodium conductance channel is inhibited or the manner in which synaptic transmission is modified. Otherwise, if the hypothesis merely shows a correlation of anaesthetic potency with some physiochemical parameter, the hypothesis is essentially only a rule of anaesthesia". Since disruption of the sodium channel by anaesthetic interaction occurs, wholly or in part, within the lipid component of the membrane, it is not surprising that most investigations in this area have focused their

attention on the lipid element in efforts to determine the anaesthetic mechanism. Many of these studies have demonstrated the ability of anaesthetics to increase the fluidity (decrease the gel-liquid crystalline hydrocarbon transition temperature) of the hydrophobic enviroment of the bialyer, leading to suggestions that this effects either a protein or lipoprotein conformation with subsequent inactivation of the sodium channel. Models, such as that due to Lee (4), have suggested that the sodium channel is surrounded by an annulus of gel state lipid, and penetration of anaesthetic molecules into this annulus causes an increase in fluidity leading to disruption and inactivation of the channel. This sort of model seems unlikely considering the unsaturated nature of nerve membrane lipid (5) (causing greater fluidity) as well as the fact that there is no evidence for the existence of gel-state lipids in eukaryotic cell membranes in general. Secondly, the anaesthetic concentrations required to induce significant "fluidization" are usually an order of magnitude larger than those required to inhibit the action potential in vivo (6). In fact, such concentrations often have lytic effects.

All anaesthetics whether general or local inhibit sodium conduction at concentrations low enough not to affect the resting potential of the nerve cell (1). By this general mechanism anaesthetic agents, in some way, disrupt the sodium channel and drastically alter the function of the neuronal membrane. Anaesthetics are of particular interest to membrane biologists because of their apparent ability to radically

perturb membrane structure and function and consequently they provide a useful tool to explore the possible functional roles of lipids in biological membranes.

In recent years it has become increasingly evident that models for membrane structure such as the fluid mosaic model of Singer and Nicholson (13) or the earlier unit membrane model (14) do not explain the many observed membrane functions. It is implied in the fluid mosaic model that the lipid component assumes a closed bilayer structure, thus realizing both a structural matrix with which functional proteins may be associated as well as an internal enviroment which may be regulated and controlled. This model also provides for lateral diffusion of membrane components in the plane of the membrane, as well as penetration of proteins into or through this matrix. This concept of membrane structure does not, however, allow explanation of certain membrane-mediated processes such as cell fusion, exo- and endocytosis, transbilayer movement of lipids ('flip-flop'), facilitated transport as well as protein insertion and orientation. More recently, alternative explanations to some functional aspects of membrane biology have become possible through advances in techniques such as X-ray and nuclear magnetic resonance which have shown membrane dynamics previously unknown.

For the past twenty years it has been known that hydrated lipids have the ability to adopt a variety of phases in addition to the commonly accepted bilayer phase. Particularly significant contributions to this research

area have been made by Luzzatti and coworkers (7-9) employing X-ray techniques to solve in detail the structural characteristics of these alternatives. In addition, these (10) and other (11 and 12) investigators immediately recognized the possibility that these non-bilayer structures may be related to membrane structure and function. Introduction of ^{31}P NMR added a new dimension to membrane research in terms of lipid polymorphism and allowed further reevaluation of existing membrane theories. In addition, more sophisticated freeze-fracture techniques, improved isolation and purification of lipids, plus better defined model systems has allowed confirmation of earlier observations made with X-ray and NMR, thus helping to provide more direct evidence for the existence on non-bilayer phases in biological membranes.

Biological membranes are composed of more than a hundred different species of lipid. That all these molecular types are required to maintain the structural integrity of the membrane bilayer seems unlikely when this same structure can be formed by a single lipid species such as phosphatidylcholine. This structural view seems even more unlikely considering the findings that a number of lipid species, including unsaturated phosphatidylethanolamines (15), cardiolipin and phosphatidic acid in the presence of calcium ion (16, 17), preferentially adopt non-bilayer phases when hydrated (as illustrated with corresponding ^{31}P NMR spectra in Figure 1) and it is logical to assume that these phases may be vital to such membrane functions as 'flip-flop' (18),

membrane fusion (19), exo- and endocytosis, as well as facilitated transport (20). Additionally, the existence of non-bilayer lipids in biological membranes has been strongly suggested with ^{31}P NMR and freeze-fracture techniques in the (rat liver) endoplasmic reticulum (21,22) and fusion of erythrocyte membranes (23).

In view of the proposed general mechanism of anaesthetic action, that is, the ability of anaesthetic molecules (so-called membrane perturbants) to radically alter membrane function, it became clear that a study of anaesthetic effects on lipid polymorphism would lend valuable insight to the possible functional role of non-bilayer lipids as well as a better understanding of anaesthetic action.

Lipids used for models in this study were: egg phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, and phosphatidylglycerol. These lipids, except phosphatidylethanolamine, spontaneously adopt and maintain a bilayer configuration when hydrated. The latter, however, undergoes a bilayer to hexagonal (H_{II}) phase transition at $30^{o}C$. A number of representative anaesthetics were chosen or the study including the series of n-alcohols and n-alkanes; the local anaesthetics, dibucaine, tetracaine, and procaine; the tranquilizer, chlorpromazine; plus the general anaesthetic, chloroform.

The series of n-alcohols and n-alkanes were found to convert bilayer phosphatidylethanolamine to the hexagonal phase at high concentrations, with the exception of ethanol

and butanol, which produced stabilization of the bilayer. The charged local anaesthetic dibucaine stabilizes the bilayer structure of egg phosphatidylethanolamine above the control bilayer to hexagonal phase transition temperature, but has no effect on egg phosphatidylcholine, phosphatidylserine, or phosphatidylglycerol. The other charged anaesthetics tested: procaine, tetracaine, and chlorpromazine also produced bilayer stabilization similar to the effects of dibucaine and this ability was found to correlate with the anaesthetic potency of the individual drugs. Furthermore, in a mixed lipid system (80% PE/ 20% PS), addition of small amounts of Ca^{++} induces the hexagonal phase, whereas subsequent addition of dibucaine at pharmacologically relevent concentrations was found to reestablish a bilayer structure.

The above results are discussed in the context of recent evidence for the existence of non-bilayer phases in biological membranes plus their possible relevance to anaesthetic mechanisms. A model for lipid/anaesthetic action is proposed in terms of the molecular shapes of these molecules, and additionally, speculations made on the possible roles of non-bilayer phases in ion transport and how this function may be disrupted by anaesthetic molecules. Also, the possibility of lipid-protein interactions being disrupted by anaesthetic molecules is discussed.

CHAPTER 2

MATERIALS and METHODS

2a. General

Egg phosphatidylethanolamine and egg phosphatidylcholine were purified from a total egg lipid extraction using high pressure liquid chromatography (Waters Prep LC 500) with silic acid providing the stationary phase, the lipid being separated with a mixture of chloroform and methanol. Egg phosphatidylserine and phosphatidylglycerol were prepared by a conversion technique according to Comfurius and Zwaal (24) using phosphalipase D to change the headgroup from egg PC to PG or PS. The individual lipids were further purified by column chromatography using silic acid.

In each case the purity of the lipid was assessed by thin-layer chromatography and, for egg PE, by consistency in the bilayer to hexagonal phase transition temperature of the lipid which is very sensitive to PE degradation. Storage of pure phosphatidylethanolamine in chloroform at -20^{o}C is not sufficient to maintain purity (ie. stable Tbh) and within three days the phase transition temperature can increase as much as 10^{o}C. The reason for degradation of phosphatidylethanolamine, in particular, is not known, however, it is reasonable to suspect that it may be a result of oxidation of a relatively large portion of the highly unsaturated 22:6 fatty acid chains found in egg PE (approximately 15% by weight). Phosphatidylethanolamines from other sources which do not contain such highly unsaturated fatty acids are less subject to such effects. To avoid this problem of degradation the lipid was kept under nitrogen as much as possible during preparation, and

immediately after purification was sealed in plastic cyro-tubes and immersed in liquid nitrogen.

2b. Experiments with Neutral Anaesthetics

Samples were prepared from dry 100 mg. portions of purified egg phosphatidylethanolamine. The lipid was dissolved in 1 ml. chloroform and transferred to a 10 mm. NMR tube and subsequently dried under a stream of nitrogen. All traces of chloroform were removed by further drying the lipid under high vacuum for a minimum of 2 hours. To test an individual concentration of chloroform, alcohol, or alkane, 0.6 ml. $^{2}H_{2}O$ containing 100 mM NaCl, 10 mM Tris/acetic acid, 2 mM EDTA, p^{2}H 7.0 was added to the dry lipid. A small amount of anaesthetic was injected directly into this solution using a Hamilton micropipette to make up the required concentration, and the entire mixture was Vortex mixed to form liposomes and ensure maximum partition of anaesthetic into these structures. Equivalent results were obtained when longer chain length anaesthetics were added in chloroform, which was subsequently evaporated.

2c. Experiments with Charged (Amine) Anaesthetics

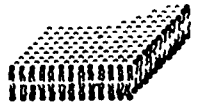
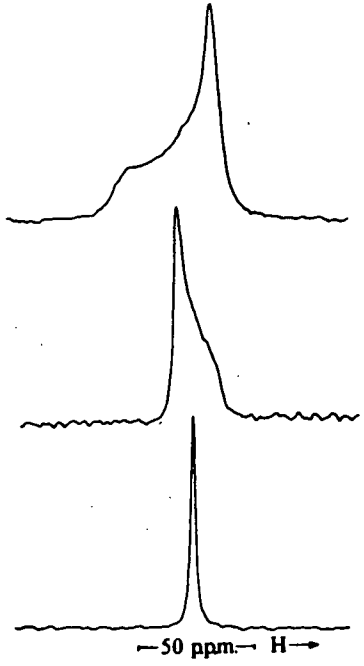
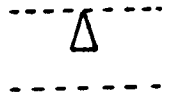
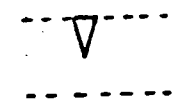
Preparation of samples for these experiments was slightly different than with the neutral anaesthetics. Large volumes of buffer was used to ensure maximum partition of dibucaine into the lipid during liposome formation. Samples containing PC, PS, and PG were prepared with dibucaine solutions

only, whereas PE was made up for experiments with dibucaine, plus procaine, tetracaine, and chlorpromazine.

Dibucaine HCl was purchased from ICN Pharmaceuticals; Procaine and Tetracaine HCl from Winthrop Laboratories; and Chlorpromazine HCl from Poulenc Ltd. Lipids were dissolved in chloroform and transferred to 50 ml. SS-34 centrifuge tubes, dried under nitrogen, then high vacuum for a minimum of 2 hours. The dry lipids were dispersed by Vortex mixing in 4 ml. aqueous solutions of appropriate anaesthetic containing 2 mM EDTA and 100 mM NaCl. The hydrated lipid was centrifuged at 3000 rpm for 10 minutes and the pellet resuspended in 0.6 ml. of fresh incubation medium plus 0.1 ml. D_2O. The pH was neutralized and the sample sealed in a 10 mm. NMR tube. Samples containing PC, PS, and PG were made in dibucaine preparations only. By the same procedure, the PE/PS mixtures (125 mg.) containing 20 mol% PS and 80 mol% PE were dispersed in 200 ml. aqueous solutions of 100 mM NaCl, 10mM Tris/acetic acid (p^2H 7.0), 5 mM $CaCl_2$ plus the required concentration of dibucaine. The samples were centrifuged and pellets resuspended in 0.6 ml. of the original solution plus 0.1 ml. D_2O.

For all experiments ^{31}P NMR spectra were made using a Bruker WP 200 Fourier transform spectrometer operating at 36.4 MHz. Temperatures were raised incrementally through the bilayer to hexagonal transition allowing 10 minutes equilibriation time for each experiment. ^{31}P NMR spectra obtained form phospholipid membranes are interpreted on the

FIG. 1. Representative lipids and corresponding phases plus schematic molecular shapes and ^{31}P NMR spectra. The bilayer spectrum was obtained from aqueous dispersions of egg yolk phosphatidylcholine, whereas the hexagonal (H_{II}) phase spectrum was obtained from soya phosphatidylethanolamine. The "isotropic motion" spectrum was obtained from a mixture of 85 mole % soya phosphatidylethanolamine and 15 mole % egg yolk phosphatidylcholine. All spectra were taken at 30°C.

Lipid	Phospholipid Phases	Molecular Shape	Corresponding ^{31}P NMR Spectra
Phosphatidylcholine Sphingomyelin Phosphatidylserine Phosphatidylglycerol	Bilayer 	Cylindrical 	
Phosphatidylethanol-amine (unsaturated) Cardiolipin - Ca^{++} Phosphatidic acid - Ca^{++}	Hexagonal (H_{II}) 	Cone 	
Lysophospholipids Detergents	Phases where isotropic motion occurs a, Micellar, inverted micellar b, Vesicles c, Cubic Rhombic	Inverted Cone 	

basis of a number of established principles. A typical bilayer spectrum (Fig. 1) results from the large chemical shift anisotropy of lipid phosphorus in large (radius 2000 A) liquid-crystalline bilayer systems. The primary mode of motion available to these systems is rapid rotation of the molecules about their long axis so that only partial averaging occurs (20, 25-27). In the presence of proton decoupling, this results in a characteristic broad spectrum with a low field shoulder and high field peak, which are separated by $\sigma_{CSA}^{EFF} \simeq -40$ ppm. Similar values are found for all glycerol based phospholipids (25), (with the possible exception of phosphatidic acid) including the mammalian phosphosphingolipid, sphingomyelin; this results in almost equivalent line shapes for these different species when in the liquid-crystalline bilayer configuration. Therefore in mixed lipid systems and biological membranes effectively all the endogenous phospholipids contribute to a composite bilayer lineshape if they are in the bilayer phase. In the case of small sonicated lipid vesicles lateral diffusion of lipid around the vesicle and vesicle tumbling occur rapidly on the NMR timescale (10^{-5} sec.) such that motional averaging occurs resulting in line-narrowing effects (28). Lipids in the hexagonal (H_{II}) phase experience additional motional averaging because motional averaging due to lateral diffusion around the small (20 A diameter) aqueous channel occurs. This effect results in characteristic ^{31}P NMR lineshapes which have reversed asymmetry compared to bilayer spectra

and are narrower by a factor of two (20, 25, 29). Furthermore, lipids in micellar, inverted micellar, or other phases (such as the cubic or rhombic) configurations allow effectively isotropic motion to occur, as lateral diffusion results in averaging over all orientations, leading to a narrow, symmetric ^{31}P NMR spectrum. A summary of lineshapes observed is presented in Figure 1.

CHAPTER 3

RESULTS

3a. General

Hydrated egg phosphatidylethanolamine with no anaesthetic present displays a bilayer to hexagonal phase transition near 30°C (Fig.2a). This value, termed Tbh, is defined as the temperature at which the lipid is both 50% in the bilayer phase and 50% in the hexagonal (H_{II}) phase (Fig.3). As the temperature is increased from 10°C (Fig. 2a bottom) the corresponding ^{31}P NMR spectrum changes from a broad asymmetric peak with a defined low field shoulder, representing lipid in the bilayer phase, to a narrow spectrum with a high field shoulder and reversed asymmetry corresponding to lipid in the hexagonal (H_{II}) phase (25). Phosphatidylcholine, phosphatidylserine, and phosphatidylglycerol which were used in experiments with dibucaine, spontaneously adopt a bilayer configuration and maintain this phase throughout increasing temperatures (data not shown).

3b. Neutral Anaesthetics

In comparison to the polymorphic phase behavior of egg phosphatidylethanolamine (Fig.2a) the spectra obtained for the same lipid in the presence of 1M ethanol and 100mM decanol are markedly different (Fig.2b and 2c). The estimated Tbh (Fig.3) for these experiments is raised to 41°C for 1M ethanol and lowered to 5°C for 100mM decanol. Thus a substantial shift in the Tbh of phosphatidylethanolamine was produced by these anaesthetics and the degree and direction of phase shift was dependent on the alcohol employed.

FIG. 2. 81.0 MHz ^{31}P NMR spectra of egg phosphatidyl-ethanolamine at indicated temperatures (a) in the absence of alcohols; (b) in the presence of ethanol (1 M) (c) in the presence of decanol (100 mM).

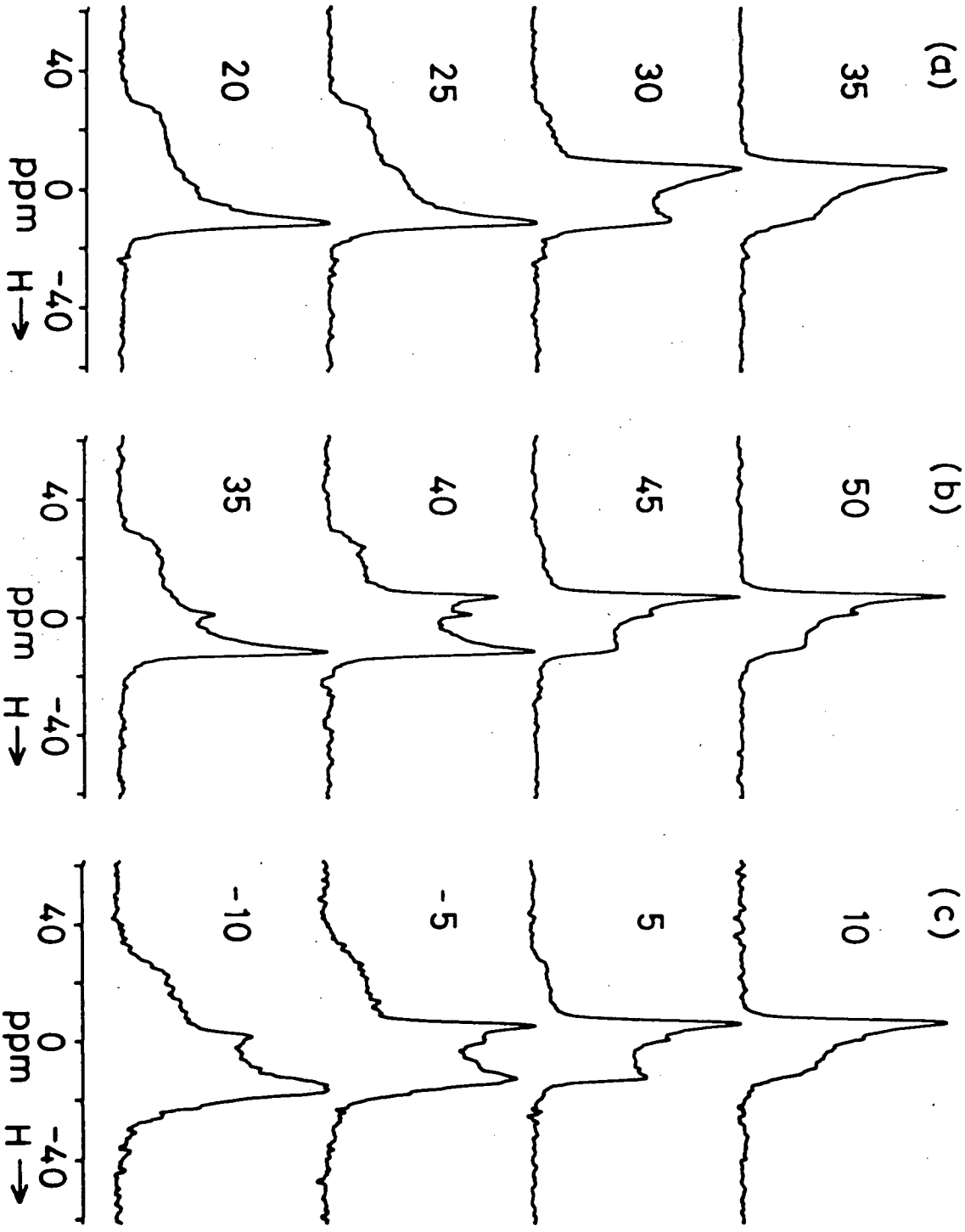
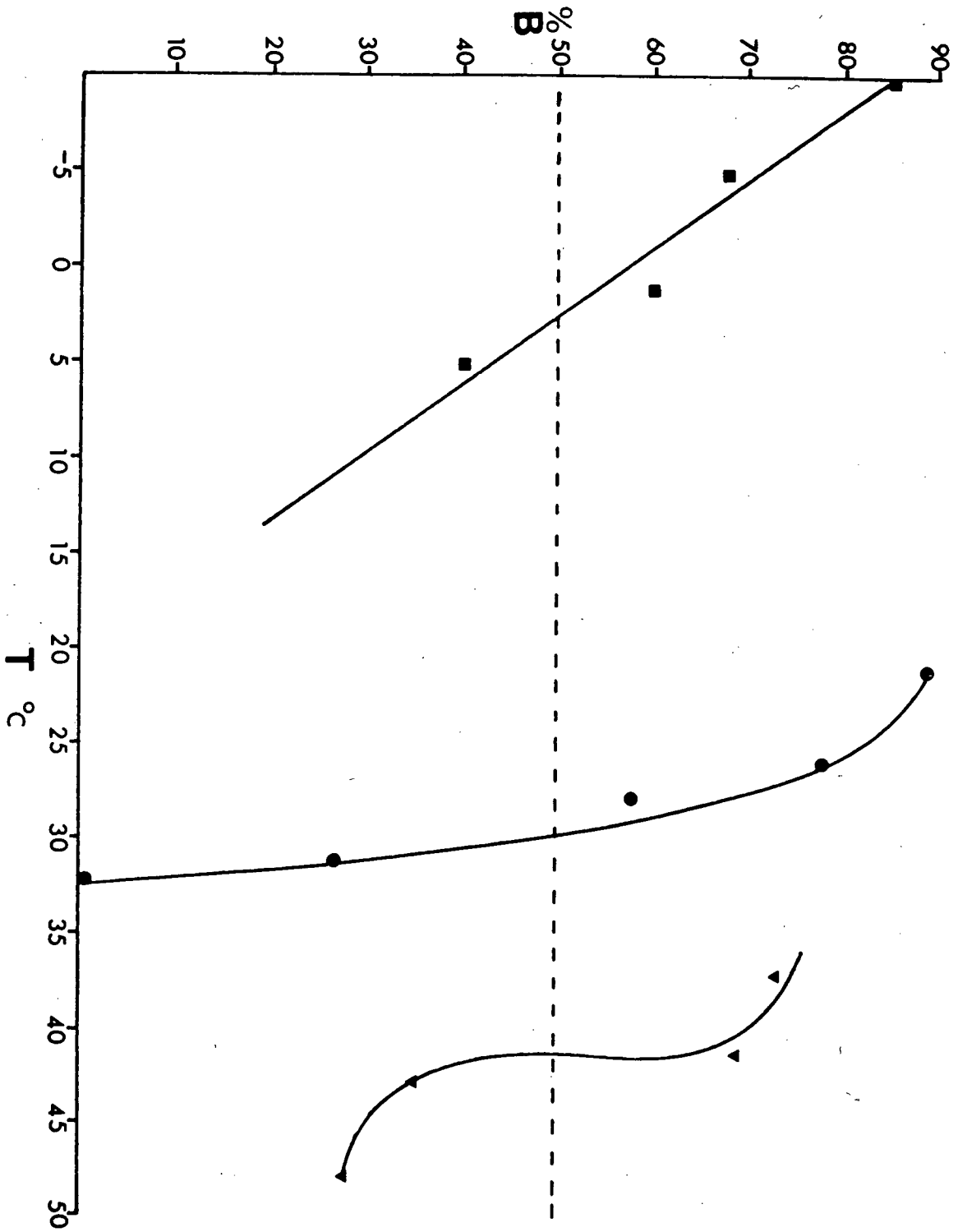

(a)
35
30
25
20
(b)
50
45
40
35
(c)
10
5
-5
-10
40
0
-40
ppm
H

Fig. 3. Method for determining the bilayer to hexagonal (H_{II}) phase transition temperature (Tbh) for egg phosphatidyl-ethanolamine. Tbh is estimated as that temperature where 50% of the lipid is in the bilayer organization and 50% is in the hexagonal (H_{II}) phase:

■- decanol (100 mM); ●- control; ▼- ethanol (1M).


B %
10
20
30
40
50
60
70
80
90
T °C
-5
0
5
10
15
20
25
30
35
40
45
50

The series of even numbered n-alcohols from ethanol (C_2) to dodecanol (C_{12}) were added to individual egg PE preparations and each alcohol was tested over a range of concentrations (Fig.4). The ability of a particular alcohol to alter the phase properties of the lipid increased with increasing hydrocarbon chain length, such that dodecanol (C_{12}) caused a 20^{o} drop in the Tbh at a concentration of 50 mM whereas the same 20^{o} decrease in Tbh required 120 mM concentration of hexanol (C_6).

The same protocol as above was used for the series of n-alkanes (Fig.5). However, because of the difficulty in handling the gaseous alkanes, only those with chain lengths greater than pentane (C_5) were tested. The results for this series are not as well defined as those for the alcohols, although their ability to decrease the Tbh of the PE resembles that for the alcohols; similarly, an increase in potency with increasing chain length is observed. By comparison to the alcohols the alkanes appear to be approximately two times more potent in their ability to initiate the hexagonal phase, where 120 mM hexanol produces a 20^{o} drop in Tbh, only 70 mM hexane is required to produce the same decrease in Tbh.

Similar to the effects of the longer chain length alcohols and alkanes, the gaseous anaesthetic chloroform causes reduction in the Tbh of Soya PE and initiation of the hexagonal (H_{II}) phase at lower temperatures as compared to controls (Fig.6). The spectra shown are taken at approximately $15^{o}C$ below the control Tbh of the lipid and demonstrate that 5 and 10 mM

FIG. 4. Influence of varying amounts of the normal alcohols on the bilayer to hexagonal (H_{II}) phase transition temperature of egg phosphatidylethanolamine.

● - ethanol; □ - butanol; ■ - hexanol

△ - octanol; ▼ - decanol; ○ - lauryl alcohol

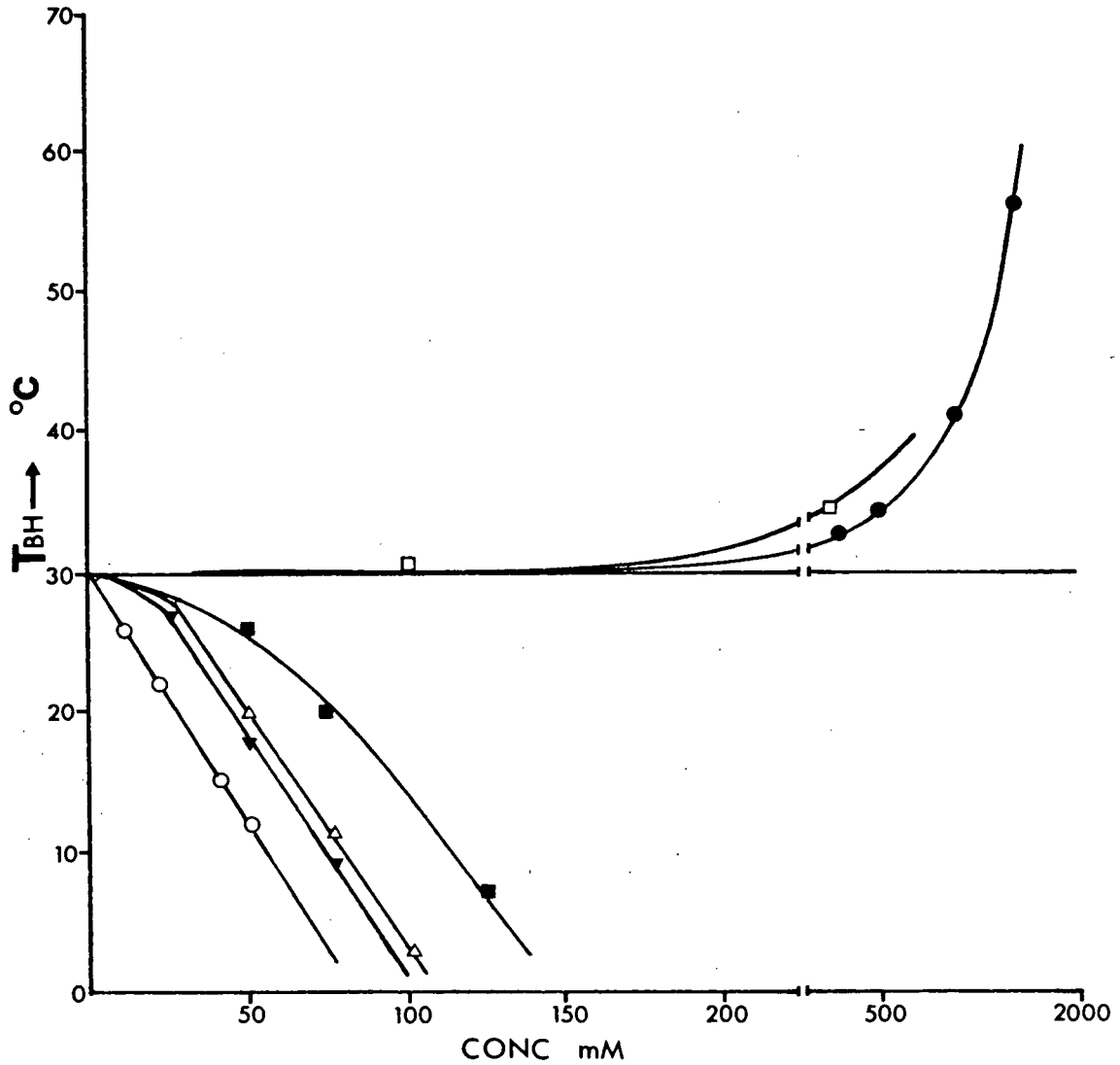

70
60
50
40
30
20
10
0
°C
T_{BH} ⟶
50
100
150
200
500
2000
CONC mM

Fig. 5. The influence of increasing amounts of normal alkanes of various chain lengths on the bilayer to hexagonal (H_{II}) transition temperature of egg phosphatidylethanolamine: ● - hexane; ▼- heptane; ○- octane; ■- decane.

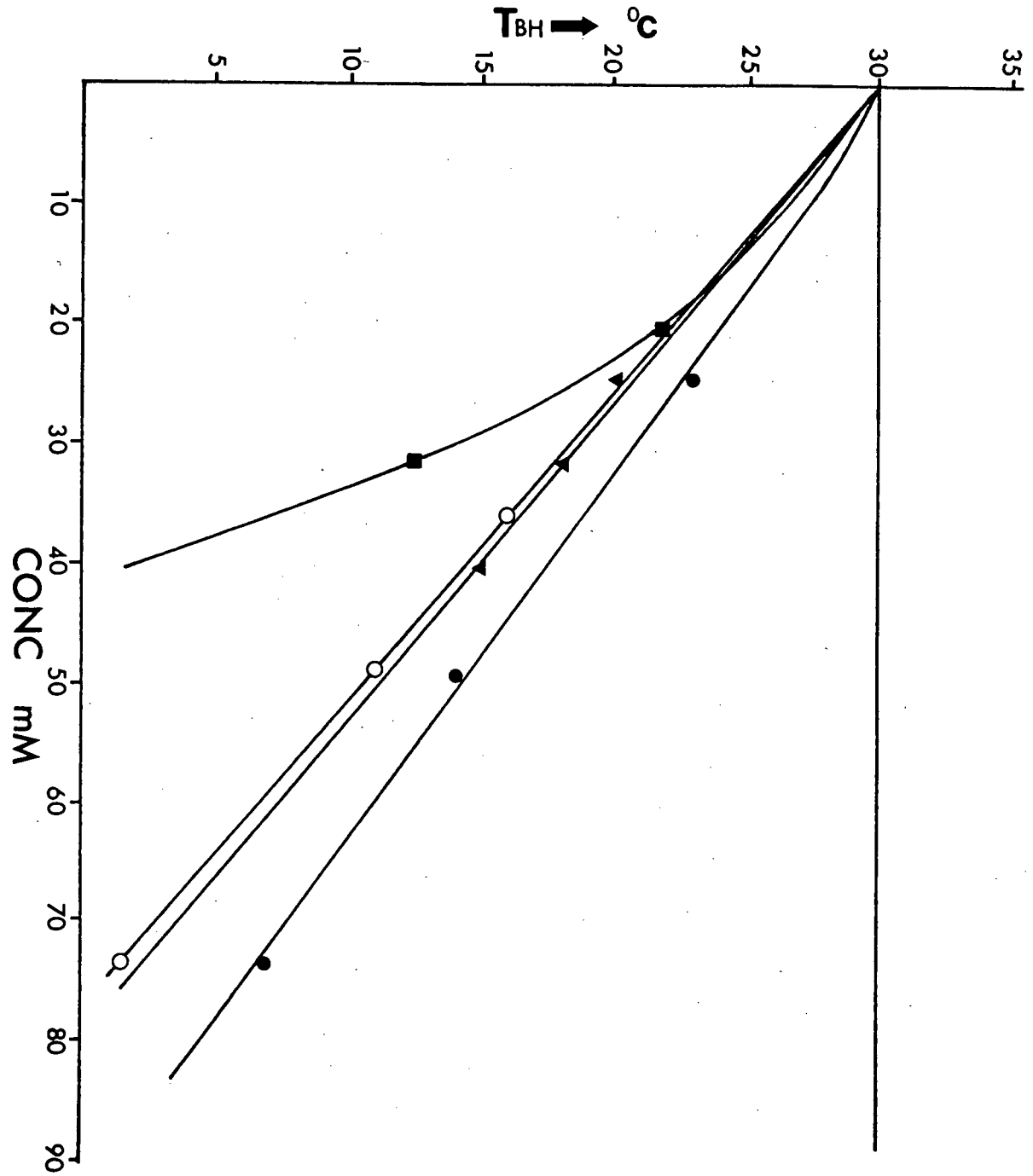

T_{BH} → °C
5
10
15
20
25
30
35
CONC mM
10
20
30
40
50
60
70
80
90

FIG. 6. 81.0 MHz ^{31}P NMR spectra of Soya phosphatidylethanolamine at $-5^{o}C$ in the presence of varying concentrations of chloroform.

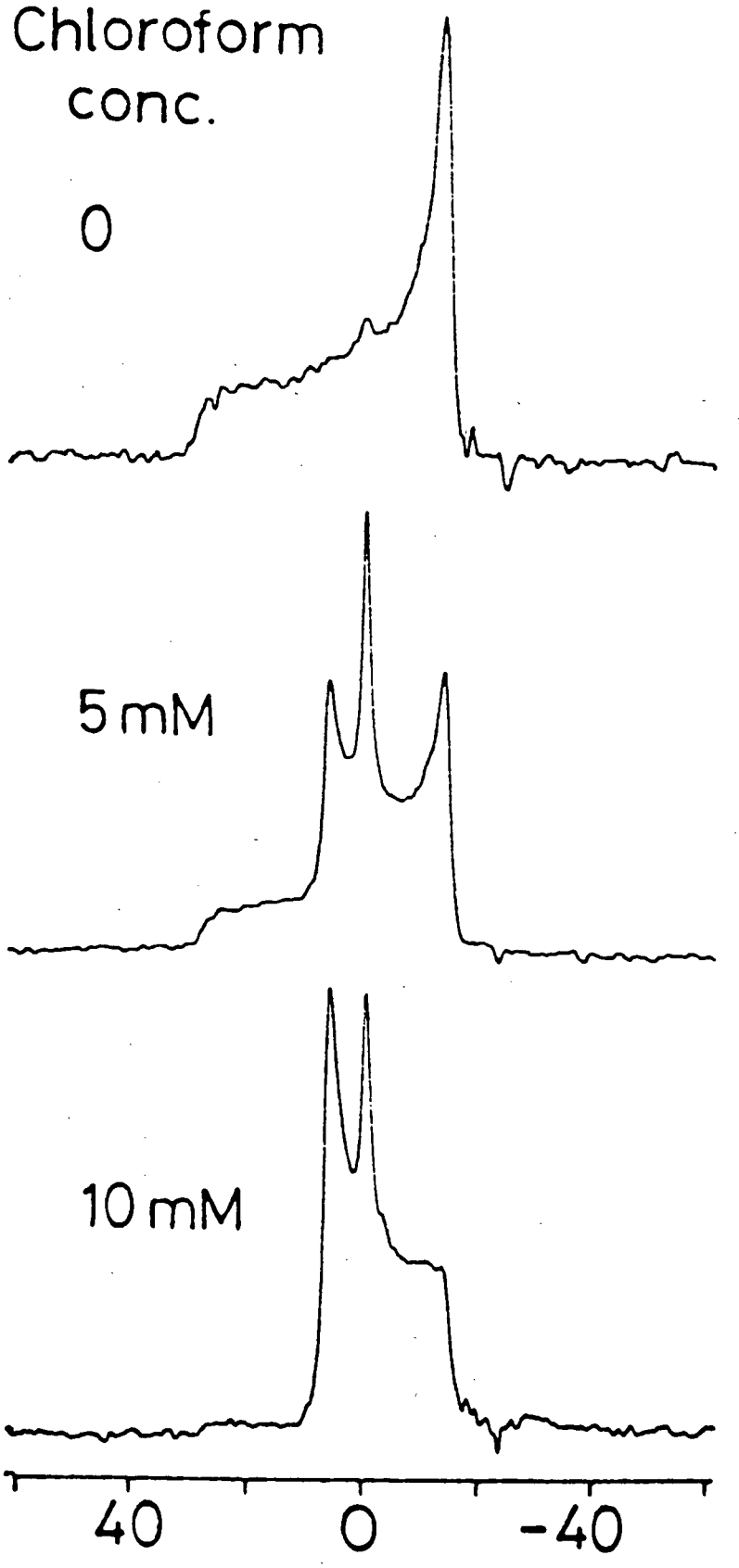

Chloroform
conc.
0
5 mM
10 mM
40
0
-40

FIG. 7. 81.0 MHz ^{31}P NMR spectra of egg phosphatidyl-ethanolamine at 40^{o}C in the presence of varying concentrations of (a) chlorpromazine; (b) dibucaine; (c) tetracaine and (d) procaine. For details of sample preparations see Methods.

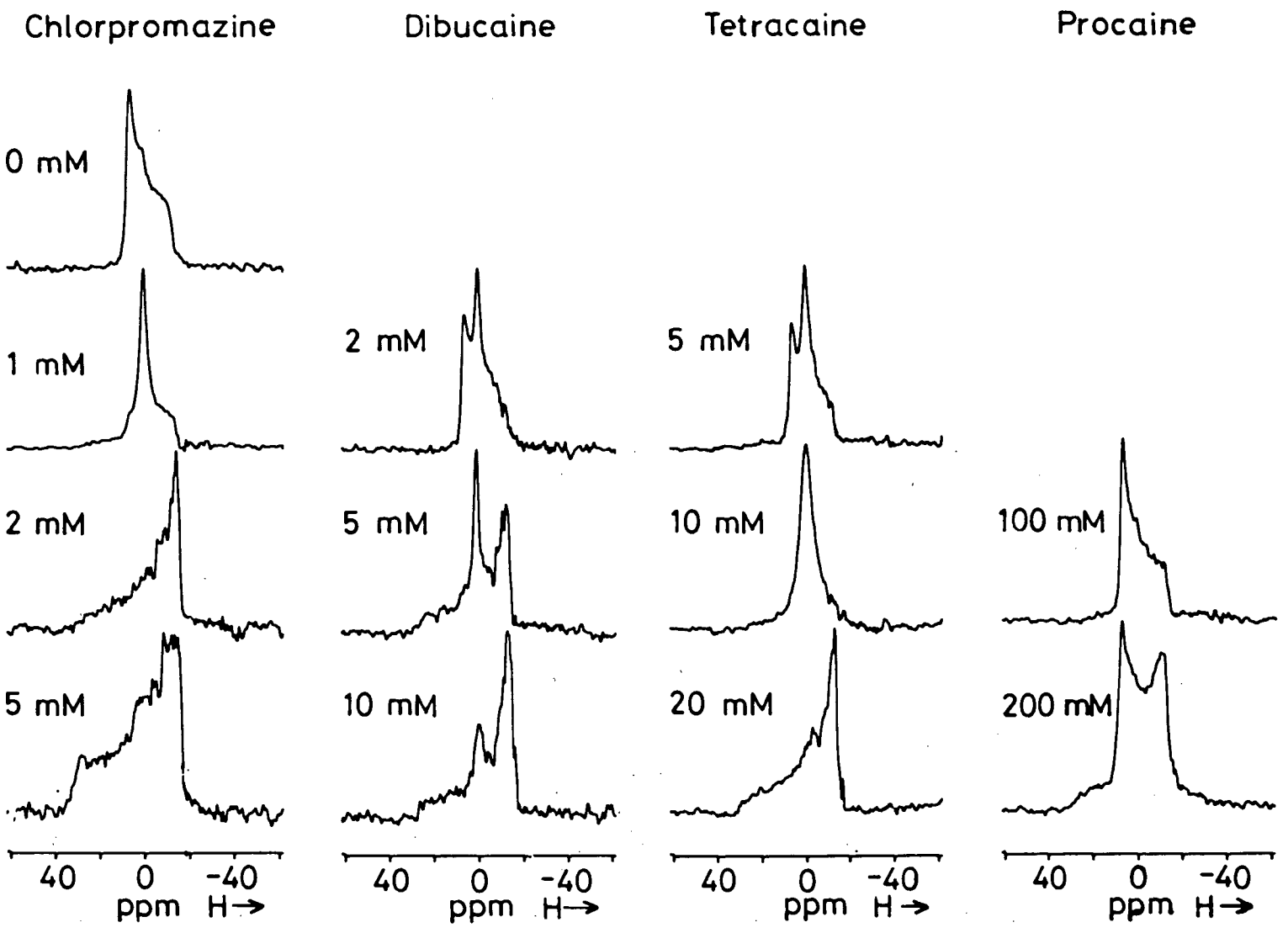
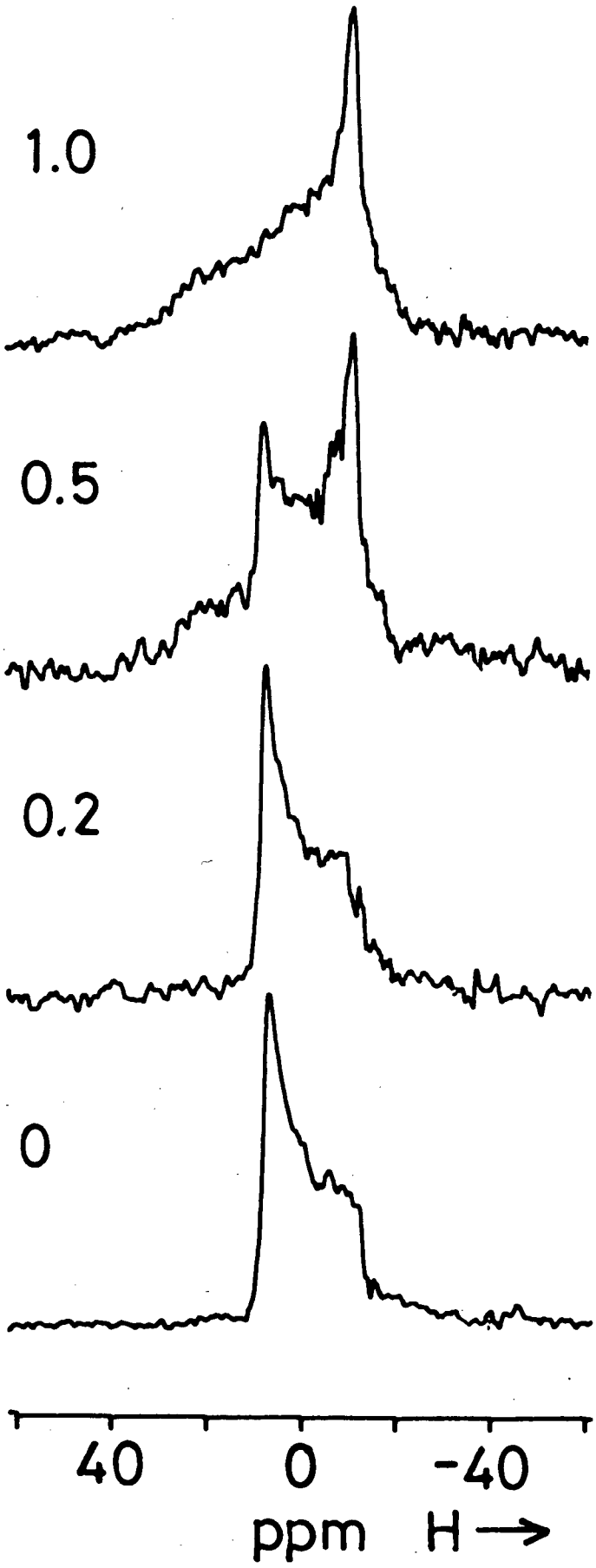

Chlorpromazine
Dibucaine
Tetracaine
Procaine
0 mM
1 mM
2 mM
5 mM
2 mM
5 mM
10 mM
5 mM
10 mM
20 mM
100 mM
200 mM
40
0
-40
ppm
H→

FIG. 8. Influence of dibucaine on the 81.0 MHz ^{31}P NMR spectra at 40°C of an aqueous dispersion of 20 mol % phosphatidylserine and 80 mol % egg phosphatidylethanolamine in the presence of 5 mM $CaCl_2$.


1.0
0.5
0.2
0
40
0
-40
ppm H →

concentrations of this anaesthetic can produce non-bialyer structures as indicated by an increase in the hexagonal and isotropic component of the ^{31}P NMR spectra. Furthermore these effects are obtained at near clinical concentrations of the anaesthetic. This latter point is an important consideration since little can be said about anaesthesia unless pharmacologically relevent concentrations of anaesthetic are being employed; this is true regardless of whether the system is a model or biological. Since the initial results with the neutral anaesthetics indicated that there was some correlation between the potency of an anaesthetic and its ability to alter membrane stability, whether through bilayer stabilization or destabilization, it was of interest to observe the effects of some local anaesthetics on lipid polymorphism and additionally to produce these effects at clinical concentrations.

3c. Charged (Amine) Anaesthetics

It was found that all the charged anaesthetics tested show an ability to stabilize the bilayer configuration of egg PE above the control Tbh of 30°C. This effect correlates with the relative potency of these anaesthetics: chlorpromazine >dibucaine >tetracaine >procaine. The spectra for experiments carried out at 40° are shown in Fig.7. Egg phosphatidylethanolamine with no anaesthetic present normally begins its bilayer to hexagonal phase transition between 20 and 25°C, such that the bilayer spectra shown in Fig. 7 are approximately 20° above those for control conditions. It is apparent that a

higher concentration of anaesthetic is required to preserve the bilayer structure at 40^{o} with a less potent anaesthetic such as procaine. That is, where a partial bilayer structure is observed with 200 mM procaine, less than 2 mM chlorpromazine was found to stabilize the bilayer configuration of egg PE above 60^{o}C (data not shown).

The effects of dibucaine on a mixed lipid system (80 mol% PE/ 20 mol% PS) in the presence of 5 mM Ca^{++} were observed (Fig. 8a). The spectra shown are those obtained at 40^{o}C, which is 5^{o} above where the control PE/PS mixture in the presence of Ca^{++} is normally hexagonal (8b). An increased stability of the bilayer structure was observed for 0.5 and 1.0 mM dibucaine, however, 0.1 and 0.2 mM concentrations had no effect on stabilizing the bilayer above control values.

CHAPTER 4

DISCUSSION

It is clear from this and other studies (30) that a number of lipid species have the ability to adopt non-bilayer phases when pure and hydrated. This property, plus the observation that a large variety of lipid types with diverse structures and shapes are present in biological membranes suggests that lipids may serve functional roles in membranes rather than simple maintainence of their structural integrity (30). Lipids which prefer to be non-bilayer at physiological temperatures may therefore be performing a functional rather than a structural role. This was the basic premise of this study along with the notion that anaesthetics, (so-called 'membrane perturbants') which presumably interact with the lipid element of biological membranes may, in some way, influence the polymorphic phase behavior of non-bilayer lipids. This possibility had been indicated previously by work carried out in this laboratory: model membrane systems comprised of cardiolipin, a major component of the inner mitochondrial membrane, can be converted from the bilayer to hexagonal phase by the addition of equimolar Ca^{++} at $30^{o}C$. In the same study it was noted that both dibucaine and chlorpromazine trigger formation of the hexagonal phase at anaesthetic/cardiolipin ratios of 2:1. Some preliminary experiments had also been made using ethanol and chloroform (31).

In this study the effects of anaesthetics on lipid polymorphism has been expanded to a number of other lipid species, particularly egg phosphatidylethanolamine. In addition, a number of representative anaesthetics have been tested

including charged, neutral and gaseous species, the purpose being to obtain a comprehensive overview of anaesthetic effects on lipid polymorphism. From the results obtained it is clear that a number of anaesthetic agents have profound effects on the polymorphic phase behavior of egg phosphatidyl-ethanolamine. In all the experiments presented above the egg PE sample used had a bilayer to hexagonal (H_{II}) phase transition temperature of approximately 30°C. This property of egg PE remains consistent by the technique described, however initial difficulties were enountered in varying Tbh's with slightly different purification techniques and preparations.

The usefulness of the series of n-alkanes and n-alcohols in studies of anaesthetic mechanism (or in effecting the polymorphic phase behavior of egg PE) is a result of the increasing potency of these molecules with increasing hydrocarbon chain length. Experiments with the series, therefore, provides a comparison of increasing potency effects using analogous molecules. Generally the alcohols and alkanes have anaesthetic effects up to carbon number 10 in a physiological system, after which the longer chain molecules are no longer anaesthetic. This so-called 'cut-off' effect occurs because the longer chain length anaesthetics are too insoluble to have any effect <u>in vivo</u>; the cut-off effect, therefore, is not relevent in a model system where physiological partition parameters do not exist. The results in Figure 4, however, do show a type of 'cut-off' effect between the ability of the n-alcohols up to C_4 to stabilize

the bilayer configuration of egg PE, whereas C_6 alcohol, and greater, cause destabilization and initiation of the hexagonal phase. This capacity to trigger hexagonal phase formation increases with increasing hydrocarbon chain length for both alcohols and alkanes and in this way correlates with the anaesthetic potency of the individual drugs. It is further observed that the series of alkanes shows a greater potency than the alcohols which is also the case in physiological studies. The shift away from the temperature at which the bilayer to hexagonal phase transition occurs increases with greater concentrations of the particular anaesthetic. Although these concentrations are not pharmacologically relevent with respect to anaesthesia, with exception of ethanol and butanol, the aim of these experiments was merely to demonstrate the ability of these anaesthetic agents to alter egg PE polymorphic phase behavior. Since the capacity of an alkane or alcohol to effect hexagonal phase formation appears to be a functicn of the hydrocarbon chain length of the anaesthetic species, this property will be discussed later in terms of the molecular shape of these molecules.

The hexagonal phase is also effected by chloroform at near clinical concentrations (10mM). The results shown are for Soya phosphatidylethanolamine but identical effects have been observed with egg PE. Converse to the effects of those agents discussed above, the local anaesthetics tested stabilize the bilayer structure of the liposomes and this effect too, is a function of the anaesthetic potency of the

individual species. In comparing the effects of the local anaesthetics on egg PE concentrations higher than those required to produce nerve block in vivo were used, however experiments with dibucaine on a mixed lipid system of PE/PS plus Ca^{++} show bilayer stabilization at pharmacologically relevent concentrations (0.5 - 1.0 mM) (Fig.8 and 9).

To recap on the above observations, it is clear that a number of representative anaesthetic agents can markedly affect the polymorphic phase behavior of phosphatidylethanolamine singularly and in a mixed lipid system and that the degree of effect directly relates to the physiological anaesthetic potency of the agent being used. Additionally, similar effects can be achieved at pharmacologically relevent concentrations of the anaesthetic.

A rather naive but illustrative interpretation can be made for the mechanism whereby anaesthetic molecules alter the polymorphic phase behavior of certain lipids based on the dynamic molecular shape of the molecules involved. Other authors (32) have invoked similar considerations to rationalize the behavior of particular lipid systems. Briefly, as indicated in Figure 1, lipids assuming the hexagonal (H_{II}) phase may be considered to exhibit a 'cone' shape, where the polar headgroup region is at the smaller end of the cone. Alternatively, lysophospholipids may be suggested to display an 'inverted cone' shape where the cross-sectional area of the polar region is larger than that subtended towards the end of the acyl chain. This shape would be

compatible with the micellar phase adopted by these lipids. Finally, lipids which assume a more cylindrical shape would be most easily accomodated in the familar bilayer phase.

In terms of this proposal, the smaller headgroup of PE (as compared to PC) as well as the possibility of intermolecular hydrogen bonding (33) would be expected to result in a reduced area per molecule at the lipid-water interface. This would be compatible with a 'cone' shape configuration and preference for hexagonal phase formation commonly found for PE. By the same premise, increased unsaturation of the acyl chain region of the molecule would effectively increase the cross-sectional area of this portion of the molecule as would increased thermal motion at higher temperatures, again resulting in a 'cone-shaped' configuration and liability toward an hexagonal arrangment (15). These molecular shape considerations can also be extended to acidic phospholipids for which the area per molecule at the lipid-water interface is sensitive to the net charge in the polar region (34). Cardiolipin, isolated from mitochondria, would be expected to exhibit a cone shaped structure considering the relatively small headgroup region and the large cross-sectional area of four (usually very unsaturated) acyl chains (35). This lipid adopts a bilayer configuration when hydrated, however, when in the presence of divalent cations, charge neutralization occurs and presumably reduction in the effective size of the headgroup area, consequently triggering formation of hexagonal (H_{II}) phase (19).

Similarly, at pH values above 5, unsaturated phosphatidylserine adopts the bilayer phase, but a pH=2.5 (below the pK of the carboxyl group) the hexagonal phase is observed (36), which again may be attributed to reduced charge repulsion effects.

A relationship between anaesthetic mechanism and the observations made in this study can be made in terms of the molecular shape of the anaesthetics employed (30). Ethanol, for example, being relatively polar, would be expected to reside at the lipid-water interface with the hydrocarbon region extended toward the interior of the bilayer. This interaction with PE would result in a 'cylinder' shaped complex resulting in an increase in bilayer stability. Increasing the hydrocarbon chain length of the alcohol to decanol produces a further integration into the acyl chain region of the phospholipids, effectively expanding this region and initiating an hexagonal arrangment. Similarly, chloroform would be expected to reside in the hydrophobic region of the membrane producing a similar effect to that of the longer chain alcohols.

This effect may be interpreted by the same model, however, in the presence of the acidic PS, it is expected that an ion-ion (charge neutralization) or ion-dipole interaction of the local anaesthetic with acidic phospholipid occurs. It has been demonstrated that addition of local anaesthetics to an acidic phospholipid dispersion produces a decrease in bulk pH, indicating an interaction with either

phosphate or carboxyl groups (37). In the mixed lipid system studied local anaesthetics may displace Ca^{++} from sites on the PS which initially produces the hexagonal arrangment and consequently reform a bilayer structure and at the same time produce their effects by net expansion of the headgroup region of this mixture.

To expand slightly on the properties of local anaestheitcs, some common generalizations are that the useful local anaesthetics, with few exceptions, are composed of three constituents: 1.) a carbocyclic or heterocyclic ring of the aromatic type (lipophilic portion) 2.) an intermediate chain, and 3.) an amino group (hydrophilic portion) (37). It is further indicated that the aromatic ring and its electron-donating substituents conjugate with the adjacent carboxyl group rendering it more electronegative and capable of forming hydrogen bonds. The electronegativity of the carbonyl oxygen is thus correlated with the intensified local anaesthetic activity (38). This theory readily accounts, for instance, for the local anaesthetic activity of procaine (p-NH_2 group) and tetracaine (p-C_4H_9-NH group), where the introduction of an electron-donating substituent results in a marked intensification of the weak effect of the parent compound. In general, therefore, compounds of the type in which resonance effects results in an active dipole carbonyl oxygen, and particularly those in which the negativity of the carbonyl oxygen is partially intensified by electron-donating substituents, are potent local anaesthetics (38). A correlation

between anaesthetic potency and the ability to stabilize hydrated PE into a bilayer may be the result of an ion-ion, or ion-dipole interaction of the local anaesthetic and the phospholipid headgroup; such an association would produce a net expansion of this headgroup region consequently effecting bilayer stability with greater expansion of this region being due to greater electronegativity of the carbonyl oxygen. Given the above mechanism of anaesthetic effects on lipid polymorphism the question arises of how this relates to a biological system.

Non-bilayer phases have not been proven to exist in biological membranes however, strong evidence exists to support this contention (see Ref. 30). Erythrocyte ghosts, for example, normally show ^{31}P NMR spectra representing a bilayer configuration even after treatment with phospholipases which produce a large percentage of non-bilayer lipids in the membrane. The erythrocyte membrane, therefore, exhibits a strong bilayer stability. Introduction of oleic acid however (a so-called 'fusogen') can cause a wholesale disruption of bilayer structure, promoting formation of the hexagonal phase (23). This behavior has been used to suggest the involvement of non-bilayer phases as intermediate during fusion events. Additionally, non-bilayer phases may be present in endoplasmic reticulum preparations where isotropic motion has been observed with ^{31}P NMR in rat, bovine, and

rabbit liver endoplasmic reticulum (21, 22). Although it is not clearly established what these isotropic spectra represent, they diminish below 37°C, such that a greater percentage of this lipid contributes to the bilayer component of the spectra: the same behavior is observed in model systems where it is thought that the isotropic motional averaging may represent inverted micellar or (short) cylindrical (H_{II}) arrangments of lipids inside the bilayer (30). ^{31}P NMR evidence is now available which indicates that the temperature dependent phase change observed in model systems and endoplasmic reticulum preparations also occurs in the endoplasmic reticulum of intact rat liver (41). As this isotropic motion does not arise from microsomal tumbling (21,22) it is tempting to ascribe it to non-bilayer lipid structures. However, the possibility that lateral diffusion in the bilayer produces the observed averaging cannot be excluded. Similar observations of isotropic motion have been obtained for sarcoplasmic reticulum membrane (39) as well as inner mitochondrial membrane (40). The evidence suggests that non-bilayer phases may exist in biological membranes and that these phases may play some functional part in membrane processes. Likely functional roles for these phases may be membrane fusion phenomena (including related processes such as exo- and endocytosis) and transbilayer transport processes (including lipid 'flip-flop' and facilitated transport).

It is not unlikely that phospholipids are involved in

membrane transport of ions such as Ca^{++} (8) or, indeed, Na^{+} and K^{+} during neuronal excitation (17). This contention requires a transport process whereby ions may be transported in a polar enviroment through the hydrocarbon matrix of the membrane. This could occur by non-bilayer phase formation such as inverted micelles or (short) hexagonal arrangements. This would not be expected to be a localized phenomenon in a nerve membrane where propagation of the action potential requires virtually the entire membrane surface, however, the effect may be very transient and occur in a facilitated fashion such as destabilization of non-bilayer ('cone-shaped') lipids by the temporary increased binding of Ca^{++} to acidic phospholipids during excitation. This effect has been observed in model systems and has a strong possibility of occurring in biological systems. If, indeed, this were the case the mechanism of anaesthetic action (based on the results obtained here) would be one of disruption of lipid polymorphism, either by bilayer stabilization or destabilization. The ability of anaesthetic molecules to strongly interact with lipids and the demonstration that they have profound effects on polymorphic phase behavior would suggest that they could easily disrupt a functional aspect of lipids in the membrane.

An alternative model for anaesthetic action could regard the sodium channel as having a protein construction rather than being facilitated totally by lipids. This hypothesis seems more realistic considering the specificity of blocking agents such as tetraodotoxin and saxitoxin for a

a finite number of sodium conduction sites on axon membranes, plus the selectivity of the channel for sodium ions. Such a protein channel may obtain all, or a portion, of its stability from surrounding lipids. As viewed by Israelachvili (33) proteins may have varying shapes in the bilayer, requiring 'cone' or 'inverted-cone' shaped lipids to provide optimal packing and sealing at the protein-lipid interface. Evidence for such an interaction is given by the recent observation (41) that reconstituted glycophorindioleoyl phosphatidyl-choline membranes require the addition of small quantities of cone shaped lipid in order to render the membrane impermeable to shift reagents. Anaesthetic molecules partitioning into a cell membrane will disrupt all protein functions when present in high enough concentrations. The 'sensitivity' to partitioning anaesthetic agents in the region of the sodium channel may be such that an 'inverted-cone' shaped alcohol, for example, would disrupt the packing around the protein sufficiently to prevent its function.

The ability of anaesthetics to alter the phase properties of specific lipids has been clearly demonstrated in this study; disruption of the viable phase properties in a biological system by anaesthetics would be expected to markedly affect the membrane's function. With further understanding of the roles of non-bilayer lipids in biological membranes more exact testing of anaesthetic mechanisms can be made. This possibility is near, considering the very rapidly accumulating information in this field. Already the findings

of studies discussed here have provided a new dimension to membrane biology and, additionally, anaesthetic mechanism.

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