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EFFECTS OF GENERAL ANESTHETICS ON LIPID PROTEIN INTERACTIONS AND ATPASE ACTIVITY IN MITOCHONDRIA

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Abstract—We have investigated the effect of general anesthetics (the normal alcohol series up to pentanol, halothane, pentrane, diethyl ether and ketamine) on lipid fluidity of phospholipid vesicles and mitochondrial membranes and on the kinetics of mitochondrial ATPase, a lipid-requiring enzyme.

Lipid fluidity has been studied by using spin labels (5- and 16-doxyl stearic acids) and the fluorescent probe *N*-phenyl naphthylamine (NPN). The spin labels show that all anesthetics tested have a slight fluidizing effect on lipid vesicles but induce a very strong increase in mobility in mitochondrial membranes. Similar effects have been found by analysing the decrease in fluorescence of NPN induced by anesthetics: the decrease is due to decreased quantum yield as shown by double reciprocal plots of probe fluorescence against membrane concentration. The fluorescence decrease is interpreted as an increase in fluidity of the bilayer and not as an increase in polarity of the probe environment, since there is no significant shift in the fluorescence spectra induced by anesthetics. The effects are stronger in mitochondrial membranes than in lipid vesicles, showing a labilization of lipid–protein interactions induced by anesthetics.

The mitochondrial ATPase is inhibited by anesthetics at concentrations of the order of those found to affect lipid–protein interactions. The inhibition appears usually uncompetitive with respect to ATP, with a decrease of both V_{max} and K_M, indicating a possible stabilization of the enzyme–substrate complex. Arrhenius plots of ATPase activity show a striking increase in activation energy below 17–20°; anesthetics affect the temperature dependence in a variable way depending on the specific anesthetic used; most increase the activation energy above the break or abolish the break completely. Anesthetics make the ATPase insensitive to the energy transfer inhibitor oligomycin. At low anesthetics concentration the oligomycin inhibition curve is changed from sigmoidal to hyperbolic, showing a loss of cooperativity in the inhibition.

It has long been recognized that the activity of general anesthetics is related to their hydrophobicity, i.e. it increases with increasing oil/water partition coefficient [1, 2]; according to Meyer [2], narcosis results when any chemically indifferent substance has attained a certain molar concentration in the cell lipids.

It has been found that anesthetics enhance the fluidity of model lipid membranes [3–7]; the idea that anesthesia is the result of a change in lipid fluidity of neuronal membranes involved in nerve-impulse propagation has been therefore advanced. Hill [8] has theoretically shown that the concentration of anesthetic increases the disorder or entropy of the membrane and has generalized the Meyer rule in the following way: anesthesia will be produced when the free energy of a non-aqueous phase has been changed by a critical amount, independent of the method used to change free energy itself.

It was postulated very early that the action of anesthetics is a general phenomenon not confined to specific nervous membranes, but it is in nervous membranes that the consequences of such changes will give rise to phenomena resulting in anesthesia.

It is generally assumed that general anesthetics act by depressing synaptic transmission within the central nervous system [9, 10]. Field potential studies [11] suggest that all anesthetics decrease synaptic transmission by interference with the process of chemical transmission, either by reducing the output of transmitter from the presynaptic nerve terminals or by reducing the sensitivity of the postsynaptic membrane to the released transmitter or by both effects.

The recognition that anesthetics perturb all membranes allows the possibility to use model systems for the study of the molecular events associated with anesthesia.

We have previously enunciated the working hypothesis that general anesthetics, through perturbation of membrane fluidity, may induce conformational changes in membrane proteins, which are on the basis of changes in the catalytic power of membrane enzymes or carriers [12]. A similar model has been postulated by Lee [13] for the action of local anesthetics at the level of the sodium channels in nerve membranes: an increase in fluidity of the surrounding lipids allows the channels to close.

In this investigation we use mitochondrial membranes as a model system and produce experimental evidence that anesthetics induce a disorganization of lipid–protein interactions. The evidence is supported by (a) the differential sensitivity of mitochondrial membranes and artificial lipid vesicles to perturbation

by anesthetics, probed by ESR spin labels and fluorescent probes, and (b) the changes in the kinetics of a lipid-dependent membrane enzyme, the mitochondrial Mg^{2+}-activated ATPase. We also discuss the available data as indicating a conformational change of the enzyme which is secondary to primary changes in the lipid environment. Conformational changes were proposed in the action of anesthetics by other authors [14, 15] but were considered as directly produced by anesthetics on the membrane proteins [16, 17].

Mitochondrial ATPase is a very complex enzyme system consisting of an extrinsic portion (F_1) [18] containing the catalytic site for ATP hydrolysis, and an intrinsic portion [19], which are linked together by a small protein called OSCP (oligomycin sensitivity conferring protein) [20]. The enzyme undergoes an interesting phenomenon called allotopy [21]; the isolated F_1, which is water-soluble, is active in absence of lipids and does not bind lipids, is cold-labile and insensitive to the energy-transfer inhibitors, oligomycin and dicyclohexyl carbodiimide (DCCD) [18, 22]. The membrane-bound enzyme requires phospholipids for activity, is cold-stable, and is inhibited by oligomycin and DCCD. The above inhibitors do not bind to F_1 but to intrinsic polypeptide chains in the ATPase complex [19, 23]. The conformation and environment of ATPase in the intact membrane must therefore be different from the conformation in isolated F_1.

Lipid requirement for a membrane-bound enzyme. according to our hypothesis [12] may be the result of inducing in the enzyme the optimal conformation for its activity. The choice of ATPase is valuable in our opinion, because this enzyme has many important parameters which can be tested in relation to its lipid environment, and the enzyme is relatively well known in its kinetic and structural properties [16, 17, 24].

For the investigation of the physical state of lipids we have used both spin labels [25] and fluorescent probes [26], which have been widely employed in the literature as extrinsic probes of both molecular motion and polarity of their environment.

To this purpose, we have probed two different depths of the lipid bilayer by using stearic acid derivatives having paramagnetic nitroxide groups at positions 16 and 5 respectively, and the fluorescent probe, *N*-phenyl naphthylamine (NPN), which is located in the hydrophobic interior of the membrane.

We have found that anesthetics affect the mobility of the probes to a greater extent in membranes than in lipid vesicles, suggesting a perturbation of the lipids that are in closer contact to proteins.

As for ATPase activity and kinetics, anesthetics have effects comparable with those of lipid depletion by means of phospholipase A_2, described elsewhere [28, 29]. We believe that changes in the kinetic properties of the enzyme are related to conformational changes in its active site. Preliminary reports of part of this study has been presented elsewhere [28, 30].

EXPERIMENTAL METHODS

Preparative procedures. Beef heart mitochondria (BHM) were prepared by a small scale procedure [31] and submitochondrial particles ETP by sonication with a Branson sonifier with the method of Beyer [31]. Lipids were extracted from BHM with the procedure of Folch *et al.* [33] and phospholipids were purified from the total extract [34]. Lipid vesicles were prepared [35] from soybean phospholipids (Asolectin) or mitochondrial phospholipids using a Branson sonifier under nitrogen at full power for a total time of 8–12 min.

Spin label studies. The spin labels used were 5- or 16-*N*-oxyl-4',4'-dimethyloxazolidine derivatives (doxyl) of stearic acid (designated 5-NS and 16-NS, respectively) obtained by Synvar. The labels were added to lipid vesicles corresponding to 150 μg of phosphorus, or membranes (2.6 to 7.5 mg of particle protein), taking care that the label to phospholipid molar ratio was in the range of 1:100. To avoid reduction of the nitroxide, ensuing in contact with mitochondrial membranes, all the samples contained 5 mM K ferricyanide. Under these conditions the ESR signals were stable for many hours. Several controls have shown that incorporation of the label was complete and no free label was detectable (either in supernatant after centrifugation or by examination of the typical signals of unincorporated label).

The ESR spectra were recorded by using either a DECCA Radar XI Spectrometer with a Newport Instruments 7-in magnet or a Varian E-4.

The freedom of motion of the spin labels in the membrane was calculated as discussed previously [36]. The rotational correlation times τ_c can be calculated only in the rapid tumbling limit ($\tau_c < 10^{-9}$ sec) [37], and therefore were applied only in the case of 16-NS. For 5-NS which is more immobilized according to the fluidity gradient in the membrane described by several authors [38], the splitting between the hyperfine extremes ($2T_{\parallel}$) was taken as a measure of the freedom of motion of the label in the membrane.

The spectrum of a spin label in buffer shows a high rotational mobility, evidenced by three symmetrical peaks at increasing magnetic fields; incorporation in lipid bilayers shows a spectrum characterized by a decreased τ_c and a decreased hyperfine splitting. Incorporation in membranes induces spectra showing even larger immobilization.

Fluorescence studies. *N*-phenyl-naphthylamine (NPN) was added to different membranes at a concentration of 5×10^{-5} M. Fluorescence was measured with a Hitachi–Perkin Elmer spectrofluorimeter Model MPF 3, using excitation at 345 nm and emission at 425 nm. The fluorescence spectra were recorded after excitation at the above-mentioned wavelengths and corrected for light scattering (scanning without probe addition). The background to be substracted was usually very low. The fluorescence of NPN is strong in hydrophobic media, but is largely quenched in water with a red shift in the emission maximum [39]. For this reason, NPN and other fluorescent probes have been widely used to probe the polarity of their environments when bound to protein molecules or membranes [39, 40]; their fluorescence is also sensitive to the viscosity of the medium [27, 41, 42] and has been used to detect phase changes in lipid vesicles and in membranes [27].

For fluorescence studies, the concentration of phospholipid vesicles was 40 μg of lipid P/ml and that of membranes of 0.5 mg of protein/ml.

The interaction of fluorescent probes with the

membrane systems has been considered as a partition phenomenon [12, 43, 44]. It has been suggested that such interaction does not involve binding to specific membrane sites, but rather partition between aqueous and membrane phases. According to this view, the pattern of a double reciprocal plot of fluorescence against membrane concentration is linear in the case of a partition phenomenon and the intercept at the ordinate gives the value of $1/F_0$ (maximal fluorescence at infinite membrane concentrations, which is a function of the quantum yield of the probe in the membrane when all probe is bound). For operational purpose, such plots are best suited for expression of fluorescence yields under our experimental conditions.

ATPase activity. ATPase activity was assayed in two ways. The current procedure was the colorimetric assay already described [45]. For the determination of K_M, since we are measuring very low activities at low substrate concentations, we have preferred a titrimetric method by titration of H^+ produced by using a pH-stat Radiometer. ATPase activity in presence of volatile anesthetics (halothane and pentrane) was assayed by adding different proportions of the anesthetics in nitrogen by means of appropriate vaporizers (Fluotec Mark 3 and Pentec).

Analytical procedures. Lipid phosphorus was determined according to Marinetti [46] and protein either with a biuret method [47] or according to Lowry *et al.* [48].

RESULTS

EPR Studies. The series of anesthetics studied induce similar effects on liposomes and mitochondria, and are summarized in Table 1. As an example, Fig. 1 shows the effect of increasing halothane concentrations in vesicles and membranes.

The effects of the different anesthetics are strikingly similar qualitatively, although they are elicited at different concentrations.

In all cases, a slight effect in lipid vesicles is in contrast with stronger "fluidization" in mitochondrial membranes; the difference among vesicles and membranes is less pronounced near the surface (5-NS) than in the membrane core (16-NS).

The results with commercial soybean phospholipids and phospholipids extracted from mitochondria are almost superimposable.

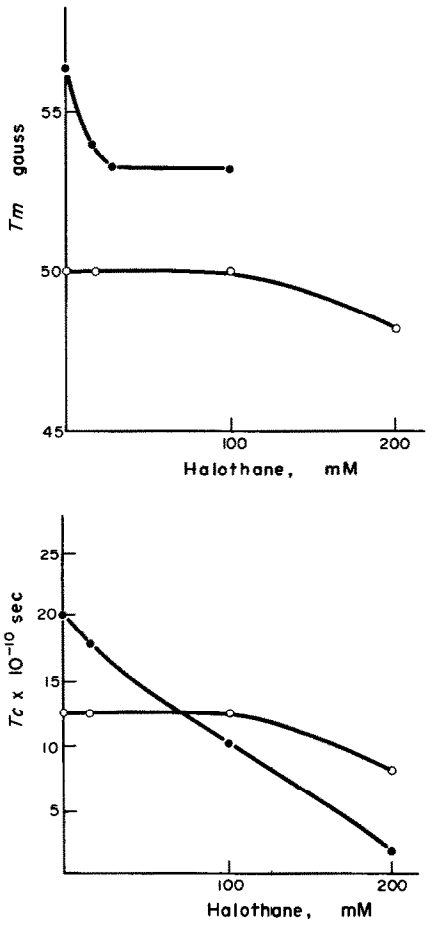
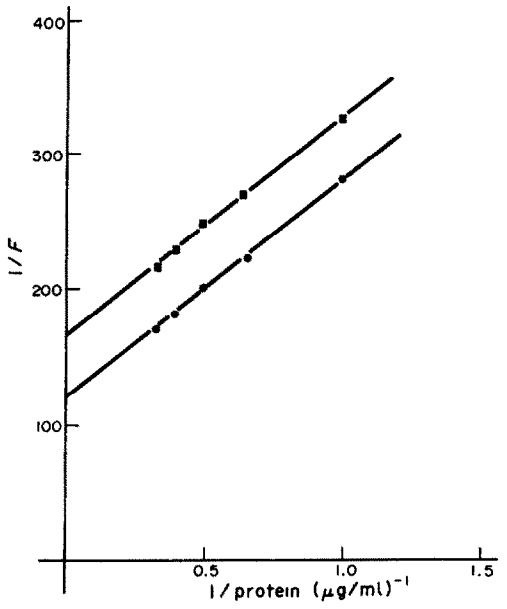


Fig. 1. Effect of halothane on lipid fluidity in Asolectin and BHM. (A) 5-NS. (In the ordinate the hyperfine splittings $2T_{\parallel}$). O——O, Asolectin; ●——●, BHM. (B) 16-NS. (In the ordinate the rotational correlation time τ_c); O——O, Asolectin; ●——●, BHM.

Comparison of the effect of anesthetics in lipid vesicles and in mitochondria indicate that they make the spectra of protein-containing membranes approach those of spin-labeled lipid vesicles. Such differences in responsiveness to anesthetics are considered by us

Table 1. Effect of anesthetics on the mobility of stearic acid spin labels in phospholipid vesicles and membranes

Anesthetic	Concentration (mM)	5-NS Vesicles	5-NS BHM	16-NS Vesicles	16-NS BHM
		Change in $2T_{\parallel}$ (gauss)		Change in τ_c(sec × 10^{-10})	
Butanol	50	−3	−2	−3	−6.5
	150	−4	−6	—	—
Halothane	10	0	−2.5	0	−2.5
	100	0	−3.1	0	−11
Pentrane	10	0	−1	0	−6
	100	−1.7	−3.5	−4.5	−13
Ketamine	20	−0.5	−0.7	−3	−4.5



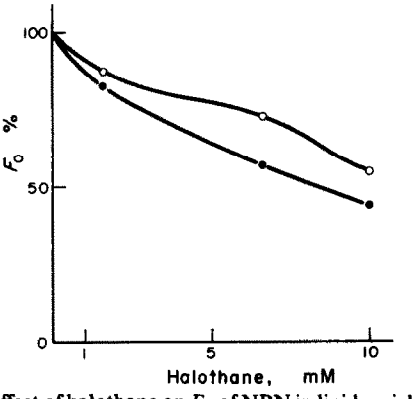


Fig. 3. Effect of halothane on F_0 of NPN in lipid vesicles and in mitochondrial membranes. O——O, lipid vesicles (Asolectin); ●——●, Mitochondrial membranes (ETP). The maximal fluorescences at infinite membrane concentration (F_0) (see Experimental Methods), obtained from the data in Fig. 2 and from similar plots, were normalized to 100 per cent for the controls and replotted as a function of halothane concentration.

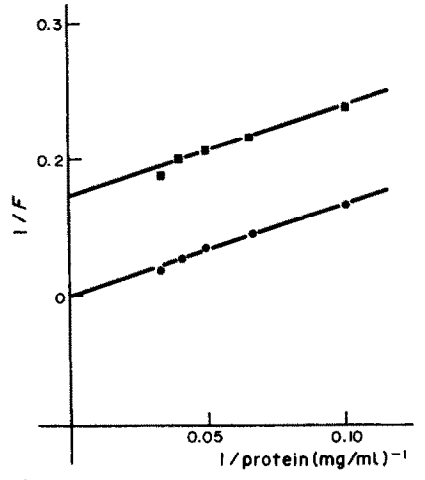


Fig. 2. Effect of halothane on a double reciprocal plot of NPN fluorescence in lipid vesicles and mitochondrial membranes. (A) Asolectin. ●——●, No halothane; ■——■, halothane 1.66 mM. (B) ETP. ●——●, no halothane; ■——■, halothane, 6.6 mM.

as resulting from disruption of lipid–protein interactions.

Effect of anesthetics on NPN fluorescence. All anesthetics decrease NPN fluorescence, without affecting emission maxima (413 nm for Asolectin, 425 nm for mitochondrial membranes); the effects are due to decreased maximal fluorescence at infinite membrane concentration. As an example, the effect of halothane on a double reciprocal plot of fluorescence against membrane concentration is shown in Fig. 2.

The decrease of F_0 induced by butanol and other anesthetics is usually stronger in mitochondria than in lipid vesicles, on a percentage basis (Fig. 3).

Effects of anesthetics on ATPase activity. All anesthetics tested inhibit ATPase activity in both intact mitochondria and submitochondrial particles (Table 2) in spite of a large variability of the initial (non-inhibited) ATPase activity, due to the state of the particles (freezing and thawing, ageing, etc.).

In mitochondria there is a concentration lag for the action of the different solvents. The results in mitochondria are shown for ketamine in Fig. 4. Anesthetics

Table 2. Effect of anesthetics on ATPase activity in mitochondria

Anesthetics	Concentration	ATPase activity* — (μmoles Pi/min. mg)	Oligomycin† inhibition (%)
Butanol	—	1.25	69.6
	0.29 M	1.00	−16.0
Halothane	—	0.70	75.7
	0.5%	0.39	0
Pentrane	—	0.63	88.0
	0.1%	0.30	12.0
Ketamine	—	0.40	86.4
	1.8 mM	0.11	13.0

*The results for each anesthetic refer to a different experiment using a different mitochondrial preparation; the concentration of anesthetics reported in the table is that giving maximal resistance to oligomycin inhibition.

†Oligomycin concentration was 1 μg/ml.

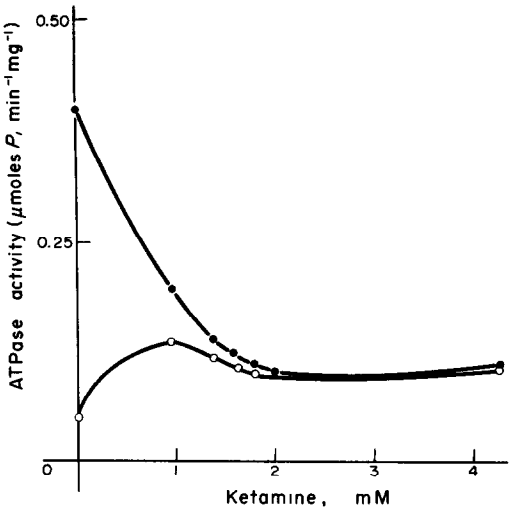


Fig. 4. Effect of ketamine on ATPase activity in BHM. ●——●, Without oligomycin; O——O, in presence of oligomycin, 1 μg/ml.

also induce large changes in the enzyme kinetics. Figure 5 shows the Lineweaver–Burk plot of ATPase inhibition exerted by ketamine. With this and the other anesthetics an uncompetitive type of inhibition is apparent: there is a decrease of both V_{max} and K_M for ATP.

The implications of an uncompetitive type of inhibition have been tentatively ascribed to stabilization of the enzyme–substrate complex.

Effect of anesthetics on the temperature dependence of ATPase. In mitochondrial ATPase, a break in Arrhenius plots is found at 17–20°. Solvents and anesthetics modify the temperature dependence curves: the result for methoxyflurane is shown in Fig. 6.

As shown in Table 3, the results are not univocal. Diethyl ether modifies the transition temperature, which decreases to 12°, without significantly changing activation energies, whereas butanol abolishes the break by increasing activation energies above the

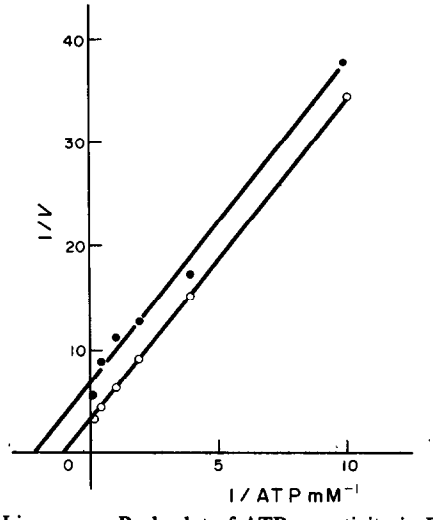


Fig. 5. Lineweaver–Burk plot of ATPase activity in BHM treated with ketamine. O——O, Control; ●——●, ketamine 1 mM.

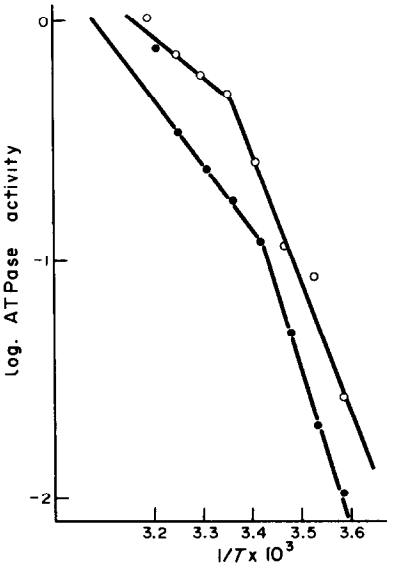


Fig. 6. Arrhenius plot of ATPase activity in BHM treated with pentrane. O——O, Control; ●——●, pentane 0.5%.

Table 3. Effect of anesthetics on temperature dependence of ATPase.

Exp.	Anesthetic added	Break temperature (°)	E_A Above break (kcal/mole)	E_A Below break (kcal/mole)
1	—	18	9.7	36
	Butanol (0.35 M)	—	31.3	
	Ether	12	2.7	40
	Pentrane (0.25%)	12	22	35
	Triton X-100 (2.5 mg/ml)	22	22.6	31
2	—	19	6.8	11.4
	Halothane (0.5%)	24	7.8	10.5
	Ketamine ($1.2.10^{-2}$ M)	30	6.4	9.1
3	—	18	9.4	25.2
	Phospholipase A_2 (5 min digestion)	18	14.7	25.2

break temperature to the same values observed below the break temperature. Pentrane shows both effects at the same time (decrease of break temperature and increase of activation energy). Halothane and ketamine give intermediate effects. The results are compared with the effect of the detergent, Triton-X-100, that is similar to that of butanol.

Effect of anesthetics on oligomycin sensitivity. All anesthetics tend to make the ATPase oligomycin insensitive; the different extents of this effect depend on the extent of inhibition of ATPase activity induced by the given anesthetic (Table 2). There is, however, for each anesthetic tested, a concentration where the ATPase is completely oligomycin insensitive. The nature of this effect had been studied in particular for *n*-butanol [49].

The ATPase activity is not inhibited by oligomycin in the presence of butanol, when oligomycin is added at 1 μg/mg of protein; however, very large concentrations of oligomycin still inhibit butanol-treated ATPase; in other words, butanol induces a large increase of the K_i of oligomycin inhibition. The effect does not appear to be the result of displacement of oligomycin by butanol for the membrane site of action of the inhibitor; in fact, DCCD, which is covalently bound to a subunit of the ATPase complex [23], shows a behaviour comparable to that of oligomycin, even if it cannot be displaced by the alcohol. Similar effects are shown by other anesthetics.

Anesthetics do not detach oligomycin insensitive F_1 from the membrane: in fact treatment of ETP with butanol or other solvents makes the ATPase oligomycin insensitive but still recovered in the particulate fraction after centrifugation, whereas no ATPase is detected in the supernatant [50].

The inhibition curve of ATPase by oligomycin in ETP is sigmoidal [51] and this shape has been ascribed to a change in conformation induced by the inhibitor. The cooperativity approaches the value of $n(-2)$ in a Hill plot of oligomycin inhibition.

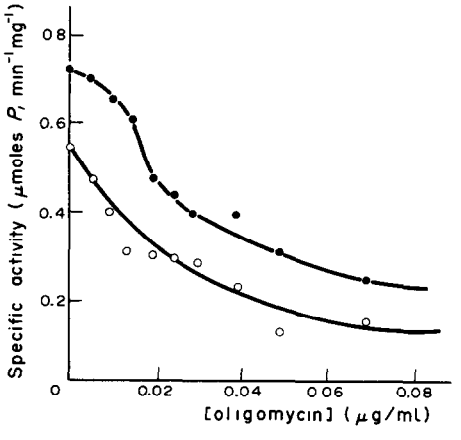


Fig. 7. Effect of *n*-butanol on oligomycin inhibition curve of ATPase in ETP. ●——●, No butanol; ○——○, butanol 100 mM.

Anesthetics at lower concentrations abolish the cooperativity of the oligomycin inhibition curve. Figure 7 shows the inhibition curve of oligomycin sensitivity of ATPase in ETP, induced by butanol.

DISCUSSION

Spin label and fluorescence studies. We have previously shown that mitochondrial proteins exert strong immobilization at all levels of phospholipid bilayers, as shown by the changes of ESR spectra of 3 stearic acid spin labels having nitroxides in position 5, 12, and 16 respectively [52]. The mobility of the fatty acyl chain increases from the surface to the bilayer core, in accordance with several findings in bilayers and membranes [38, 53, 54].

An immobilizing effect of intrinsic proteins on phospholipids has been observed in several membranes [55–58]. It has been postulated that the hydrophobic surfaces of proteins are solvated by an immobilized layer of lipid [56]; these "boundary lipids" slowly exchange with surrounding free bilayer lipids [59, 60].

The results of addition of anesthetics to different membranes confirm our working model showing a labilization of lipid protein interactions. Anesthetics have a slight fluidizing effect on phospholipid vesicles, as indicated by increased rotational motion, but have a very strong effect in enhancing the mobility of phospholipids in BHM.

Our interpretation is that anesthetics abolish the immobilization induced on phospholipids by the mitochondrial intrinsic proteins. In other words, whatever is the precise molecular effect induced by proteins on lipid bilayers, this effect is abolished by anesthetics, indicating a labilization of lipid protein interactions. Many anesthetics are organic solvents which at high concentrations are capable *to extract* lipids from membranes; this effect is apparently induced to a smaller extent at lower concentrations, i.e. those concentrations that induce general anesthesia. We can postulate that the decrease of rigidity is an indication of higher escape or exchange of phospholipids from the annulus to the surrounding bilayer, as suggested by Hesketh *et al.* [59] for the effect of benzyl alcohol on the Ca^{2+}-ATPase incorporated into dipalmitoyl lecithin.

Similar results are obtained by using the fluorescent probe NPN, localized in the hydrophobic core of the membrane [27].

All anesthetics have been found to decrease NPN fluorescence without any change in its partition from medium to the lipid phase, nor in emission maximum.

The effects of anesthetics on NPN fluorescence indicate changes in the chromophore environment in the membrane. These changes may be interpreted to represent either changed polarity or changed fluidity of the environment (in that a fluorescence increase reflects decreased fluidity or decreased polarity, and vice-versa). We like to favor the first possibility, i.e. changes in fluidity induced by anesthetics. This conclusion is supported by the fact that the fluorescence decrease is not accompanied by a significant red shift of the emission maximum, as it would have been if the chromophore environment had become more polar.

A quantitative study of the effects of anesthetics on NPN fluorescence reveals that they are not usually

identical in phospholipid vesicles and in mitochondrial membranes. Maximal fluorescence intensity at infinite membrane concentration (F_0) appears to be decreased by anesthetics more in membranes than in vesicles, supporting the conclusions, derived by the spin label studies, that anesthetics disrupt lipid–protein interactions.

The effect of a local anesthetic (tetracaine) on lipid dispersions and natural membranes was studied by Feinstein *et al.* [61] by fluorescence polarization of perylene; a comparison however is not possible because there is no concentration dependence of the anesthetic in the two systems.

We conclude therefore that the general anesthetics investigated by us decrease the overall fluidity in lipid bilayers, in accordance with the findings of several authors [3–8, 62, 63] but this effect is rather small when compared with the increase in fluidity of a natural membrane (the mitochondrial membrane); the decrease of attraction exerted by the proteins on the surrounding lipids appears the primary action of anesthetics. A similar conclusion is reached by Hesketh *et al.* [59] for the effect of benzyl alcohol; a model of local anesthetic action involving a primary effect on the lipid annulus surrounding the sodium channel in nerves is discussed by Lee [13, 64].

In our studies the fluidizing effects is stronger in the bilayer core (16-NS, NPN) and weaker near the surface (5-NS).

A word of caution must be put forward in the discussion of data obtained by extrinsic probes such as spin and fluorescence labels, since they themselves are known to induce perturbation in model and natural membranes [65–69]. Therefore the effect of membrane perturbers such as anesthetics is investigated by means that can perturb the membrane themselves; since, however, our main goal is comparison between lipid vesicles and membranes, we feel that the perturbing effect of the probes should be comparable in the two systems.

Furthermore the data on ATPase, to be discussed in the following section, point to the same conclusions, that anesthetics perturb lipid–protein interactions.

Kinetics of ATPase. The modifications of the kinetic properties of mitochondrial ATPase induced by anesthetics may be best interpreted as resulting from conformational changes in the enzyme, following a primary effect on lipid–protein interactions.

Kinetic parameters of other membrane-bound enzymes have been found to be modified by alcohols and other solvents. Grisham and Barnett [70] found that loss of (Na^+–K^+)ATPase activity induced by alcohols is related to the alcohol hydrophobicity in the same way as the decreased ability to orient an androstane spin label. Hégyvary [71] has shown that organic solvents inhibit (Na^+–K^+)ATPase; inhibition is uncompetitive with respect to ATP; in the presence of the solvents the apparent affinity of the enzyme increases for Na^+ and ATP and decreases for K^+. It was shown that solvents enhance interaction of ATP with the enzyme prior to phosphorylation of the enzyme, but decrease the rate of dephosphorylation of phosphoenzyme in presence of K^+. The author concludes that these modifications are partly due to conformational changes of the enzyme and partly to changes in water structure around the active site.

In our study we have shown that anesthetics induce uncompetitive inhibition of Mg^{2+}-ATPase from mitochondria, suggesting an increased stability of an enzyme–substrate complex. The finding that all anesthetics tested have similar effects on the kinetics of ATPase, suggests that a common mechanism is operative for ATPase inhibition. We can exclude that the K_M decrease induced by anesthetics is the result of increased accessibility of the substrate ATP to the active site due to membrane disruption, since in submitochondrial particles the active site is exposed to the outer medium (contrary to intact mitochondria) [72]. It has been postulated by Lenaz [73] that phospholipids are required for allowing the conformational change accompanying the formation of an enzyme–substrate activated complex. Anesthetics could favor enzyme–substrate stabilization by allowing the active site to receive the substrate in a more "open" conformation, but at the same time preventing a further change to an activated complex. It is of considerable interest that a decrease of both K_M and V_{max} has been observed for several enzymes [74–76] after lipid depletion of membranes. We have observed the same type of inhibition in ATPase by treatment of the membrane with phospholipase A_2, which removes large amounts of phospholipids from the membrane [28]. The similarity of the effect of anesthetic addition and delipidation points out on an indirect effect mediated through the lipids in accordance with our working model.

Breaks in Arrhenius plots. Discontinuities or breaks in Arrhenius plots of membrane-bound enzymes are interpreted as the result of conformational changes of the enzymic proteins at temperatures above and below the critical temperature [77]; the origin of the conformational change has to be ascribed to a phase change of the lipids of the membrane [78, 79]. An alteration of the temperature dependence curves induced by anesthetics in a membrane-bound enzyme can be a very suitable system to study the effect mediated through physical changes of the lipids.

Sullivan *et al.* [80] have found that the rate of transport of *O*-nitrophenyl-D-galactopyranoside in *E. coli* is increased below the apparent phase transition in presence of *n*-alkanols. The degree of activation is determined by both concentration and chain length of the alcohol. Arrhenius plots of the kinetic constants indicate that the K_M shows discontinuity with increasing temperature, while the slope of V_{max} changes only gradually.

In our study we have shown that anesthetics modify the temperature dependence of mitochondrial ATPase. In presence of butanol, no break is apparent in the Arrhenius plot of ATPase and the activation energy is increased at all temperatures to values in the range of those below the break in unperturbed ATPase, suggesting disorganization of lipid-protein interactions, in agreement with the spin label studies. Other anesthetics modify the temperature dependence of ATPase in different ways. Diethyl ether decreases the break temperature without affecting activation energies; pentrane both decreases break temperature and increases activation energies; other solvents and

anesthetics have intermediate effects. It is possible that different anesthetics affect fluidity and lipid–protein interactions to different extents at different concentrations because of polarity differences. Diphasic effects at different concentrations in the case of *n*-alkanols [73, 81] can be explained as an increase in lipid fluidity and interchain distance at low alcohol concentration, as one would induce in bilayers by increasing the temperature, followed by loss of optimal lipid–protein interactions at high alcohol concentrations; at such higher concentration, conformational changes or changed intersubunit relationships in ATPase complex may also be expected. Changes in slopes of Arrhenius plots are also induced by lipid removal (by phospholipase A_2 digestion) [28]. The effect is in favor of changes mediated through the lipids only, since also Triton-X-100, which breaks lipid protein interactions but does not affect protein structure [82], abolishes the break in Arrhenius plot of ATPase [83, 84]. The interpretation is already supported by the knowledge itself that breaks in Arrhenius plots are a consequence of lipid fluidity changes [78, 85].

Oligomycin inhibition. Finally, inhibition of ATPase activity by oligomycin, although a complex phenomenon, may give important hints on the effects of anesthetics on conformation induced changes of enzyme activity.

The oligomycin inhibition of membrane-bound ATPase is explained by constraints induced by the intrinsic subunits of the ATPase complex on F_1; changes in the relative position and optimal distance or in the conformation of the subunits will induce loss of oligomycin inhibition.

All anesthetics tested abolish oligomycin and DCCD sensitivity of ATPase. The concentration of anesthetics required for this effect is in the range of concentration which disrupts lipid–protein interactions in mitochondria as probed by spin labels. The ATPase is not detached from the membrane [50] suggesting that a dislocation of F_1 from the membrane factors has occurred. Montecucco and Azzi [86] have shown by means of EPR techniques that the paramagnetic DCCD analog, NCCD and Mn^{2+}-ATP are within a distance of about 20 Å in normal submitochondrial particles, but ether treatment [87] increases this distance to >35 Å.

It is tempting to suggest that the loss of oligomycin sensitivity and disolocation of ATPase subunits are a consequence of changes in membrane lipids.

Lower concentrations of anesthetics, which do not inhibit ATPase activity significantly, change the oligomycin sensitivity curve from sigmoidal to hyperbolic, with a change of Hill coefficients from about -2 to -1, indicating a complete loss of cooperative character of the interaction of the oligomycin binding site to F_1-ATPase. The cooperativity is abolished by anesthetics (as it is by phospholipase A_2 digestion of membrane lipids) again indicating changes in the mutual interactions between ATPase subunits. Similar conclusions must be derived from the effect of anesthetics upon the changes of fluorescence of bound aurovertin induced by oligomycin (unpublished observations).

All of the effects point to conformational changes of ATPase as a consequence of anesthetics; such changes are considered conformational in the broadest meaning, indicating possible changes of secondary up to quaternary structure. Direct studies with physical means (circular dichroism) have confirmed that anesthetics induce impressive changes of secondary structure [88].

The important dilemma is whether the conformational changes detected after anesthetic addition are the result of lipid changes or of direct interaction of anesthetics on the ATPase protein [14, 15]. The available data do not allow a clearcut answer to this question, although the above interpretation is strongly favored by the fact that the same concentrations of anesthetics which induce changes in proteins also induce changes in membrane lipids. Moreover, lipid removal induces the same changes given by anesthetic addition [28]. A possible answer is given by a study of the erythrocyte membrane acetylcholinesterase (unpublished observations from our laboratory). Anesthetics have been found to inhibit the membrane-bound enzyme, but not the enzyme after detachment from the membrane; addition of phospholipids to the solubilized enzyme restores sensitivity to anesthetics. In such case we can conclude that anesthetic sensitivity of an enzyme is conferred indirectly through the lipids.

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