THE LOCALISATION OF SMALL MOLECULES IN LIPID BILAYERS

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1. Introduction

The use of small molecules as empirical spectroscopic probes to detect changes in membrane structure has been largely superseded by direct observation of labelled membrane components. For example, phospholipids carrying reporter groups or nuclei for spin label, fluorescence and NMR studies [1–3] have been used on the assumption that they will reflect the behaviour of unmodified lipids in bilayers and membranes. At the same time, these probes are able to provide more direct and detailed information about the perturbation of lipids in bilayers and membranes by small molecules such as anaesthetics and ionophores.

In this paper we illustrate the use of proton NMR measurements on the $-\overset{+}{N}Me_3$ resonance from the polar headgroup of unmodified lecithins to provide information about the localisation of aralkyl alcohols and fluorescence probes in lecithin bilayers, and the relationship between the structure of the alcohol and the perturbation which it induces in the bilayer. To analyse the distribution of the molecules quantitatively between the polar headgroup region and the hydrocarbon chain region of the bilayer, binding data for the alcohol/lecithin systems is also described. A full account of these studies will be presented elsewhere; here we emphasise that we are exploiting only a small part of the information which can be obtained using lipids systematically labelled with ^{19}F or ^{13}C nuclei [4].

2. Materials and methods

Hen egg lecithin was prepared by the method of Dawson [5] and dipalmitoyl lecithin (DPL) was obtained from Koch Light. The equilibrium dialysis measurements of alcohol partition coefficients in lecithin vesicles, and the preparation of sonicated aqueous suspensions of lecithin vesicles are described elsewhere [6, 7].

Proton NMR spectra were obtained on a Varian HA 100 spectrometer locked on tetramethyl silane as an external reference. Shift measurements of the lecithin $\overset{+}{N}Me_3$ resonance were made with respect to an internal reference signal of 30 mM internal acetate, which was shown to be negligibly affected by variation in the alcohol concentration. Line width measurements of the $-\overset{+}{N}Me_3$ resonance ($\Delta\nu_{1/2}$) were made by sweeping through the resonance at 0.4 Hz/sec at a radiofrequency power which caused no significant saturation broadening, and were corrected for instrumental broadening estimated from the line width of the internal acetate.

3. Results and discussion

3.1. *Binding of benzyl alcohol to lecithins*

The binding of benzyl alcohol to dipalmitoyl lecithin (DPL) and egg lecithin as a function of tempe rature and concentration are shown in fig. 1 a, b. The most striking effect is the very sharp increase in alcohol binding to DPL between 35° and 37° at which the partition coefficient increases by 150%, but remains constant either side of this transition.

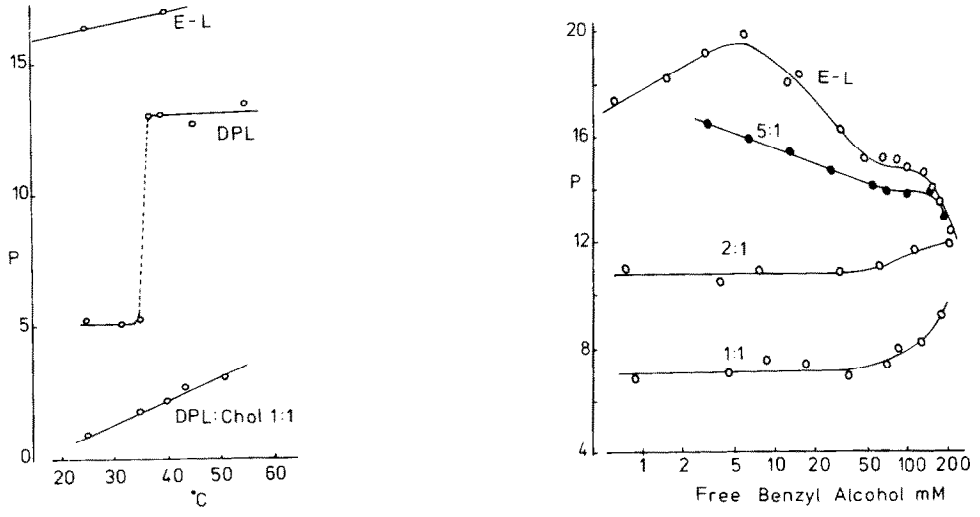

Fig. 1. (a) The effect of temperature on the partition of benzyl alcohol into dipalmitoyl lecithin (DPL) and 1:1 DPL:cholesterol at 40 mM total benzyl alcohol. The partition into egg-lecithin (E-L) is also shown.
(b) The partition of benzyl alcohol into egg lecithin/cholesterol mixtures at 25°. Cholesterol concentrations are given as molar ratios of lecithin:cholesterol.

There is a similar increase in the binding of the spin label TEMPO (2,2,6,6-tetramethyl piperidine-1-oxyl) to DPL [8] through the thermal transition which occurs at approx. 41°. Binding studies of TEMPO to DPL indicate that this transition temperature is decreased from 41° in the absence of benzyl alcohol to approx. 33° at 80 mM alcohol [9]. It is concluded from the NMR studies described later that this can be most simply explained if the alcohol disrupts the packing of the chains into the crystalline form below the transition, and a lower temperature is required before chain interactions are strong enough to expel the alcohol from the chain region to allow crystallisation. Polyvalent cations bound to the lipid phosphate group have the opposite effect of increasing the transition temperature. The increase in binding of small molecules above the thermal transition can be attributed to the greatly increased fluidity of the chains [1], allowing partition of small molecules into the hydrophobic interior of the bilayer.

Cholesterol has the opposite effect of increasing chain packing and displacing benzyl alcohol from lecithin vesicles. There is a marked depression in alcohol binding by DPL in the presence of equimolar cholesterol, and there is a gradual increase in binding between 25° and 45° with no indication of a sharp increase in binding at the DPL transition temperature (fig. 1a). Apparently cholesterol prevents the crystallisation of the chains which normally occurs at the transition, since the cholesterol remains within the bilayer and is not expelled as a separate phase.

The partition of benzyl alcohol into egg lecithin shows no transition and is slightly higher than in DPL above the transition (fig. 1a). At 25° the concentration dependence of the alcohol binding is complex (fig. 1b); below 5 mM alcohol there is an increase in partition with increasing alcohol concentration implying the appearance of new binding sites or an increase in affinity caused by the presence of the alcohol. At approx. 80 mM alcohol, there is an inflection in the binding curve, at which there is a structural reorganisation of the bilayer leading to an increase in the number of binding sites. Cholesterol causes a marked depression in alcohol binding over the whole alcohol concentration range, but there is an increase in partition at concentrations above 80 mM benzyl alcohol (fig. 1b). There is some indication that cholesterol separates into a

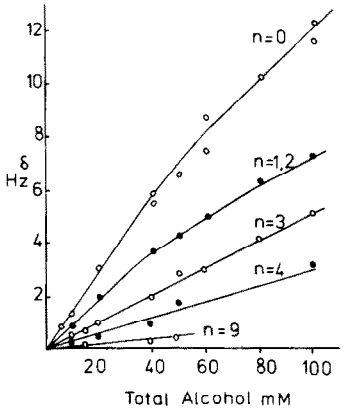

Fig. 2. The observed shift (δ) of the egg lecithin (65 mM) $-\overset{+}{N}Me_3$ resonance at 25°as a function of the total concentation of ω-phenyl alcohols ($C_6H_5{\cdot}(CH_2)_n{\cdot}OH$).

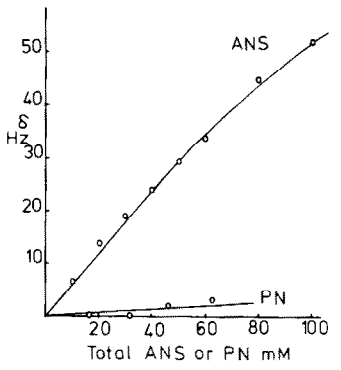

Fig. 3. The observed shift (δ) of the egg lecithin (65 mM) $-\overset{+}{N}Me_3$ resonance at 25° as a function of the total concentration of ANS or phenylnaphthylamine (PN).

separate phase at higher alcohol concentrations, and the partition coefficients tend towards a similar value at the highest alcohol concentrations, corresponding to that of lecithin in the absence of cholesterol.

Quantitative analysis of the binding by a simple mass action treatment of independent binding sites is obviously inadequate to account for the complex concentration dependence of the binding. Approximate treatment of the data for the series of aralkyl alcohols $C_6H_5(CH_2)_nOH$ indicates that the maximum theoretical binding capacity of the lecithin is likely to be $\leqslant 2$ alcohol molecules per lecithin and the experimental data indicate that the bilayer structure is radically disrupted at much lower levels of saturation. For example the molar ratio of bound alcohol:lecithin is 0.6:1 at 80 mM alcohol, where the lecithin vesicles begin to flocculate.

3.2. *NMR studies of alcohol interactions with lecithin*

After sonication to optical clarity, egg lecithin and DPL above the thermal transition yield high resolution proton spectra [10, 11] in which the major well-defined resonances are from the $-\overset{+}{N}Me_3$ polar headgroup and the $-(CH_2)_n-$ and $-CH_3$ groups of the fatty acid chains. When benzyl alcohol is added to sonicated egg-lecithin vesicles at 25°, an upfield shift of the $-\overset{+}{N}Me_3$ headgroup relative to the internal acetate reference was observed. The shift increased with increasing total alcohol concentration (fig. 2) and a series of control experiments established that the shift was due to binding the alcohol to the lecithin vesicles. Using the binding data already described, it was found that the upfield shift of the $-\overset{+}{N}Me_3$ resonance was proportional to the bound alcohol concentration up to 0.5 moles of alcohol bound per mole of lecithin. The shift as a function of bound alcohol concentration was independent of the lecithin concentration.

The $-\overset{+}{N}Me_3$ shift can be attributed to the effect of the aromatic ring current of the benzyl alcohol molecules in the immediate vicinity of the $-\overset{+}{N}Me_3$ headgroup. Thus other aromatic alcohols produce upfield shifts of the $-\overset{+}{N}Me_3$ resonance whereas the alkyl alcohols produce no significant shift. The observed shift decreases as the number of methylene groups (n) between the aromatic ring and the terminal hydroxyl group increases in the series $C_6H_5{\cdot}(CH_2)_n{\cdot}OH$, at the same total alcohol concentration (fig. 2). Since increasing the chain length increases the partition coefficient (n = 1–5), the decrease in $-\overset{+}{N}Me_3$ *shift per molecule of alcohol bound* for the ω-phenyl alcohols as n increases is more pronounced than the decrease in the *observed shift* in fig. 2. In contrast the 1-phenyl alcohols all produced similar shifts; for example at a concentration of 40 mM, the shifts produced by the alcohol series $C_6H_5{\cdot}CH{\cdot}OH\,(CH_2)_n{\cdot}CH_3$ were between 2.5 Hz and 3.9 Hz with no systematic changes in shift with increasing chain length (n = 1–6).

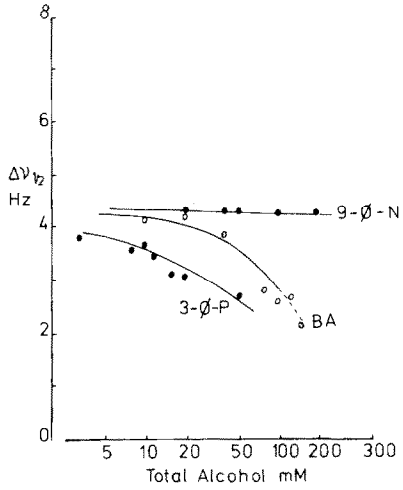

Fig. 4. The effect of benzyl alcohol (BA), 3-phenyl-1-propanol (3-Φ-P) and 9-phenyl-nonanol (9-Φ-N) on the linewidth ($\Delta\nu_{1/2}$) of the $-\overset{+}{N}Me_3$ resonance of egg lecithin (65 mM) at 25°.

The qualitative interpretation of these shift measurements is straightforward. The $-\overset{+}{N}Me_3$ shift per molecule of phenyl alcohol bound to the lecithin vesicles represents some averaged ring current effect of all the alcohol molecules associated with the lecithin. Differences in this shift for a particular alcohol under different conditions can be interpreted as changes in the proportion of the bound alcohol molecules which are in the headgroup region of the lecithin bilayer as opposed to the chain region away from the headgroups. For example the small increase in shift per molecule of benzyl alcohol bound to egg lecithin vesicles which occurs when the temperature is lowered suggests a redistribution of the bound alcohol population in favour of the headgroup region, because the chain region is less fluid. When the $-\overset{+}{N}Me_3$ shifts per molecule of alcohol bound are compared for the ω-phenyl alcohols, it is found that phenol, benzyl alcohol and 2-phenyl ethanol all produce very similar shifts, suggesting that for these three molecules the disposition of the aromatic rings relative to the $-\overset{+}{N}Me_3$ group and the proportion of molecules in the headgroup region are probably rather similar. However, the shift per molecule bound decreases by about 70% for 3-phenyl propanol and is negligible for 9-phenyl nonanol, indicating that these molecules are bound with the aromatic ring preferentially localised in the chain region, away from the $-\overset{+}{N}Me_3$ group. The 1-phenyl alcohols all appear to have the phenyl ring sufficiently close to the $-\overset{+}{N}Me_3$ group for all the analogues to produce substantial $-\overset{+}{N}Me_3$ shifts irrespective of their chain lengths. Presumably the hydroxyl group orientates the molecules with the ring in the headgroup region. The importance of a hydrophilic group as a localising factor in amphiphilic molecules is demosntrated by the very large difference in shifts produced by the fluorescence probe ANS (1-anilino-naphthalene-8-sulphonate) and the uncharged analogue 1-phenyl naphthylamine which lacks the sulphonic acid group. ANS has three aromatic rings and produces very large shifts of the $-\overset{+}{N}Me_3$ resonance (fig. 3), whereas phenyl napthylamine with the same aromatic ring system produces very small shifts although it is almost totally bound to the lecithin in the NMR experiments.

A more quantitative analysis of the distribution of benzyl alcohol within the bilayer is suggested by the $-\overset{+}{N}Me_3$ shift data for DPL. The observed upfield shift of the $-\overset{+}{N}Me_3$ at a fixed total alcohol concentration is very similar over the temperature range 25° to 50° for both DPL and egg lecithin, in spite of the abrupt increase in alcohol binding to DPL above the thermal transition. For example at 40 mM benzyl alcohol, the observed shift for DPL and egg lecithin is between 2.8 Hz and 3.2 Hz over this temperature range. This suggests that at a fixed total alcohol concentration, the number of binding sites occupied in the headgroup region is the same for DPL and egg lecithin and is independent of temperature. It also implies that the upfield shift of the $-\overset{+}{N}Me_3$ resonance per molecule of alcohol bound in the headgroup region is constant under this range of conditions. The interaction of the alcohol with the headgroups can be regarded as a weak complex in which the aromatic rings remain in a similar spatial relationship to the $-\overset{+}{N}Me_3$ groups. The ring shift produced by aromatic ring currents become negligible at distances greater than ~ 8 Å. Models indicate that the $-\overset{+}{N}Me_3$ group must lie between 6 and 10 Å from the chain region depending on the conformation of the headgroup, so it is unlikely that molecules bound in the chain region can make any contribution to the $-\overset{+}{N}Me_3$ shift. The $-\overset{+}{N}Me_3$ shift

measurements therefore distinguish a class of weak binding sites in the polar region which produce a constant shift of the $-\overset{+}{N}Me_3$ headgroup, and other binding sites distributed in some unspecified way within the chain region which produce no shift.

There is some evidence that below the thermal transition in DPL, the alcohol molecules are bound only to the headgroup region and are virtually excluded from the chain region. The value of the shift per molecule of alcohol bound below the transition is therefore the maximum value found in any lecithin/benzyl alcohol system (~ 23 Hz per molecule bound per lecithin). This shift is unaffected by the presence of cholesterol below the transition although the concentration of bound alcohol is depressed. Under these conditions cholesterol simply displaces alcohol from the headgroup region, but does not affect the shift per molecule produced by the remaining bound alcohol. Above the thermal transition in DPL, the number of bound alcohol molecules is approximately doubled, while the shift per molecule bound per lecithin falls from ~ 23 Hz to ~ 12 Hz. This is interpreted as binding of all of the additional alcohol molecules within the fluid chain region which becomes accessible above the transition, while binding to the headgroup region remains unaltered. Above the transition the shift per molecule bound per lecithin is increased by the presence of cholesterol, whereas the observed shift is decreased, suggesting that cholesterol preferentially displaces nearly 2 alcohol molecules from the chain region for each alcohol molecule displaced from the headgroup region.

The measurement of a single shift parameter therefore allows the distinction of two binding regions in the bilayer and estimation of the relative proportions of alcohol molecules in the two regions. It does not give any indication of the gradient of alcohol distribution within the chain region. This can be examined by using lecithins labelled with single ^{19}F nuclei at different positions along the chains, which indicate that the very fluid region at the centre of the bilayer is preferentially occupied by small hydrophobic molecules.

3.3. *Perturbations induced in the bilayer*

In addition to shifting the $-\overset{+}{N}Me_3$ resonance upfield, benzyl alcohol also causes the line width of the resonance to decrease (fig. 4). Similar effects are observed for the chain $-(CH_2)_n-$ and terminal methyl resonances, although these effects are too small for quantitative analysis. Although the linewidths of resonances from the bilayer structure do not represent true spin–spin relaxation times [12], they have always been found to chainge in the same direction as the corresponding spin-lattice relaxation times and can therefore be taken as a qualitative measure of changes in the fluidity of the bilayer. Other short chain aralkyl and aliphatic alcohols have a similar effect in narrowing the $-\overset{+}{N}ME_3$ resonance and causing a fluidising effect on the bilayer as a whole. For the short chain members of the ω phenyl alcohol series ($n < 5$), the narrowing of the $-\overset{+}{N}Me_3$ resonance occurred at lower total alcohol concentrations with increasing chain length. However the long chain homologues (e.g. 9-phenylnonanol) had no effect on the $-\overset{+}{N}Me_3$ line widths, and similar changes were observed with chain length for the n alkyl alcohols. Cholesterol caused some broadening of the $-\overset{+}{N}Me_3$ resonance, but the effect on the chain methylene resonance was much greater, consistent with parallel changes in the spin-lattice relaxation data for the same system [11]. Benzyl alcohol partially reverses the effect of cholesterol on the $-\overset{+}{N}Me_3$ line width.

The data are interpreted to imply that whatever the primary region of localisation in the bilayer, the effect of the alcohols in fluidising the structure, or the cholesterol in increasing the packing of the bilayer of the bilayer is sensed by the whole of the lipid molecule in its steric interactions with neighbouring lipids.

Finally, the perturbation produced depends on the structure of the small molecule and is not simply dependent on the number or molecular volume of the bound molecules. Thus the long chain alcohols have only small effects in fluidising the bilayer structure even at high molar ratios in the bilayer. The correlation between the perturbation induced by a molecule and the corresponding permeability changes strongly support the conclusion that the structural features of small molecules are of major importance in determining their effects on the lipid bilayer [9].

References

[1] W.L. Hubbell and H.M. McConnell, J. Am. Chem. Soc. 93 (1971) 314.
[2] L. Stryer, in: Molecular Properties of Drug Receptors, eds. R. Porter and M. O'Connor (J. and A. Churchill, London, 1970) p. 133.
[3] Y.K. Levine, N.J.M. Birdsall, A.G. Lee and J.C. Metcalfe, Biochemistry 11 (1972) 1416.
[4] N.J.M. Birdsall, A.G. Lee, Y.K. Levine and J.C. Metcalfe, Biochim. Biophys. Acta 241 (1971) 693.
[5] R.M.C. Dawson, Biochem. J. 70 (1958) 559.
[6] C.M. Colley, S.M. Metcalfe, B. Turner, A.S.V. Burgen and J.C. Metcalfe, Biochim. Biophys. Acta 233 (1971) 720.
[7] J.C. Metcalfe, N.J.M. Birdsall, J. Feeney, A.G. Lee, Y.K. Levine, P. Partington, Nature 233 (1971) 199.
[8] J.C. Metcalfe, N.J.M. Birdsall and A.G. Lee, FEBS Letters 21 (1972) 335.
[9] J.R.S. Hoult and J.C. Metcalfe, unpublished data.
[10] N.J.M. Birdsall, A.G. Lee, Y.K. Levine and J.C. Metcalfe, Chem. Comm. (1971) 1171.
[11] A.G. Lee, N.J.M. Birdsall, Y.K. Levine and J.C. Metcalfe, Biochim. Biophys. Acta 255 (1972) 43.
[12] A.F. Horwitz, W.J. Horsley and M.P. Klein, Proc. Natl. Acad. Sci. U.S. 69 (1972) 590.