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Lipid Protein Interactions in Mitochondria. VIII. Effect of General Anesthetics on the Mobility of Spin Labels in Lipid Vesicles and Mitochondrial Membranes

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Abstract

We have studied the effect of general anesthetics on the mobility of two stearic acid spin labels (5-doxyl stearic acid and 16-doxyl stearic acid) in bovine heart mitochondria and in phospholipid vesicles made from either mitochondrial lipids or commercial soybean phospholipids. The general anesthetics used include nonpolar compounds (alcohols, halothane, pentrane, diethyl ether, chloroform) and the amphipathic compound, ketamine. All anesthetics tested increase the mobility of the spin labels in phospholipid vesicles to a limited extent up to a concentration where the ESR spectra become those of free spin labels. On the other hand, anesthetics have a pronounced effect on mitochondrial membranes at concentrations as low as those known to produce general anesthesia; the effect is lower near the bilayer surface (5-doxyl stearic acid) and very strong in the bilayer core (16-doxyl stearic acid). The effects of anesthetics are mimicked by the detergent, Triton X-100. We suggest that the discrepancy between the action of anesthetics in mobilizing the spin labels in lipid vesicles and in membranes results from labilization of lipid protein interactions.

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Introduction

During the course of our investigations on lipid protein interactions in biomembranes (1–5), we have studied the effect of the series of *n*-alkanols on membrane lipid fluidity, probed by stearic acid spin labels and by the fluorescent probe ANS. We have found that alcohols enhance the fluidity of membrane lipids; in particular, the spin label studies have given strong indications that alcohols disrupt lipid protein interactions (5), since they appear to abolish the immobilization induced by intrinsic membrane proteins on the lipid bilayer.

A consequence of this effect was the finding that alcohols induce kinetic changes in the mitochondrial ATPase, which have been attributed to changes in membrane lipids (4, 6, 7).

Alcohols are usually included among general anesthetics (8); we have therefore considered it of interest to investigate whether compounds belonging to the class of clinically useful anesthetics induce the same or similar changes as those induced by alcohols. The Meyer rule of anesthesia (9) states that narcosis is the result of attaining a certain molar concentration of any chemically inert substance in the cellular lipids; according to Hill (10), this will increase the entropy of the system, and anesthesia is the result of this entropy increase.

It has been found that anesthetics increase the fluidity of model lipid membranes (8, 11, 12); it is therefore plausible that anesthesia is the result of a change in lipid fluidity of neuronal membranes involved in the transmission of nerve impulses (13).

It is well known, however, that anesthetics act on all membranes (8) although local quantitative differences may be present. For this reason it is customary to investigate physical changes induced by anesthetics on several non-neuronal membranes, e.g., erythrocyte ghosts. In such membranes, expansion by anesthetics has been directly related to a fluidity increase.

We have advanced a working hypothesis (13), based on the results obtained with *n*-alkanols, that anesthetics change the physical state of membrane lipids leading to an alteration in normal lipid protein interactions, which in turn induces conformational changes in membrane proteins. Such changes in neuronal membranes may be directly related to the mechanism of anesthesia. A similar model has been postulated by Lee (14) for the action of local anesthetics at the level of the sodium channel in nerve membranes. There are data in the literature that anesthetics affect lipid fluidity; such data are obtained mainly on lipid bilayer vesicles or oriented multibilayers (11, 12, 15). Few data are available on natural membranes, and there are indirect indications that natural membranes may have a quantitatively different

behavior. For example, the expansion of lipid vesicles caused by anesthetics is much less than that of a natural membrane (16), suggesting that proteins are largely involved in this effect. Moreover, Augustin and Hasselbach (17), using the fluorescent probe ANS, have questioned whether anesthetics affect lipids primarily, and they have indicated membrane proteins as the primary site of action of anesthetics. That the primary site of action of anesthetics is represented by membrane proteins has also been suggested by others (18–20). Many data are also available on effects of anesthetics on the conformation of nonmembrane proteins (21, 22). The problem of the primary site of action of anesthetics is still largely unsolved.

In addition, often the data in the literature concern scattered examples of different anesthetics in different studies, and direct comparisons are not always available. Furthermore, little is known of the effects exerted on membranes by ketamine (2-chlorophenyl-2-methylaminocyclohexanone) which is a powerful anesthetic in many respects different from the inhalation anesthetics or the barbiturates more usually investigated. Ketamine, contrary to inhalation anesthetics, which are largely hydrophobic, is an amphipathic molecule which is charged at physiological pH. For this reason we have investigated the effect of selected compounds belonging to the category of anesthetics on the lipid phase of both lipid bilayer vesicles and natural membranes, in order to establish a comparison and to directly assess the influence of membrane proteins.

The lipid phase was probed by using both spin lables (23), which have paramagnetic groups producing characteristic ESR spectra sensitive to the molecular order, viscosity, and polarity of their environment, and fluorescent probes (24), which give emission spectra also sensitive to the characteristics of their microenvironment. The probes were chosen so that the lipid bilayer could be investigated at different depths, according to the location of the chromophores and the polarity of the molecules.

The spin labels used were two derivatives of stearic acid, having the nitroxide paramagnetic groups in the 5 and 16 positions, respectively.

This paper describes the results obtained with a series of anesthetics on the mobility of spin labels in lipid vesicles and in mitochondrial membranes as a model system.

Materials and Methods

Preparative Procedures

Bovine heart mitochondria (BHM) were prepared by a small-scale procedure (25) and submitochondrial particles ETP by sonication (26).

Lipid vesicles were prepared from soybean phospholipids (Asolectin from Associated Concentrates, Long Island, New York) or from mitochondrial phospholipids extracted according to Folch Pi et al. (27) and purified according to Marks et al. (28), using a Branson sonifier at full power for a total time of 8–12 min under nitrogen (29).

Spin Label Studies

The spin labeles used were 5- or 16-N-oxyl 4′,4′-dimethyloxazolidine(doxyl)derivatives of stearic acid (abbreviated 5-NS and 16-NS, respectively) obtained from Synvar Co., Palo Alto, California. The experimental details about spin label studies were described in a previous paper (5). The ESR spectra were recorded by using a Varian E-4 spectrometer at 20°C (microwave frequency, 9.52 GHz; amplifier gain, 1.6×10^3; modulation amplitude, 1 G; time constant, 0.3–1.0). The incorporation of spin labels into the membranes was accomplished as described previously (5); the phospholipid to spin label molar ratio was about 100:1. Under such conditions controls showed that all the label was incorporated into the membranes.

The freedom of motion of spin labels in the membranes was calculated by measuring the following parameters. The pseudoisotropic rotational correlation times τ_c were calculated (30) for 16-NS by using the following equation:

$$\tau_c = 6.5 \cdot 10^{-10} W_0 \left[(h_0/h_{-1})^{1/2} - 1\right]$$

where W_0 is the width of the medium field line, and h_0 and h_{-1} are the heights of the medium and high field lines, respectively. The values of τ_c calculated from the spectra were valid within 10.5×10^{-10} sec, as shown by results obtained by repetition of the spectra. The equation is valid only in the rapid tumbling limit, i.e., $<10^{-9}$ sec (31). The empirical ratio h_0/h_{-1} was often taken as an indication of rotational freedom (32).

The spectra with 5-NS, which probe the outer region of the bilayer, exhibit probe ordering proportional to the separation of the two hyperfine extrema; the maximal splitting between the hyperfine extremes ($2T_{11}$ or $2T_m$) was usually taken as a measure of the freedom of motion of the label in the membrane (33); in our experimental conditions $2T_m$ was significant within the limit of 0.5 G.

The EPR spectrum of a spin label in water or buffer shows high rotational mobility; incorporation in lipid bilayers gives rise to increased τ_c and increased $2T_m$; incorporation into membranes induces larger immobilization, according to the known effect of membrane proteins on lipids (34–37).

Analytical Procedures

Protein was determined by a biuret method (38). Phosphorus was tested according to Marinetti (39).

Materials

The anesthetics used in this study were the following: halothane (2-bromo-2-chloro-1,1-trifluorethane) purchased from Hoechst A.G.; pentrane (2-2-dichloro-1,1-difluoro ethyl methyl ether) purchased from Habbot; ketamine (2-chlorophenyl-2-methylamino cyclohexanone) purchased from Parke-Davis; *n*-butanol and chloroform purchased from Merck.

The anesthetics were added directly into stoppered tubes containing the spin-labelled membranes by means of Hamilton microsyringes and incubated for 30 min with shaking.

Results

Effects of Neutral (Solvent) Anesthetics

Figures 1–4 show selected effects of hydrophobic anesthetics (halothane, pentrane, chloroform among others studied) on the mobility of 5-NS and 16-NS in lipid vesicles of Asolectin and mitochondrial membranes. The series of anesthetics studied induce similar effects, although elicited at different concentrations. The results are summarized in Tables I and II.

The results with commercial soybean phospholipids and phospholipid extracted from mitochondria are superimposable (Table III) and show only moderate effects induced by the volatile anesthetics. On the other hand, the ESR spectra of BHM show strong increases of mobility of the spin labels induced by all anesthetics. Comparison of the effect of each anesthetic in lipid vesicles and in mitochondrial membranes indicates that the anesthetic makes the spectra of spin labels in protein-containing membranes quite similar to those of protein-free vesicles. There are, however, individual differences among different anesthetics. High anesthetic concentrations induce large increases of probe mobility in both vesicles and membranes, and the spectra may indicate extraction of the label from the membrane. The effects of all anesthetics are quantitatively different near the bilayer surface (5-NS) and in the hydrophobic core (16-NS): in general, the difference between vesicles and membranes is more pronounced for 16-NS than for 5-NS.

Calculations of the isotropic hyperfine splitting constants (a') and of the

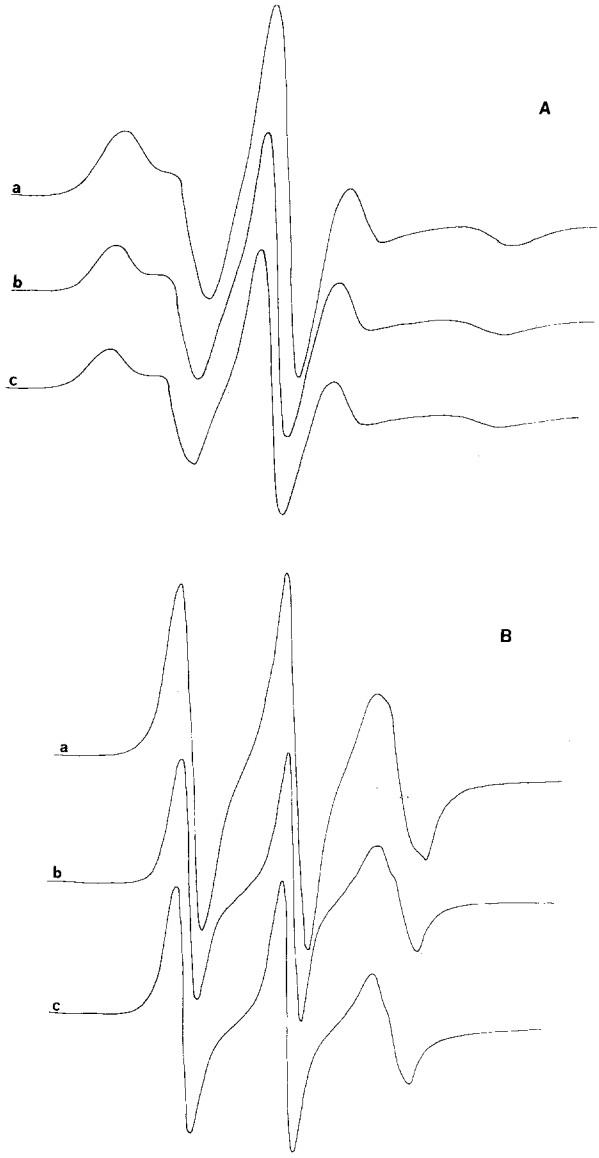

Fig. 1. Effect of halothane on the ESR spectra of 5-NS (A) and 16-NS (B) in BHM. (a) Control; (b) halothane, 0.37 mM; (c) halothane, 1 mM.

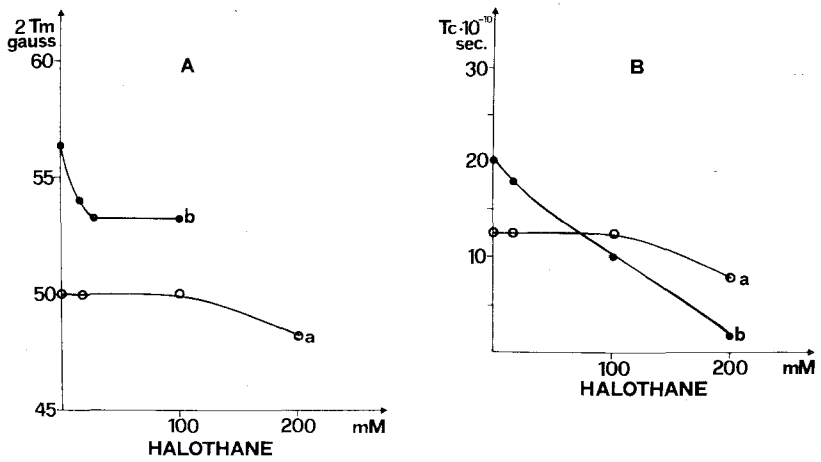

Fig. 2. Effect of halothane on lipid mobility in Asolectin vesicles and BHM. (A) 5-NS: (a) Asolectin; (b) BHM. (B) 16-NS: (a) Asolectin; (b) BHM. The mobility was measured as $2T_m$ for 5-NS and as τ_c for 16-NS.

order parameters (S_n) (40) for 5-NS showed that the decreases of $2T_m$, when present, were not due to polarity increases, but only to increase of fluidity.

The concentrations of anesthetics used in this comparison are of the order of magnitude of those inducing local anesthesia (4). In accordance with the work of Boggs et al. (41), we do not see any "fluidization" of lipid bilayers

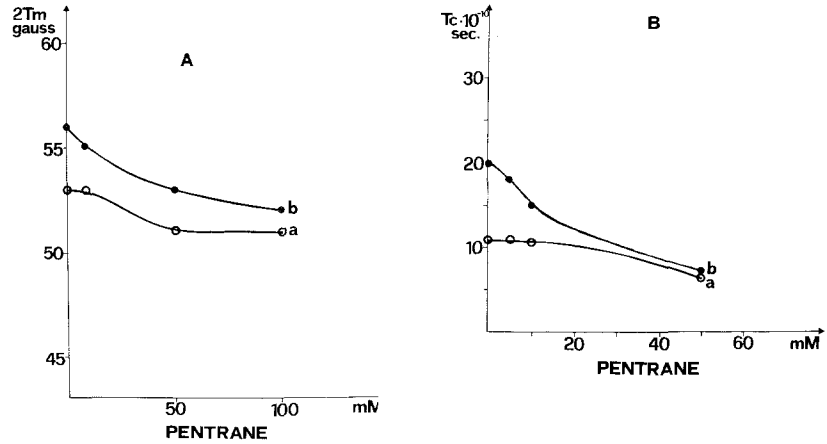

Fig. 3. Effect of pentrane on lipid mobility in Asolectin vesicles and BHM. (A) 5-NS: (a) Asolectin; (b) BHM. (B) 16-NS: (a) Asolectin; (b) BHM. See caption to Fig. 2.

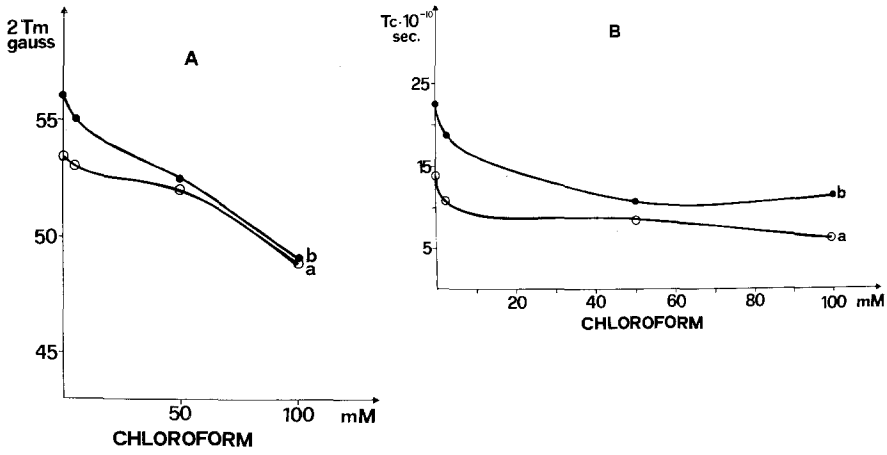

Fig. 4. Effect of chloroform on lipid mobility in Asolectin vesicles and BHM. (A) 5-NS: (a) Asolectin; (b) BHM. (B) 16-NS: (a) Asolectin; (b) BHM. See caption to Fig. 2.

Table I. Effect of Anesthetics on the Mobility of 5-Doxyl Stearic Acid Spin Labels in Phospholipid Vesicles and Membranes

Anesthetic	Concentration, mM	5-NS Change in $2T_m$ (G)[a]	
		Vesicles	BHM
Butanol	50	−3	−2
	150	−4	−6
Halothane	10	0	−2.5
	100	0	−3.1
Pentane	10	0	−1
	100	−1.7	−3.5
Chloroform	50	−1.5	−3.5
	100	−5.7	−7
Ketamine	20	−0.5	−0.7
Triton X-100	2% (v/v)	−1	−2.5
	10% (v/v)	−3.5	−7.5

[a]Controls were 50.5 G in lipid vesicles and 54.5 G in mitochondria.

Table II. Effect of Anesthetics on the Mobility of 16-Doxyl Stearic Acid Spin Labels in Phospholipid Vesicles and Membranes

Anesthetic	Concentration, mM	16-NS Change in τ_c (sec · 10^{-10})[a]	
		Vesicles	BHM
Butanol	50	−3	− 6.5
Halothane	10	0	− 2.5
	100	0	−11
Pentrane	10	0	− 6
	100	−4.5	−13
Chloroform	50	−4.5	−11.5
	100	−6.5	−11
Ketamine	20	−3	− 4.5
Triton X-100	2% (v/v)	−2.5	− 6.5
	10% (v/v)	−1	− 7.5

[a]Controls were 12.48 sec · 10^{-10} for lipid vesicles and 20.4 sec · 10^{-10} for BHM.

at these or even greater concentrations; the effect in mitochondrial membranes is, however, quite clear. We have subsequently screened the range of concentrations known to produce general anesthesia (4), and the results are shown in Table IV: the motion increase is already clearly shown at concentrations as low as 5 mM for butanol, 0.5 mM for chloroform, and 0.37 mM for halothane (these concentrations are lower than those inducing a 50% decrease of the righting reflex in the newt).

Table III. Effect of Anesthetics on the Mobility of Stearic Acid Spin Labels in Mitochondria and Phospholipids Extracted Therefrom

Anesthetic	Concentration, mM	5-NS Change in $2T_m$ (G)		16-NS Change in τ_c(10^{-10} sec)	
		Extracted lipids	BHM	Extracted lipids	BHM
Butanol	50	−0.5	−2	−0.3	− 6.5
	100	−0.5	−4	−0.5	− 8
Halothane	10	−0.5	− 2.5	0	− 2.5
	100	−1.75	−3.1	−2.7	−11
Ketamine	8×10^{-2}	−0.25	0	−0.1	0
	4.8×10^{-1}	−0.25	−0.7	−0.3	− 4.5

Table IV. Effect of Anesthetics on the Mobility of Stearic Acid Spin Labels in Mitochondria

Anesthetic	Concentration, mM	5-NS Change in $2T_m$ (G)	16-NS Change in τ_c (sec · 10^{-10})
Butanol	5	0	−1.3
	10	−0.5	−2.3
	20	−1	−2.6
Ketamine	8×10^{-2}	−0.5	−1.3
	4.8×10^{-1}	−1	−2.7
Halothane	0.37	−1	−1.1
	5	−2	0
	10	−3.5	−4.1
Chloroform	0.5	−0.5	−2.2
	1	−0.5	−2.4
	2	−1	−3.1

The effects of the different anesthetics are related to both anesthetic hydrophobicity and to their partition coefficients between water and lipids. The first point was shown in a previous paper (5) for the series of alcohol homologs from methanol to pentanol.

Ketamine

The fluidizing effect of this amphipathic cationic anesthetic is more evident in the bilayer core (16-NS) as in the case of neutral anesthetics; there is, however, a smaller, but appreciable difference between lipid vesicles and mitochondrial membranes, indicating a more indirect fluidizing effect independent of the presence of proteins (Fig. 5). The quantitative values for ketamine are also shown in Tables I–IV.

Studies with Detergents

Certain detergents act preferentially by detaching lipids from proteins, e.g., Triton X-100 (42). In order to compare the results obtained with anesthetics, we have considered it of interest to investigate the action of certain detergents in lipid vesicles and in membranes. It has to be pointed out, however, that detergents are classified as general anesthetics by Seeman (8). The results with Triton X-100 are shown in Fig. 6. The effect of the detergent on the mobility of spin labels is strikingly similar to that shown by anesthetics; in particular, the fluidizing effect is stronger in membranes than in lipid

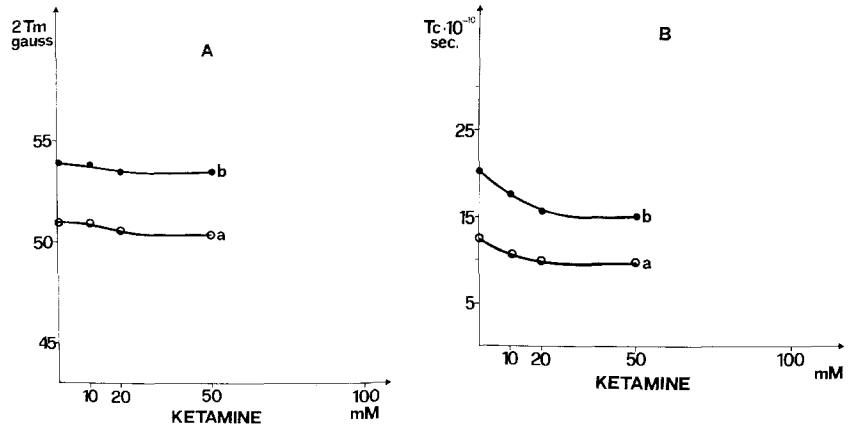

Fig. 5. Effect of ketamine on lipid mobility in Asolectin vesicles and BHM. (A) 5-NS: (a) Asolectin; (b) BHM. (B) 16-NS: (a) Asolectin; (b) BHM. See caption to Fig. 2.

vesicles. Also for Triton X-100 the quantitative values are shown in Tables I and II.

The EPR spectra of spin labels in these experiments do not result from incorporation of the spin label into free Triton micelles, since in that case we would expect comparable results in phospholipid vesicles and in membranes. Apparently, at the concentrations used, no free micelles are formed.

Discussion

The results of this study suggest that the general anesthetics investigated induce a fluidization of biological membranes and disrupt lipid protein interactions.

An immobilizing effect of proteins on membrane lipids has been observed in several membranes. The studies of Jost et al. (34) for cytochrome oxidase, showing a layer of strongly immobilized boundary lipids surrounding the protein, have been confirmed by several authors for other integral proteins in different membranes (35–37). The boundary lipids do not undergo the normal phase transition (40–43); they are in a very viscous, although disordered state (44); their precise significance and their extension far away from the protein are not clarified. According to Seelig and Seelig (44) the *disordering* effect of the protein on the surrounding lipids is exerted on three layers within a time of 10^{-3} sec. We have observed a similar immobilization of the residual lipids of lipid-depleted mitochondria (6) after acetone extraction.

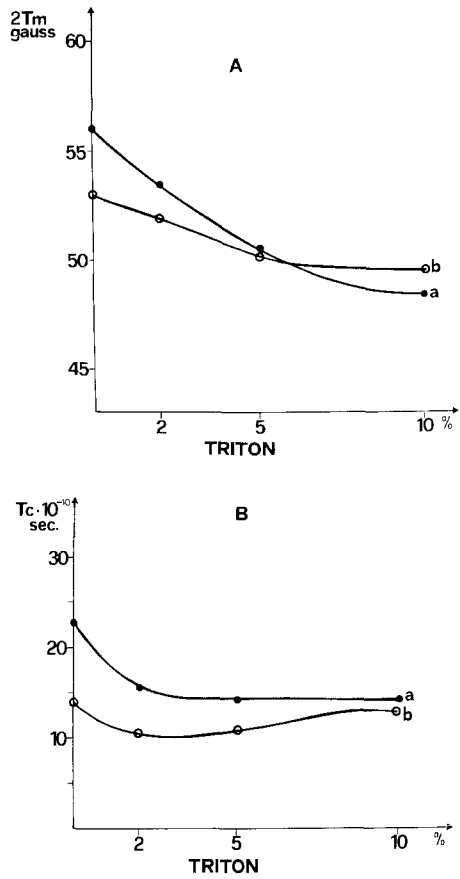

Fig. 6. Effect of Triton X-100 on lipid mobility in Asolectin vesicles and BHM. (A) 5-NS: (a) Asolectin; (b) BHM. (B) 15-NS: (a) Asolectin; (b) BHM. See caption to Fig. 2.

The results of addition of anesthetics to different membranes confirm what we have previously seen with the series of *n*-alkanols (5). Anesthetics have a moderate fluidizing effect on phospholipid vesicles, as indicated by the decrease of the rotational correlation times of 16-NS and of the hyperfine splitting of 5-NS. Consistent with this, Paterson et al. (15) found that aliphatic alcohols produce changes in the structural arrangement of phospholipids in oriented multibilayers. It was found that halothane and diethyl ether also enhance rotational mobility of spin labels in lipid bilayers (45). Boggs et

al. (41), however, find that the fluidization of lipid bilayers is produced only at much higher concentrations than those required to produce anesthesia.

On the other hand, in our study, we find that the anesthetics tested strongly enhance the mobility of phospholipids in natural membranes (mitochondria) containing their protein complement. The effects were exerted by different anesthetics at different concentrations; however, in all cases the effect was relatively stronger in mitochondrial membranes than in lipid vesicles, indicating that proteins are involved in this effect. The concentrations of anesthetics found to increase the mobility of spin labels in mitochondrial membranes are quite low, and in the range known to produce general anesthesia (41). As in the case of alcohols previously described (5), we believe that anesthetics abolish the immobilization induced by membrane proteins on phospholipids; in other words, in our interpretation, anesthetics labilize lipid protein interactions and fluidize the boundary layer of lipids. The differences are not due to the types of lipids used, since mitochondrial phospholipids behave as Asolectin in undergoing only a slight fluidization by anesthetics (cf. Table III and Ref. 5). Therefore, a decrease of rigidity only in membranes may be thought of as the result of higher escape or exchange of phospholipids from the annulus, as postulated by Hesketh et al. (46) in Ca-ATPase by benzyl alcohol: in other words, a labilization of lipid protein interaction.

It could be thought that anesthetics enhance the partition of spin labels from immobilized to fluid areas of the lipid bilayer, in accordance with the notion that fatty acid spin labels are preferentially incorporated in fluid rather than crystalline lipids (47). However, immobilized lipids are not crystalline (14). Furthermore, in experiments of Jost et al. (34), a steroid spin label, which according to Butler et al. (48) is equally distributed in all membrane areas, gives superimposable results in comparison with the stearate labels.

The idea that anesthetics labilize lipid protein interactions is strengthened by the observed effect of detergents which are known to break such interactions (42); a similar stronger fluidizing effect in membranes than in bilayers has been observed upon addition of Triton X-100.

Many organic solvents (and anesthetics), at high concentrations, effectively extract lipids from membranes (49). The effects of low anesthetic concentrations is apparently quantitatively, and not qualitatively, different. Anesthetics at very low concentrations may dislocate integral proteins from their natural interactions within the lipid bilayer; we are of the opinion that this effect, in neuronal membranes, is related to the phenomenon of anesthesia. A slight dislocation of proteins within the hydrophobic lipid milieu, by changing their environment, may induce conformational changes which will

reflect strongly on their particular functions; if the transmission of nervous impulses is linked to protein channels in neuronal membranes (50–52), it is conceivable that conformational changes in the channels would lead to impairment of nerve transmission (53).

Acknowledgments

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