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THE BINDING OF BENZYL ALCOHOL TO ERYTHROCYTE MEMBRANES

C. M. COLLEY, S. M. METCALFE*, B. TURNER, A. S. V. BURGEN AND J. C. METCALFE

Medical Research Council, Molecular Pharmacology Unit, Medical School, Hills Road, Cambridge and Department of Biochemistry, Tennis Court Road, Cambridge (Great Britain)*

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SUMMARY

1. The partition of benzyl alcohol into erythrocyte membranes at 25° has a slight negative dependence on concentration up to approx. 80 mM. At higher concentrations there is a sharp increase in partition.

2. Below 80 mM, the membrane has a slightly lower partition coefficient than the separated lipid, which is much lower than the membrane protein. The binding of the alcohol is reversible and is restricted by interactions between the membrane components essential for the assembly of the structure.

3. Above 80 mM, the alcohol competes with the essential interactions between the membrane components and replaces them with alcohol interactions, resulting in the increase in partition. This is a progressive irreversible change in the membrane which at the limit approaches the binding due to the separated components.

4. The maximum binding capacities of the separated components are very high, approx. 1 alcohol for every 2–3 amino acid residues, and 1 alcohol per lipid. The high binding capacity of the proteins probably results from a distinctive feature of their primary structure.

5. Comparable studies at 5° and 40° gave qualitatively similar relationships, although the absolute values of the partition coefficients increased with temperature for membranes and the separated components.

INTRODUCTION

A number of physical techniques involving the use of probe molecules have recently proved to be useful for following changes in membrane structure. For example, spin-labels have been used by HUBBELL AND MCCONNELL[1–3] to probe the structure of nerve and erythrocyte membranes and TASAKI *et al.*[4] used a fluorescence probe to follow changes in neural membranes during conduction. We have introduced the use of nuclear magnetic relaxation measurements of probe molecules bound to membranes[5,6] to provide information about molecular motion within the membrane. For erythrocyte membranes the anaesthetic benzyl alcohol is a sensitive probe for changes in steric interactions with the membrane components when the membrane structure is perturbed. To compare directly the relaxation rates of bound alcohol molecules under different conditions it is necessary to know the fraction of molecules

bound to the membrane. The practical reason for selecting benzyl alcohol and erythrocyte membranes for the first detailed binding study of a probe molecule was to provide the partition data necessary for a full analysis of the relaxation measurements to be described in a later paper. However, it became clear that the binding data itself provide useful information about the interaction of the alcohol with the membrane.

MATERIALS AND METHODS

Membrane preparations

Haemoglobin-free human erythrocyte membranes were prepared from outdated blood as described previously[5] and stored at 4° in a buffer containing 5 mM Tris–HCl, 1 mM EDTA, and 1 mM NaN_3 (pH 7.5). The membranes were used within 7 days; no significant change in binding properties was detected over this period.

For some experiments membranes were pretreated with benzyl alcohol. A volume of stock membrane suspension was dialysed for 24 h at 25° against 19 vol. of 300 mM benzyl alcohol in the Tris buffer, to give a final overall concentration of 285 mM benzyl alcohol. The membranes were then dialysed against several changes of the same buffer solution at 25° until the residual benzyl alcohol concentration was less than 1 mM, and stored at 4°.

Erythrocyte lipids and protein were separated by the method of MADDY[7] as modified by REGA *et al.*[8]. The membranes were thoroughly washed with deionised water adjusted to pH 7.5 with NaOH and shaken with 0.5 vol. of butanol at 4°. After centrifugation there was usually a small layer of undissolved material at the solvent interface which was dispersed by replacing the aqueous protein layer with an equal volume of deionised water and repeating the above procedure. Provided that the membranes were freshly prepared and the pH was maintained at 7.0–7.5, all of the membrane preparation was distributed into the two solvent layers (approx. 60 % by weight into the aqueous phase and 40 % into the butanol phase).

The aqueous protein fraction, saturated with butanol, was dialysed exhaustively against deionised water. Precipitation of the protein occurred unless the pH was close to 7.0. The protein was concentrated by ultrafiltration to 3–5 % (w/w) and dialysed against the standard buffer solution (45 mM NaCl, 30 mM sodium acetate, 5 mM NaH_2PO_4–Na_2HPO_4, 1 mM NaN_3, in 99.7 % ^2H_2O), which was used for all the partition measurements. The uncorrected "pH" was adjusted to 7.4. Good preparations of membrane proteins remained optically clear and yellowish when the dialysis against the standard ^2H_2O buffer was complete, but precipitation occurred if there was residual butanol in the preparation. The protein preparations normally contained approx. 5 % lipids (w/w) but up to 15 % lipid has been detected in poor preparations which remain cloudy after removal of butanol. If the aqueous protein fraction was re-extracted with fresh butanol, the lipid content was reduced to less than 1 % (w/w) and was indetectable by thin-layer chromatography of the protein solution.

The lipid fraction was evaporated under nitrogen at 35° and washed repeatedly with water to remove all traces of butanol. The lipids were finally resuspended in vesicle form at approx. 5 % (w/w) by agitation with the standard ^2H_2O buffer solution. The vesicles were stable under nitrogen and did not require sonication.

For all preparations of membranes and components, the final stock concentration (w/w) used for partition measurements was measured by drying at 105° to

constant weight and corrected for the dry weight of the standard buffer measured in the same way.

Partition coefficients of membranes

Membrane partition coefficients were measured by a centrifugation technique. This proved to be more convenient than the equilibrium dialysis method used for the separated membrane components, because of the long equilibration times required for the viscous membrane suspensions.

Membrane suspensions and pretreated membranes were washed repeatedly with the standard ^2H_2O buffer containing the required concentration of benzyl alcohol (analytical grade), and concentrated by centrifugation to approx. 5 % (w/w). Three parts by weight of membrane suspension were diluted with one part of standard buffer containing the same concentration of benzyl alcohol, and in addition 6 nC of [^{14}C]-benzyl alcohol per ml. The diluted suspension was left to equilibrate for 2 h at the required temperature. A control sample was prepared in the same way, but without the membranes present. Five 0.55-ml samples each of membranes and controls were centrifuged for 12 h at 100 000 $\times$ *g* at the required temperature ($\pm$ 1°C), in solid aluminium tubes containing five cylindrical sample compartments (45 mm long, 4 mm diameter). After centrifugation 200 $\pm$ 1 mg aliquots of the supernatants from each tube were weighed into counting vials and 10 ml of scintillation medium added. Samples of uncentrifuged membranes and controls were prepared for counting in the same way. The aluminium tubes absorb less than 0.5 % of the benzyl alcohol over the experimental concentration range. Three 10-min counts were made on each sample (about 30 000 counts) and the partition coefficient was calculated separately from each set of counts. The partition coefficients of membranes pretreated with benzyl alcohol were measured in the same way.

Partition coefficients of the separated membrane components

The partition coefficients of the separated lipids and proteins could not be measured conveniently by the centrifugation technique, and were determined by equilibrium dialysis in chromium plated brass cells. Two parts by weight (approx. 1.5 g) of lipid or protein solution was dialysed against one part of buffer containing [^{14}C]benzyl alcohol. For final concentrations of benzyl alcohol above 100 mM, benzyl alcohol was also added to the sample suspension. Four sample cells and four controls in which buffer replaced the sample suspension were set up in each determination and allowed to equilibrate at the required temperature for 24–48 h. The cells were rotated continuously to aid mixing. After equilibration, the controls and depleted buffer samples were withdrawn and counted as before. It should be noted that equilibration takes longer in the sample cells which contain more viscous solutions than the controls. Depletion of benzyl alcohol by absorption to the cells and dialysis membranes was negligible.

The calculation of the partition coefficient

To achieve the maximum accuracy in the determination of the partition coefficients it was preferable to make the relevant experimental measurements on a weight basis rather than by volume. The partition coefficient is then defined as the ratio of the weights of benzyl alcohol per unit weight of membrane and buffer solution,

rather than the more conventional definition based on unit volumes of each phase. This also facilitates the direct comparison of the partition coefficients for the different membrane preparations, since it is not necessary to know their densities. The partition coefficient for all preparations has been calculated from the expression:

$$P = \frac{C_c - C_s(1-m)}{C_s m}$$

where m is the fractional membrane concentration measured as the weight of membrane preparations per unit weight of suspension, and C_c and C_s are the counting rates per unit weight of control and depleted buffer solutions, respectively, in either centrifugation or equilibrium dialysis experiments.

We find that for intact membrane and the lipid preparations, the standard error in separate determinations on different samples is about $\pm$ 10 %, whereas pretreated membranes and protein partition coefficients vary with standard errors of the order of 20 %. This variation is not due to larger standard errors in C_c and C_s for a single preparation, but is presumably due to variations between different sample preparations.

The solubility of benzyl alcohol in the standard buffer was determined at 5, 25 and 40° and the values found were 349 $\pm$ 2.7, 339 $\pm$ 2.4, and 359 $\pm$ 0.6 mM, respectively. The temperature dependence is small and the limiting solubility was taken to be the mean of these values, *i.e.* 349 mM.

RESULTS

The partition coefficient (p) of benzyl alcohol for erythrocyte membranes is nearly constant over the alcohol concentration range 5–80 mM at 25°; there is actually a small decrease from $p = 3.4$ at 5 mM to $p = 3.1$ at 80 mM. Above this concentration p increases sharply and reaches 4.9 at 200 mM and 6.4 at 300 mM (Fig. 1).

A transition at 80 mM benzyl alcohol has been found previously in a study of the relaxation of the alcohol by nuclear magnetic resonance; it is also the concentration at which lysis of intact erythrocytes occurs[5]. It was proposed that the changes above the transition point were due to an irreversible partial dissociation of the membrane into its lipid and protein components. It was readily shown that the increased partition above 80 mM was also due to an irreversible change. The membranes were pretreated with benzyl alcohol at a concentration of 285 mM, and the alcohol removed by dialysis before partition measurements were made. The partition was elevated (Fig. 1) and this affected mainly the low concentration range. For instance at 5 mM, p was increased to 6.6, and at 80 mM to 5.8, but at 300 mM, p was only increased from 6.4 to 6.8. Pretreatment with concentrations of alcohol below 80 mM had no effect on the partition, but if concentrations between 80 and 285 mM were used, intermediate increases in partition occurred. For instance pretreatment at 180 mM increased the value at 10 mM to $p = 4.7$ compared with $p = 6.4$ after pretreatment at 285 mM. The irreversible changes produced by pretreatment are thus graded with concentration.

The separated lipid exhibited a dependence of partition on alcohol concentration which was quite unexpectedly similar in shape to that of the membrane, having a negative slope region and a transition to a positive slope at about 80 mM (Fig. 2).

The partition coefficient was higher than for the membrane (mean ratio 1.33). The protein partition curve was also similar with a transition at about 80 mM but p was considerably higher, and the mean ratio of protein/membrane partitions was 2.60. The protein partition is therefore approximately twice that for lipid, but both are higher than for the membrane. Since the membrane contains protein and lipid in the approximate weight ratio 6:4 we may obtain a weighted average partition for a protein–lipid mixture corresponding to a membrane fully dissociated into its components. The values obtained are in a ratio of 2.05 to the partition of the native membranes, and are up to 15 % higher than the partition coefficient of pretreated membranes up to 80 mM, but the discrepancy is greater at higher alcohol concentrations (Fig. 3).

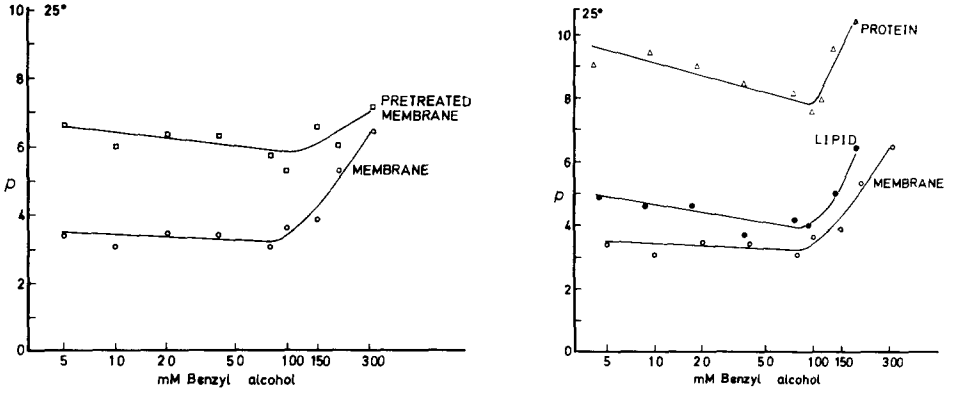

Fig. 1. The partition of benzyl alcohol at 25° into erythrocyte membranes and membranes pretreated with 285 mM benzyl alcohol. Note that in all the figures the alcohol concentration refers to free alcohol in the aqueous phase [A].

Fig. 2. The partition of benzyl alcohol at 25° into separated erythrocyte lipid and protein fractions. The membrane curve is also included for comparison.

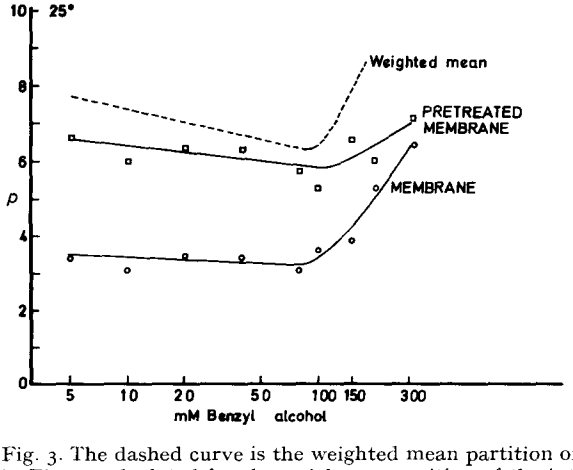

Fig. 3. The dashed curve is the weighted mean partition of the separated lipid and protein curves in Fig. 2, calculated for the weight composition of the intact membrane (4 lipid: 6 protein). The calculated curve lies above and close to the pretreated membrane curve.

The experiments so far discussed were carried out at 25°, but measurements at 5 and 40° gave basically similar relationships, although the absolute values of the partition coefficients were changed (Fig. 4). It proved difficult to obtain reproducible results with pretreated membranes at 40° and these results have not been included. It is clear that there is a larger increase in partition coefficient with temperature for the lipid component than the membrane; at 5° the mean partition ratio is 1.07, whereas the ratio is 1.33 at 25° and 1.48 at 40°. A systematic study of the effect of temperature on partition was carried out at a fixed concentration of 10 mM which confirmed that the lipid and membrane partition coefficients have approximately linear temperature dependence.

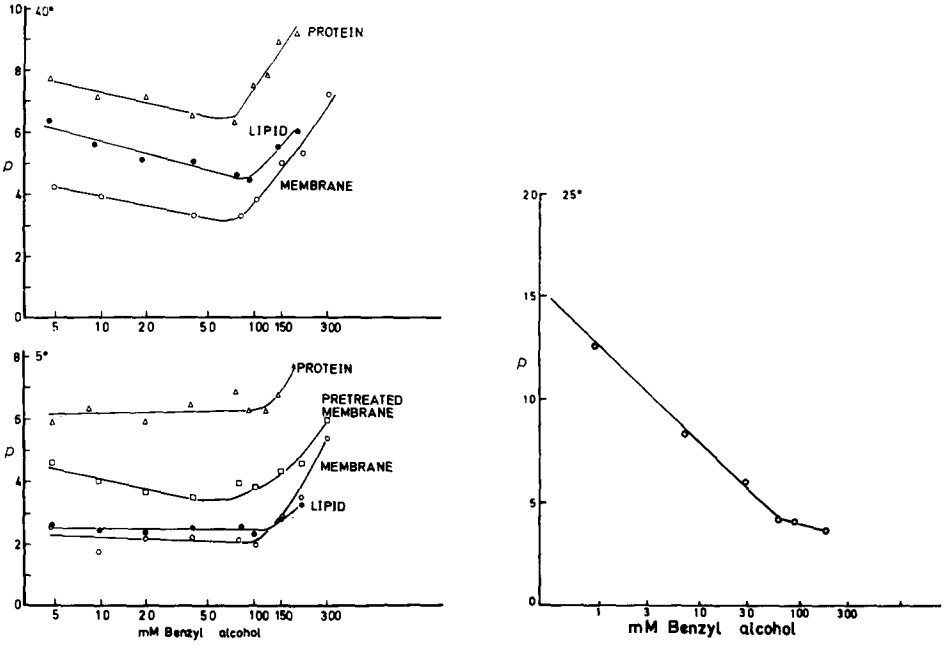

Fig. 4. The partition of membranes, lipid protein, and pretreated membranes at 5 and 40°. (The curve for pretreated membranes at 40° is omitted because these membranes gave variable results.)

Fig. 5. The partition of benzyl alcohol into bovine serum albumin (fatty acid free) at 25°.

For comparison with the results on membrane protein we have also measured the partition into bovine serum albumin (Fig. 5). There is a more pronounced decrease of partition with concentration than with membrane protein, and an inflection, but no upswing, at higher concentration. The partitions are of the same order; at 10 mM for bovine serum albumin $p = 8.3$ compared with $p = 9.3$ for membrane proteins and at 80 mM for bovine serum albumin $p = 4.5$ compared with $p = 8.1$. The binding capacity of bovine serum albumin is high; at 200 mM, 480 moles of alcohol are bound per mole of protein.

DISCUSSION

The binding of benzyl alcohol has been presented as partition coefficients without making explicit assumptions about the nature of the binding site. We now compare two idealised models of the binding in the 5–80 mM prelytic concentration range, first in terms of the binding of the alcohol A to a macromolecule M according to the mass action equation

$$A + M \rightleftharpoons AM$$

with a single affinity constant K for the alcohol. The data for the membranes and the derived preparations were treated as Scatchard plots ($\bar{v}/[A]$ *vs.* $\bar{v}$), where $\bar{v}$ is the moles of A bound per g of M and [A] is the concentration of free alcohol, and fitted linearly by least squares analysis to give K and M_{max}, the maximum binding capacity of M (Table I). Within the accuracy of the data, the figures suggest that the main difference in binding for the membranes, protein and lipid is the higher binding capacity of the membrane protein rather than differences in affinity. The weighted mean binding capacity of the mixture of lipid and protein corresponding to the membrane composition is 3.1 moles/kg which is more than 50 % higher than the value for the intact membrane. Table I also compares the amount of alcohol bound by each preparation at 80 mM free alcohol with the maximum binding capacity from the Scatchard plots. The binding at the critical concentration lies in the range 10–20 % of the saturated binding capacity, so that a radical change occurs in binding profile of each preparation at a low level of saturation.

TABLE I

THE BINDING OF BENZYL ALCOHOL AT 25°

	K^* (M^{-1})	*Maximal binding capacity** (*moles A/kg*)	*Amount bound at upswing*** (*moles A/kg*)
Membranes	1.7 ± 0.7	2.0 ± 0.8	0.26
Lipid	2.2 ± 0.3	2.1 ± 0.3	0.32
Protein	2.5 ± 0.4	3.7 ± 0.6	0.65

* From Scatchard plots.
** From p at upswing.

Translated into molecules of alcohol bound per amino-acid residue, the binding concentrations in Table I correspond to approx. 1 molecule per 14 residues at 80 mM, and a saturated binding capacity of 1 molecule per 2–3 residues for the membrane protein. Such a large binding capacity cannot be accounted for easily as binding to discrete hydrophobic areas or centres in the protein structure, but must be distributed over most of the protein structure. The comparison with bovine serum albumin is of interest; the Scatchard plot does not fit a single straight line, but can be approximated by two linear portions intersecting at about 60 mM. Below this concentration the maximum binding capacity extrapolates to approx. 1 alcohol molecule to 20 amino acid residues, but at higher concentrations the saturated capacity is approx. 1 alcohol

molecule to 3 residues. Preliminary experiments indicate that these binding similarities of membrane protein and serum albumin for benzyl alcohol are not common to a range of proteins, but probably reflect some distinctive feature of the primary structure of these proteins which results in a high hydrophobic binding capacity. However, it is significant that REYNOLDS AND TANFORD[9] find a similar binding pattern for sodium dodecyl sulphate to membrane proteins, serum albumin, and a diverse range of other proteins. Below a critical sodium dodecyl sulphate monomer concentration of 1 mM, sodium dodecyl sulphate is bound at a ratio 1 sodium dodecyl sulphate: 7 amino acid residues; at higher concentrations there is a transition to a higher binding ratio of about 1 sodium dodecyl sulphate: 2 amino acid residues. The generality of this binding pattern is striking and presumably only occurs with amphiphilic molecules of rather precisely specified character; our results suggest that the benzyl alcohol binding profile is only similar to sodium dodecyl sulphate for a limited number of proteins with appropriate structure. The ability to discriminate proteins by their general hydrophobic binding capacity may be useful in defining a distinctive structural feature of the membrane proteins.

The alcohol binding capacity of the lipids at 80 mM is equivalent to 1 alcohol: 5 lipids (assuming a mean lipid mol. wt. of 600) and the maximum binding capacity is 1 alcohol: 1.3 lipids. The data for the lipid is sufficiently precise to indicate that the increase in binding with temperature is probably due to increased affinity without a significant change in the number of binding sites (Table II).

TABLE II

EFFECT OF TEMPERATURE ON THE BINDING OF BENZYL ALCOHOL TO LIPID

Lipid binding parameters.

Temp.	*K* (M^{-1})	*Maximal binding capacity* (*moles A/mole lipid*)*
5°	1.4 ± 0.4	1.1 ± 0.3
25°	2.2 ± 0.3	1.3 ± 0.2
40°	2.7 ± 0.5	1.3 ± 0.2

* The mean lipid molecular weight is assumed to be 600.

In view of the heterogeneity of the membrane and its components, the mass action treatment must certainly be over-simplified since it implies independent binding sites of uniform affinity. The satisfactory fit of the binding data to linear Scatchard plots probably occurs because of the combination of low affinity with a low level of saturation; the plot is relatively insensitive to heterogeneity within these limits and does not enable us to estimate the heterogeneity of the binding parameters.

An alternative model for the binding is to regard it as analogous to partition between liquid phases, in which specific structural binding sites are assumed not to exist, because the molecules in the liquid are assumed not to form any persistent lattice structure. In general, partition between such phases is insensitive to the concentration of the solute, provided that its concentration expressed as a mole fraction is small[10]. At the upper limit of the range of alcohol concentration being considered (*i.e.* 80 mM) the corresponding mole fraction (in ^2H_2O) is 0.0016 so that this condition

is satisfied for the aqueous phase, but the mole fraction in the membrane and components is not readily evaluated. However, if we take as an example the alcohol partitioned into lipid, at 80 mM in the aqueous phase the mole fraction in the lipid phase will be almost 0.2. It would not be surprising therefore if there were concentration dependence of partition due to the relatively large mole fraction in the non-aqueous phases. For this model this could account for the negative dependence of partition on concentration.

The activity coefficients of benzyl alcohol calculated from the partition coefficients at 80 mM and corrected for the effects of dimerisation in the pure alcohol[11] are 2.8, 1.8, 0.9 and 1.4 for the membrane, lipid, protein, and pretreated membrane, respectively. These values are quite close to unity, expected for an ideal solution. This is perhaps surprising in view of the chemical complexity of the structures in which the alcohol is dissolved, but does agree very well with deductions about the thermodynamic state of alcohols and similar small hydrophobic ligands in membranes based on physiological studies[12–14].

The two models of alcohol binding are not really in conflict, since the distinction between them hinges on the degree of organisation of the binding sites. We prefer the approach based on saturable binding because it focuses attention on the structural features in membranes, lipids and membrane proteins which are responsible for binding the alcohol.

We have seen that when the alcohol concentration is increased beyond the critical value of 80 mM a substantial increase in partition developes which coincides with an irreversible change in the membrane. This is evidenced by the increased partition coefficient at concentrations below 80 mM, in membranes pretreated with higher concentrations of alcohol; the extent of this pretreatment effect depends on the concentration of alcohol used in the pretreatment. A simple explanation is that above the critical concentration a progressive disruption of the structural organisation of the membrane occurs which may be extrapolated to the limit of total dissociation of the membrane into separated lipids and proteins. Pretreatment with 285 mM benzyl alcohol (82 % satn. in the buffer) seems to fall short of producing such a complete dissociation as judged by the partition values, but this is hardly surprising in view of the obvious retention of some residual structure in the pretreated membrane.

It is a striking feature of the results reported that at concentrations below 80 mM the binding of the alcohol to the membrane is less than to either membrane lipid or protein. One explanation of this could be that some of the locations in the separated lipid and protein available for alcohol binding have become inaccessible in the native membrane because they are used in the assembly of the membrane. This suggests that binding occurs below 80 mM alcohol to sites not essential for membrane integrity, but at higher concentrations of the alcohol above the critical level, the alcohol is able to compete with essential interactions of the membrane components and replace them with alcohol interactions. We consider that the most probable candidate for this essential interaction is a hydrophobic interaction between lipids in direct contact with hydrophobic regions of membrane proteins, and that the disruption of these interactions, which normally seal the membrane proteins in a defined conformation into the intact membrane structure, leads to a breakdown in the structural integrity of the membrane. These interactions may be supposed to remain intact in the organised structure until binding at non-essential sites to lipid and protein so

perturbs the structure that the alcohol molecules compete with the essential lipid–protein interactions. The progressive and irreversible character of this change suggests that the assembly of the membrane involves detailed molecular correspondences between the components which cannot reform spontaneously on removal of the perturbant, or alternatively, that the dissociated components exist in altered conformations that cannot spontaneously revert to the conformations in the native membrane when the components are brought together. This is of interest for the problem of initial membrane assembly in cells and the question of the feasibility of membrane reassembly *in vitro*.

Finally it should be emphasised that the binding capacity of the lipid in the membrane is grossly inadequate to account for the alcohol binding capacity of the native membrane, whereas the binding capacity of the protein is more than adequate to account for all the membrane binding. The evidence is therefore weighted in favour of a major contribution from the membrane protein to the total binding in the intact membrane.

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