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INTERACTIONS BETWEEN PHOSPHOLIPIDS AND BARBITURATES

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SUMMARY

The effects of a number of barbiturates on the temperature of the lipid phase transition have been studied using chlorophyll *a* as a fluorescence probe. The barbiturates cause a reduction in the temperature of the phase transitions of dipalmitoyl phosphatidylcholine and dipalmitoyl phosphatidylethanolamine, the effects being greatest at lower pH values where more of the barbiturate is present in the uncharged form. There was no significant interaction between the barbiturates and dipalmitoyl phosphatidylserine.

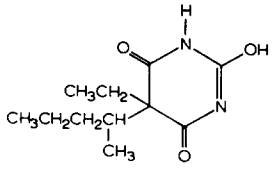
These and other observations on the actions of local anaesthetics are used to develop a model for local anaesthesia. It is suggested that the sodium channel is surrounded by an annulus of lipid in the gel state, this rigid microenvironment preventing the sodium channel relaxing from its active configuration to an inactive one. Local anaesthetics, which reduce the temperature of lipid phase transitions, trigger a change of the annular lipid from the gel to the liquid-crystalline state, with a consequent relaxation of the sodium channel to an inactive configuration, in which the sodium current is reduced or blocked.

INTRODUCTION

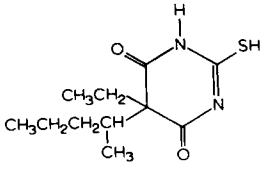
The mode of action of hypnotics such as the barbiturates is still uncertain. However, the fact that the hypnotic activities of +- and −-isomers of several series of *N*-substituted barbiturates are either equal or differ by only a factor of two or three, together with the fact that no specific antagonist of any hypnotic is known, makes it unlikely that hypnotics act via any specific receptor site [1]. Rather, effects of the hypnotics seem to follow from a general binding of the hypnotic to the membrane. At low concentrations where they produce sleep, the barbiturates have been suggested to affect either the pre-or post-synaptic nerve membrane [2, 3]. At higher concentrations they produce local anaesthesia by blocking sodium conductance [4–8].

In previous papers we have reported on the effects of alcohols [9] and amines [10] on the temperatures of the gel to liquid-crystalline phase transitions in phosphatidylcholines and phosphatidylethanolamines. Here we report similar studies on the effects of barbiturates, and show how the data can be fitted to a unified model for local anaesthetic action.

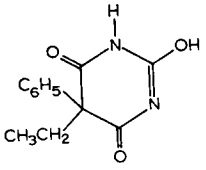
The structures of the barbiturates used are listed below:



pentobarbital



thiopental



phenobarbital

EXPERIMENTAL

Dipalmitoyl phosphatidylcholine was obtained from Koch-Light, dipalmitoyl phosphatidylethanolamine from Fluka, dipalmitoyl phosphatidylserine from Serdary and myristic acid from Sigma. Chlorophyll *a* was prepared as previously reported [9]. Samples were prepared by dissolving lipids plus chlorophyll *a* in chloroform in 10-ml stoppered flasks and evaporating to dryness under a stream of N_2. For the fluorescence measurements, buffer (0.01 M Tris · HCl) of the appropriate pH was added together with the anaesthetic and the mixture shaken on a Vortex mixer. Fluorescence measurements were made on an Aminco Bowman SPF fluorimeter, exciting fluorescence at 420 nm and recording at 670 nm. Samples were continuously stirred, and the temperature was monitored with a thermocouple inserted into the fluorescence cell.

The phase transition in dipalmitoyl phosphatidylserine was detected by an optical method, similar to that of MacDonald et al. [11]. Dipalmitoyl phosphatidylserine at a concentration of 1 mg/ml in buffer (0.01 M Tris · HCl, 0.1 M NaCl, 10 mM EDTA, pH 7.2), was sonicated in glass vials in a Megason sonicating bath. Absorbance measurements were performed on a Cary 14 spectrophotometer at 400 nm.

RESULTS

It has been shown elsewhere [12, 13] 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 lipid phase transition, as a result of the formation of non-fluorescent aggregated chlorophyll *a* species. Upper and lower transition temperatures, corresponding to the onset and completion of gel phase formation, respectively, are given experimentally by the intersections of the straight lines that can be drawn through the three distinct portions of each fluorescence vs. temperature curve. The midpoint of the transition is defined as previously [14].

Fig. 1 shows the effect of addition of pentobarbitone on the mid-point transition temperature of the main gel to liquid-crystalline phase transition of dipalmitoyl phosphatidylcholine at pH 7.3 and 8.5. The relative proportion of ionised [A^-] and unionised [A] pentobarbitone can be calculated at these pH values from the Henderson-Hasselbalch equation

$$\log \frac{[A^-]}{[A]} = pK_a - pH$$

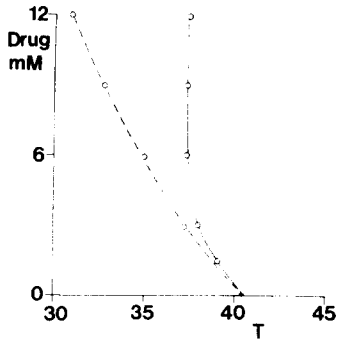


Fig. 1. The effect of pentobarbitone on the phase transition temperature (°C) of dipalmitoyl phosphatidylcholine. Solid line at pH 8.5, broken line at pH 7.3.

The pK_a values of pentobarbitone and phenobarbitone have been given as 8.0 and 7.3, respectively [8]. At pH 8.5, when 76 % of the pentobarbitone is ionised, the effect on the transition temperature is very non-linear, due to the build-up of negative charge on the liposomes as a result of the binding of negatively charged barbitone: very similar non-linear effects were observed due to the build-up of positive charge with the tertiary amine anaesthetics [10]. At pH 7.3, when only 17 % of the pentobarbitone is ionised, effects on the transition temperature are more marked, and more nearly linear. Studies at lower pH were not possible because of rapid precipitation of the barbiturate.

Incorporation of 11 mol % of myristic acid into liposomes of dipalmitoyl phosphatidylcholine increased the phase transition temperature in the absence of pentobarbitone to 41.3 °C, but had little effect on the interaction with barbiturate. In the presence of 1.5 mM pentobarbitone, the phase transition temperature was approx. 1 °C higher in the presence of myristic acid than in its absence, but at 6 mM pentobarbitone, the phase transition temperatures were equal in the two systems. The presence of 2 mM or 10 mM Ca^{2+} had no effect on the phase transition temperature in the presence of pentobarbitone at pH 8.5.

Fig. 2 shows that pentobarbitone has a much smaller effect on the phase transition temperature of dipalmitoyl phosphatidylethanolamine at pH 8.5 than it did with phosphatidylcholine. However, addition of 10 mM Ca^{2+} significantly increases the effect to a level comparable to that with phosphatidylcholine at the same pH.

The effects observed with the other barbiturates were similar, and the data is summarised in Table I. Interestingly, at pH 8.5 the effect of phenobarbitone was much less marked than that of the other barbiturates. Because of its low pK_a (7.3), approx. 94 % of the phenobarbitone will be in the ionised form at this pH, but this cannot be the only reason for its small effect, since at pH 6.6 where only 17 % is ionised its effect is still very much less than for pentobarbitone at the same ionisation level (Table I).

Attempts to measure phase transition temperatures in liposomes of dipalmitoyl phosphatidylserine using fluorescence techniques were unsuccessful: chlorophyll *a* appeared to be degraded in these highly negatively charged liposomes, and studies using *N*-phenyl naphthylamine as fluorescence probe gave very unclear results.

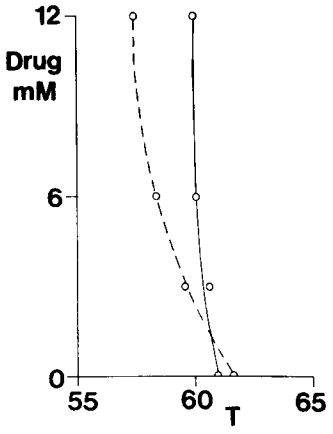


Fig. 2. The effect of pentobarbitone on the phase transition temperature (°C) of dipalmitoyl phosphatidylethanolamine at pH 8.5, in the absence of Ca^{2+} (solid line), and in the presence of 10 mM Ca^{2+} (broken line).

TABLE I

THE EFFECT OF BARBITURATES ON THE LIPID PHASE TRANSITION

Lipid	Anaesthetic	pH	Concentration (mM) for a 3°C drop in T_c
Dipalmitoyl phosphatidylcholine	pentobarbitone	8.5	4.5
		7.3	2.5
Dipalmitoyl phosphatidylcholine+10 mM Ca^{2+}	pentobarbitone	8.5	4.5
Dipalmitoyl phosphatidylcholine+11 % myristic acid	pentobarbitone	8.5	4.5
Dipalmitoyl phosphatidylethanolamine	pentobarbitone	8.5	>12
Dipalmitoyl phosphatidylethanolamine +10 mM Ca^{2+}	pentobarbitone	8.5	5
Dipalmitoyl phosphatidylcholine	thiopentone	8.5	3
Dipalmitoyl phosphatidylcholine +11 % myristic acid	thiopentone	8.5	4
Dipalmitoyl phosphatidylcholine+10 mM Ca^{2+}	thiopentone	8.5	4
Dipalmitoyl phosphatidylcholine	phenobarbitone	8.5	35
		6.6	8
Dipalmitoyl phosphatidylcholine+11 % myristic acid	phenobarbitone	8.5	35

However, from measurements of light scattering a phase transition temperature of approx. 57 °C was measured at pH 7.3 and 8.5, as previously reported by MacDonald et al. [11]. Addition of 12 mM pentobarbitone at either pH 7.3 or pH 8.5 had no significant effect, presumably because of the high negative charge on the liposomes.

In the presence of 2 mM Ca^{2+}, no transition could be observed, presumably because of the formation of "cochleate" cylinders as observed by Papahadjopoulos et al. [15].

DISCUSSION

The results given here clearly show that the barbiturates bind to phosphatidylcholines and phosphatidylethanolamines, causing a reduction in the phase transition temperatures. For uncharged molecules such as the alcohols [9] and benzocaine [10], plots of anaesthetic concentration against transition temperature are linear as expected for ideal or close-to-ideal behaviour. For the barbiturates at pH values where they are largely in the ionized form, however, effects are non-linear, clearly as a result of the build-up of negative charge on the liposomes. Similar effects for positively charged tertiary amines have been described earlier [10]. As the pH is lowered, the proportion of uncharged barbiturate increases, and the effect of the barbiturate on the temperature of the lipid phase transition becomes more marked and more nearly linear.

The charge-charge interactions within lipid bilayers tend to be rather complex [10], perhaps because of discrete charge effects [16]. Incorporation of 11 mol % myristic acid into the bilayers had little effect on the interaction with phenobarbitone. There appears, however, to be no interaction between phenobarbitone and phosphatidylserine, presumably because of the high negative charge on the bilayers. It was not possible to study any possible effect of Ca^{2+} on the binding of barbiturates, because in the presence of Ca^{2+}, liposomes of phosphatidylserine transform into new structures with strong bilayer-bilayer interactions [15] of uncertain relevance to studies of biological membranes containing mixtures of phosphatidylserine with zwitterionic lipids.

The presence of Ca^{2+} up to 10 mM had no effect on the binding of barbiturates to bilayers of dipalmitoyl phosphatidylcholine. There was, however, a very marked increase in the interaction between dipalmitoyl phosphatidylethanolamine and pentobarbitone at pH 8.5 in the presence of 10 mM Ca^{2+}. These effects can be understood if the phosphatidylethanolamine were partly negatively charged at pH 8.5: measurements of the electrophoretic mobility of liposomes of phosphatidylethanolamine as a function of pH do in fact show that ionisation of the ethanolamine group is significant at this pH [17]. Interaction with Ca^{2+} would then reduce the negative charge on the liposomes of phosphatidylethanolamine at pH 8.5 and allow further interaction with pentobarbitone.

The annular transition model for local anaesthesia

It has now been amply demonstrated that local anaesthetics block conduction of the nervous impulse by blocking the sodium currents in nerve, any effects on potassium current being secondary [18]. Four theories of these effects seem to be current. Firstly, local anaesthetics could block conduction by altering the surface charge of the membrane. Secondly, there could be specific receptors for local anaesthetics in the nerve membrane. Thirdly, it has been suggested that anaesthetics interact with the lipid component of the membrane to cause an expansion of the membrane [19] and, fourthly they could interact with the sodium channel itself and cause a change in conformation [20]. Although there is no a-priori reason why all local anaesthetics should act by a single mechanism, any model that could account for all the observations would, at the very least, be more parsimonious than a multiple-site theory.

Clearly, neither of the first two theories can account by a single mechanism for the anaesthetic action of neutral, positively charged and negatively charged molecules.

Again, interaction of anaesthetics with membrane proteins would be expected to show charge effects, which must count against the fourth theory .Further, it is often observed that in a homologous series of compounds such as the alcohols, as the chain length increases, a point is reached where the compounds become non-anaesthetic [19]. It is difficult to account for this cut-off effect in terms of anaesthetic-membrane protein interactions. As for the third theory, it is not clear how an expansion of the membrane leads to a loss of sodium current, and, in fact, it has recently been observed that an increase in pressure can cause an increase in the effect of a local anaesthetic [20].

The only obvious similarity between compounds with local anaesthetic action is their hydrophobicity, and it is this observation that underlies the membrane expansion theory of local anaesthesia. Here we present a modified form of this theory. The essential point of the model is that the sodium channel is postulated to be surrounded by an annulus of lipid in the gel, or crystalline state. If the sodium channel consists of an oxygen-lined slit through which the Na^+ pass, as pictured by Hille [21], then the rigid environment provided by the gel-state lipid prevents the sodium channel relaxing to a more stable state in which the slit has closed up, preventing the passage of Na^+. The effect of local anaesthetics is then to trigger a transition of the lipid from the gel state to the liquid-crystalline state, leading to a relaxation of the sodium channel, and a reduction in the sodium current.

In this paper we show that barbiturates will interact with phosphatidylcholines and phosphatidylethanolamines, also causing a decrease in the temperature of the lipid phase transition. Because of the build-up of negative charge, the barbiturates produce a larger effect at lower pH values where more of the barbiturate is present in uncharged form. Although Blaustein [5] suggested that the barbiturates are more effective local anaesthetics at high pH, in more recent studies Narahashi and coworkers [7, 8] found that the barbiturates were more effective at low pH, which would agree with the ideas developed here. Further, they found that pentobarbitone was more effective than phenobarbitone, and that concentrations in the mM range were required to produce block of the action potential in squid axon [8], concentrations comparable to those reported here to produce significant decreases in lipid transition temperatures. The barbiturates have also been reported to show a cut-off effect, the hypnotic activity increasing with lipid solubility until the total number of carbon atoms of both groups substituted in the 5-position reaches eight. Further lengthening of the chains gives compounds that are either convulsants or are inactive [1].

The time course for the effect of barbiturates on nerve action potentials was found to be faster when the barbiturate was applied to the inside of the axon [8]. This could reflect an easier access of the barbiturate to the axolemma from the inside of the axon, or it could mean that the barbiturates are more active when bound to the inside half of the lipid bilayer membrane. If the latter were true it could be a reflection of the asymmetry of the membrane, with more of the negatively charged lipid on the outside face of membrane than on the inside as has been suggested, by, for example, Hille et al. [22]: it has been shown here that the barbiturates do not interact appreciably with phosphatidylserine.

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