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THE INTERACTION OF SMALL MOLECULES WITH SPIN-LABELLED ERYTHROCYTE MEMBRANES

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SUMMARY

The spectra of non-covalent spin labels have been examined in the presence of erythrocyte membranes and their separated lipid and protein components. Evidence has been obtained for the perturbations induced in the membrane structure by a range of extraneous molecules. The perturbations are detected either as changes in the spectra of the bound spin label, or as changes in the partition of the spin label into the membrane. A comparison of the perturbations detected by different spin labels provides preliminary evidence for the localisation of the perturbing agents in the membrane. With steroid spin labels it is possible to detect the onset of irreversible structural breakdown in the membrane at high concentrations of perturbing agents. This results in the exposure of new protein binding sites for the steroid spin labels which are not accessible in the intact membrane.

The interpretation of the spin label experiments is supported in detail by directly comparable studies of the nuclear magnetic relaxation of the same perturbing agents in erythrocyte membranes.

INTRODUCTION

Recently we have examined the interaction of extraneous molecules with neural membranes, using the spin label 2,2,6,6-tetramethyl piperidine-1-oxyl (TEMPO) to report on the structural perturbations which are produced[1]. For example, a number of anaesthetics were found to increase the fluidity of the membrane, whereas other molecules, including cholesterol and Ca^{2+}, had the opposite effect. The changes in the packing or ordering of the membrane lipids were interpreted from changes in the partition coefficient of TEMPO in the neural membrane which were induced by the perturbing agents. A similar description of the effect of local anaesthetics on erythrocyte membranes has been obtained from nuclear magnetic relaxation measurements of anaesthetics bound to erythrocyte membranes[2]. In these experiments the anaesthetic molecule itself was used as a probe to report on the viscosity of its immediate environment in the membrane. As the anaesthetic concentration was increased the

Abbreviation: TEMPO, 2,2,6,6-tetramethyl piperidine-1-oxyl.

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environment within the membrane became more fluid until at a critical concentration irreversible structural changes were produced. Thus a similar description of membrane perturbations was obtained using independent techniques in different membrane systems.

Here we examine the interaction of anaesthetics with erythrocyte membranes using a range of spin labels. The experiments were designed to determine whether the various spin labels provided evidence for a consistent pattern of structural perturbations in a single well-defined membrane. With different spin labels the nitroxide group is localised at distinct regions of the membrane structure, and a comparison of the spectral changes induced by a particular perturbing agent provides evidence for the localisation of the perturbations in the membrane. A more difficult problem is to distinguish the interactions of both spin labels and perturbing agents with membrane lipids and proteins in the intact membrane.

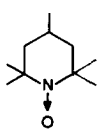
Nuclear magnetic relaxation data for the same anaesthetics in erythrocyte membranes is available[3] and enables a detailed comparison to be made of the techniques. The similar perturbations detected by both techniques in a range of membranes suggest that they reflect common structural features of the membranes which depend on the organisation of the membrane components.

MATERIALS AND METHODS

The syntheses and properties of labels I, IV, V and VI have been described elsewhere (refs. 4–7).

Label II. Benzyl tempoyl ether.* Potassium *tert.*-butoxide (13.5 g; 0.12 mole) was dissolved in 200 ml of *tert.*-butanol with warming and 10 g (0.058 mole) of 2,2,6,6,-tetramethyl piperidinol-1-oxyl was added. When the alcohol had completely dissolved, 6.7 ml (0.058 mole) of benzyl chloride were added. The solution was left to stand overnight, warmed to 50° for 12 h and then cooled before adding 100 ml of diethyl ether. After exhaustive washing with water (additional ether was added during the washing to maintain volume), the ether solution was dried over anhydrous Na_2SO_4 and the ether removed under reduced pressure. The solid was recrystallized from hexane and chromatographed on 250 g of silica gel, yielding 6.1 g of reddish-orange plates. Analysis gave C, 73.05; H, 9.21; N, 5.30%. The composition calculated for $C_{16}H_{24}NO_2$ is C, 73.24; H, 9.21; N, 5.34%.

Label III. N,N-dimethyl-N-dodecyl-N-tempoyl ammonium bromide. 10 g (0.064 mole) of 4-amino-2,2,6,6-tetramethylpiperidine were added with cooling and rapid stirring to 15.2 g of 98% formic acid solution (0.32 mole). After addition was complete, 11.4 g of a 37% aqueous solution of formaldehyde (0.14 mole) were added. The solution was heated on a steam bath under reflux for 10 h, cooled, 7.3 g of concentrated HCl added, and the formic acid and any excess formaldehyde were evaporated under reduced pressure. The reddish-brown oily residue was dissolved in approximately 150 ml of water and made strongly alkaline by addition of solid NaOH. The solution separated into two layers. The red, oily upper layer was collected,

* Tempoyl ≡ 

dried over $Ba(OH)_2$ and distilled under reduced pressure. This product was used in the next step without further purification, as the NMR spectrum indicated that the desired *N,N*-dimethyl-4-amino-2,2,6,6-tetramethylpiperidine was obtained.

3 g of the above product (0.016 mole) and 4.65 g of 1-bromododecane (0.019 mole) were mixed, heated in the dark to 75° for 6 h, cooled and triturated with diethyl ether. The resulting white solid was collected by vacuum filtration and rinsed exhaustively with diethyl ether. This compound was dissolved in 75 ml of water and 0.5 g of sodium tungstate and 0.5 g of disodium EDTA were added. After solution was complete, 10 ml of 30% H_2O_2 were added, the reaction allowed to stand for 12 h, and the water carefully removed under reduced pressure. The sticky residue was dissolved in excess hot benzene and filtered. The filtrate was collected and the benzene removed under reduced pressure. The semi-solid residue was again dissolved in hot benzene and the benzene removed under reduced pressure. This process was repeated until the residue was solid. The compound was then recrystallized once from benzene. The crystalline material was dissolved in a minimum amount of water and chromatographed on 50 g of AG 2-X 8 anion-exchange resin in the bromide form (Bio-Rad Laboratories), by eluting with distilled water. After evaporation of the water under reduced pressure, the solid was recrystallized from water and dried under vacuum, yielding 3 g of hydroscopic plate-like orange crystals.

Analysis gave C, 61.19; H, 10.79; Br, 18.73%. The calculated composition for $C_{23}H_{48}NOBr$ is C, 61.58; H, 10.78; Br, 17.81%.

Haemoglobin-free human erythrocyte membranes were prepared according to DODGE *et al.*[8] from outdated blood. Erythrocyte lipids and protein were separated by the method of MADDY[9] as modified by REGA *et al.*[10]. The membranes were washed thoroughly with deionised water adjusted to pH 7.0 and shaken briefly with a half-volume 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 that the pH was carefully controlled, all of the orginal membrane material is separated into the two fractions (approximately 55% by weight into the aqueous phase and 45% into the butanol phase).

The aqueous protein fraction, saturated with butanol, was dialysed exhaustively against deionised water. Good preparations are optically clear and only faintly yellow. Provided that all the butanol has been removed the ionic strength may be increased without causing precipitation of the protein. The protein fraction contains less than 5% phospholipid by weight; if necessary this can be reduced to less than 1% by treating the aqueous solution with fresh butanol.

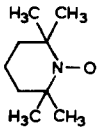
The lipid fraction was evaporated under nitrogen at 30° and washed repeatedly with water to remove all traces of butanol. The lipids were finally resuspended in vesicle form by agitation with the required buffer solution. The vesicles are stable under nitrogen for several days and do not require sonication. Unless otherwise indicated the buffer used in all experiments was 45 mM NaCl; 30 mM sodium acetate; 5 mM sodium phosphates; 1 mM NaN_3; pH 7.2.

Spin labels soluble in water to at least 1 mM were added to the membrane preparations by direct mixing with rapid stirring. Insoluble labels were dissolved in an organic solvent and then dried as a thin film in a flask. The aqueous membrane pre-

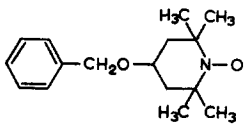
paration was then added and the mixture agitated until all the label was taken up. For preparations containing lipids, the final label concentration was usually less than a molar ratio of 100:1 in lipid:spin label. Water-insoluble perturbing agents were added by the same technique. The ESR spectra were generally recorded at 25°.

RESULTS

(a) *Labels I and II*



I (TEMPO)



II

TEMPO does not bind to the intact erythrocyte membrane significantly and the spectrum observed is simply that of TEMPO in aqueous solution. In the presence of high concentrations of local anaesthetics such as benzyl alcohol (200 mM), or tetracaine (5 mM), a small but detectable amount of TEMPO is bound in the membrane, and of the agents examined butane thiol was the most effective. It has been shown previously that TEMPO is a specific probe for fluid hydrophobic regions of membrane structure, and that the partition coefficient of TEMPO increases with the fluidity of the hydrophobic medium in which it is dissolved[1]. Thus TEMPO was found to bind to intact neural membranes and also showed an increased partition coefficient in the presence of local anaesthetics. The inability of TEMPO to bind to the unperturbed erythrocyte membrane suggests that it is inherently a more tightly packed structure than the neural membrane. On the other hand the binding of TEMPO in erythrocyte membranes induced by anaesthetics implies that they fluidise hydrophobic regions of this membrane, as well as in the neural membrane.

Consistent data was obtained with the related spin label II, which was designed to have a higher partition coefficient than TEMPO. This label is bound by erythrocyte membranes (Fig. 1) although the resonance of the bound component is much broader than the corresponding signal for the same label, or TEMPO, in neural membranes

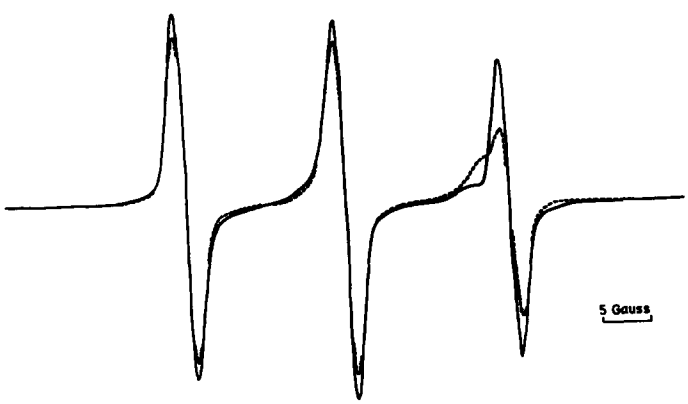


Fig. 1. $2 \cdot 10^{-4}$ M label II + 3.0% erythrocyte membranes. ———, zero benzyl alcohol; -----, 200 mM benzyl alcohol. Temperature 25°.

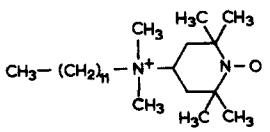
This indicates a more immobilised environment for label II in the erythrocyte membrane than in neural membranes, consistent with the behaviour of TEMPO and a range of other spin labels[6,7].

Fig. 2. $2 \cdot 10^{-4}$ M label II + 3.0% erythrocyte membranes. ———, 25°; ------, 45°.

In the presence of increasing concentrations of benzyl alcohol, the partition coefficient of label II in the membrane increases (Fig. 1). This is again analogous to the response of TEMPO in neural membranes. Increasing the temperature has the same effect of increasing the partition coefficient of label II (Fig. 2). However, it is not immediately clear whether this effect results from a change in the state of the membrane, where the increased thermal motion of the membrane components may provide a more fluid environment, or whether it is the result of a change in the activity of label II with temperature and is independent of the state of the membrane structure.

In these experiments both TEMPO and label II provide two distinct types of information. The first is the change in the partition coefficient as a response to membrane perturbation; this data could of course be obtained independently, without making use of the nitroxide spectra of the labels. On the other hand the information about the molecular motion of the labels in the membrane depends directly on properties of the nitroxide spectra. This information is more readily obtained from labels with high partition coefficients, which are almost entirely bound to the membrane, so that the spectrum of the bound component is not obscured by the free label. All the subsequent labels are of this kind and all show spectra in the intermediate range of immobilisation (τ approx. $1 \cdot 10^{-8}$ sec), which is the most sensitive range to changes in molecular motion.

(b) Label III



III

The spectrum of label III in erythrocyte membranes is shown in Fig. 3a. The high field resonance of the residual unbound spin label is resolved. It has been

confirmed that this represents unbound spin labels rather than a weakly bound fraction by measuring the nitroxide concentration in the supernatant of centrifuged membrane suspensions.

It is expected that this label will be preferentially oriented in the membrane with the quaternary charge of the polar interface region of the membrane. This is supported by the observation that the nitroxide group of this label bound to phosphatide vesicles and various membranes is rapidly reduced by aqueous reducing agents[6].

With increasing concentrations of benzyl alcohol and all the other neutral anaesthetics examined, there was a progressive sharpening of the spectrum, corres-

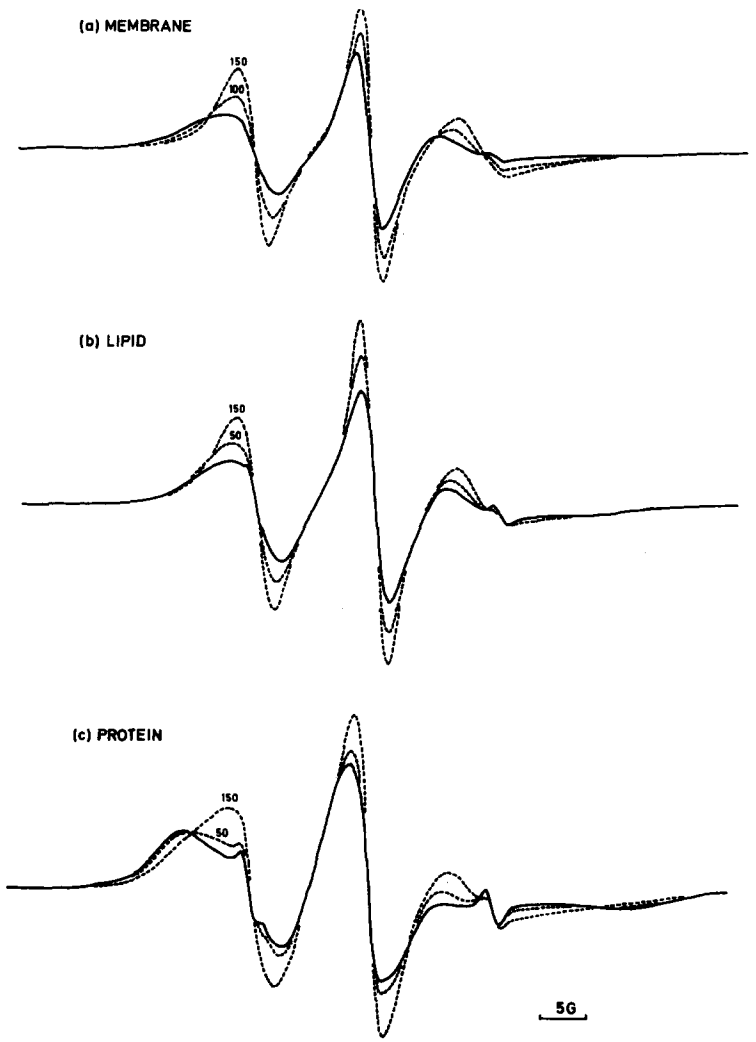


Fig. 3. Effect of benzyl alcohol on membrane preparations containing $1 \cdot 10^{-4}$ M spin label III. ———, zero benzyl alcohol; ------, with benzyl alcohol. Temperature 25°. (a) 1.0% erythrocyte membranes, with 0, 100, and 150 mM benzyl alcohol. (b) 1.0% membrane lipids with 0, 50, and 150 mM benzyl alcohol. (c) 0.3% membrane protein, with 0, 50 and 150 mM benzyl alcohol.

ponding to a fluidising effect of these agents on the environment of the spin label (Fig. 3a). As a general rule this label is most affected by agents with a strong hydrophilic group which have relatively low partition coefficients (< 10). Although the rotation of the tempoyl group about the C–N bond will be hindered in aqueous solution[11], it is probable that this motional restriction is small compared with that imposed by the membrane structure. The main factor determining the spectrum from label III in the membrane will be steric restriction of the rotation of the tempoyl group about its C–N bond, due to direct interaction of this group with its immediate environment. The tempoyl group is therefore expected to report primarily on perturbations at the membrane interface where it is localised by the influence of the quaternary charge. The freedom of rotation of the tempoyl group with respect to the rest of the molecule probably accounts for the sensitivity of this label to perturbation by hydrophilic agents, and its relatively modest response to the more hydrophobic agents. Direct evidence for the preferential localisation of the more hydrophilic members of homologous series of *n*-alkyl and $C_6H_5(CH_2)_nOH$ alcohols at the interface region of lecithin vesicles is presented elsewhere[12].

The erythrocyte membrane is reversibly stabilised against hypotonic haemolysis by benzyl alcohol at concentrations up to 80 mM. At higher concentrations the alcohol causes haemolysis, irrespective of the ionic strength[2]. With label III there is no evidence of a discontinuity to mark the onset of the irreversible structural changes in the membrane induced by lytic concentrations of benzyl alcohol, and the spectral sharpening occurs continuously over the entire alcohol concentration range. The reason for this became apparent on comparing the effects of benzyl alcohol on the spectra of label III bound to the separated membrane lipids and protein (Figs. 3b, 3c). Both components have a high binding capacity for the label; the spectrum from the membrane protein has a component which is strongly immobilised compared with the membrane lipids, and contains a heterogenous range of binding sites. Benzyl alcohol progressively fluidises the environment of the spin label bound to both components and this probably accounts for the continuous nature of the spectral changes in the membrane over the whole alcohol concentration range. In the absence of benzyl alcohol, there is no highly immobilised component of the spectrum from the intact membrane, suggesting that the strongly immobilised binding sites on the separated membrane protein are not accessible to the spin label in the intact membrane. Thus the spectrum from the intact membrane is readily distinguished from the composite spectrum of the separated components. While the spin label may bind to both lipid and protein binding sites in the intact membrane, the interaction is modified and restricted by the structure of the membrane itself.

The spectral changes induced by inorganic cations and cationic anaesthetics are entirely different from those described for neutral molecules. Instead of a smooth change in the whole spectral envelope, there is a progressive increase in the concentration of free spin label, superimposed on the spectrum of the bound component. This is entirely free spin label, which can be measured independently in the supernatant of centrifuged membrane suspensions. All the cations which have been examined cause the displacement of the spin label (Fig. 4), but this does not occur with neutral or anionic molecules. The effectiveness of simple cations depends on the valency and follows the order $Ce^{3+} > Ca^{2+} > Na^{+}$. The ability of hydrated cations such as Ca^{2+} to displace the spin label from the membrane suggests that the cationic group on

the label must be accessible to the aqueous medium and is consistent with the expected orientation of the label. For monovalent cations in molecules with hydrophobic groups the displacing activity appears to depend on the partition coefficient and hence

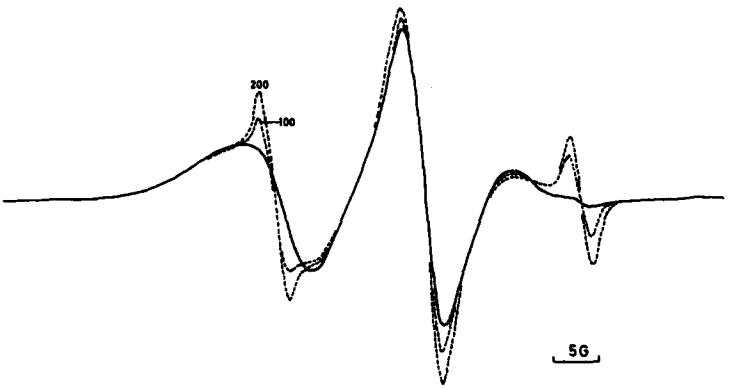
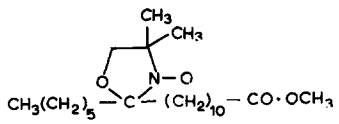


Fig. 4. Same as Fig. 3a, but with 0, 100 and 200 mM xylocaine.

on the local concentration of the agent in the membrane. For example, both chloropromazine and the dodecyl trimethyl ammonium ion are much more effective as displacing agents than xylocaine, which has a relatively low partition coefficient. The alkyl ammonium compound is an analogue of label III in which the tempoyl group carrying the nitroxide function is replaced by a methyl group.

Label IV

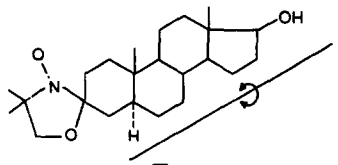


IV

This label is oriented in the membrane with the nitroxide group buried in the hydrophobic interior, where it is inaccessible to the solvent[6]. In contrast to label III it was more sensitive to perturbations by hydrophobic agents which lack a strong hydrophilic group. For example butane thiol was much more effective at perturbing label IV than label III, whereas the relative effectiveness was reversed for *n*-butanol. However, the perturbations of both labels in the presence of the same agent were qualitatively similar. Agents which fluidise the environment of label IV consistently showed the same effect with label III, although the effects may differ quantitatively to a considerable extent. The technique therefore provides a consistent description of membrane perturbation which can be localised within the structure by the use of appropriate labels.

Label IV is a neutral molecule and is not displaced from the membrane by cationic molecules. Cationic anaethetics, such as xylocaine and tetracaine, produce only a modest fluidising effect on the environment of label IV, which is similar to that obtained with benzyl alcohol.

Label V



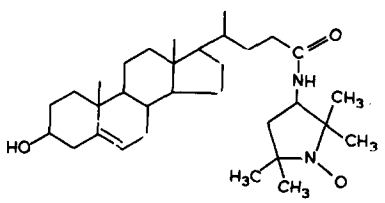
V

Label V shows significant anisotropy in its motion in neural membranes and in erythrocyte membranes oriented by hydrodynamic shear[7]. It rotates preferentially about its long axis which is perpendicular to the membrane surface, and is restricted in its rotation about the other two axes.

The spectrum of label V in erythrocyte membranes is shown in Fig. 5a. Prelytic concentrations of benzyl alcohol produce progressive sharpening of the spectrum similar to that observed with the previous labels. The environment of the spin label becomes more fluid and at the same time less anisotropic. Since the anisotropy must be imposed on the label by the organisation of the membrane components, it is not surprising that a fluidising effect on the membrane structure is associated with decreased motional anisotropy.

At high (lytic) concentrations of benzyl alcohol, a new spectral component appears, which corresponds to a fraction of the spin label molecules which are now in a highly immobilised environment (Fig. 5a). The spectra of label V bound to the separated membrane components are shown in Figs. 5b and 5c. It is immediately apparent that there is a highly immobilised component in the membrane protein spectrum which is similar to that observed in the whole membrane at lytic concentrations of benzyl alcohol. However there is little change in the spectrum of this component in the presence of benzyl alcohol, in marked contrast to the fluidising effect on the spectrum of label III bound to membrane protein in Fig. 3c. The highly immobilised component of the spectrum of label V in the membrane therefore remains clearly resolved in the lytic concentration range, and can be attributed to new protein binding sites for the spin label which were inaccessible at prelytic concentrations. On the other hand, the response of lipid-bound label V to benzyl alcohol is very similar to that observed with label III and there is simply a progressive fluidising effect over the whole concentration range.

Label VI



VI

Very similar spectral changes were observed with a second steroid label VI which also showed a highly immobilised component of the spectrum in the lytic concentration range of benzyl alcohol. Other molecules (*e.g.* xylocaine and trichloro-

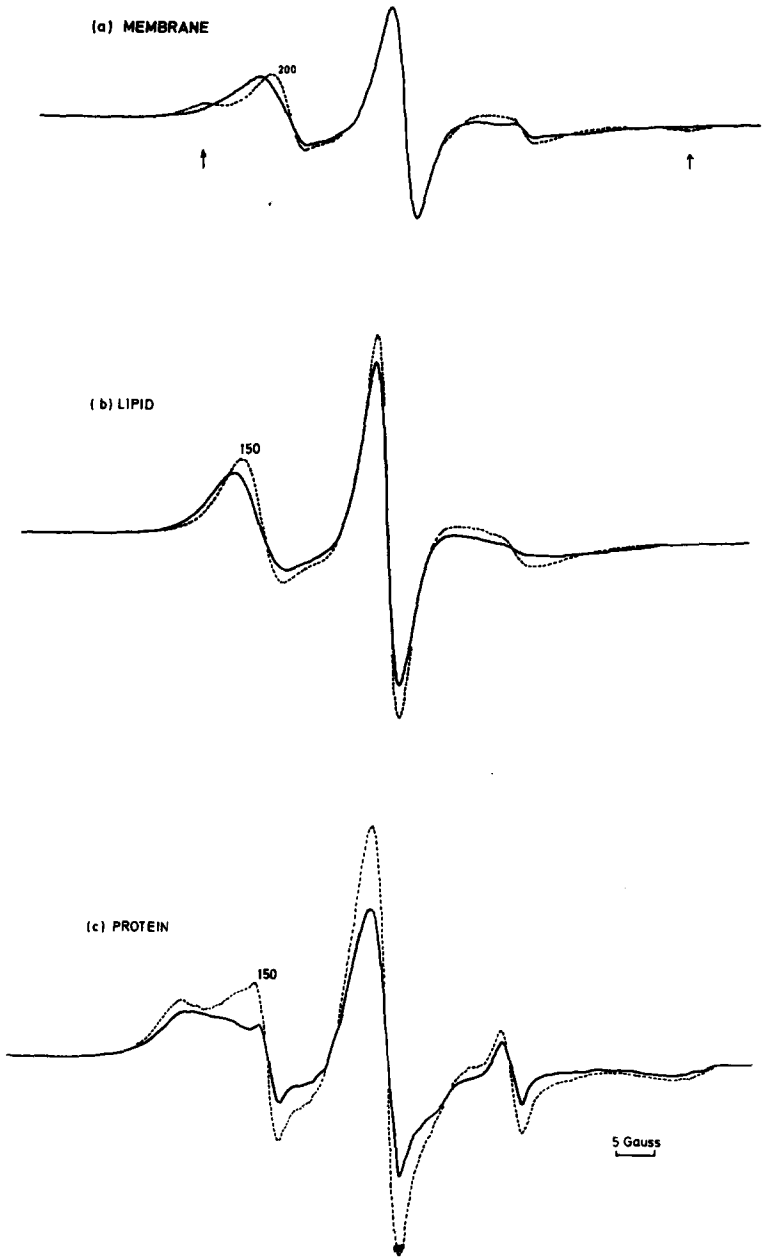


Fig. 5. Membrane preparations containing $1 \cdot 10^{-4}$ M spin label V. ———, zero benzyl alcohol; ------, with benzyl alcohol. Temperature 25°. (a) 1.0% erythrocyte membranes with 0, and 200 mM benzyl alcohol. The arrows indicate the strongly immobilised component of the spectrum which is only observed in the lytic concentration range. (b) 1.0% membrane lipids with 0 and 150 mM benzyl alcohol. (c) 0.3% membrane protein with 0 and 150 mM benzyl alcohol. Note the strongly immobilised component present in this spectrum.

ethanol) which cause lysis of intact erythrocytes also produced the highly immobilised spectral component in the lytic concentration range.

DISCUSSION

Taken together the non-covalent spin labels provide a consistent description of the perturbations of erythrocyte membranes by anaesthetics. The predominant effect is a fluidising of the environment of the spin label in the membrane, and by inference of the membrane components themselves. For example, in model systems of lecithin vesicles it has been shown that the rotational motions of both spin labels and perturbing agents are directly determined by the fluidity of the lipid molecules[12,13].

The various labels differed in their sensitivity to the perturbations caused by a particular agent. These differences reflect the different localisation of the nitroxide group in the membrane structure, which is determined by the structure of the spin labels. This implies that the perturbations occuring within the membrane are attenuated at sites which are distant from the regions where the perturbing agent is localised, and that spin labels can be used to define the perturbed regions of the structure. For example, the relatively high sensitivity of label IV to hydrophobic perturbing agents suggests that such agents may be preferentially distributed within the hydrophobic interior of the membrane. In contrast relatively hydrophilic agents perturb label III strongly and appear to be mainly localised at the interface region of the membrane. Again it is possible to confirm these interpretations directly for lecithin vesicles by observing the nuclear magnetic resonance spectra of the phospholipid and the perturbing agent simultaneously.

A more precise description of the interaction of membranes with perturbing agents hinges upon knowing how the spin label is oriented with respect to the membrane–solvent interface, and also how the label is distributed between protein and lipid binding sites. For spin labels in which the nitroxide group is rigidly fused to the parent structure (as in label V, for example), the motion of the nitroxide group is determined by the motion of the whole spin label, and the rotational anisotropy and orientation of the label can be determined unambiguously[6,7]. On the other hand if the nitroxide group is able to rotate freely with respect to the rest of the spin label, as for example in label III, then the spectrum will also depend on the steric interactions of the nitroxide group with its immediate environment, and anisotropic motion has not been observed with labels of this kind.

The problem of distinguishing interactions between the spin label and lipid or protein in the intact membrane is more difficult. All the spin labels examined were bound by both membrane lipids and proteins when separated, so that it cannot be assumed that any of these labels are bound specifically by one or other component in the intact membrane. Although the binding sites on the separated membrane protein are generally more strongly immobilised than in the lipid vesicles there is no immediate method of distinguishing protein and lipid binding sites in the intact membrane. At present this limits the usefulness of the labels in localising membrane perturbations, and as structural determinants. Potentially it should be possible to design labels which will have distinct spectra when bound to lipids or proteins in the intact membrane. For example the anisotropy of motion at the two groups of

binding sites may be distinct and related to the organisation of the membrane components.

In addition to the general perturbations of structure detected by these spin labels, the labels also provide information directly related to their particular chemical structures. For example, the cationic spin label III with a quaternary ammonium group provides a simple method of following cation displacements in the membrane.

As another example the steroid spin labels V and VI are especially useful in providing a marker of the irreversible structural changes which occur at lytic concentrations of perturbing agents. The highly immobilised component of the spectrum is clearly due to the exposure of new protein binding sites, which are exposed only when essential interactions between the membrane components have been disrupted by the perturbing agent. When these sites are exposed, there is extensive evidence that the membrane interacts as the sum of its separated components[13]. This effect is not reversible on the removal of benzyl alcohol, and the essential interactions between the membrane components which maintain the structure in a reversibly perturbable state are not reformed. In the prelytic concentration range the structural perturbations are fully reversible, and the structure of the membrane itself modifies and restricts the interaction with extraneous molecules. This provides sensitive criteria for an intact membrane structure; for example, the absence of the highly immobilised component of the spectrum with steroid spin labels provides a simple condition for structural integrity.

The type of perturbation produced by a chemical agent also depends on its structure. For example, the fluidising effect of the *n*-alkyl alcohols on label IV in erythrocyte membranes decreases with increasing chain length and beyond hexanol any effect is marginal. However, no agent has been found which has the effect of increasing packing or ordering on the erythrocyte membrane. Even cholesterol, which has a pronounced effect of this kind on the neural membrane, has little effect on the erythrocyte membrane. It is likely that this membrane is so tightly packed that any further increase in ordering can only be marginal. On the other hand increasing packing is readily demonstrated in lecithin vesicles, with cholesterol[1, 12]. Labels with the nitroxide group in the interior of the membrane which are inaccessible to the aqueous medium are most sensitive at detecting increased packing of the vesicle structure.

Neural and erythrocyte membranes are clearly distinguished in their interactions with spin labels by the greater fluidity of the neural membrane. For example the neural membrane binds TEMPO while the intact erythrocyte membrane does not, and a range of spin labels indicate a more immobilised and anisotropic environment in the erythrocyte membrane. However the pattern of perturbations induced in the two structures is remarkably similar. A striking example is the observation of the highly immobilised component of steroid spin label spectra in the lytic concentration range of perturbing agents (*e.g.* above 100 mM benzyl alcohol). The appearance of the highly immobilised component in the spectra from both membranes coincides with well-defined irreversible structural changes, namely lysis of intact erythrocytes and toxic anaesthesia in nerves. In the prelytic concentration range, comparable fluidising effects are observed in both membranes over the same concentration range. This provides a structural explanation for the empirical observations of SEEMAN[14], that the stabilisation of erythrocyte membranes against hypotonic haemolysis in the

prelytic concentration range correlates remarkably accurately with the anaesthetic action of a very diverse range of chemical structures on the neural membrane. In fact a similar pattern of structural perturbations has been observed in a range of different membrane structures by nuclear magnetic relaxation measurements[3].

Finally it is worth emphasising that the spin label experiments on erythrocyte membranes described here provide powerful support for the previous interpretation of NMR relaxation changes in terms of an increasing fluidisation of the membrane with increasing anaesthetic concentrations, followed by the exposure of new protein binding sites in the lytic concentration range. The general description of structural perturbations by both techniques is therefore well established.

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