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The Influence of Anesthetics and Cholesterol on the Degree of Molecular Organization and Mobility of Ox Brain White Matter

Lipids in Multibilayer Membranes: a Spin Probe Study Using Spectral Simulation by the Stochastic Method

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SUMMARY

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The effects of some anesthetics on the molecular arrangement in multibilayer of brain white matter lipids (of low cholesterol content) were studied by observing the ESR spectra of a spin probe derivative of cholestane intercalated in the lipid multilayers. ESR spectra were simulated by a stochastic method, with explicit inclusion of the molecular order parameter and two correlation times. By this method some insight was gained into the arbitrary index or ordering commonly used in these systems. Tetracaine, promazine, chlorpromazine, pentobarbitone, and mepivacaine increased the degree of order in multibilayers containing 5% cholesterol, but did not induce the same high degree of order found at high concentrations of cholesterol. Increasing the concentration of anesthetics eventually produced disorder in the multilayers. The ordering effects of tetracaine and pentobarbitone were reversible. The ordering effect of tetracaine, but not that of pentobarbitone, was potentiated by calcium ions at a concentration (5 mM) which had no intrinisc ordering effect. Multilayers containing up to 5% cholesterol showed a very low degree of order at the molecular level, but above this concentration striking changes in the ESR spectra indicated that the lipid molecules were tending to arrange their long axes close to the normal to the plane of the films. Multilayers of higher cholesterol content showed almost perfect order. The response to cholesterol took place over a very small range of cholesterol concentration, suggestive of a cooperative response. Despite the very large change in order parameter, cholesterol induced no change in the rotational correlation time (mobility).

INTRODUCTION

The conduction block in nerves produced by local anesthetics is well understood in terms of alterations of nerve membrane conductance; however, the mechanism by which these alterations are produced is unknown. Many cationic, neutral, and anionic drugs have local anesthetic actions, and this property seems to be due to a balance of hydrophobic and hydrophilic groups in the molecule. It is possible that local anesthetics accumulate in the nerve membrane and produce changes in the or-

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ganization of the membrane, usually referred to as swelling, packing or stabilization. Such actions might reduce the size of the sodium channels or could possibly cause spatial separation of molecules or structures essential for the as yet unknown molecular reactions responsible for excitation. (For reviews on the mechanisms of local anesthetics, see refs. 1–3.)

Cholesterol is a principal component of mammalian membranes. Although its exact role has yet to be elucidated, it is known to have a strong influence on the packing properties of the fatty acyl chains of phospholipids (4). Surface pressure studies have shown that it condenses, or reduces the mean chain-chain distance of, phospholipids above their gel–liquid crystal transition temperature. Below this temperature it has the opposite effect (5–7). A high cholesterol content has been shown to remove the gel–liquid crystal transition by fluidizing the gel phase (6). An excellent correlation has been found between the condensing effect of cholesterol and reduction in the permeability of phospholipid vesicles (7, 8). It thus appears that one role of cholesterol in biological membranes is to regulate the degree of order and mobility of fatty acyl chains.

The influence of cholesterol on phospholipids has been investigated at the molecular level by use of nitroxide-labeled lipids, spin probes (8–14). Thorough treatments of the technique are presented in recent books (15, 16) and several review articles (17–19).

The lipids of the white matter of bovine brain are a complex mixture (20) of phospholipids, cerebrosides, and cholesterol (approximately 20%). It is therefore difficult to predict the order and mobility properties of such a mixture in terms of those of its components. The spin probe method provides a sensitive and rigorously interpretable means of determining these properties, and the influence on them of anesthetics and cholesterol.

The cholestane spin probe CSL[2] has the advantage that the nitroxide group is firmly attached to the steroid nucleus. Thus the ESR spectra reflect the order and motion of the entire steroid nucleus. Furthermore, surface pressure measurements have shown that CSL interacts with phospholipids in a manner very similar to that of cholesterol (21). If the long axes of the probe molecule are randomly oriented, there is no difference between the spectra observed with the film perpendicular and parallel to the magnetic field of the spectrometer (22). However, if all the steroid molecules are arranged normal to the plane of the film and are rotating rapidly about their long axes, spectra consisting of three lines with separations of about 6 and 19 G are observed with the film perpendicular and parallel to the magnetic field, respectively. The narrower the distribution of CSL long axes about the normal to the plane of the film, the greater becomes the angular dependence. Thus the degree of angular dependence reflects the degree of order in the film. A convenient "measure" of order which has been used in earlier studies is the ratio of the heights of the peak 6–8 G downfield from the center peak and the center peak of the spectra taken with the magnetic field perpendicular to the lipid film (*B/C* ratio, Fig. 1). For perfect order this ratio approaches 1; for complete disorder it approaches 0 (22, 23). By spectral simulation we show here that the *B/C* ratio can be directly related to the molecular order parameter of the spin probe, as well as to the rate of rotation of CSL about its long axis.

[2] The abbreviations used are: CSL, 3-spirodoxylcholestane; doxyl, 2′-(*N*-oxyl-4′,4′-oxazolidine)-.

METHODS

The methods of film preparation (22, 24) and lipid isolation (25–27) have been described in detail. Films were hydrated with buffer (controls) or with buffer containing anesthetic and allowed to equilibrate at room temperature (22°) for 30 min. The cells were then drained to remove any vesicles, and the ESR spectra were recorded at 22° with a Varian E-9 ESR spectrometer.

The films were hydrated either with sodium phosphate buffer (150 mM) at several pH values or with Tris-HCl buffer (5 mM), pH 7.4, containing sodium chloride (140 mM). The cholestane spin probe CSL was prepared from cholestan-3-one (28), and the doxyl derivatives of 5- and 12-keto-

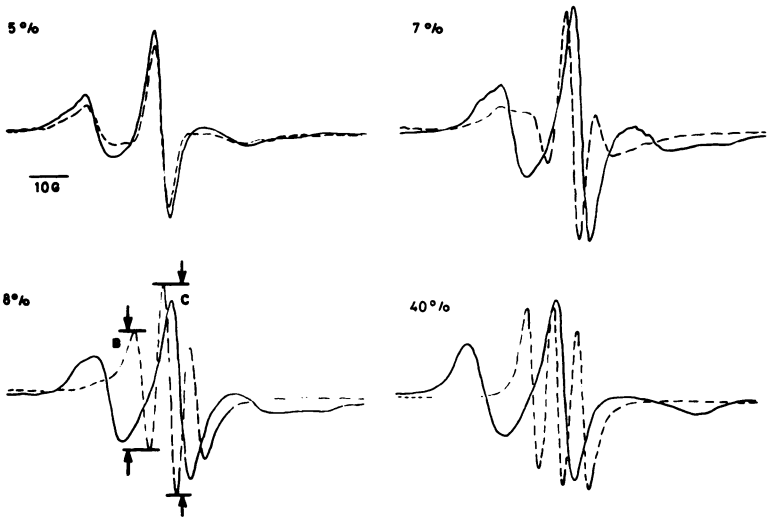

FIG. 1. *Effect of different concentrations (grams per 100 g) of cholesterol on spectrum of cholestane spin probe (CSL, 0.5%) in films of white matter lipid hydrated with phosphate buffer (150 mM), pH 6.8*
Solid spectra were taken with the film parallel to the magnetic field; dashed spectra were taken with the film perpendicular to the magnetic field.

stearic acid (5-doxylstearic acid and 12-doxylstearic acid) were purchased from Syva, Palo Alto, Calif. Cholesterol (Steraloids, Inc., Pawling, N. Y.) was recrystallized from methanol. Tetracaine was purchased from Mann Research Laboratories, and mepivacaine, from Winthrop Laboratories, Aurora, Ontario.

ESR spectra were simulated by the stochastic method of Freed (29) with a distribution of spin probe orientations (30). The method is outlined in a recent review by Polnaszek (31). Correlation times for the rotation of CSL about its long axis were estimated from the ESR spectra obtained with the magnetic field parallel to the plane of the film, using the jumping spin formalism of Mailer *et al.* (13). The value of the z component of the hyperfine splitting tensor a_z was obtained from the ESR spectrum of CSL in hydrated lipid films formed on glass wool, measured at −70°.

RESULTS

Effect of Cholesterol on Organization of Lipid Multibilayers

The spectra of hydrated films containing less than 5 g/100 g of cholesterol indicate an almost isotropic distribution of CSL long axes (Fig. 1); the only motion apparent is rotation about the CSL long axis. This motion is rapid enough to average the x and z components of the hyperfine splitting tensor (6 and 32 G, respectively) to give an effective hyperfine component of 19 G. However, when the concentration of cholesterol is increased above 6% there occurs an abrupt and striking increase in degree of anisotropy of the films; i.e., the difference between the parallel and perpendicular hyperfine splitting increases (Fig. 1). In addition, the asymmetry of the hyperfine lines in the parallel direction increases (Fig. 1). These spectral changes develop progressively with increase in the concentration of cholesterol (Fig. 2), and in films containing 8% cholesterol a high degree of order is observed; the B/C ratio is 0.75, corresponding to a molecular order parameter of 0.78 (see below). Further increasing the cholesterol concentration gradually raises the degree of order, until at 40% cholesterol the B/C ratio is about 0.9, equivalent to a molecular order parameter of 0.88. Note that the order parameter used here is a measure of the distribution of orientations of the long axis of the spin probe. The limiting values of S, 0 and 1, correspond to a random distribution of orientations and to all the molecules being mutually parallel, respectively.

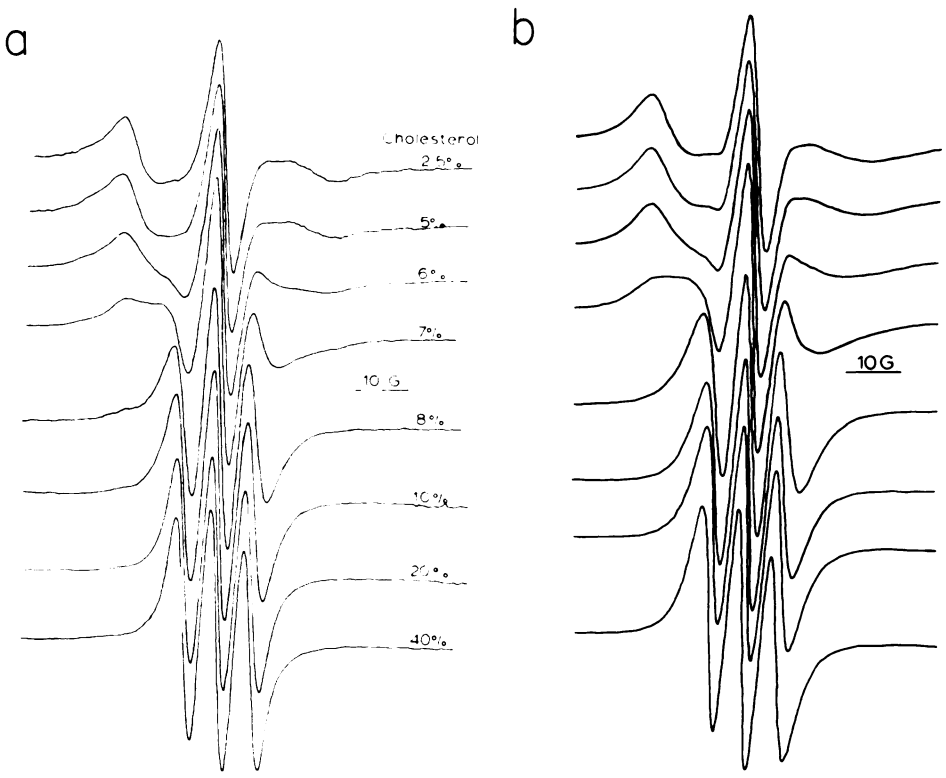

FIG. 2. *Cholesterol effects on brain lipid films*

a. Influence of increasing amounts of cholesterol on the ESR spectra of spin probe CSL in hydrated films of decholesterolized brain lipid. The spectra were taken at 23° with the applied magnetic field perpendicular to the plane of the multibilayer membranes.

b. ESR spectra of CSL simulated by the stochastic method (29–31). The input parameters were: $a_x = 5.3$ G; $a_y = 5.3$ G; $a_z = 33.3$ G; $g_x = 2.0084$; $g_y = 2.0060$; $g_z = 2.0022$; center of spectrum, 3300 G; $\tau_{R\perp} = 3 \times 10^{-7}$ sec; $\tau_{R\parallel} = 1.2 \times 10^{-9}$ sec; X_2 (orientation-dependent part of the line width) = −1.5 G. The values for λ, S, and X_0 are given in Table 1.

In the experiments involving anesthetics, films containing 5% cholesterol were used, i.e., a concentration of cholesterol just below that which caused large changes in the angular dependence of the spectra of the films. Such films are subsequently described as low-cholesterol films.

Approximate correlation times for rotation *about* the cholestane long axis, $\tau_{R\parallel}$, calculated using the jumping spin method (13) with the magnetic parameters given in the legend in Fig. 2, are given in Table 1. The values calculated show that one cannot use the rapid rotation model discussed by Van *et al.* (32) because the rate of axial rotation is too slow. In the rapid rotation model the nature of the motion about the long axis is not specified, except that it is rapid on the ESR time scale (32); spectral simulations have shown that this corresponds to $\tau_{R\parallel} \leqq 5 \times 10^{-11}$ sec (31). Thus it was necessary to use the stochastic method for simulating ESR line shapes, as it includes correlation times and distributions of spin probe orientations explicitly. Attempts to fit the spectra corresponding to low degrees of order (2.5–5% in Fig. 2a) showed that the rotational rate *of* the steroid long axis was very slow on the ESR time sclae. Thus $\tau_{R\perp}$ is greater than or equal to 3×10^{-7} sec; this value was used for all theoretical spectra. It was found that increasing the values of $\tau_{R\parallel}$ given in Table 1 resulted in better fits for less ordered spectra. Thus it was decided to use the mean value of $\tau_{R\parallel}$ (1.2×10^{-9} sec) calculated from the spectra due to a high degree of order (8–40% cholesterol), as the jump-

TABLE 1

Parameters used in or determined from Fig. 2

Cholesterol	λ[a]	S[b]	X_0[c]	τ_R[d]	Order parameter from B/C ratio[e]
%			G	sec × 10^9	
2.5	0.25	0.03	3.5	0.9	<0.2
5	0.5	0.07	3.5	1.0	<0.2
6	1.1	0.16	3.8	1.1	0.35
7	2.5	0.37	3.9	1.2	0.53
8	7.5	0.78	4.0	1.3	0.84
10	10.5	0.85	3.8	1.1	0.91
20	12.5	0.87	3.9	1.2	0.92
40	13.0	0.88	3.9	1.4	0.95

[a] Orientation distribution parameter.
[b] Order parameter from simulations.
[c] Residual line width.
[d] Jumping spin method (13).
[e] Using rapid rotation model (10, 32).

ing spin method is valid only for highly ordered systems. All spectra were fitted quite well by varying only the orientation distribution parameter λ (and hence the order parameter S) and the instrinsic line width X_0, as shown in Fig. 2b. The relationship between S and λ and the definition of X_0 and X_2, the residual line widths are given by Polnaszek (31). The values of λ, S, and X_0 which gave the best fits are listed in Table 1. The relative error in S is ±0.02, and the value of $\tau_{R\parallel}$ used is accurate to ±20%.

In earlier studies of CSL in oriented systems the *B/C* parameter was used as an arbitrary index of order. In fact, the *B/C* parameter also depends upon the correlation time for motion about the CSL long axis, $\tau_{R\parallel}$, as shown in Fig. 3.

The effect of increasing $\tau_{R\parallel}$ while keeping *B/C* constant is to decrease the calculated order parameter S. This is evident in Fig. 3, where the *B/C* ratios vs. the order parameter S are plotted for the *rapid rotation model* (32) and the case observed here, $\tau_{R\parallel} = 1.2 \times 10^{-9}$ sec. In general there will be a different curve for *B/C* vs. S at different values of $\tau_{R\parallel} > 5 \times 10^{-11}$ sec. Thus, although *B/C* is a function of both S and $\tau_{R\parallel}$, it will not be accurately indicative of the ordering in a system unless $\tau_{R\parallel}$ remains nearly constant, as was observed here. *B/C* is also slightly dependent upon the intrinsic line widths X_0 and X_2. All the simulations were performed using Brownian rotational diffusion; the use of strong or intermediate jump models (29, 30) changed the spectra very little. Recently ESR spectra of CSL in smectic liquid crystals were fitted using an axis tilted somewhat from the nitroxide y axis as the principal axis of ordering and rotation (33). For the present data, simulations of ESR spectra using a tilt angle of 20° did not agree as well as those with the nitroxide y axis parallel to the long axis of the molecule. The values of S required for the simulated ESR spectra of Fig. 2b are shown in Fig. 4

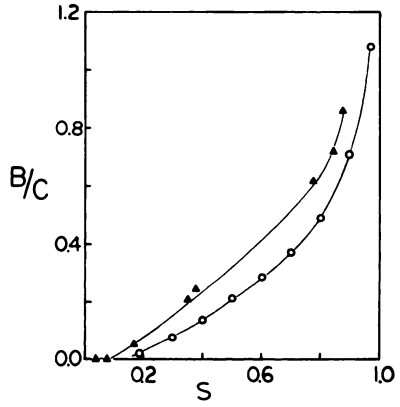

FIG. 3. *Dependence of arbitrary ESR order index B/C on molecular order parameter S for spin probe CSL*

O——O, curve valid for any value of the rotational rate about the CSL long axis, $\tau_{R\parallel} \lesssim 5 \times 10^{-11}$ sec, i.e., the rapid rotation model; ▲——▲, curve for $\tau_{R\parallel} = 1.2 \times 10^{-9}$ sec.

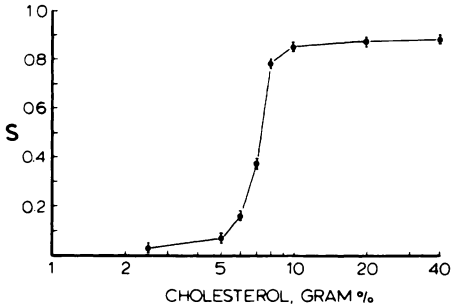

FIG. 4. *Values of order parameter S of CSL in brain lipids as a function of cholesterol content (grams per 100 g)*

The values of S were determined from the simulations in Fig. 2.

as a function of cholesterol content.

The theoretical simulations of the CSL spectra show that the effect of adding cholesterol to brain lipids is to increase the ordering of the system, with a sharp transition from an almost random to a well-ordered system occurring near 7% cholesterol. However, the fluidity or microviscosity sensed by the spin probe is apparently independent of the cholesterol concentration and hence of the ordering of the system. Similar observations have recently been noted in ^2H NMR studies of model systems (34).

Stearic Acid Spin Probes in Brain Lipids

Substituted stearic acids have provided valuable insight into the motion and order of fatty acyl chains in membranes (11, 35, 36). In most membranes rapid chain flexing motions lead to ESR spectra which can be interpreted in terms of order parameters. In oriented films interpretation is even more straightforward (11):

$$S = \frac{T_{\perp}' - T_{\parallel}'}{T_{\perp} - T_{\parallel}}$$

where $T_{\perp}'$ and $T_{\parallel}'$ are the hyperfine splittings observed with the magnetic field perpendicular and parallel to the plane of the film, respectively, and $T_{\perp}$ and $T_{\parallel}$ are the values expected for perfect order, approximately 32 and 6 G, respectively. Usually a large decrease in order parameter is observed as the position of the nitroxide label is moved down the fatty acid chain and away from the anchoring carboxyl head group (11, 35, 36).

The ESR spectra for the 5-doxylstearic acid spin probe in oriented multibilayers of brain lipid are shown in Fig. 5. The spectra at pH 4.5 in the absence of chlorpromazine are indicative of a low degree of order and a low rate of chain flexing. The order parameters can be estimated by measuring the distances between crossing points (where the first derivative curves cross the baseline), but can only be accurately determined by spectral simulation. A most interesting observation, however, is that at pH 4.5 the estimated order parameters for both 5-doxyl- and 12-doxylstearic acid probes are essentially equal (Table 2). This is in sharp contrast to liquid crystalline systems, in which differences as high as a factor of 2 are seen (11, 35, 36). At pH 7.2 the difference in order parameter between positions 5 and 12 is significant. Notice that the order parameter for CSL (Table 1) is lower than that for 5-doxylstearic acid but higher than that for 12-doxylstearic acid (Table 2) at approximately the same pH (6.8 for CSL, 7.2 for the stearic acids). This is due to the rigidity of the CSL steroid nucleus; its order parameter represents an average value for the first ≅20-Å depth of the bilayer. The data for the stearic acid probes are thus in excellent agreement with those for the cholestane spin probe: at low cholesterol concentrations bilayers of brain lipid exist in a highly disordered, gel-like state, with slow rates of fatty acyl chain motion.

Effects of Anesthetics on Organization of Multibilayers

Tetracaine. The effects of hydrating films prepared from white matter lipids containing 5% cholesterol (low-cholesterol films) with tetracaine (5 mM) is illustrated in Fig. 6. This concentration of tetracaine produced a massive increase in anisotropy, indicating that the anesthetic was causing increased order of the lipid molecules in the film. The effect of different concentrations of tetracaine on the *B/C* ratio is shown in Fig. 7. The threshold concentration of tetracaine was 0.8–1.0 mM, and a maximum ordering effect was reached at about 4 mM. Increasing further the concentration of drug caused a progressive decrease in the order of the films (Fig. 7). When these experiments were repeated using the 5-doxylstearic acid spin probe, corresponding changes in anisotropy between spectra taken in the parallel and perpendicular orientations were noted over the same range of tetracaine concentrations.

The ordering effect of tetracaine was reversible. Washing films which had been hydrated previously with tetracaine (2 mM) resulted in a decrease in anisotropy and ESR spectra indistinguishable from the controls (Fig. 6). The effect of pH on the ordering effect of tetracaine was investigated. In the concentration range 1–2

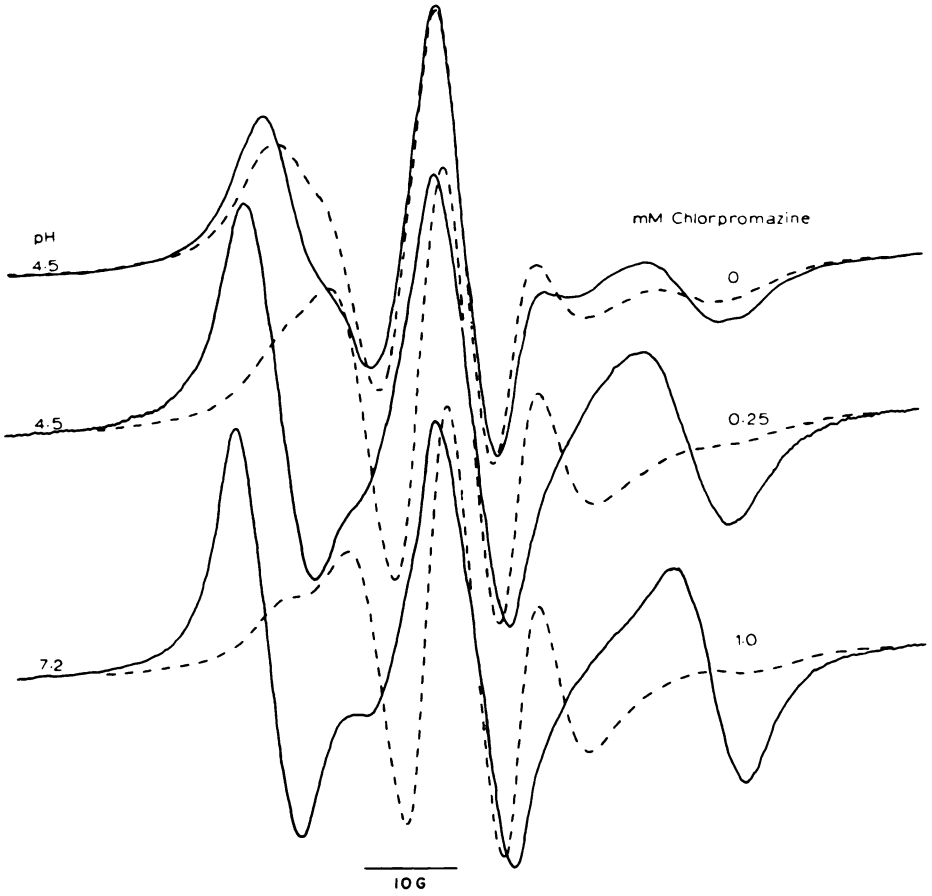

FIG. 5. *Spectra of 5-doxylstearic acid spin probe in multibilayer films of white matter lipids with reduced cholesterol concentration, hydrated with phosphate buffer 0.15 N in sodium*

The pH values and chlorpromazine concentrations were as noted. Low degrees of anisotropy were observed in the absence of chlorpromazine at any pH.

mM, tetracaine had about 2–3 times the ordering effect at pH 6.8 as at pH 7.4. When the ordering effect of tetracaine was measured as a function of concentration at several pH values, it was found that the maximal order varied with pH, but that the concentration of tetracaine yielding maximal order remained in the 2.5–5 mM range. Similar effects have been noted with procaine (26).

Mepivacaine, promazine, chlorpromazine, and pentobarbitone. These anesthetics also produced anisotropy in the low-cholesterol lipid bilayers. The effects of different concentrations of these drugs and tetracaine at pH 7.4 on the order of lipid bilayers are shown in Fig. 8. At pH

TABLE 2

Estimated order parameters for stearic acid spin probes in oriented multibilayers of decholesterolized brain lipids

Probe position	pH	Chloropromazine	S
		mM	
5	4.5	0	0.14
	4.5	0.25	0.35
	7.2	0	0.23
	7.2	0.25	0.27
	7.2	1.0	0.45
12	4.5	0	0.15
	4.5	0.20	0.22
	7.2	0	0.11
	7.2	0.8	0.23

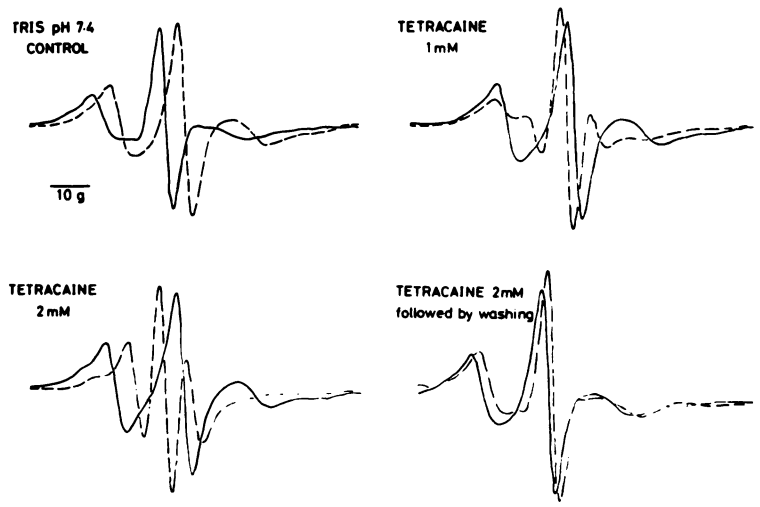

FIG. 6. *Spectra of CSL in low-cholesterol brain lipid films hydrated with (a) Tris-HCl buffer, pH 7.4 (control); (b) tetracaine, 1 mM; (c) tetracaine, 2 mM; (d) tetracaine, 2 mM, and then washed with buffer*

Solid and dashed spectra were taken with the film parallel and perpendicular to the magnetic field, respectively.

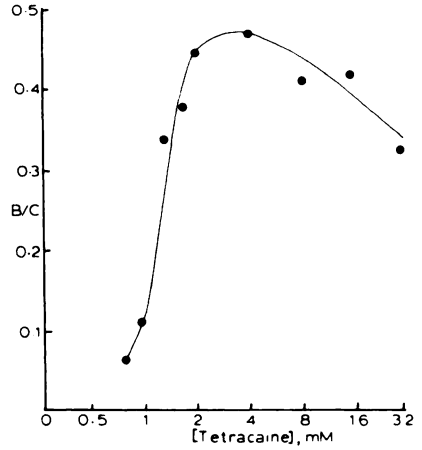

FIG. 7. *Effect of different concentrations of tetracaine on degree of order (B/C ratio) of low-cholesterol multibilayers produced from white matter lipids*

Sodium phosphate buffer, pH 6.8, 150 mM. Each point is the mean of three to six determinations.

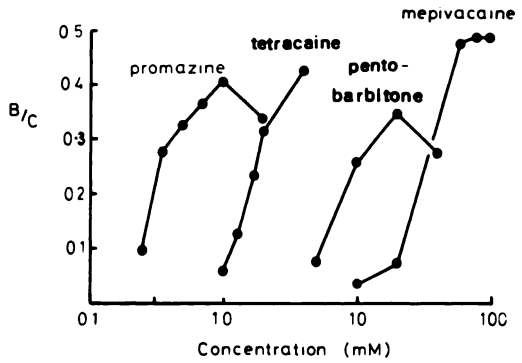

FIG. 8. *Effects of different anesthetics on order (B/C ratio) of low-cholesterol multibilayers produced from white matter lipids, using Tris-HCl buffer, pH 7.4, 5 mM, containing sodium chloride, 140 mM*

Each point is the mean of two to six results.

7.4 promazine was the most potent drug, producing half-maximal order (EC_{50}) at about 0.3 mM. Tetracaine, pentobarbitone, and mepivacaine were less potent, the EC_{50} values for these drugs being respectively 1.5, 7, and 30 mM. However, it should be stressed that these values give only an approximate indication of the relative potencies of the drugs, because in some cases the maximum response was poorly defined. The ordering effect of pentobarbitone was found to be completely reversible. The pH dependence of the ordering effect of chlorpromazine was investigated on several decholesterolized brain lipid preparations. It was found that the concentration required to yield maximal order increased from 0.25–0.5 mM at pH 4.5 to 1–2 mM at pH 7.2, whether measured as an increase in the *B/C* ratio with the cholestane spin probe or as an increase

in order parameter for the 5- (Fig. 5) or 12-doxylstearic acid probe (Table 2). Chlorpromazine, like the local anesthetics, was capable of disordering films formed from brain lipid with its normal cholesterol content when applied at much higher concentrations. Unlike the results with local anesthetics, increases in pH did not increase the disordering effect of chlorpromazine.

Effect of calcium ions. Calcium ions significantly potentiated the ordering effect of tetracaine (Fig. 9). Thus, in the presence of 5 mM calcium choloride, the order produced in multibilayers by tetracaine was approximately doubled over the concentration range for tetracaine of 1–2 mM. However, the maximum ordering effect of the anesthetic was not increased by the presence of calcium ions. A similar synergistic effect on the permeability of bilayers to sodium ascorbate has been detected by spin probes (37).

Calcium (5 mM) did not significantly potentiate the ordering effect of pentobarbitone (Fig. 9). In the absence of anesthetic, hydration with buffer (pH 7.4) containing calcium chloride (5 mM) did not produce any appreciable ordering.

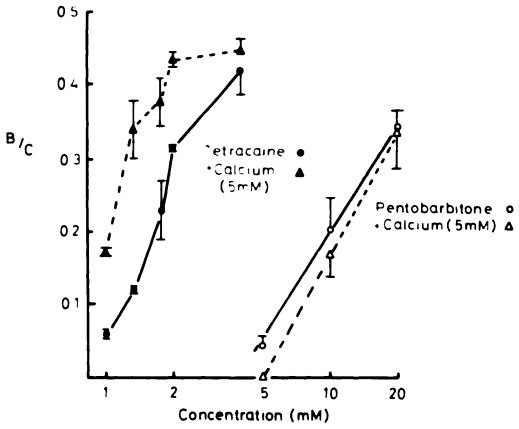

FIG. 9. *Effect of calcium (5 mM) on ordering effects (B/C ratio) of tetracaine and pentobarbitone*

The anesthetics were dissolved in Tris-HCl buffer, pH 7.4, 5 mM, containing sodium chloride, 140 mM. Each point is the mean of four to six determinations. The vertical bars are the standard errors of the means. ●, tetracaine; ▲, tetracaine plus calcium; ○, pentobarbitone; △, pentobarbitone plus calcium.

DISCUSSION

Cholesterol Effect

Although the influence of cholesterol has been investigated by a spin probe in a wide variety of lipids (8–14, 38), no system reported to date has shown as sharp a response to cholesterol as the brain lipid system described here which goes from extremely low order to essentially perfect order over a range of 3 gram % cholesterol. This is undoubtedly due to a delicate balance of intermolecular interactions resulting from the variety of phospholipid head groups, acyl chain lengths, and degrees of saturation. The combination of a high degree of order and low degree of mobility found at the natural cholesterol concentration (20 g/100 g) is quite unlike the situation found in most biological membranes. Normally one finds a balance of forces leading to intermediate degrees of order with rather high mobility. This has been referred to as the "fluid" state of the membrane bilayer (39). Phospholipid bilayers are very sensitive to perturbations by ions (23), proteins (40, 41), temperature (26), pH (26), and local (26) as well as general (27, 42) anesthetics. It is conceivable that the gel-like state of myelin lipids at physiological temperatures decreases the sensitivity of this system to such perturbations.

Anesthetic Effect

The present spin probe study confirms previous reports that in multibilayers of brain lipids with a low cholesterol content some anesthetics are able to produce an ordering effect of the lipid molecules; i.e., the anesthetics cause a tendency for the lipids to arrange their long axes perpendicular to the plane of the film (22, 26). The relative potency of the drugs in producing this ordering effect at pH 7.4 roughly paralleled their local anesthetic action: promazine > tetracaine > pentobarbitone > mepivacaine. The effectiveness in promoting order of all these drugs depended upon the pH of the hydrating medium. At a constant concentration, the amount of order promoted by either tetracaine or chlorpromazine declined when the pH was increased. With tetracaine, the concentration necesary to produce maximal ordering did not depend on pH. Further increases in

concentration at pH values led to a decrease, rather than an increase, in order. However, increasing the concentration of chlorpromazine at pH 7.2 above the range promoting optimal order at lower pH values did lead to an increase in order. Thus the concentration of chlorpromazine required for maximum ordering effect increases with increasing pH. The difference in ordering behavior of these two drugs is no doubt related to the fact that increasing the pH increases the effectiveness of tetracaine in disordering films formed of brain lipid containing normal cholesterol concentrations (26), but does not increase the disordering ability of chlorpromazine.

Although promazine, tetracaine, pentobarbitone, and mepivacaine produced ordering effects in the multilayers similar to those seen with increasing concentrations of cholesterol, the degree of order produced was never as great as that produced by cholesterol. The highest *B/C* ratio obtained with tetracaine was about 0.5, and further increasing the drug concentration produced a progressive decrease in the *B/C* ratio. These results suggest that the anesthetics have both ordering and disordering actions and that the onset of the disordering effect prevents the anesthetics from producing the very highly ordered films obtained with cholesterol. A disordering or fluidizing effect of anesthetics on various membranes has been reported in several studies (3, 27, 42–44). Also, Butler *et al.* (26) found that when films of white matter lipids containing their normal amount of cholesterol were hydrated with anesthetics in concentrations which produce order in the low-cholesterol films, no changes in the ESR spectra were observed, although higher concentrations of drugs caused a disordering effect. Presumably the lack of ordering effect under those circumstances was due to the high degree of order initially present in such films. Local anesthetics at anesthetic dose levels do not alter the resting potential of the nerve (45, 46). They may act by stabilizing the nerve membrane, preventing the conduction changes necessary for the propagation of action potentials (37).

The ordering effects of both tetracaine and pentobarbitone on the multilayers were reversible, and washing the anesthetic-treated low-cholesterol films produced ESR spectra identical with those of the controls. However, once the films were hydrated with buffer alone, rehydrating them with buffer containing anesthetic had only a minimal ordering effect. This presumably was due to a decreased permeability of the bilayers, once hydrated, to the local anesthetic. A similar observation has been reported for the ordering effect of polylysine (41).

Calcium ions had a striking effect on the concentration-response curve of tetracaine. At low concentrations of tetracaine, which produced very little order in the films, calcium (5 mM) increased the order at least 3-fold. Although calcium ions are known to stabilize membranes, and have been shown by ESR spectroscopy to increase the order in lipid multilayers (23), the concentration used in the present study (5 mM) did not produce any observable effect on the spectra of the low-cholesterol films. The simplest explanation of these results is that in the absence of tetracaine, in the low-cholesterol films, the lipid molecules are too far apart or too randomly distributed for interaction with the calcium ions. However, with increasing order as a result of tetracaine effects, the calcium ions are able to exert an additional stabilizing effect by interaction with the anionic head groups of 2 or more neighboring lipid molecules. However, this does not explain why the effect of pentobarbitone was not potentiated by calcium ions. It seems unlikely that in the present lipid system calcium ions compete with tetracaine molecules for single anionic sites in the bilayers, since increasing the calcium concentration to 50 mM, which in the absence of tetracaine had little effect on the degree of order in the bilayers, did not antagonize the ordering effect of the anesthetic.

Other studies of the interaction of drugs with membranes using the spin label technique have employed a variety of model systems (22, 26, 27, 42, 44, 47). However, it is not yet clear which particular model system is most suitable for studies of drugs. Although defined model membranes have obvious advantages, it was

decided in the present experiments to use extracts of brain lipid, since it seemed possible that multilayers produced from such a source might be more likely to reflect the action of drugs in the mammalian nervous system. It has been possible to demonstrate an ordering effect of anesthetics in multibilayers which were artificially disordered by the removal of the cholesterol which is normally present in brain lipids. Their effects on normal membranes may be more subtle.

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