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INTERACTIONS BETWEEN ANAESTHETICS AND LIPID MIXTURES AMINES

A. G. LEE

Department of Physiology and Biochemistry, University of Southampton, Southampton SO9 3TU (U.K.)

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SUMMARY

The effect of a number of amine anaesthetics related to procaine on the temperature of lipid phase transitions has been studied using chlorophyll *a* as a fluorescence probe. The amines cause a reduction in the temperature of the phase transition of dipalmitoyl phosphatidylcholine and dipalmitoyl phosphatidylethanolamine and of mixtures of these lipids. The binding of charged amines causes a build up of positive charge on the membranes, limiting the binding. Incorporation of negative charge into the lipid bilayers causes a considerable increase in the binding of the charged amines, and the effect is reversed by addition of Ca^{2+}. Anaesthesia is suggested to arise from an increase in the proportion of lipid in the liquid crystalline phase, resulting in a conformational change in the sodium channel. Effects of the tertiary amines on nerve conduction can be understood if the negatively charged lipid in the membrane is concentrated around the sodium channel: positively charged anaesthetics will have a greater effect when applied to the inside of a nerve because of the low Ca^{2+} concentration inside the nerve.

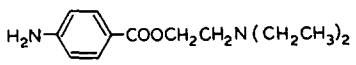
INTRODUCTION

Local anaesthetics are drugs which have the ability to block conduction in nerves. This they do by preventing the influx of Na^+ during impulse propagation [1]. Drugs such as tetrodotoxin and saxitoxin appear to block conduction by direct combination with the sodium channels in nerves [2]. Alcohols, on the other hand, are thought to interact with the lipid component of the nerve membrane, and so to effect the sodium channels indirectly through their effect on the lipid [3]. The mode of action of the amine anaesthetics such as procaine and its derivatives is much less clear.

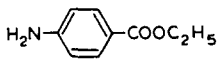
At physiological pH the secondary and tertiary amines will be present predominantly in a charged form, and Ritchie and Greengard [4] have shown that it is the cationic form of the anaesthetic which is the most active. Nevertheless, benzocaine, a neutral molecule at physiological pH, is also an effective anaesthetic [5]. It has been suggested that whereas the uncharged form may interact with the lipid

component of the membrane the charged form interacts directly with the sodium channel [2, 6].

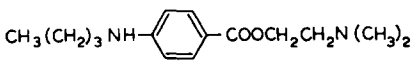
One approach to this problem is to study the interaction between the amine anaesthetics and lipid bilayers, so that effects not seen in the simple system can be more firmly attributed to specific interactions between the anaesthetic and sodium channels. In a previous paper [7] it has been suggested that the primary effect of the anaesthetic alcohols is to increase the proportion of membrane lipid in the fluid phase. Here, we report similar studies of the effects of amines on phosphatidylcholines and phosphatidylethanolamines, and on mixtures of these lipids. The structures of the anaesthetics used are listed below.



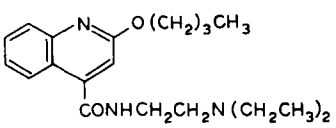
procaine



benzocaine



tetracaine



dibucaine

EXPERIMENTAL

Dipalmitoyl phosphatidylcholine was obtained from Koch-Light, dipalmitoyl phosphatidylethanolamine from Fluka, and myristic acid from Sigma. Chlorophyll *a* was prepared as reported previously [7]. Samples were prepared by dissolving lipids plus chlorophyll *a* ($1.6 \cdot 10^{-9}$ mol) in chloroform in 10-ml stoppered flasks and evaporating to dryness under a stream of nitrogen. Buffer (4 ml; 0.01 M Tris · HCl pH 7.2; NaCl 0.1 M) was added together with the anaesthetic and the mixture shaken on a Vortex mixer.

Fluorescence measurements were made on an Aminco Bowman SPF Fluorimeter. Chlorophyll *a* fluorescence was excited at 420 nm and recorded at 670 nm, with continuous stirring of the sample. Although dibucaine is itself fluorescent, there was no interference in these experiments, since its excitation maximum is at approx. 325 nm.

DL-12-(9-Anthroyl) stearic acid was obtained from Sigma. Fluorescence measurements were taken with anthroyl stearic acid incorporated into liposomes in

4 ml buffer, the amount of acid being $1.4 \cdot 10^{-9}$ mol. Fluorescence was excited at 385 nm and recorded at 440 nm.

RESULTS

Effects of anaesthetics on uncharged lipid bilayers

In earlier studies [8, 9] it has been shown that plots of fluorescence intensity for chlorophyll *a* in liposomes as a function of temperature show abrupt decreases in magnitude at temperatures corresponding to the phase transition, as a result of the formation of non-fluorescent aggregated chlorophyll *a* species. Fig. 1 shows the effect of addition of dibucaine on the mid-point temperature of the main gel to liquid-crystalline phase transition. With increasing anaesthetic concentrations, the transition moves to lower temperature, with the width of the transition remaining fairly constant. The effect on the transition is clearly non-linear. With the other charged anaesthetics, very similar non-linear plots are obtained: the data are presented in Table I. The effect of the uncharged anaesthetic benzocaine, however is linear up to 3 mM.

The effect of dibucaine on the transition temperature of dipalmitoyl phosphatidylethanolamine is shown in Fig. 2, and appears to be linear up to 2 mM dibucaine although the effect on the transition temperature is very much less marked than for dipalmitoyl phosphatidylcholine. Plots of fluorescence intensity of chlorophyll *a* as a function of temperature when incorporated into lipid mixtures, provides a series of temperatures corresponding to the onset and completion of gel phase formation. These can be plotted in the form of phase diagrams, and Fig. 3 shows the effect of dibucaine on the phase diagram for a mixture of dipalmitoyl phosphatidylcholine and dipalmitoyl phosphatidylethanolamine.

Mixtures of alcohols and amines appear to have close to additive effects on the phase transition temperature of dipalmitoyl phosphatidylcholine. Thus addition

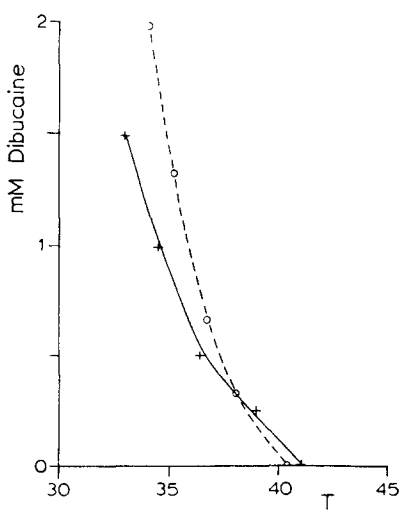


Fig. 1. The effect of dibucaine on the phase transition temperature (°C) of: – – –, dipalmitoyl phosphatidylcholine; —, dipalmitoyl phosphatidylcholine containing 11 mol % myristic acid.

TABLE I

THE EFFECT OF AMINES ON THE LIPID PHASE TRANSITION

Lipid	Anaesthetic	Concn. (mM) for a 4° drop in T_c
Dipalmitoyl phosphatidylcholine	Dibucaine	0.7
	Tetracaine	2.5
	Procaine	>70
	Benzocaine	1.5
Dipalmitoyl phosphatidylcholine +11 % myristic acid	Dibucaine	0.5
	Benzocaine	1.5
Dipalmitoyl phosphatidylethanolamine	Dibucaine	2.2
Dipalmitoyl phosphatidylethanolamine +11 % myristic acid	Dibucaine	0.6

of *n*-octanol up to 1.6 mM causes a linear decrease in transition temperature in the presence of tetracaine up to 2.5 mM: the concentration of *n*-octanol required to produce a 5 °C drop in transition temperature is approx. 1.5 mM, comparable to the 1.2 mM required in the absence of tetracaine [7].

No fluorescence quenching was observed on addition of either dibucaine or tetracaine to these lipid mixtures containing chlorophyll *a*: this contrasts with the fluorescence quenching observed for the fluorescent stearic acid derivative anthroyl stearic acid incorporated into red blood cells [10].

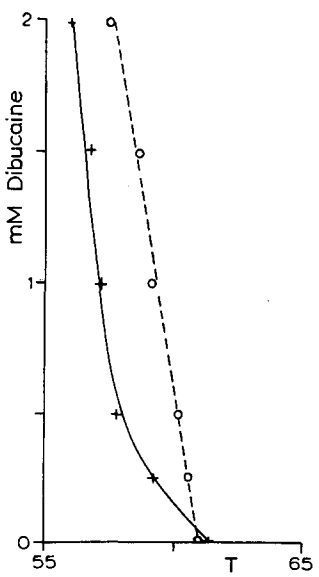


Fig. 2. The effect of dibucaine on the phase transition temperature (°C) of: – – –, dipalmitoyl phosphatidylethanolamine; —, dipalmitoyl phosphatidylethanolamine containing 11 mol % myristic acid.

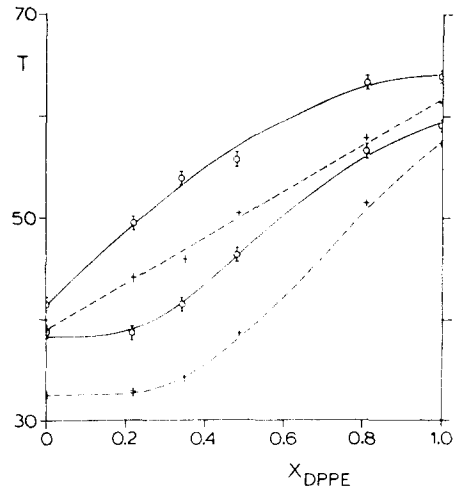


Fig. 3. Temperatures (°C) of onset and completion of solid lipid separation in mixtures of dipalmitoyl phosphatidylcholine and dipalmitoyl phosphatidylethanolamine, in the absence (—) and presence (– – –) of 1 mM dibucaine.

The phase transition in charged lipid bilayers

Most biological membranes contain negatively charged phospholipids together with phosphatidylcholine and phosphatidylethanolamine. In the studies reported here, fatty acids were added to the lipid bilayers to create a negative charge.

Addition of 11 mol % of myristic acid to dipalmitoyl phosphatidylcholine in Tris buffer at pH 7.2 increased the phase transition temperature as detected by chlorophyll *a* fluorescence to 41.3 °C. Interestingly, the transition temperature was independent of NaCl concentration from 0.001 M to 1 M, and was unaffected by the addition of 25 mM Ca^{2+}.

To investigate the organization of the fatty acids within the lipid bilayers, studies were also performed with the fluorescent anthroyl stearic acid analogue. Fig. 4 shows the changes in fluorescence intensity of anthroyl stearic acid caused by changing the anthroyl stearic acid: lipid molar ratio, whilst keeping the amount

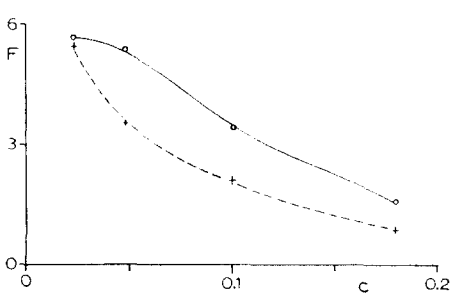


Fig. 4. Fluorescence intensity (arbitrary units) of the fluorescent anthroyl stearic acid vs. lipid/anthroyl stearic acid molar ratio $1/c$, in liposomes of dipalmitoyl phosphatidylcholine. The amount of anthroyl stearic acid was maintained constant at $1.4 \cdot 10^{-9}$ mol, and the lipid/anthroyl stearic acid molar ratio was varied by varying the amount of lipid present. —, 50 °C, – – –, 30 °C.

of anthroyl stearic acid constant. In the absence of any concentration quenching the fluorescence intensity in this experiment would remain constant: in fact, the fluorescence intensity is greater for those liposomes containing a higher proportion of lipid. A similar concentration quenching has been observed for many fluorescent dyes in solution, and attributed to deactivating collisions between excited molecules and molecules in the ground state. In such a case the fluorescence intensity should follow the Stern-Volmer relation, and a plot of the reciprocal of fluorescence intensity against anthroyl stearic acid: lipid molar ratio would be a straight line [11]. This is not so for the data of Fig. 4. The data can, however, be fitted to the following equation,

$$\frac{F_0}{F} = 1 + \left(\frac{C}{C_{\frac{1}{2}}}\right)^2$$

where F_0 is the fluorescence in the absence of quenching (at infinite dilution), F is the fluorescence actually observed at the anthroyl stearic acid: lipid molar ratio of C, and $C_{\frac{1}{2}}$ is the value of C at which $F = F_0/2$. At 50 °C the data of Fig. 4 gives $C_{\frac{1}{2}} \approx 0.1$.

Plots of fluorescence intensity vs temperature for anthroyl stearic acid incorporated into liposomes of dipalmitoyl phosphatidylcholine show breaks at approx. 40.5 °C, with lower fluorescence at lower temperatures. At 30 °C, the data can be fitted with $C_{\frac{1}{2}} \approx 0.07$.

Addition of Ca^{2+} to anthroyl stearic acid containing liposomes above the phase transition temperature causes a considerable decrease in fluorescence intensity. Thus addition of 2 mM Ca^{2+} to liposomes of dipalmitoyl phosphatidylcholine containing 10 % anthroyl stearic acid causes a 50 % reduction in fluorescence at 50 °C.

Effects of anaesthetics on charged lipid bilayers

Addition of 11 mol % of myristic acid to dipalmitoyl phosphatidylcholine and dipalmitoyl phosphatidylethanolamine causes a considerable increase in the effect of dibucaine on the phase transition temperature (Figs. 1 and 2). Data obtained with 15 mol % of myristic acid were not significantly different from those obtained with 11 mol %. The effect of the negatively charged fatty acid could be reversed by the addition of divalent metal ions. Addition of 0.5 mM $Ca^{2+}$to dipalmitoyl phosphatidylcholine containing 11 mol % myristic acid in the presence of 0.5 mM dibucaine and the absence of monovalent ions, increased the phase transition temperature

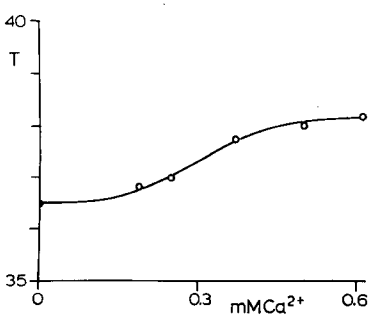


Fig. 5. Phase transition temperature (°C) in liposomes of dipalmitoyl phosphatidylcholine containing 11 mol % myristic acid in the presence of 0.5 mM dibucaine, as a function of Ca^{2+} concentration.

TABLE II

PHASE TRANSITION TEMPERATURES IN DIPALMITOYL PHOSPHATIDYLCHOLINE BILAYERS CONTAINING 11 mol % MYRISTIC ACID IN THE PRESENCE OF 0.5 mM DIBUCAINE

Metal ions present	Phase transition temperature
None	36.5
0.5 mM Ca^{2+}	38.2
0.5 mM Ca^{2+}, 25 mM Na^{+}	37.6
0.5 mM Ca^{2+}, 50 mM Na^{+}	37.3
0.5 mM Ca^{2+}, 200 mM Na^{+}	36.2

from 36.5 °C to 38.2 °C (Fig. 5). The effects of Mg^{2+} and Ca^{2+} were identical. Addition of monovalent ions reversed the effect caused by the divalent ions, and, as shown in Table II, a monovalent ion concentration of approx. 200 mM would reverse the effect of 0.5 mM Ca^{2+}.

For the neutral anaesthetic benzocaine, addition of myristic acid had no significant effect (Table I).

DISCUSSION

Whereas the interaction of alcohols with lipid bilayers is clearly dominated by hydrophobic interactions, the interaction of charged amines with lipid bilayers is likely to be more complex. Feinstein [12] emphasized ionic interactions between the charged groups of the amine anaesthetic and charged groups at the lipid bilayer surface, and assumed no penetration of the anaesthetic into the fatty acid chain region of the bilayer. However, recent studies have suggested that both ionic and hydrophobic interactions are important. Thus it has been found for a series of spin-labelled intracaine analogues that increasing hydrophobicity increases binding to phosphatidylcholine bilayers although binding to phosphatidylcholine bilayers is always less than that to bilayers of phosphatidylserine [13]. Similarly, it has been suggested on the basis of proton nmr spectra that a local anaesthetic such as dibucaine can interact hydrophobically with a phosphatidylcholine bilayer [14]. Further, it has been shown that whereas tetracaine can bind to phosphatidylcholine and displace Pr^{3+} bound to the surface, the less hydrophobic procaine cannot [15].

It has been suggested elsewhere [7] that anaesthesia results from a process of fluidus extension: addition of anaesthetic increases the proportion of lipid in a fluid state, and this in turn effects the ionic permeability properties of the membrane and effects the activities of proteins in the membrane. The increase in fluid lipid could be caused by a reduction in the proportion of lipid present as clusters [16] or by a reduction in the lipid phase transition temperature, so that the proportion of lipid in the liquid crystalline phase increases at the expense of that in the gel phase.

There appear to be only two earlier studies of the effects of the amine anaesthetics on lipid transition temperatures. Jain et al. [17] reported effects on the width of the phase transition in dipalmitoyl phosphatidylcholine and Papahadjopoulos et al. [18] described studies with dibucaine.

The experiments reported here show that the amine anaesthetics bind to

dipalmitoyl phosphatidylcholine and dipalmitoyl phosphatidylethanolamine, causing a reduction in the phase transition temperature. However, whereas for alcohols and benzocaine plots of anaesthetic concentration against transition temperature are linear as expected for ideal or close-to-ideal behaviour [7], for the tertiary amines the plots are non-linear. This clearly results from a build-up of positive charge on the liposomes due to the binding of positively-charged anaesthetic. The binding constant for the anaesthetic can be written as

$$K = K_0 \exp(F\psi_0/kT) \tag{1}$$

where K_0 is the binding constant at high ionic strength, F is the Faraday and ψ_0 is the surface potential. It has been suggested (see, for example, ref. 19) that the surface potential ψ_0 can be calculated from the Guoy-Chapman equation which, for monovalent ions is

$$\sigma = \frac{N_0}{F}\left(\frac{2\varepsilon RTc}{\pi}\right)^{\frac{1}{2}} \sinh\frac{F\Delta\psi}{2RT} \tag{2}$$

where σ is the surface charge density (charges/cm^2), c is the electrolyte concentration in mol/cm^3, ε is the dielectric constant and N_0 is Avogador's number.

Incorporation of negative charges into bilayers of phosphatidylcholine and phosphatidylethanolamine causes a very large increase in the effect of the positively charged anaesthetics, as expected from Eqn. 1. The quantitative fit of the data to the Gouy-Chapman theory is however not very good. Firstly, Trauble and Eibl [21] have shown that in charged bilayers, the transition temperature is expected to decrease with increasing surface charge, because the electrostatic free energy of a fluid bilayer is less than that of an ordered one (see also 22). However, addition of up to 15 mol % of myristic acid to dipalmitoyl phosphatidylcholine or dipalmitoyl phosphatidylethanolamine causes a slight increase in transition temperature. Secondly, varying the monovalent metal ion concentration from 1 mM to 1 M would be expected to cause a considerable reduction in the surface potential of the bilayer, and hence cause an increase in transition temperature, whereas no change in transition temperature could be detected. Thirdly, the effect of Ca^{2+} is much more marked than expected from the Gouy-Chapman theory (see below) and suggests a direct binding of Ca^{2+} to the negative charges in the bilayer, rather than a simple screening effect.

Since the apparent failure of the Gouy-Chapman theory could be due to a non-random distribution of the fatty acid molecules within the lipid bilayer, the distribution of a fluorescent analogue of stearic acid was studied by fluorescent quenching. Although the quenching was marked it did not occur until an anthroyl stearic acid: lipid ratio of approx. 0.02, a molar ratio much higher than that found for concentration quenching of, for example, chlorophyll *a* [8]. The simplest explanation of the second power dependence on concentration is as follows. Two anthroyl stearic acid molecules closer than some minimum interaction distance constitute a non-fluorescent 'dimer', and the number of such dimers will obviously increase with concentration. Fluorescence transfer could then occur from fluorescent monomers to the non-fluorescent 'dimer' where there would be rapid quenching. The overall effect would be to give something close to a second-order dependence on concentration. A detailed analysis does not seem worthwhile, since it is clear that any aggregation of anthroyl stearic acid molecules must be slight. The increase in fluorescence

quenching caused by the transition of the lipid to the gel state can be attributed to the decrease in area occupied by a lipid at the bilayer surface. The average distance d between anthroyl stearic acid molecules at some particular molar ratio c can be written as

$$d = \left(\frac{2F(1+c)}{c3^{\frac{1}{2}}}\right)^{\frac{1}{2}} \tag{3}$$

where F is the area occupied by a lipid molecule and where, for simplicity it has been assumed that the area occupied by an anthroyl stearic acid molecule and by a lipid are equal. In the liquid crystalline phase, the lipid molecular area is 58 $Å^2$ [23] so that at the anthroyl stearic acid: lipid molar ratio for half quenching ($c_{\frac{1}{2}} = 0.1$), the average distance between labels is calculated as approx. 25 Å. In the gel state, with a lipid molecular area of 48 $Å^2$ and a molar ratio of half quenching of $c_{\frac{1}{2}} = 0.07$, the average distance is approx. 28 Å. Thus the increased quenching in the gel state can be attributed largely to the decreased molecular area.

The presence of Ca^{2+}, however, does cause an aggregation of the fatty acid, as shown by the increased fluorescence quenching for anthroyl stearic acid. Similar aggregation has been established for negatively charged, spin-labelled lipids [24, 25].

The various charge-charge interactions mean that the binding of tertiary amines to lipid bilayers will be more complex than the binding of alcohols. Binding of the charged amines to phosphatidylcholine or phosphatidylethanolamine will be restricted because of the build-up of positive charge. Incorporation of negatively charged fatty acids causes a considerable increase in binding of the positively charged amines. Papahadjopoulous [18] has shown by direct measurement that the partition coefficient of 0.2 M dibucaine into phosphatidylserine is considerably greater than that into phosphatidylcholine, but this does not necessarily reflect any difference in 'intrinsic' partition coefficient, but merely the charge effects predicted by Eqn. 1.

The presence of Mg^{2+} or Ca^{2+} reduces the effect of incorporated fatty acids. Normally the incorporation of 11% myristic acid into dipalmitoyl phosphatidylcholine causes a considerable increase in the binding of dibucaine, and so a considerable further reduction in the temperature of the lipid phase transition. However, this effect of the negatively charged fatty acids is completely masked by 0.5 mM Ca^{2+} or Mg^{2+}, because the divalent metal ions bind to the fatty acids and thus reduce the negative charges on the bilayer. The effect of divalent metal ion can be reversed by high concentrations of monovalent ions: 200 mM Na^+ will reverse the effect of 0.5 mM Ca^{2+}. Divalent and monovalent cations thus have opposing effects, with increasing Ca^{2+} reducing the binding of positively charged anaesthetic, and Na^+ reversing the inhibitory effect of Ca^{2+}.

Fluidus phase lipids and charged amines as anaesthetics

A very wide range of compounds have been shown to act as local anaesthetics, blocking the sodium current in nerve. For the uncharged, lipophilic anaesthetics, the most likely cause for anaesthesia is through an increase in 'fluidity' for the lipid component of the nerve membrane, this, in turn, affecting the functioning of the sodium channel [3]. A number of experiments have shown the sensitivity of membrane-bound proteins to the state of the surrounding lipids, major changes in activity occurring at the lipid gel to liquid crystalline phase transition [23]. It has

therefore been suggested elsewhere [7] that the major effect of anaesthetics such as the alcohols is to decrease the proportion of lipid present as clusters in the liquid crystalline phase, and to increase the proportion of lipid in the liquid crystalline phase at the expense of that in the gel phase. The increase in proportion of fluid lipid then results in changes in rates of action of membrane proteins and changes in the permeability properties of the membrane. These ideas successfully account for the 'cut-off' effect often observed when comparing the anaesthetic potency of a homologous series of compounds such as the alcohols [7].

Although there is no a priori reason why the mechanisms of action of all local anaesthetics should be similar, it will now be argued that the facts regarding the anaesthetic action of the amines can be interpreted in the same terms as previously applied to the alcohols.

Benzocaine has a pK_a of 3.19, and so will be uncharged in all the experiments reported here. It acts to lower the temperature of lipid phase transitions, with a linear relationship between lowering of temperature and anaesthetic concentration. Similar relationships were found for *n*-alcohols up to *n*-octanol and are analogous to a classical 'depression of the freezing point'. The concentration of benzocaine used for nerve conduction block is typically 1 mM [26], a concentration which causes a significant decrease in the temperature of a lipid phase transition (Table I).

Binding of the charged tertiary amines to lipid bilayers also results in a reduction of the phase transition temperature, but, because of the build-up of positive charge, effects are non-linear with respect to concentration. Dibucaine has a smaller effect on the phase transition in dipalmitoyl phosphatidylethanolamine than in dipalmitolyphosphatidylcholine (Table I). A similar effect was observed for the alcohols [7] and can be attributed to a tighter packing in the ethanolamine, leading to smaller partition coefficients for the anaesthetics.

Since biological membranes contain complex mixtures of lipids, these studies were also extended to mixtures of phosphatidylcholines and phosphatidylethanolamines, the most abundant phospholipids in most membranes. The effect of dibucaine on mixtures of lipids can be plotted in the form of a 'phase diagram', as in Fig. 3, although, as discussed elsewhere [7, 23] these diagrams are not strictly analogous to phase diagrams as defined for macroscopic systems. Clearly, the primary effect of dibucaine is to shift both the upper, fluidus, curve and the lower, solidus curve to lower temperatures. The overall effect is to increase the amount of fluid lipid present. Thus, for example, in an equimolar mixture of dipalmitoyl phosphatidylethanolamine and dipalmitoylphosphatidylcholine at 45 °C, all the lipid is in the gel phase in the absence of anaesthetic, but in the presence of 1 mM dibucaine, 40 % of the lipid is in the fluid state.

Because of the positive charge carried by the primary and secondary amines, their binding to lipid bilayers is much affected by negatively charged lipids. Incorporation of 11 % fatty acid into bilayers of dipalmitoylphosphatidylcholine or dipalmitoylphosphatidylethanolamine considerably increases the effect of dibucaine (Figs. 1 and 2) due to an increased partitioning of the anaesthetic into the bilayer. However, the concentration of dibucaine required for nerve conduction block is only ca. 0.02 mM [26], considerably lower than the concentration required to produce a significant change in the transition temperature for bilayers of phosphatidylcholine or phosphatidylethanolamine. This suggests that the approx. 15 % of

negatively charged lipid in the nerve membrane is not randomly distributed. If the negatively charged lipid were concentrated into distinct patches, then the local negative charge would be considerably higher and the partitioning of dibucaine into these patches would be higher than into the rest of the membrane. Papahadjopoulos et al. [18] have shown that dibucaine has a large effect on the transition temperature of pure phosphatidylserine at 0.1 mM.

Interestingly, it has been shown that the charged form of the anaesthetics is most active when applied to the inside surface of the nerve [2]. This can be explained since, although it seems that negatively charged lipid is present on both sides of the membrane close to the sodium channel [27], the concentration of Ca^{2+} inside the nerve has been estimated [28] to be only a few μM. Thus charged anaesthetics will partition most strongly into patches of uncomplexed, negatively charged lipids on the inside surface.

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