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INFLUENCE OF LOCAL AND NEUTRAL ANAESTHETICS ON THE POLYMORPHIC PHASE PREFERENCES OF EGG YOLK PHOSPHATIDYLETHANOLAMINE

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(1) The polymorphic phase preferences of egg phosphatidylethanolamine have been examined in the presence of normal alcohols and alkanes of varying chain length, as well as charged amine anaesthetics. (2) It is shown that the charged anaesthetics, ethanol and butanol can stabilize a bilayer arrangement for egg phosphatidylethanolamine. In contrast, longer chain ($C \geqslant 6$) normal alcohols and alkanes induce the hexagonal (H_{II}) phase. (3) The relative potency of local anaesthetics in vitro (chlorpromazine, dibucaine, tetracaine and procaine) is mirrored by their relative ability to stabilize bilayer structure for hydrated egg phosphatidylethanolamine. Further, the aqueous concentrations of anaesthetic required to affect phospholipid polymorphism is sensitive to the lipid composition. For example, the inclusion of 20 mol% egg phosphatidylserine in egg phosphatidylethanolamine dispersions can reduce the aqueous concentrations of dibucaine required to induce appreciable bilayer stabilization effects from 5.0 mM to 0.5 mM. (4) It is suggested that the ability of amphipathic molecules such as anaesthetics to influence phosphatidylethanolamine polymorphism arises from their molecular shape. The possibility that anaesthetic molecules may exert their effects by virtue of this shape property is raised.

Introduction

The molecular mechanisms whereby anaesthetics inhibit Na^+-channel function in excitable membranes are not well understood. However, the diverse structures of molecules which can induce anaesthesia and the strong correlation between solubility in a hydrocarbon environment and anaesthetic potency [1] suggests a non-specific interaction at the membrane level. In particular, such observations suggest that the Na^+ channel is a lipid-protein complex and the action of anaesthetics corresponds to a non-specific disruption of the lipid participating in that complex.

It is logical that these observations have led to investigations of the effects of anaesthetics on those physical properties of lipids thought to be of importance in vivo. Particular attention has been paid to the influence of anaesthetics on membrane fluidity [2,3], and it has been shown that anaesthetics increase the fluidity of model membranes. Such observations are difficult to relate to in vivo mechanisms however as anaesthetic concentrations required to induce significant fluidization are usually an order of magnitude higher than those required to inhibit passage of the action potential in vitro [3,4].

In previous work [5] we have shown that the polymorphic phases (i.e. bilayer, hexagonal H_{II}) assumed by hydrated lipid dispersions are sensitive to representative anaesthetics, and that strong effects can be observed at or near pharmacologically relevant aqueous concentrations of these agents. In the present work we extend these observations to include the homologous series of the normal alcohols and alkanes of various chain lengths as well as the charged amine anaesthetics. The model system chosen is egg phosphatidylethanolamine, which exhibits a bilayer to hexagonal (H_{II}) transition as the temperature is increased through 30°C [7]. By monitoring this tran-

sition temperature (T_{BH}) in the presence of anaesthestic, effects corresponding to bilayer stabilization (increase of T_{BH}) or destabilization (decrease of T_{BH}) can be obtained. We show that the shorter chain alcohols ($C < 6$) stabilize the bilayer, whereas the longer chain ($C \geqslant 6$) alcohols and alkanes induce H_{II} phase structure. Alternatively, the amine anaesthetics stabilize the bilayer at lower concentrations and can progressively solubilize the phospholipid at higher concentrations. These results are discussed in terms of the molecular 'shape' of anaesthetic agents.

Materials and Methods

Egg phosphatidylethanolamine was purified according to standard procedures [6] employing a Waters Prep 500 LC apparatus. In all cases the lipid was more than 99% pure as indicated by thin-layer chromatography. A final check on purity was made by observing the bilayer to hexagonal (H_{II}) transition temperature (T_{BH}) (see Results) which is very sensitive to phosphatidylethanolamine degradation. It was found, for example, that storage of egg phosphatidylethanolamine in chloroform at −20°C for as little as 3 days commonly resulted in an increase of T_{BH} from 30°C [7] to as high as 40°C. This was often accompanied by a noticeably more yellow coloration. The nature of the breakdown is not known, however it is likely associated with oxidation of highly unsaturated 22 : 6 fatty acid chains found in egg phosphatidylethanolamine (approx. 15% by wt.). Phosphatidylethanolamines from other sources which do not contain such highly unsaturated fatty acids are less subject to such effects. In order to avoid these problems the egg phosphatidylethanolamine was immediately stored in a dry form in 100 mg ampoules at liquid nitrogen temperatures after isolation. Phosphatidylserine and phosphatidylglycerol (sodium salt) were obtained from egg phosphatidylcholine employing the headgroup exchange capacity of phospholipase D [8] as indicated elsewhere [9,10].

The *n*-alcohols and alkanes (Sigma, St. Louis, MO) were added directly to prehydrated egg phosphatidylethanolamine (dry lipid dispersed in 0.6 ml 100 mM NaCl, 10 mM Tris-HAc (pH 7.0), 2 mM EDTA by vortex mixing) employing a Hamilton micropipette. In some cases for those alcohols which are relatively insoluble in water ($C \geqslant 6$) the alcohol was added to the lipid (in chloroform) prior to dispersal in the aqueous medium. Equivalent results were obtained.

The local anaesthetics (hydrochloride form) were prepared in appropriate concentrations in 40 ml of 100 mM NaCl, 2 mM EDTA where the pH of the medium was adjusted to 6.0 employing HCl subsequent to addition of the anaesthetic. (At higher pH values the local anaesthetics precipitate.) The dry lipid film was then hydrated employing this solution by vortex mixing. The final pH was 6.0. The lipid was then concentrated for NMR studies by centrifugation (10 min at 3 000 rev./min). Dibucaine was obtained from ICN Pharmacenticol, procaine and tetracaine from Winthrop Laboratories and chlorpromazine from Poulene Ltd.

^{31}P-NMR spectra were obtained employing a Bruker WP 200 Fourier Transform spectrometer operating at 81.0 MHz. Free induction delays were obtained from 500 transients employing an 11 μs 90° radiofrequency pulse, a 0.8 s interpulse time and gated proton decoupling.

Results

The influence of a short and a long chain alcohol (ethanol and decanol) on the polymorphic phase preferences of egg phosphatidylethanolamine are illustrated in Fig. 1. In the absence of anaesthetic (Fig. 1(a)) egg phosphatidylethanolamine exhibits a T_{BH} of approx. 30°C as indicated by the transition from a 'bilayer' [11] ^{31}P-NMR lineshape (with a low field shoulder and high field peak) to a lineshape which has reversed asymmetry and is a factor of two narrower. This lineshape is characteristic of the hexagonal (H_{II}) phase [11]. The addition of ethanol to obtain an anaesthetic to phospholipid ratio $R = 4.5$ results in an increase of T_{BH} to approx. 42°C (Fig. 1(b)). Conversely, the presence of decanol at a molar ratio of $R = 0.45$ results in a marked decrease in T_{BH} to approx. 5°C (Fig. 1(c)). Thus ethanol may be termed as a bilayer stabilizing agent, whereas the longer chain molecule decanol favours H_{II} phase formation. In order to quantitate these observations, T_{BH} was calculated by obtaining the amount of bilayer and the amount of H_{II} phase lipid (estimated by cutting and weighing the bilayer and H_{II} phase components in the ^{31}P-NMR spectrum) as the temperature was varied. This procedure was performed

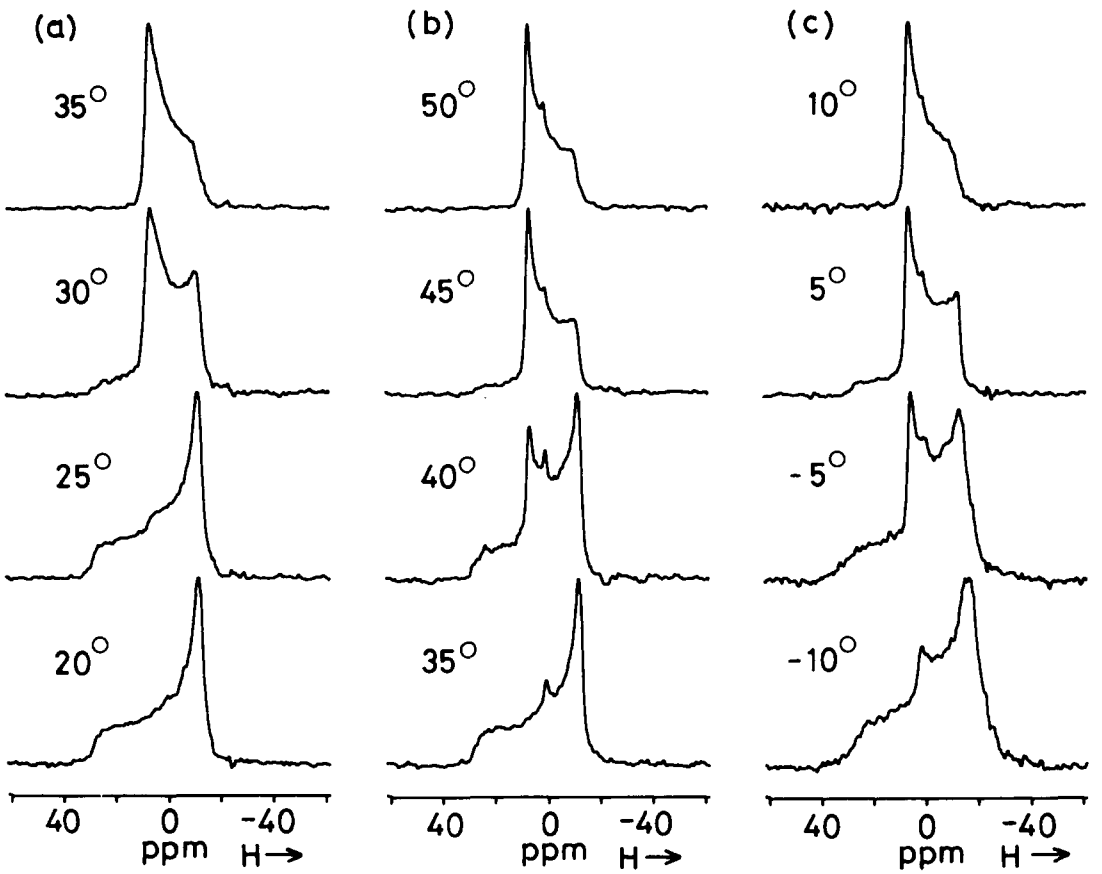

Fig. 1. 81.0 MHz ^{31}P-NMR spectra of egg phosphatidylethanolamine at indicated temperatures (a) in the absence of alcohols; (b) in the presence of ethanol (ethanol to phospholipid molar ratio = 4.5) (c) in the presence of decanol (decanol to phospholipid molar ratio = 0.45).

for data obtained from egg phosphatidylethanolamine in the presence of a variety of concentrations of normal alcohols of differing chain length, and the results are presented in Fig. 2. These results show that ethanol, and butanol can stabilize a bilayer organization, but the longer chain alcohols ($C \geqslant 6$) clearly induce a preference for the hexagonal (H_{II}) organization in egg phosphatidylethanolamine. It also appears that the longer the chain length the smaller the molar ratio of the alcohol required to produce equivalent bilayer destabilization. It should be noted that the molar ratios of anaesthetic to phospholipid given in these and subsequent figures may not reflect the actual amounts of anaesthetic present in the phospholipid matrix. For example, although the partition coefficients of the alcohols for phosphatidylethanolamine have not been determined, partition coefficients (P) determined for other membrane systems [1] would suggest membrane associated ethanol to phosphatidylethanolamine molar ratios of 0.02 for $R = 4.5$. Alternatively, decanol concentrations corresponding to $R = 0.45$ likely reflects the actual membrane bound decanol to phosphatidylethanolamine ratio due to the very high membrane partition coefficient ($P > 1\,000$ [1]) for these hydrophobic molecules.

Similar experiments were performed for the normal alkanes, which can also have anaesthetic properties [1]. Due to difficulties in handling the shorter chain (gaseous) alkanes these were not investigated, however Fig. 3 clearly shows the very strong bilayer

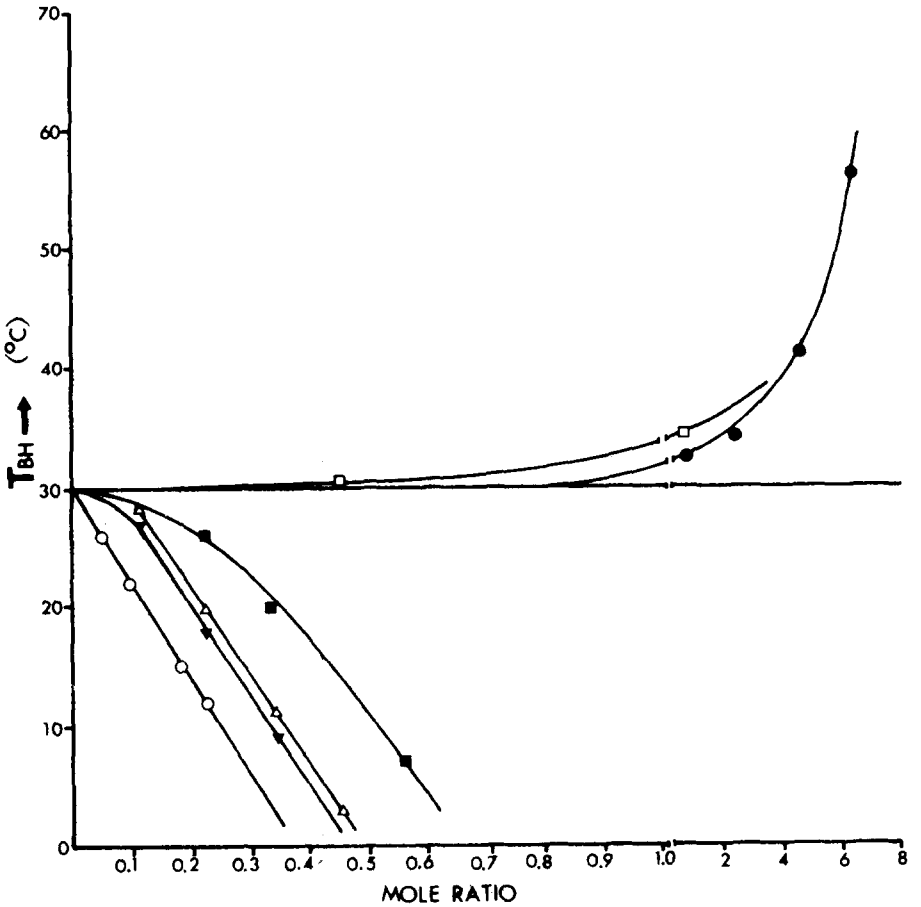

Fig. 2. Influence of varying amounts of the normal alcohols on the bilayer to hexagonal (H_{II}) phase transition temperature (T_{BH}) of egg phosphatidylethanolamine. T_{BH} is estimated as that temperature where 50% of the lipid is in the bilayer organization, and 50% is in the hexagonal (H_{II}) phase: •———•, ethanol; □———□, butanol; ■———■, hexanol; ▼———▼, octanol; △———△, decanol; ○———○, lauryl alcohol.

destabilization obtained for alkanes with $C \geqslant 6$. It may be noted that alkanes appear to be approximately twice as potent as the corresponding alcohols in this regard.

It is of interest to extend these studies to the positively charged local anaesthetics in an effort to determine first whether such compounds can be classified as bilayer stabilizing agents, whether their bilayer stabilizing or destabilizing potency can be correlated to their relative potencies in vitro [12], and whether effects are observable at pharmacologically relevant aqueous concentrations of anaesthetic. It was found that all these agents had an ability to stabilize a bilayer organization for egg phosphatidylethanolamine at various concentrations. In particular, as indicated in Fig. 4, approx. 2 mM chlorpromazine was required to stabilize relatively complete bilayer organization at 40°C for egg phosphatidylethanolamine, whereas 5 mM dibucaine, 20 mM tetracaine and 200 mM procaine was required to produce equivalent effects. It is of interest to note that the relative potency of these agents with regard to their ability to stabilize bilayer structure (chlorpromazine > dibucaine > tetracaine > procaine) corresponds to their relative potencies in vitro [12].

These latter results, while intriguing, would not appear to have any direct relation to anaesthesia in vivo for a variety of reasons. The primary one (aside

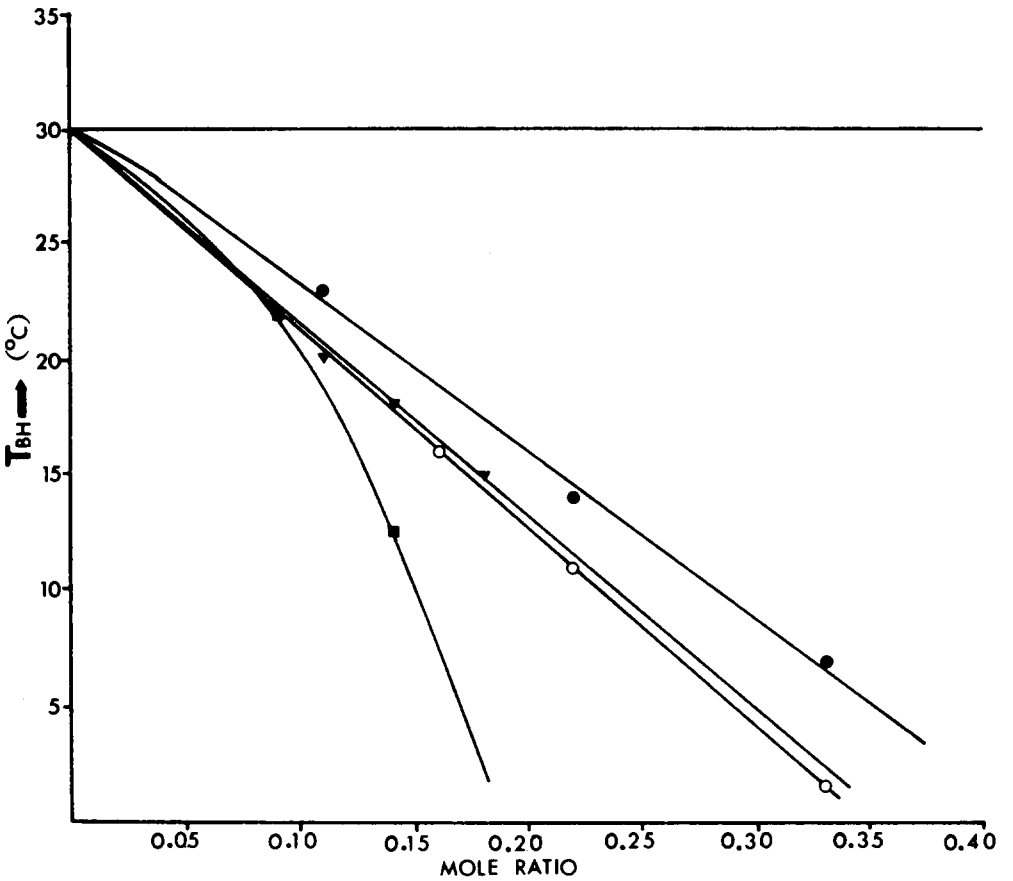

Fig. 3. Influence of increasing amounts of normal alkanes of various chain lengths on the bilayer to hexagonal (H_{II}) transition temperature T_{BH} for egg phosphatidylethanolamine: •———•, hexane; ▼———▼, octane; ○———○, decane; ■———■, dodecane.

from the fact that no biomembranes are composed entirely of a lipid which prefers a non-bilayer organization at physiological temperatures) is that the anaesthetic concentrations required to induce significant bilayer stabilization, such as reflected by an increase in T_{BH} by 10°C, are more than an order of magnitude larger than those required to induce anaesthesia in vivo. We therefore extended our studies to include mixtures of egg phosphatidylethanolamine with phosphatidylserine, as the presence of acidic (negatively charged) phospholipids can markedly increase the membrane-water partition coefficient for positively charged anaesthetics [3]. As we have shown elsewhere [13,14] the presence of phosphatidylserine stabilizes a bilayer organization for phosphatidylethanolamine, however the presence of 2 mM or more Ca^{2+} can result in a return to the H_{II} phase organization. We therefore examined the influence of dibucaine on a lipid mixture containing 20 mol% egg phosphatidylserine and 80 mol% egg phosphatidylethanolamine in the presence of 5 mM $CaCl_2$. As indicated in Fig. 5, such a system is very sensitive to the presence of dibucaine, and as little as 0.5 mM of this anaesthetic produces relatively complete bilayer stabilization at 40°C.

Discussion

The results presented here clearly establish the ability of certain anaesthetic molecules to influence the polymorphic phase preferences of egg phosphatidylethanolamine. In this discussion we do not wish

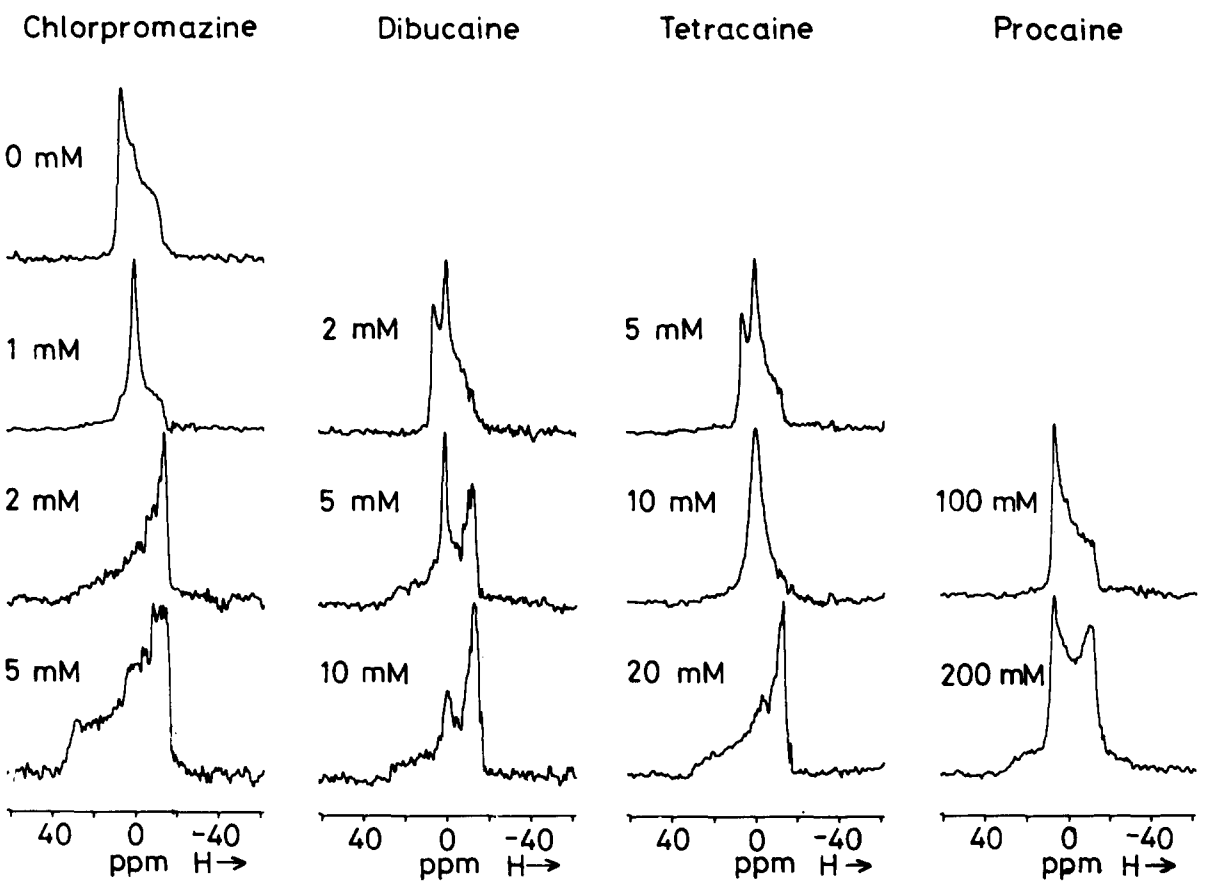

Fig. 4. 81.0 MHz ^{31}P-NMR spectra of egg phosphatidylethanolamine at 40°C in the presence of varying concentrations of (a) chlorpromazine; (b) dibucaine; (c) tetracaine and (d) procaine. For details of sample preparation see Methods.

to suggest that this ability to affect lipid polymorphism has any direct relation to mechanisms of anaesthesia. Rather, we examine how these molecules may be able to exert such effects in these model systems, which leads to some insight regarding their potential distribution when added to biomembranes.

The ability of the anaesthetics investigates have to stabilize or destabilize the bilayer organization would appear to depend on two factors. The first of these concerns the amount of anaesthetic actually associated with the lipid matrix as indicated by the membrane-buffer partition coefficient, which increases for more hydrophobic agents. As to whether this incorporation results in bilayer stabilization or destabilization may be suggested to depend on the geometry of the anaesthetic in the lipid matrix. According to this 'shape' hypothesis introduced by Israelachvili and co-workers [15,16], membrane components have characteristic shapes which determine the macromolecular structure assumed as well as the distribution of various components in mixed systems. In the case of lipids, as we have emphasized elsewhere [11], there are three types of shapes available. Lipids which adopt the hexagonal H_{II} phase on hydration are considered to have a 'cone' shape, whereas lipids adopting the bilayer are more cylindrical. Alternatively, 'detergent' type lipids prefering a micellar organization can be suggested to exhibit an 'inverted cone' shape. Evidence supporting this shape hypothesis is increasingly convincing (see Ref. 11) including the fact that mixtures of cone and inverted cone shaped lipids can exhibit bilayer structure, an organization available to neither species in isolation (see, for example, Ref. 18).

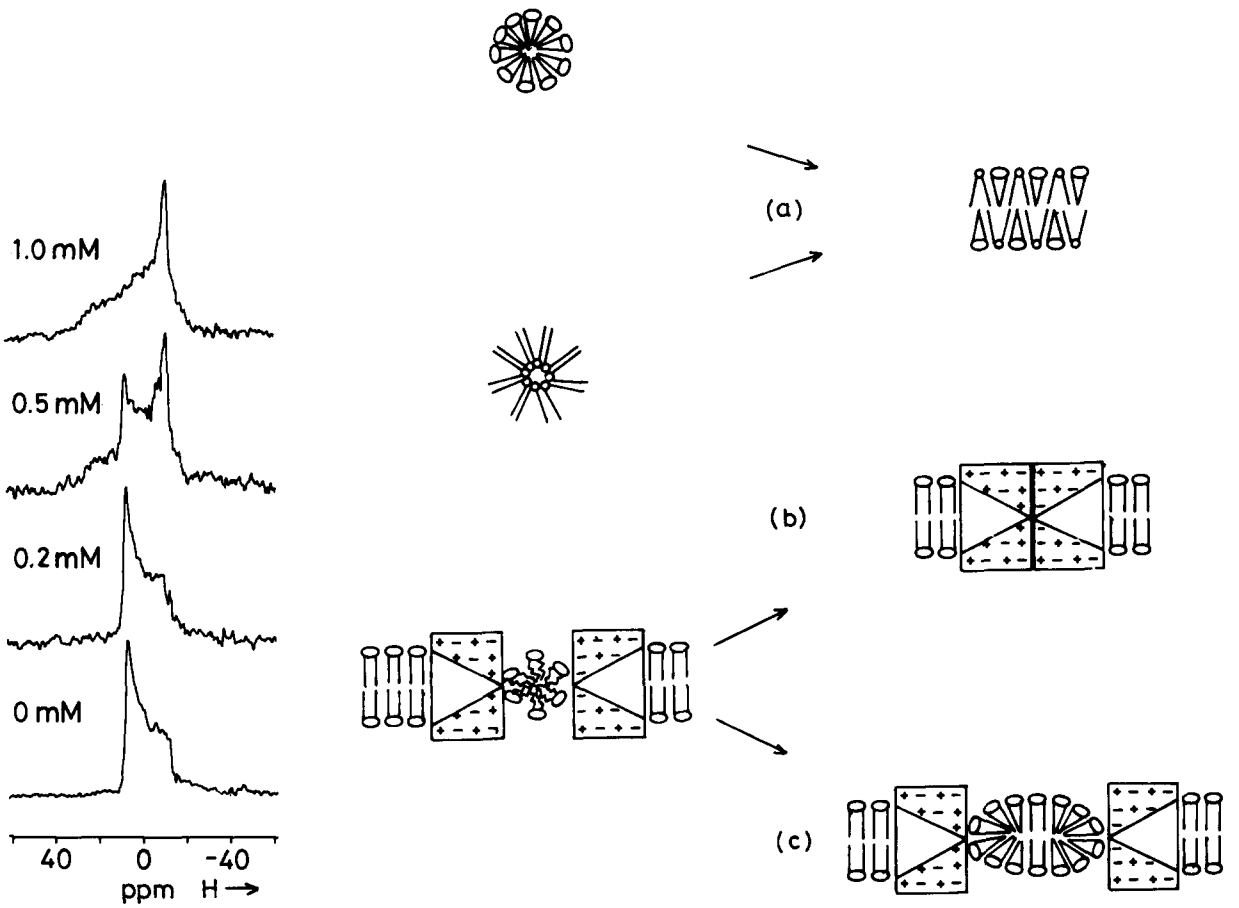

Fig. 5. Influence of dibucaine on the 81.0 MHz ^{31}P-NMR spectra at 40°C of an aqueous dispersion of 20 mol% egg phosphatidylserine and 80 mol% egg phosphatidylethanolamine in the presence of 5 mM $CaCl_2$.

Fig. 6, Lipid-lipid and lipid-protein interactions in terms of the 'shapes' of membrane components. (a) Micellar ('inverted cone' shape) and hexagonal (H_{II}) phase ('cone' shape) lipids combining to form bilayer structures due to shape complementarity. (b) Protein aggregation (dimer formation) favoured for proteins with non-polar residues in the presence of bilayer ('cylindrical' shape) lipids (see Ref. 17). (c) A potential effect of the availability of 'inverted cone' shape lipids, allowing stable monomers to be maintained.

The action of the short chain alcohols and the charged anaesthetics to stabilize bilayer structure can be rationalized in the same manner, as indicated in Fig. 6(a). It should be noted that the mechanism of bilayer stabilization for the charged anaesthetics could be 2-fold, both due to molecular shape complementarity as well as charge repulsion between bilayers resulting from the positive charge on the anaesthetic. As we have indicated elsewhere [19] there is evidence to suggest that bilayer-H_{II} transitions proceed as inter-bilayer processes, and would thus be inhibited by electrostatic factors preventing close apposition.

The influence of the longer chain ($C \geqslant 6$) alcohols and alkanes, which depress T_{BH}, can again be rationalized according to the shape concept in that the longer chain alcohols will penetrate more deeply into the hydrocarbon interior and assume more of a cone shape. It is however surprising that relatively short chain alcohols (e.g. hexanol) can exhibit such effects, which may be due to a somewhat deeper penetration into the hydrocarbon interior (to maximize hydro-

phobic interactions) than expected.

The relation of these results to mechanisms of anaesthesia are obviously exceedingly tenuous, although various speculations may be made [5]. The data indicating that the relative bilayer stabilizing ability of the charged anaesthetics corresponds to potency in vitro likely reflects a correlation between different partition coefficients, although it is satisfying that strong effects can be observed at or near pharmacologically relevant concentrations in appropriate model systems. Equally, the finding that shorter chain alcohols stabilize the bilayer, whereas longer chain ($C \geqslant 6$) destabilize such structure could be correlated with 'cut-off' effects [1] (longer chain alcohols are not anaesthetics) noted as chain length is increased. However, such cut-off behaviour in vitro may well result from the limited solubility in the aqueous phase of these increasingly hydrophobic molecules [1]. The major point we make here is that by virtue of their shape amphipathic molecules such as anaesthetics would be expected to preferentially partition (on a time-averaged basis) into local regions of membranes most compatible with that shape. Further, it is intriguing that biological membranes do not contain appreciable concentrations of 'inverted cone' shaped lipids (such as lysophospholipids) in vivo, and it is conceivable that the presence of such molecules could dramatically affect membrane protein conformation and/or distribution. An example among many possibilities is given in Fig. 6b and c. Fig. 6b is a variation on a model due to Israelachvili [17], who suggested that protein aggregation would be favoured for proteins with appropriate distributions of polar and nonpolar residues. The presence of 'inverted cone' lipids could provide an alternative low energy configuration where such aggregation does not occur (Fig. 6c).

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