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The effect of benzyl alcohol and cholesterol on the acyl chain order and alkane solubility of bimolecular phosphatidylcholine membranes

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An investigation was made of the effects of cholesterol and benzyl alcohol on the partitioning of *n*-alkanes between lipid bilayer membranes and bulk lipid/alkane solutions (in the torus). Bilayers were formed from solutions containing alkanes of different chain lengths, together with phosphatidylcholine and cholesterol in varying proportions. The partitioning of the alkanes was determined from measurements of the very low frequency (1 Hz) capacitance of the membranes. Perturbation of the internal membrane structure by the inclusion of cholesterol and benzyl alcohol produced very significant changes in the *n*-alkane partition coefficient, cholesterol causing a decrease and benzyl alcohol an increase in the alkane partitioning into the bilayer. A correlation exists between the effects of these compounds on the alkane partitioning and their effect on the segmental chain order of the acyl chains in the bilayer and this correlation is consistent with a statistical-mechanical model of the lipid/alkane bilayers in the liquid crystalline state. The perturbation by cholesterol and benzyl alcohol of the internal structure of membranes bears on the conflicting reports of the effects of these substances on artificial lipid bilayers and could also be relevant to their known physiological effects.

Introduction

The effects of the local anaesthetic benzyl alcohol on artificial lipid bilayers has been the subject of a number of studies aimed at elucidating the molecular basis of the anaesthetic action of this and other similar local anaesthetics (see, for example, Refs. 1–5). Benzyl alcohol has been shown to fluidize such membranes [2] and to modulate the thickness of planar lipid bilayers generated from lipid-in-alkane solutions [3–6]. Considerable controversy has arissen in the literature over the interpretation of the latter data (for example, see, Refs. 3 and 5). Ashcroft et al. [3] reported that benzyl alcohol at 7.5 mM concentration produced an increase of about 25% in the thickness of the hydrocarbon region of phosphatidylcholine bilayer membranes in 1 mM KCl, generated from solutions of the lipid in *n*-tetradecane. Such effects appeared much reduced and sometimes even reversed at higher KCl concentrations [12]. Similar effects were also reported by Ebihara et al. [6] and Reyes and Latorre [5]. The effect of the anaesthetic on bilayer membranes made by monolayer apposition, which are sometimes considered to be free of alkane solvent, appear much reduced [5]. On this basis it has been suggested (for example, Ref. 6) that the effect of

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benzyl alcohol on bilayers generated from solutions of the lipids in alkane solvents may be simply due to an increase in the amount of solvent retained in the membrane.

Cholesterol is also known to modulate membrane thickness and the molecular organisation of lipid bilayers (for example, Refs. 1, 10, 17, 20) although, again, some apparently conflicting results have been reported.

NMR studies have shown [1] that high concentrations of benzyl alcohol decrease the lipid acyl chain order parameters, whilst inclusion of cholesterol increases the chain order. Molecular mean-field calculations [11] predict a relation between the segmental order parameters of the acyl chains and the solubility of alkanes in the lipid bilayer. Our recent measurements [9] of the dependence of the membrane capacitance of egg phosphatidylcholine bilayers on temperature and chain length of the alkane solvent confirmed, at least qualitatively, this relationship between acyl chain order and alkane solubility. There may thus be a simple physical basis for the changes in the equilibrium concentrations of alkane in lipid bilayers generated from lipid-in-alkane solutions induced by benzyl alcohol and cholesterol.

In this study we intend to show that the temperature-dependent 'solubility' of alkanes in lipid bilayer membranes is a useful method for probing the intrinsic order/disorder in the acyl chains of the lipids in the bilayer. Here we present results of an investigation, using this approach, of the effects of benzyl alcohol and cholesterol on the lipid bilayer structure.

Methods

The methods used have been described by us previously [8–10].

Briefly, bilayer membranes were generated from solutions of egg phosphatidylcholine and cholesterol dissolved in a variety of pure *n*-alkanes using the film 'drainage' technique. The bilayers were generated at temperatures between 20 and 50°C under 1 mM KCl aqueous solutions. Benzyl alcohol was incorporated into the bilayer via adsorption from the external aqueous phase.

The mole fraction of *n*-alkane within the hydrophobic region of the bilayers was determined from four-terminal measurements of the total capacitance at a frequency of 1 Hz. We have previously established that at this low frequency the total membrane capacitance is essentially equal to the capacitance of the region which contains the acetyl and acyl portions of the lipids [8,10]. At such low frequencies we have previously established that the contribution from the capacitance of the polar head region is extremely small and the effects of cholesterol on the dipole potential [28] are not seen. In any case we are here concerned with changes in bilayer thickness due to the adsorption of alkane molecules into the bilayer and the way this adsorption is perturbed by incorporation of benzyl alcohol and cholesterol. The estimation of the amount of alkane present in the bilayers from these capacitance measurements requires that the area density of the lipids in the bilayer is unaffected by the absorption of alkane molecules; that is, alkane absorption contributes to the volume of the hydrophobic region without contributing to an increase in the area of the hydrophobic/hydrophylic interface (expressed per lipid molecule in the interface). We also have to assume that the molecular volumes of the alkane molecules themselves are essentially temperature-independent over the temperature range of interest and that the dielectric constant of the alkanes (2.02 to 2.06) is very close to that of the acyl chain region of the lipid bilayer (this is is indeed the case to within about 2%). The mole fraction of the alkane in the bilayer is then given by:

$$X_a = \frac{C_a}{C_a + C_l} \tag{1}$$

where C_a and C_l are, respectively, the molar concentrations per unit area of membrane of the alkane and lipid acyl chains. C_a is related to the membrane capacitance, C, by:

$$C_a = \frac{\rho \epsilon_0 \epsilon_H}{M}\left[\frac{1}{C} - \frac{1}{C'}\right] \tag{2}$$

where C' is the capacitance of the alkane-free bilayer, ϵ_H is the dielectric constant of the bilayer interior, ρ is the density of the *n*-alkane solvent and M is the molecular weight of the *n*-alkane.

Results

The effects of benzyl alcohol

The capacitance and hence thickness of the hydrophobic region of phosphatidylcholine bilayers generated from alkane solutions of the lipid was temperature-dependent. However, at sufficiently low temperatures (e.g., 30°C for phosphatidylcholine bilayers generated from hexadecane solutions) the capacitance was essentially temperature independent. The form of the temperature dependence was similar for both *n*-tetradecane and *n*-hexadecane, except that for the longer-chain-length alkanes the curve was shifted to higher temperatures. This result is similar to the results we previously reported [9].

Fig. 1 shows the variation of the capacitance of the hydrophobic region of egg phosphatidylcholine membranes generated from phosphatidylcholine in *n*-tetradecane solutions as a function of temperature. The corresponding capacitance in the presence of 10 mM and 30 mM concentrations of benzyl alcohol in the aqueous phase are also shown. Fig. 2 shows the results obtained with *n*-hexadecane with 30 mM benzyl alcohol (and without the benzyl alcohol).

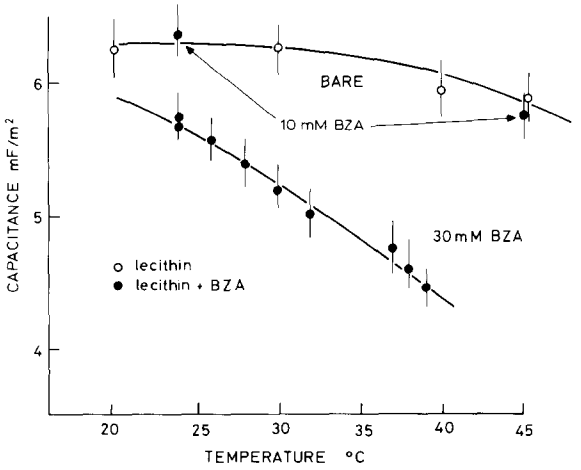

Fig. 1. The capacitance of egg phosphatidylcholine bilayers in 1 mM KCl, in equilibrium with *n*-hexadecane solutions of the lipid, as a function of temperature without benzyl alcohol (open circles) and with 30 mM benzyl alcohol (filled circles) in the aqueous phase. The measurements here refer to membranes which had been 'black' over the entire area of the aperture, apart from that region occupied by the torus, for an extended time (about 1 h) and for which the capacitance was stable to within 1–2%. The error bars indicate the errors arising from the uncertainty (2%) in estimating the area of 'black' membrane.

The addition of the local anaesthetic caused a dramatic decrease in the capacitance of the bilayers formed from both the *n*-tetradecane and *n*-hexadecane solutions of the lipid, although the dependence of the membrane capacitance on temperature remained. The effect of benzyl alcohol on the alkane mole fraction deduced via Eqns. 1 and 2 is shown in Fig. 3.

The effect of cholesterol

The incorporation of oxidized cholesterol into membranes increased the membrane capacitance over most of the temperature range investigated, although the effect of cholesterol at low temperatures ((25°C) appeared to be smaller than at elevated temperatures (40–50°C). The variation of the hydrophobic region capacitance with temperature for phosphatidylcholine and phosphatidylcholine + cholesterol membranes in 1 mM KCl in equilibrium with *n*-tetradecane are shown in Fig. 4. The low-temperature limiting value of the capacitance of the membranes was fairly reproducible, but the values obtained at higher temper-

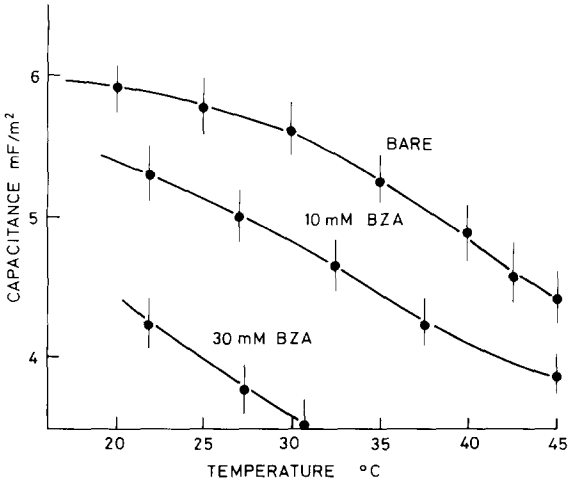

Fig. 2. The capacitance of egg phosphatidylcholine bilayers in equilibrium with *n*-tetradecane solutions of the lipid as a function of temperature for three concentrations of benzyl alcohol in the aqueous phase. The measuring conditions and error bars are as for the results presented in Fig. 1.

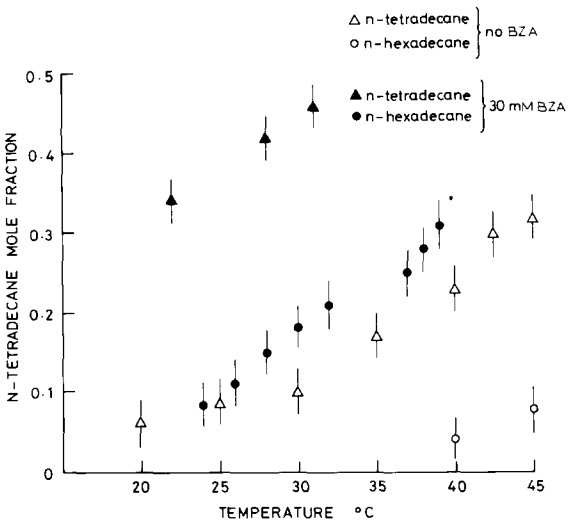

Fig. 3. The effect of benzyl alcohol on the mole-fraction of alkane in egg phosphatidylcholine bilayers. The points (○) and (△) refer to the mole fractions of *n*-tetradecane and *n*-hexadecane in the absence of benzyl alcohol. The points (●) and (▲) are the corresponding mole-fractions in the presence of 30 mM benzyl alcohol in the aqueous phase. The data presented here were calculated from the data shown in Figs. 1 and 2 using Eqns. 1 and 2 with $C' = 6.25$ mF/m^2, $\varepsilon = 2.1$ and $C_1 = 1.06 \cdot 10^{-5}$.

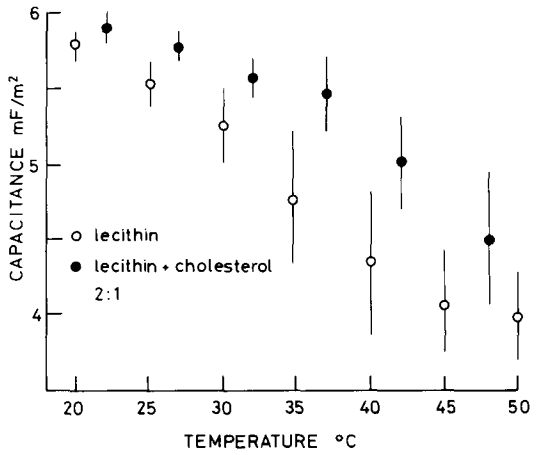

Fig. 4. The capacitance of lipid bilayers in equilibrium with *n*-tetradecane solutions of egg phosphatidylcholine (open circles) and egg phosphatidylcholine plus cholesterol (2:1 mole ratio) as a function of temperature. The aqueous solution was 1 mM KCl. The error bars refer to the total variation in the measured capacitance of ten egg phosphatidylcholine bilayers and five egg phosphatidylcholine + cholesterol bilayers. The scatter in the results increased with decreasing membrane capacitance (i.e., with increasing alkane adsorption into the membrane).

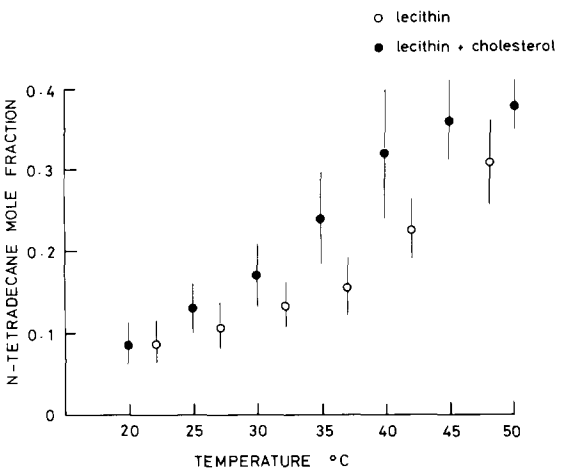

Fig. 5. The mole fraction of *n*-tetradecane in pure egg phosphatidylcholine bilayers (○) and in egg phosphatidylcholine-cholesterol bilayers (●). The data shown here were calculated from that shown in Fig. 4 using Eqns. 1 and 2 with $C' = 6.25$ mF/m^2, $\varepsilon = 2.1$ and $C_1 = 1.06 \cdot 10^{-5}$.

atures showed variation from membrane to membrane. The effect of cholesterol on the alkane mole fraction in the membrane calculated via Eqns. 1 and 2 is shown in Fig. 5.

The effect of cholesterol was dependent on the mole fraction of the steroid in the solution from which the membranes were generated. This is illustrated by the results presented in Fig. 6, which

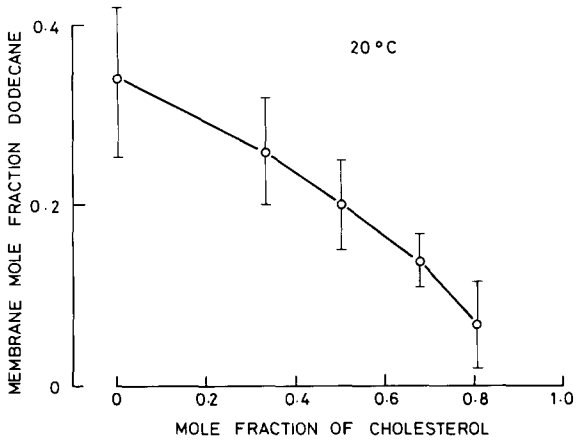

Fig. 6. The mole fraction of *n*-dodecane in egg phosphatidylcholine bilayers at 20 °C, calculated from capacitance measurements – see text, in equilibrium with solutions of the lipid in the alkane containing also cholesterol at different mole fractions (relative to the phosphatidylcholine). The error bars indicate the total variation in the measurements for 3 to 6 bilayers.

shows the mole fraction of dodecane in the membrane (calculated from the membrane capacitance with and without the alkane as described before) as a function of the mole fraction of cholesterol in the *n*-dodecane solution of the lipids. It should be noted here that the mole fraction of the steroid in the membrane is likely to be considerably less than that in the solution which comprises the membrane 'torus' from which the bilayers were generated [22,23].

Discussion

The very low frequency specific capacitance of lipid bilayers can be related to the *n*-alkane mole fraction in the bilayer (Eqns. 1 and 2). In the analysis presented here, the capacitance of a solventless bilayer, C' is taken to be approximated by the asymptotic value of the capacitance of bilayers generated from *n*-hexadecane solutions of the lipid as the temperature was decreased (that is, as the mole fraction of the *n*-hexadecane decreased). The validity of eqns. 1 and 2 requires that the dielectric constant, ϵ_H, of the acyl chain region of the bilayer be independent of temperature and membrane composition. Alkane adsorption is unlikely to affect the dielectric constant significantly, since the dielectric constant of bulk alkane and that of the lipid acyl chains in the bilayer are similar (c.f. Refs. 24 and 26). Similarly, any changes in ϵ_H for the acyl chain region produced by the inclusion of cholesterol [27] will also not be of significance in interpreting the results presented here.

The decrease in capacitance of the hydrophobic region of phosphatidylcholine bilayers generated from alkane solutions of the lipid upon the addition of benzyl alcohol implies either an increase in the thickness of the hydrophobic region or a decrease in the dielectric constant of this region. Since benzyl alcohol has a dielectric constant of 13, the decrease in capacitance would suggest that very little of the alcohol partitions into the bilayer interior (which normally has a dielectric constant around 2.1 [4,10,24]). Several other studies indicate that benzyl alcohol is adsorbed at the hydrophilic/hydrophobic interface [4,12,18] of the bilayer-electrolyte system. The increased thickness itself therefore can also not be attributed to an absorption of benzyl alcohol molecules into the hydrophobic region. The increase in the thickness of the hydrophobic region in the presence of the local anaesthetic must therefore be due to an increase in absorption of alkane into the bilayer interior.

The acyl chains in the lipid bilayer above the phase transition are partially ordered. The solubility of alkanes in the lipid bilayer involves both entropic and energy contributions. The entropic term depends on both the intrinsic order in the acyl chains and the chain length of the alkane adsorbed; this favours the adsorption of shorter chain-length alkanes which can be accommodated near the central portions of the bilayer where the acyl chain order is relatively low [11,13]. According to Gruen [11], on the basis of thermodynamic considerations, an increase in order would have the effect of increasing the free energy of solution of alkanes in the bilayer interior which would lead to an increased exclusion of the alkanes from the hydrophobic region of the membrane. In our previous study [9] we deduced that the temperature dependence of the alkane partitioning reported there, and that given in the present paper and also by others [14,15] is probably due to a decrease in the acyl chain order parameter with increasing temperature rather than due to thermal activation of the partitioning per se; a conclusion consistent with the theory of Gruen. This suggestion is also supported by NMR studies [17] of the temperature-dependence of the order parameters of the acyl chains of perdeuterated stearic acid molecules intercalated into phosphatidylcholine multilayer preparations.

Benzyl alcohol is an amphiphilic molecule which is likely to be adsorbed at the hydrophobic/polar interface of the lipid bilayer [4]. This would have the consequence of slightly reducing the lipid (area) concentration without a proportional contribution to the bilayer hydrophobic volume, since the benzyl alcohol molecule is a small molecule. Consequently, the acyl chains of the phospholipids would become more disordered as they fold around to fill the space created by the insertion of the benzyl alcohol into the bilayer/water interface. Such an increase in the disorder of the acyl chains in the presence of benzyl alcohol has also been detected using NMR methods [1]. This increase in the

disorder of the acyl chains would lead to a much increased partitioning of alkane molecules into the bilayer interior in the same way that an increase in the disorder resulting from an increase in temperature leads to an increase in the partitioning of alkane into the bilayer. This is borne out by the results presented in Fig. 4, which show the effect of benzyl alcohol on the mole fraction of various alkanes in egg phosphatidylcholine bilayers. It is immediately clear also that the increased absorption of alkane due to benzyl alcohol can be compensated by an appropriate decrease in the temperature.

In contrast with benzyl alcohol, the rigid ring structure of the cholesterol molecule decreases the number of accessible conformations of the acyl chains of lipids in its vicinity, thus increasing the order in acyl chains. This notion is confirmed by measurements of the order parameters for the acyl chains of lipid bilayers containing cholesterol [16]; the effect being more pronounced near the polar head-group and decreasing down the acyl chain. This is also borne out by our measurements (Figs. 5 and 6), which show that cholesterol reduced the mole fraction of alkanes in the bilayer interior. The effects of cholesterol were thus opposite to those of benzyl alcohol. Similar effects induced by cholesterol have also been reported previously for lipid bilayers [25] as well as for multilayer preparations [19] and vesicles [20]. The increase in alkane solubility due to benzyl alcohol could in fact be partially offset by the addition of cholesterol [12]. Indeed this may explain some of the desparate results reported in the literature.

How molecules such as benzyl alcohol act as local anaesthetics remains obscure. However, the reduction of the order parameters induced by this local anaesthetic could play an important role in determining the equilibrium between the lipids in the boundary region surrounding, for instance, the sodium channel in a nerve membrane and the bulk of the membrane. In a multicomponent system, such as that in a cell membrane, this could have very significant effects on the composition, fluidity and surface charge (and potential) in the vicinity of the excitable channels. It is of interest to note here that in neurones with a low cholesterol content such as the neurones of *Aplysia californica*, cholesterol, which increased the acyl chain order in lipid bilayers, has an anaesthetic action which is reversible on exchange of the cholesterol with phosphatidylcholine [21]. Further, it is well known that a decrease in temperature reduces the potency of many anaesthetics and it is tempting here to relate this to changes induced in the acyl chain order of the lipids.

Conclusions

The correlation between the effects of membrane-perturbing agents such as cholesterol, benzyl alcohol and changes in temperature on the acyl chain order and their effects on alkane solubility together with their 'additive' effects in egg phosphatidylcholine bilayers gives good support to the notion that the alkane solubility in lipid bilayers is a good indicator of the intrinsic acyl chain order of the lipids. It would also seem that determination of alkane mole fractions in lipid bilayers using low-frequency capacitance measurements can provide a useful and convenient technique for monitoring the relative acyl chain order in single planar bilayer membranes.

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