The Proton Relaxation of Benzyl Alcohol in Erythrocyte Membranes

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SUMMARY

The mobility of benzyl alcohol molecules in erythrocyte membranes has been measured by nuclear magnetic resonance relaxation. At low concentrations the alcohol molecules are strongly immobilized by the membrane structure, this decreases as the concentration rises but starts to increase again at 60 mM. Suspensions of membrane lipid produce similar effects on relaxation at low concentrations but show no upswing at high concentrations. Separated membrane protein has a large effect on benzyl alcohol relaxation rate as have erythrocyte membranes pretreated with 300 mM benzyl alcohol.

These results suggest that as a result of insertion of the anesthetic in the membrane the lipid becomes disordered and finally exposes the protein, making available binding sites in the protein which are occult in the untreated membrane.

INTRODUCTION

The ability to produce conduction block in nerve fibers is found in a very large number of chemical substances of extremely diverse structures. These range from noble gases, simple paraffins, alcohols, ethers, and halogenated alkanes to alkylamines, cyclic ureides, and sterols. The range of structures encompassed makes a specific chemical interaction with the membrane seem unlikely and has favored the search for physical correlates. Overton and Meyer first pointed out how well the potency of anesthetics correlated with their partition constants between lipids or lipophilic solvents and water, and subsequent work has shown that such correlations are often excellent, particularly in homologous series and when corrections have been made for thermodynamic activity and molar volume (1–5).

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The interaction of the anesthetic with the membrane involves at least two processes, a primary one which may alter the membrane organization and, secondary to this, changes in ionic permeability ensue which are directly responsible for conduction block. If we are to understand the molecular events leading to anesthesia there is a need to investigate the primary interaction. It has been known since the observations of Traube (6) that anesthetics can make erythrocytes less sensitive to hemolysis by hypotonic solutions, and recently Seeman (7–10) has made an extensive study of the effects of drugs on the lysis of red cells. With few exceptions this effect is biphasic: in low concentration the cells are made more resistant to osmotic stress but at higher concentration complete lysis occurs which is independent of osmotic stress. In the stabilizing concentrations of drug there is an increase of membrane area presumably due to insertion of the drug into the membrane, and this may account in part for the greater resistance to hemolysis. In the lytic concentrations membrane structure is disorga-

nized and finally emulsification occurs. Seeman has shown that there is a very good correlation between the concentrations of drug that produce stabilization of the erythrocyte and those which cause conduction block in nerves, so that it is likely that similar membrane changes are responsible for both phenomena. Corresponding to lysis in erythrocytes is the development of irreversible nerve block studied by Skou (11), and here again a common basis is likely.

Discussions of the molecular basis of anesthetic action have usually been based on the relationship to oil/water partition coefficient referred to previously, which can be interpreted as implying that anesthesia is the result of insertion of a fixed volume of anesthetic "atoms" into the lipid of the membrane. Featherstone and Muehlbacher (12) have been particularly at pains to point out that anesthetics also interact with proteins through residues containing nonpolar side chains and although no systematic studies have been carried out it is likely that partition into protein also correlates with the straightforward lipid/water partition. The relevance of this will appear later.

The problem of how insertion of anesthetic into the membrane leads to alterations in the complex static and dynamic ionic permeabilities that are concerned in excitation and conduction in nerve fibers is difficult to investigate experimentally. The most widely held theory is that the internal pressure in the membrane is increased and this distorts the carrier systems so that their operation is disturbed. An alternative explanation is suggested by the occurrence of lysis at higher concentration of the anesthetics. This so clearly indicates a serious disorder of membrane structure that it does not seem unlikely that a lesser degree of disordering exists in the anesthetic range and that it is this rearrangement rather than internal pressure that perturbs the ionic conducting systems.

The membrane is a highly ordered structure as demonstrated by the sharp X-ray reflections at low angle, and it is accepted that this order is due primarily to the parallel side-by-side packing of the lipids with the heads in contact with a hydrophilic environment and the hydrocarbon tails either in contact with the tails of the other half of a bimolecular leaflet or with hydrophobic residues of the stromal protein. It would be expected that an anesthetic molecule inserted into the membrane would find itself in a highly constrained environment and hence its molecular motion would be much reduced compared to that either in water or in bulk liquid or lipid. Indeed the environment would more resemble that found in the crystalline state. Measurements of the molecular motion of the anesthetic in the membrane might therefore provide a sensitive measure of the degree of ordering of the membrane and of binding to membrane components. If the insertion of the anesthetic led predominantly to an increase in internal pressure, the mobility of the anesthetics might progressively decrease as the anesthetic concentration increased; on the other hand if the predominant molecular change in the membrane is disordering, the constraint on the anesthetic molecules should decrease and hence the rate of molecular rotation should increase. There remains the possibility that the anesthetic merely fills in spaces without appreciable change in the membrane order, in which case the rotation of the anesthetic will not be markedly concentration dependent. The possibilities mentioned so far do not involve specific binding sites. If such sites are involved we may expect to see saturation of these sites with increasing concentration.

The rotational rate of molecules containing atoms with nuclear magnetic moments can be measured by the technique of nuclear magnetic relaxation because under the conditions of relatively free molecular motion in solution, the rate at which a perturbed population of nuclear spins attains equilibrium is inversely proportional to the rate of molecular motion (13, 14). The rate of attainment of equilibrium is known as the relaxation rate and may be estimated from the line width in slow passage nuclear magnetic resonance spectroscopy; when molecular motion is reduced, the spectral lines are broadened and

reduced in amplitude. In the present study the relaxation rate of the protons in a selected anesthetic has been measured in the presence of various cell membrane preparations.

METHODS

Preparation of erythrocyte membranes. Erythrocyte membranes were prepared by a procedure due to Dr. V. Marchesi (personal communication). Human blood collected in acid citrate–dextrose was centrifuged at 2000 *g* for 15 min, and the plasma and buffy coat were removed. The cells were washed twice with 4 volumes of 0.9% NaCl. The cells were then lysed in 8–10 volumes of 5 mM Tris-HCl buffer pH 7.5 containing 1 mM sodium edetate. After centrifugation at 38,000 *g* for 10 min the deposit was again treated with 10 volumes of the Tris-HCl–edetate buffer and then centrifuged again at 38,000 *g*. This step removes most of the residual hemoglobin and a single wash with 0.5 M NaCl at this stage also helped to complete the removal of adsorbed hemoglobin. Treatment with buffer was then repeated 2 or 3 times more until the centrifuged deposit was off-white or pale yellow in color. The membranes were then washed twice with distilled water and stored at 4°. Before use they were equilibrated twice with NMR buffer. Of the dry weight of the membrane preparation, 1–2% was hemoglobin; this is similar in amount to that found in the preparations of Dodge *et al.* (15) and Weed *et al.* (16). Electron microscopy of thin Epon sections of these membranes revealed closed profiles of very clean unit membranes (unpublished observations of Dr. V. Marchesi).

A further assessment of the structural integrity of these membranes was obtained by measurement of their Na-K-ATPase activity. The basal Mg-ATPase activity was of the order of 40 nmoles of PO_4 released per milligram of membrane per hour. This was increased 40–50% in the presence of optimal amounts of Na and K. This is of a similar magnitude to that reported by Dunham and Glynn (17).

Pretreatment of membranes with benzyl alcohol. A suspension of the membranes in water was brought to 300 mM benzyl alcohol by exhaustive dialysis for 24 hr at 4°C. The benzyl alcohol was reduced to less than 1.0 mM by repeated centrifugation and washing of the membranes with the D_2O buffer for NMR measurements (see below).

Preparation of membrane lipids. The CO_2 method of Parpart (18) was used to prepare large quantities of erythrocyte ghosts for lipid extraction. Plasma and buffy layer were removed as before. The packed cells were lysed in 10 volumes of CO_2-saturated distilled water at 4°. The ghosts were further treated as described by Parpart. An ethanol–ether (3:1 v/v) extract of the lipid from these ghosts was obtained by the method of de Gier and van Deenen (19). The pale yellow extract was evaporated under N_2 and reduced pressure at 18°. It was prepared for NMR studies by sonication of a 2.0% suspension in NMR buffer for 5 min with a Branson-type probe sonicator.

Preparation of erythrocyte membrane protein. A sialoprotein fraction was prepared from erythrocyte ghosts by the butanol method of Maddy (20). After exhaustive dialysis against distilled water in the cold, the protein was freeze dried. The material contained less than 5% of the lipid present in untreated membranes. Most of the dry protein readily resuspended in NMR buffer to give a slightly turbid solution, which was centrifuged before use.

NMR measurements. The membrane preparation was equilibrated with a D_2O buffer containing 45 mM NaCl, 30 mM sodium acetate, and 5 mM NaH_2PO_4/Na_2HPO_4 dissolved in 99.7% D_2O. The "pH" (uncorrected) was adjusted to 7.4. Benzyl alcohol (analytical grade) was dissolved in the same buffer. A mixture of benzyl alcohol solution and membrane suspension was made and then pipetted into standard 5 mm NMR tubes and measurements made after a minimum of 12 hr. Similar tubes were prepared with anesthetic alone and with suspensions of membrane lipid and stromal protein.

Relaxation measurements were made in a Varian A60A NMR spectrometer at 60

Mcs and at the equilibrium temperature of the insert, which was 40°. Sufficient time was allowed for the tube and its contents to come into thermal balance before measurements were commenced.

All relaxation measurements were made at a sweep span of 2 cs/cm and a sweep rate of 0.4 cs/sec. Care was taken to ensure that the radiofrequency power was below the level causing saturation. The signal from the methyl protons of the acetate was unaltered by the presence of erythrocyte material and was used for fine adjustment of the field homogeneity and for correction of the line widths for instrumental broadening (0.3–0.5 cs). The true line width of the acetate methyl was taken to be 0.1 cs.

All measurements were made at least six times and mean values were taken. Line widths $\Delta\nu_{\frac{1}{2}}$ were measured at half maximum height. The relaxation rate $(1/T_2)$ is given by $\pi\Delta\nu_{\frac{1}{2}}$.

Measurement of partition coefficients. One milliliter of membrane suspension was mixed with 0.5 or 1.0 ml of a benzyl alcohol solution containing 4 mμC benzyl alcohol-^{14}C in 45 mM NaCl, 5 mM sodium phosphate buffer pH 7, in a 7 ml polypropylene centrifuge tube closed with an aluminum centrifuge cap and left at 23° or 40° for 1 hr before centrifuging. Control tubes were treated in the same way except that 1 ml of water replaced the ghost suspension.

Using a fixed-angle rotor preheated to 23° or 43° the tubes were centrifuged at 38,000 *g* for 11 min or at 55,000 *g* for 6 min. The rotor was preheated to 43° because its temperature fell 3–4° during centrifuging. Aliquots, 0.2 ml, were added to 10 ml of scintillation medium (21) and counted in a Nuclear-Chicago liquid scintillation spectrometer. All measurements were made in triplicate.

The partition coefficient was calculated from the relation

$$P = \frac{(C_c \cdot V) - C_e[V - Mg/D]}{C_c \cdot Mg/D}$$

where C_c and C_e are the counting rates in the supernatants of the control and experimental tubes, V is the total volume of fluid in the tube, Mg is the mass of dry weight of cell membranes in the tube, and D is the density of the ghosts—this was assumed to be 1.0. Note that partition coefficients were determined in H_2O, not D_2O.

Effect of benzyl alcohol on hypotonic hemolysis. This was carried out as described previously (7–10).

RESULTS

Trials with a number of anesthetics readily showed that line broadening occurred in the presence of erythrocyte membranes. Benzyl alcohol was selected for further study because it exerts its effects on erythrocyte membranes in the concentration range 5–250 mM as judged from its effects on hemolysis in hypotonic solution; this is a satisfactory range for magnetic resonance measurements. The five protons on the aromatic ring of benzyl alcohol are magnetically equivalent and give a single resonance line well suited to line width measurements. Benzyl alcohol is also free from the complication of ionizing groups.

Figure 1 shows the signal from the aromatic protons of benzyl alcohol at a concentration of 150 mM together with the same signal in the presence of erythrocyte

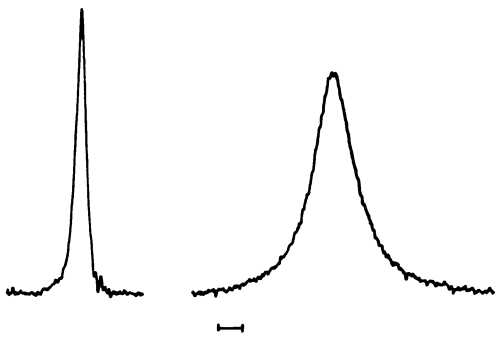

FIG. 1. *Resonance lines of the aromatic protons in benzyl alcohol*

The curve on the left shows the line obtained with 150 mM benzyl alcohol in D_2O buffer. The curve on the right the same concentration of benzyl alcohol in the presence of 1.0% by weight of erythrocyte membranes. The gain of the amplifier was increased ×4 for this curve. Temperature, 40°C.

membranes at a concentration of 1.0% by weight. The symmetrical resonance signal was considerably broadened and reduced in amplitude by the presence of the membranes.

When the concentration of benzyl alcohol was varied in the range 3–300 mM in the buffer solution in the absence of membranes the line width was unaffected and the mean value was 0.55 cs. However, in the presence of a constant concentration of membranes the line width was greatest at 3 mM and steadily decreased as the concentration increased to 60 mM, above this concentration the line width increased at least up to 150 mM (Fig. 2). At higher

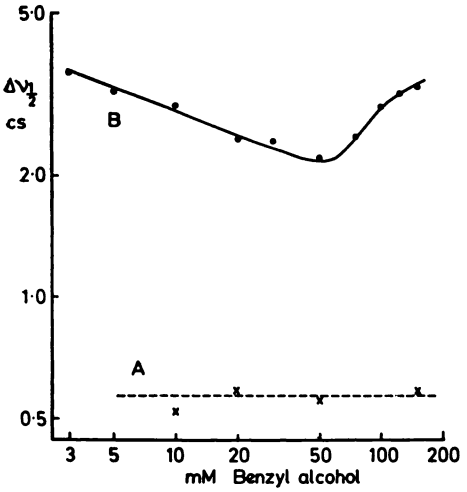

FIG. 2. *Line width of the aromatic proton line of benzyl alcohol at concentrations from 3 to 150 mM*

(*A*) In D_2O buffer; (*B*) with the addition of 1.0% by weight of erythrocyte membranes. Line widths have been corrected for instrumental broadening. Temperature, 40°C.

concentrations the line width was variable and tended to change with time. At these concentrations the physical state of the membrane suspensions was obviously altered. The membranes became agglutinated and the clumps of membranes tended to settle out. The inconsistent findings in the resonance measurements were due to the inhomogeneity of the suspension, and in all subsequent experiments the highest concentration of benzyl alcohol studied was 150 mM. The concentration series was examined in several preparations of membranes and was found to be highly consistent.

The suspension of erythrocyte membranes provides a system in which the major part of the total benzyl alcohol present remains in the aqueous phase and a minor part is dissolved in the membranes. In such a system, provided that the rate of exchange of benzyl alcohol molecules between the membrane and continuous phases is fast compared with the relaxation rates in the two phases, the measured relaxation rate will be equal to the weighted mean of the relaxation rates in free solution and in the membranes (22).

Thus

$$\frac{1}{T_2}_{\text{experimental}} = \alpha \frac{1}{T_2}_{\text{membrane}} + (1 - \alpha) \frac{1}{T_2}_{\text{free}}$$

where $1/T_2$ is the relaxation rate ($= \pi\Delta\nu_{\frac{1}{2}}$) and α is the fraction of the total benzyl alcohol which is in the membrane phase. Determination of the partition coefficient showed that it was little dependent on concentration up to 20 mM, but thereafter rose as the concentration increased so that whereas it was 1.7 at 2 mM it increased to 4.1 at 200 mM (Fig. 3).

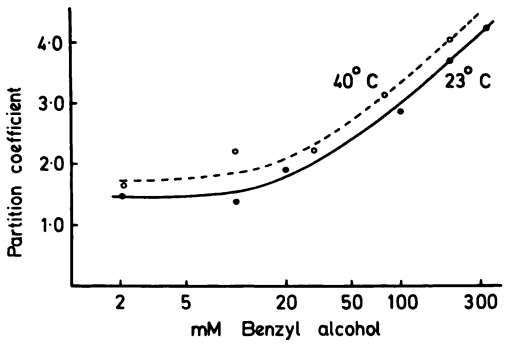

FIG. 3. *Partition coefficients of benzyl alcohol between the membrane and H_2O buffer*

When the appropriate partition coefficient was used to calculate the values of $(1/T_2)_{\text{membrane}}$ it can be seen from Fig. 4

that the general shape of the curve was similar to that in Fig. 2. At the lowest concentration (3 mM) the relaxation rate was 570 sec^{-1} and it fell to a minimum of 189 sec^{-1} at 60 mM. Since the relaxation rate in the absence of membranes was $(1/T_2)_{free} = 1.73$ sec^{-1} it can be seen that the rotation of the aromatic group was reduced by a factor of 110- to 330-fold in the membrane. For comparison the relaxation rate of benzyl alcohol dissolved in a liquid triglyceride oil (corn oil) was 8.9. sec^{-1}. The restriction of molecular motion was very

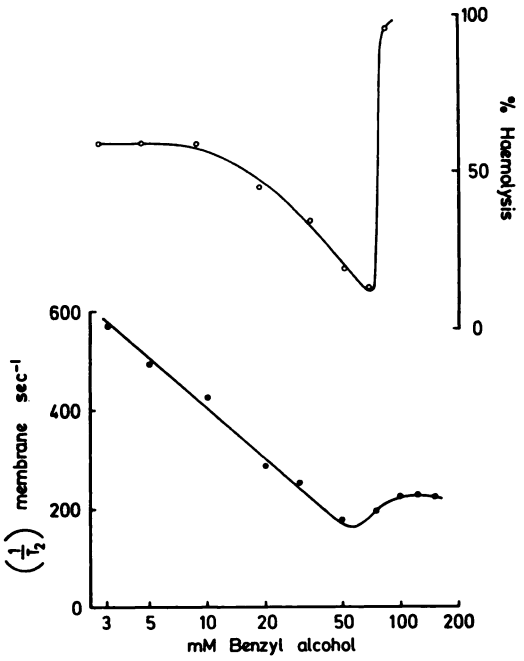

FIG. 4. *Lower curve: Relaxation rate of aromatic protons of benzyl alcohol in the membrane phase*

Calculated from the data of Fig. 2, and the partition coefficients of Fig. 3 using the equation given in the text.

Upper curve. Hemolysis of human erythrocytes In 67.5 mM NaCl, 10 mM sodium phosphates pH 7.0 at 37.5°C in the presence of benzyl alcohol. End point taken at 25 minutes.

considerable and much greater than in a liquid lipid. Since in the low concentration range the reduction in relaxation rate as the concentration is increased is not accompanied by a decrease in partition coefficient but rather by an increase, it is unlikely that saturable binding sites play any important part in the stabilization of benzyl alcohol in this range.

Comparison with the Effects of Benzyl Alcohol on Hemolysis

In Fig. 4 the effects of benzyl alcohol on hemolysis are also shown. It will be noted that the range of concentration where the membrane is progressively more stabilized against osmotic hemolysis coincides with the region where the relaxation rate is also decreasing and that there is a reasonable agreement between the concentration causing hemolysis and the upturn in the relaxation rate.

Relaxation of Benzyl Alcohol in Separated Fractions of the Erythrocyte Membrane

Lipids. The lipids obtained by chloroform methanol extraction of erythrocyte membranes were suspended in NMR buffer by sonication. The benzyl alcohol signal was broadened by the presence of lipid and in the low concentration range the curve ran parallel to that of intact membranes, but with further increase in benzyl alcohol concentration the relaxation rate continued to fall (Fig. 5). There was no exact correspondence in concentration or composition of the lipid with that in the whole membranes because part of the lipid did not enter into stable suspension on sonication, so that the magnitude of the relaxation rates should not be regarded as exactly comparable with that of the membranes.

Protein. A solution of stromal protein was prepared and in its presence the relaxation of benzyl alcohol was increased far more than by whole membranes; the relaxation rate decreased steeply with increase in alcohol concentration in a manner suggestive of saturation of binding sites at high concentrations. In the case of saturated sites the fraction of material bound decreases as the concentration rises; since the observed relaxation rate is the weighted mean of the relaxation rates in

the free and bound states, a progressive decrease in the observed relaxation rate occurs as the concentration is increased.

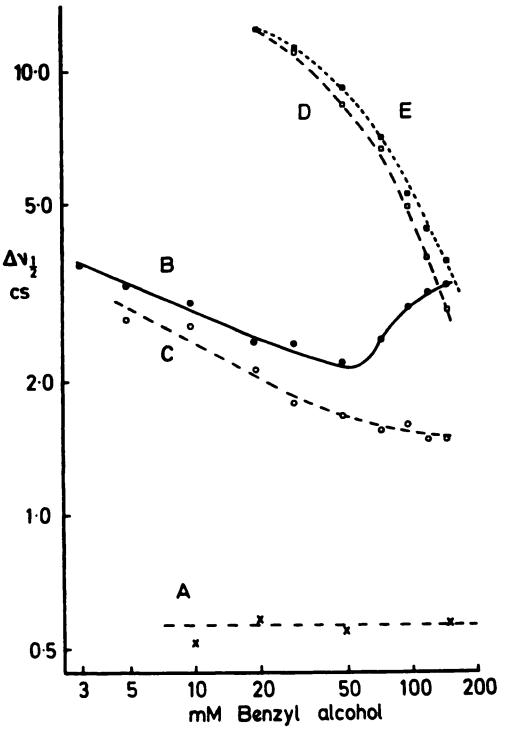

FIG. 5. *Line widths of aromatic protons of benzyl alcohol*

A and *B* are the same as in Fig. 2; *C* in the presence of 0.6% sonicated lipids extracted from erythrocyte membranes; *D* in the presence of 0.9% stromal protein; *E* in the presence of 0.85% erythrocyte membranes pretreated with 300 mM benzyl alcohol. All results obtained at 40°C and corrected for instrumental broadening.

Effect of Pretreatment of Membranes with Benzyl Alcohol

Since high concentrations of anesthetics produce morphological changes in cell membranes on the ultramicroscopic scale, it was of some interest to see whether the irreversible physiological effects produced by these concentrations were also accompanied by irreversible effects as far as the environment available to benzyl alcohol molecules was concerned. Preparations of erythrocyte membranes were exposed to 300 mM benzyl alcohol for a period of 24 hr. The membranes were washed repeatedly with buffer to remove the benzyl alcohol and then a concentration series with benzyl alcohol was prepared as with fresh membranes. The relaxation rate found in this experiment is also seen in Fig. 5. It will be seen that the curve is very similar to that obtained with the separated stromal protein. As with the separated membrane fractions the concentration of membranes solids was only approximately the same as in the control experiment with fresh membranes.

DISCUSSION

The changes in relaxation rate of benzyl alcohol found in these experiments are most readily explained on the following hypothesis. The primary site of interaction of the alcohol is with the lipid part of the membrane. As the amount of benzyl alcohol in the membrane is increased the alcohol finds itself in an increasingly more mobile environment as evidenced by the fall off in relaxation rate. This implies that the regular arrangement of hydrocarbon chains in the membrane is being increasingly disturbed. Very similar changes occur in the lipid suspension—one could imagine such changes progressing toward complete disordering of the lipid at high enough alcohol concentrations so that the environment of the alcohol would then correspond in principle to that of liquid lipid in which it has a comparatively low relaxation rate. In the whole membrane however this continuous fall in relaxation rate is reversed at about 60 mM.

The results with stromal protein showed a much greater effect on relaxation rate than by either lipid or by membrane alone except at the highest concentration. This suggests that the stromal protein has combining sites that exert a very strong immobilizing effect on benzyl alcohol. The most immediate question to be answered is why the stromal protein *in situ* does not produce such a strong effect. Two possible answers present themselves. The first is that in the intact membrane the lipid and protein are associated into lipoprotein in such a way that the protein binding sites are already used in stabilizing the lipo-

protein architecture, and hence are masked or unavailable for interaction with the anesthetic. The second possibility is that a conformation change occurs in the protein when the lipid is removed which increases the availability of hydrophobic sites. Similar changes are believed to occur in globular proteins as a result of a change which brings hydrophobic groups normally in the interior of the protein to the surface. It is not necessary to go so far as to suggest that complete denaturation or degradation of stromal protein has occurred; indeed this does not seem probable as the stromal preparations remain soluble.

We would suggest therefore that the upturn in the relaxation rate seen at higher concentrations in the presence of membranes is due to dissociation of lipid from the protein stroma with uncovering or activation of binding sites on the protein. The relatively small magnitude of the upswing is due to the fact that the concentration range where protein binding is becoming significant is also one where the effect on relaxation is small compared to that at lower concentrations. The behavior of membranes pretreated with benzyl alcohol shows clearly the irreversibility of the effects of high concentrations of anesthetics on the membrane structural organization. Despite the retention of lipid in the membrane its relationship to the protein has been disrupted so that the separated type of protein binding is evident over the full range of benzyl alcohol concentrations.

While the interpretation of results we have favored suggests that the initial and perhaps most important site of action is in the lipid moiety of the membrane, we cannot exclude the possibility of extensive interactions with the protein part of the membrane if these did not lead to strong immobilization of the alcohol molecules. By its very nature the NMR relaxation method is dominated by strongly immobilized molecules provided they are present in equivalent amounts. Thus if we were to suppose that equal amounts of alcohol were reacting with lipid and with a poorly immobilizing site in the protein, the latter would go unnoticed. Our results are quite compatible with the possibility that the interaction of benzyl alcohol in the range below 60 mM is with undissociated lipoprotein considered as a unit. Either possibility could be conceived of as leading to interference with membrane processes concerned in ionic permeation.

The method described in this paper is being extended to the study of a wide range of anesthetics. It is also a most promising method for studying alterations in cell membranes in response to a variety of circumstances.

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