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MULTIPLE SITES OF INHIBITION OF MITOCHONDRIAL ELECTRON TRANSPORT BY LOCAL ANESTHETICS *

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Local anesthetics and alcohols were found to inhibit mitochondrial electron transport at several points along the chain. The anesthetics employed were the tertiary amines procaine, tetracaine, dibucaine, and chlorpromazine, and the alcohols were *n*-butanol, *n*-pentanol, *n*-hexanol, and benzyl alcohol. Uncoupled sonic submitochondrial particles from beef heart and rat liver were studied. We report the following: (1) All of the anesthetics were found to inhibit each of the segments of the electron transport chain assayed; these included cytochrome *c* oxidase, durohydroquinone oxidase, succinate oxidase, NADH oxidase, succinate dehydrogenase, succinate-cytochrome *c* oxidoreductase, and NADH-cytochrome *c* oxidoreductase. (2) NADH oxidase and NADH-cytochrome *c* oxidoreductase required the lowest concentrations of anesthetic for inhibition, and cytochrome *c* oxidase required the highest concentrations. (3) We conclude that there are several points along the chain at which inhibition occurs, the most sensitive being in the region of Complex I (NADH dehydrogenase). (4) Beef heart submitochondrial particles are less sensitive to inhibition than are rat liver particles. (5) Low concentrations of several of the anesthetics gave enhancement of electron transport activity, whereas higher concentrations of the same agents caused inhibition. (6) The concentrations of anesthetics (alcohol and tertiary amine) which gave 50% inhibition of NADH oxidase were lower than the reported concentrations required for blockage of frog sciatic nerve.

Introduction

The objective of this work was to find out how selected anesthetics of the alcohol and tertiary amine types affect mitochondrial electron transport.

The mechanisms of action of local as well as general anesthetics remain obscure in spite of the considerable number of papers which are published each year in this area. While the physiologically most relevant action of local anesthetics is their nerve blocking ability, their effects are not limited to nerves, since they also affect a variety of other systems [1]. They have, for example, been reported to inhibit microtubule polymerization [2], to affect calcium transport or binding [3,4], to protect red blood cells from hemolysis [5], and, as reported here, to inhibit mitochondrial electron transport. The effects of anesthetics on the physical properties of lipid bialyers have also been investigated in great detail by several laboratories (e.g., Refs. 6 and 7), the principal findings being that anesthetics tend to decrease bilayer order and/or increase 'fluidity', and also to lower the phase transition temperature.

The effects of volatile (general) anesthetics on mitochondrial electron transport has been studied in some detail by others. Harris et al. [8] and Nahrwold and Cohen [9] both found that NADH oxidation in mitochondria is inhibited to a much greater degree by

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halothane and other halogenated hydrocarbons than is succinate oxidation. Those workers concluded that the principal site of inhibition is in the vicinity of Complex I (NADH dehydrogenase). Lenaz et al. [10, 11] have shown that *n*-butanol and several general anesthetics inhibit mitochondrial ATPase activity. Finally, the local anesthetic lidocaine inhibits glutamate oxidation by brain mitochondria by interfering with electron transport at the NADH dehydrogenase level [12].

We used uncoupled sonic submitochondrial particles in order to simplify the system as much as possible, short of isolating the individual enzymes. We found that all electron transport activities assayed were inhibited by each of the anesthetics tested. The latter included the tertiary amines procaine, tetracaine, dibucaine, and chlorpromazine (a tranquillizer), and the alcohols *n*-butanol, *n*-pentanol, *n*-hexanol, and benzyl alcohol. Our results were qualitatively similar to those obtained by the previous workers [8,9,12] who used halogenated hydrocarbons or lidocaine, in that we found NADH oxidation to be more sensitive to inhibition by the tertiary amines and alcohols than is succinate oxidation, but we have also shown that there are several other sites of inhibition of varying sensitivity scattered along the electron transport chain, including a site at cytochrome *c* oxidase.

Methods

Mitochondrial preparations

Rat liver [13] and beef heart [14] mitochondria were prepared by standard methods. The beef heart mitochondria were obtained in the frozen state from Dr. D.E. Green of the University of Wisconsin-Madison. Sonic submitochondrial particles were made from the liver or heart mitochondria by Method II described in Ref. 15, using a Branson Model W-350 Sonifier. The submitochondrial particles were stored at −20°C until use. Protein concentrations of the submitochondrial particles were determined by the method of Lowry et al. [16].

Enzyme assays

Succinate oxidase, NADH oxidase, durohydroquinone oxidase, and cytochrome *c* oxidase activities were measured polarographically using a Clark-type oxygen electrode (Yellow Springs Instrument Co.). The temperature of the cell was maintained within ±0.05°C of the desired temperature by a water bath. The 'standard assay medium' for oxidase determinations included 100 mM KCl, 20 mM Tris-HCl, 5 mM KH_2PO_4, 3 mM $MgCl_2$, and 0.5 mM EDTA, at pH 7.4 [17].

Cytochrome *c* oxidase was assayed in 2.8 ml standard assay medium plus 5 μl 1.6 mM cytochrome *c* (Sigma, Type III), 5 μl 50 mM carbonylcyanide *m*-chlorophenylhydrazone, and 5 μl 1 mM rotenone. 100 μl of a submitochondrial particle suspension of known protein concentration was added to this medium, followed by 30 μl of a freshly prepared solution containing 0.2 M sodium ascorbate and 10 mM *N,N,N',N'*-tetramethylphenylenediamine to start the reaction [18]. After the rate of oxygen consumption of this mixture was determined, sequential additions of the selected anesthetic were added, with the rate being determined after each addition. A typical oxygen electrode trace is shown in Fig. 1; it can be seen that after each addition of dibucaine, a new linear rate was quickly re-established. The linearity of the rate of oxygen consumption before and after addition of anesthetic was checked in selected cases by allowing the reaction to proceed under constant conditions for a longer period of time than shown in Fig. 1.

Succinate oxidase activity was determined similarly to cytochrome *c* oxidase, except that a 20 μl aliquot of 0.5 M sodium succinate was used as the reductant [17].

Durohydroquinone oxidase activity was measured by using 50 μl 63 mM durohydroquinone dissolved in ethanol as the reductant, with all other conditions being the same as for the cytochrome *c* oxidase assay [19]. This procedure resulted in the unavoidable presence of 350 mM ethanol in the reaction mixture, obviously not an insignificant amount, although control experiments in which this amount of ethanol was added to the succinate oxidase assay showed that its effect was small. Addition of 5 μl 5 mg/ml antimycin A completely inhibited durohydroquinone oxidation.

NADH oxidase was determined in a solution consisting of 2.8 ml standard assay medium plus 5 μl 1.6 mM cytochrome *c*. Carbonylcyanide *m*-chlorophenylhydrozone was omitted since its inclusion resulted in an unexplained nonlinearity of the oxygen traces.

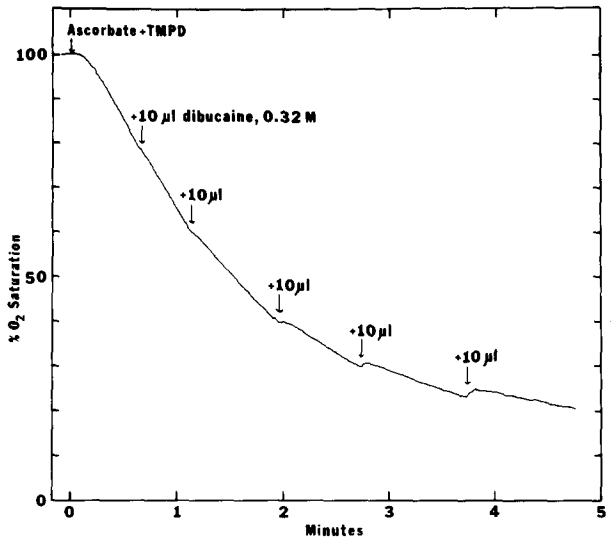

Fig. 1. A typical oxygen electrode trace showing the decrease in rate of oxygen utilization by the rat liver submitochondrial particles upon the addition of successive 10 µl increments of 0.32 M dibucaine. In this experiment, the cytochrome *c* oxidase activity was assayed, with ascorbate plus *N,N,N',N'*-tetramethylphenylenediamine (TMPD) as the electron donor. The temperature was 36°C.

The submitochondrial particles were probably completely uncoupled anyway, considering the method of preparation and the fact that they were stored in the frozen state prior to use. The reaction was started by the addition of 60 µl 45 mM NADH. Addition of 5 µl of 1 mM rotenone completely inhibited the reaction.

Spectrophotometric assays were used for succinate dehydrogenase, succinate-cytochrome *c* oxidoreductase, and NADH-cytochrome *c* oxidoreductase, using a Cary 219 spectrophotometer equipped with water-jacketed cell holders which were controlled to ±0.5°C of the desired temperature. The reaction mixture for succinate dehydrogenase [20,21] consisted of 0.75 ml 200 mM KH_2PO_4, 0.3 ml 200 mM sodium succinate, 0.1 ml 0.05% (w/v) 2,6-dichlorophenol indophenol, 0.3 ml 0.33% (w/v) phenazine methosulfate, 0.03 ml 100 mM KCN, 6 µl 5 mg/ml antimycin A, 1.5 ml deionized water, and the required amount of the selected anesthetic, giving a final volume of approx. 3 ml and a pH of 7.5. The solution was protected from light until immediately before use [20]. Enzyme activation was carried out as described by Ackrell et al. [20] by incubating the stock submitochondrial particle suspension in the presence of succinate at 37°C for 6 min. The assay was started by the final addition of 5 µl activated submitochondrial particles to the reaction mixture. The rate in the presence of each anesthetic concentration was determined in triplicate, in separate experiments, by following the change in absorbance at 600 nm.

The reaction mixture for the succinate-cytochrome *c* oxidoreductase determination [22] included 1.5 ml 200 mM KH_2PO_4, 0.3 ml 3 mM EDTA, 0.1 ml 0.6 M sodium succinate, 0.3 ml 1 mM cytochrome *c*, 30 µl 0.1 M KCN, and 0.75 ml deionized water, at pH 7.4. The required amount of the selected anesthetic was added to this mixture. The enzyme reaction was started by the final addition of 5 µl of the submitochondrial particle suspension. The rate was determined by following the absorbance at 550 nm. Addition of 5 µl 5 mg/ml antimycin A was shown to completely inhibit the reaction.

The spectrophotometric determination of the NADH-cytochrome *c* oxidoreductase activity was carried out according to the procedure given in Ref. 23, except for the omission of phospholipid suspension. Cytochrome *c* reduction was followed by the absorbance change at 550 nm. The pH of the assay medium was 8.0. A rotenone-insensitive contribution to the overall rate was noted; this was not subtracted out of the reported results.

All biochemical reagents were obtained from Sigma except for durohydroquinone, which was purchased from ICN Pharmaceuticals. The alcohols were spectral grade and were obtained from J.T. Baker.

Results

Anesthetic concentrations required for 50% inhibition. Procaine, tetracaine, dibucaine, chlorpromazine, as well as the normal alcohols C_4 to C_6 and benzyl alcohol, caused inhibition of the electron transport segments studied. Table I gives the 50% inhibitory concentrations at 25°C for each activity and anesthetic. The concentrations given in Table I were estimated either directly from plots of relative rate vs. inhibitor concentration, or by plotting log [(1/relative rate) – 1] vs. log (concentration). The latter plot serves to linearize the data [24], with the 50% inhibitory concentration being given by the intercept on the log (concentration) axis. (The 'relative rate' is defined as

TABLE I

CONCENTRATIONS OF ANESTHETICS REQUIRED FOR 50% INHIBITION OF ELECTRON TRANSPORT IN UNCOUPLED SUBMITOCHONDRIAL PARTICLES AT 25°C

The error of these data is estimated to be about 10% of the given values. 350 mM ethanol was present in the DHQ oxidase assays. Values are expressed in mM. Abbreviations: Succinate-cyt. *c*, succinate-cytochrome *c* oxidoreductase; NADH-cyt. *c*, NADH-cytochrome *c* oxidoreductase; DHQ, durohydroquinone; n.d., not determined; non. lin., nonlinear data.

Anesthetic	Rat liver			Beef heart					
	Succinate oxidase	DHQ oxidase	Cyt. *c* oxidase	NADH oxidase	Succinate oxidase	Cyt. *c* oxidase	Succinate dehydrogenase	Succinate--cyt. *c*	NADH--cyt. *c*
n-Butanol	170	300	450	45	420	700	630	90	35
n-Pentanol	65	40	160	20	125	270	n.d.	n.d.	20
n-Hexanol	45	30	80	13	95	220	n.d.	n.d.	1.5
Benzyl alcohol	80	non. lin.	130	7	130	255	190	40	non. lin.
Procaine	35	15	40	2.9	90	90	n.d.	n.d.	0.10
Tetracaine	1.0	2.5	5.0	0.1	7	25	7.6	5	0.01
Dibucaine	1.0	0.35	2.7	0.25	1.5	9	3.2	1.8	0.01
Chlorpromazine	0.12	0.4	1.0	non. lin.	0.13	1.7	n.d.	n.d.	n.d.

the ratio of reaction rate in the presence of inhibitor to that in its absence.)

Reversibility studies. A dilution method was employed to test for reversibility of the anesthetic inhibition of cytochrome *c* oxidase activity in the rat liver submitochondrial particles. Pairs of identical samples were prepared containing the complete cytochrome *c* oxidase assay medium (but omitting substrate) and submitochondrial particles. To one of these tubes was added a sufficient quantity of *n*-butanol, procaine or tetracaine to give 50–75% inhibition at the selected temperature (25 or 35°C), as shown in separate experiments on the same preparation. Following a 30-s incubation period, the two samples were diluted 1 : 1 with assay medium, causing the concentration of anesthetic in the one tube to decrease by half. After another 30-s equilibration, substrate was added to the diluted samples and the rates of oxygen consumption were determined. Following the dilution, the samples containing anesthetic showed only 10–20% inhibition in each case. These results confirmed that inhibition of cytochrome *c* oxidase by the three agents tested is reversible.

The reversibility of the inhibition of the other segments of the electron transport chain was not unambiguously established. The fact that the oxygen electrode traces were usually linear and that new (linear) slopes were rapidly attained following the addition of anesthetic (Fig. 1) suggests, but does not prove, that the reactions were reversible. Occasionally, however, the recorder traces following the addition of anesthetic were distinctly nonlinear, clearly indicating an irreversible, time-dependent loss of activity. This behavior was observed for a few cases even at low concentrations of anesthetic, and these are indicated in Table I. High concentrations of benzyl alcohol (more than that required for 50% inhibition) gave nonlinear traces with several of the assays, including cytochrome *c* oxidase.

Evidence for multiple sites of inhibition. The actual rates of oxygen consumption are given as a function of the tetracaine concentration in Fig. 2 for succinate oxidase, durohydroquinone oxidase and cytochrome *c* oxidase in the rat liver submitochondrial particles. This figure shows that cytochrome *c* oxidase has the highest activity of three systems, but also requires the greatest concentration of tetracaine for inhibition. The same relationship also holds for the oxidase activities of the beef heart submitochondrial particle, as one can see from Table I. Thus, there must be a site of tetracaine inhibition in the terminal cytochrome *c* oxidase segment of the electron transport chain. In addition, since the succinate oxidase activity is both slower than the cytochrome *c* oxidase activity in the absence of tetracaine, and is also more sensitive to inhibition by tetracaine, it means that

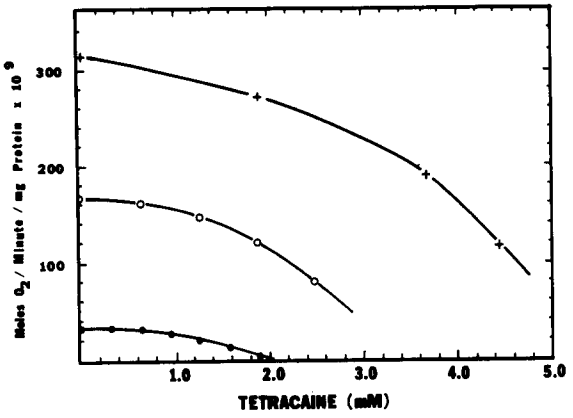

Fig. 2. Comparison of the actual rates of oxygen consumption by the rat liver submitochondrial particles at 20°C as a function of the tetracaine concentration, for cytochrome *c* oxidase (+———+); durohydroquinone oxidase (○———○) and succinate oxidase (●———●).

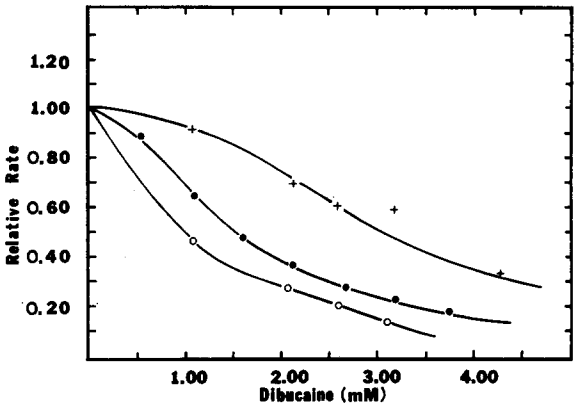

Fig. 4. The relative rates of succinate oxidation in the beef heart submitochondrial particles at 25°C as a function of the dibucaine concentration. +, succinate dehydrogenase; •, succinate oxidase; ○, succinate-cytochrome *c* oxidoreductase.

there must be another site (or sites) of inhibition along the succinate to oxygen portion of the chain. A similar argument can be made if one compares the durohydroquinone oxidase and cytochrome *c* oxidase results (Fig. 2).

The relative rates of three oxidation-reduction reactions in the beef heart submitochondrial particles having succinate as the reductant are given in Figs. 3 and 4 as a function of the concentrations of tetracaine and dibucaine, respectively. The succinate dehydrogenase and succinate oxidase activities are equally sensitive to inhibition by tetracaine (Fig. 3), suggesting that the rate limiting inhibitory reaction for succinate oxidase occurs in the succinate dehydrogenase (Complex II) segment of the chain. This conclusion is not supported by the results with dibucaine, *n*-butanol and benzyl alcohol, in all of which cases succinate oxidase is more sensitive than succinate dehydrogenase to inhibition (see Fig. 4 and Table I). Thus, there evidently is an inhibitory site within the part of the chain measured by the succinate dehydrogenase assay, and also another site or region somewhere between succinate dehydrogenase and cytochrome *c* oxidase.

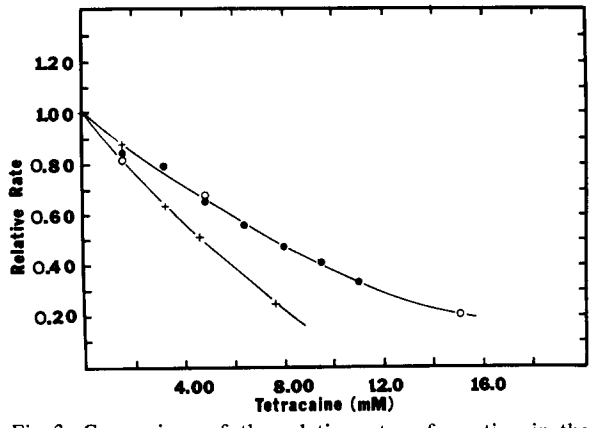

Fig. 3. Comparison of the relative rates of reaction in the beef heart submitochondrial particles at 25°C for three systems having succinate as the reductant, as a function of the tetracaine concentration. ○, succinate dehydrogenase; •, succinate oxidase; +, succinate-cytochrome *c* oxidoreductase.

The sensitivity of succinate-cytochrome *c* oxidoreductase to inhibition is shown in Figs. 3 and 4 and is seen to be greater for all agents tested than that of either succinate dehydrogenase or succinate oxidase. This is a paradoxical result, since the succinate-cytochrome *c* oxidoreductase segment is a part of the complete succinate oxidase chain, and thus the latter activity should be of equal or greater sensitivity than the former. Perhaps the explanation lies in the role played by cytochrome *c* in the two assay systems; for succinate oxidase, cytochrome *c* merely transports electrons, but for succinate-cytochrome *c* oxidoreductase, it acts stoichiometrically as the terminal electron acceptor.

The oxidation reactions having NADH as reductant are inhibited by much lower concentrations of all

anesthetics than the corresponding succinate oxidations (see Table I). We report data only for beef heart NADH oxidase and NADH-cytochrome *c* oxidoreductase. The concentrations of anesthetic which give 50% inhibition of NADH-cytochrome *c* oxidoreductase are lower than those for NADH oxidase, just as succinate-cytochrome *c* oxidoreductase is more sensitive to inhibition than succinate oxidase (see previous paragraph). (One should take note, however, that the NADH-cytochrome *c* oxidoreductase assay was carried out at pH 8.0, whereas the NADH oxidase assay was at pH 7.4. We did not investigate the possible pH dependence of the anesthetic effects.)

Since NADH oxidase is much more sensitive to inhibition than is succinate oxidase, it appears that the site primarily responsible for NADH oxidase inhibition must lie either in Complex I or between Complex I and Coenzyme Q.

Tissue source comparison. Relative rate plots are given in Fig. 5 for the inhibition of succinate oxidase in rat liver and beef heart submitochondrial particles by two alcohols. It can be seen that the rat liver submitochondrial particles are appreciably more sensitive to inhibition by these agents than are the beef heart submitochondrial particles. Examination of Table I shows that this is also true for the other anesthetics tested. Cytochrome *c* oxidase of rat liver is likewise inhibited at significantly lower concentrations of all agents than is the beef heart enzyme.

Enzyme activation by anesthetics. Low concentrations of most of the anesthetics tested were at times observed to give enzyme activation in an intermediate (20–35°C) temperature range. Examples of this are shown in the *n*-butanol curve in Fig. 5 for beef heart succinate oxidase, and in Fig. 6 for the *n*-butanol activation of rat liver cytochrome *c* oxidase. The activation of rat liver succinate oxidase by *n*-butanol at 20°C was reported previously [17]. For rat liver cytochrome *c* oxidase, activation of up to 10% was given in some (but not all) submitochondrial particle preparations by low concentrations of *n*-butanol, benzyl alcohol, procaine, tetracaine, chlorpromazine and possibly *n*-pentanol, but not dibucaine. We have found the activation phenomenon to be poorly reproducible, and have not as yet been able to clearly identify the conditions necessary for it.

Temperature dependence. Relative rate plots are given in Figs. 6 and 7 showing the temperature dependence of the effects of *n*-butanol and dibucaine on rat liver cytochrome *c* oxidase. It can be seen that the inhibitory potency increases markedly for both agents as the temperature is raised above 35°C, but that below 35°C the dibucaine inhibition is much less sensitive to temperature than is the *n*-butanol inhibition. While the examples shown in Figs. 6 and 7 represent the extremes of thermal behavior which we have observed to date, it can be said that in all systems studied thus far and with all anesthetic agents, the potency increases appreciably when the temperature is raised above about 35°C. In a few systems,

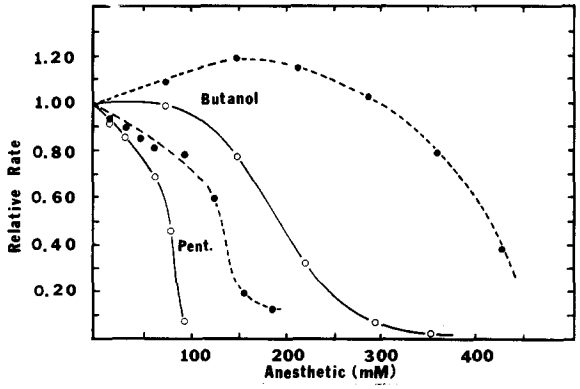

Fig. 5. Comparison of the inhibitory effects of *n*-butanol and *n*-pentanol on rat liver and beef heart submitochondrial particles at 25°C, for succinate oxidase. (●------●), beef heart submitochondrial particles; (○———○), rat liver submitochondrial particles. Pent., *n*-pentanol.

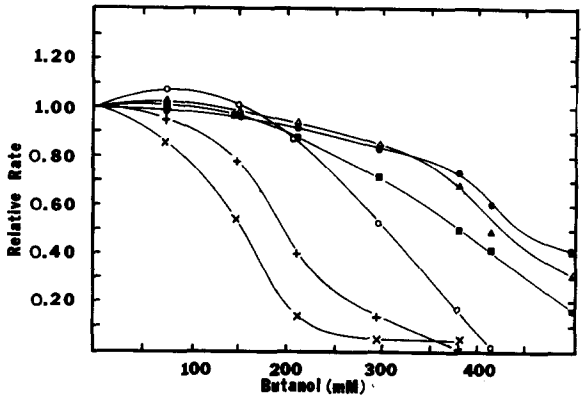

Fig. 6. The temperature dependence of cytochrome *c* oxidase inhibition in the rat liver submitochondrial particles by *n*-butanol. ×, 45°C; +, 40°C; ○, 35°C; ■, 30°C; △, 25°C; ●, 20°C.

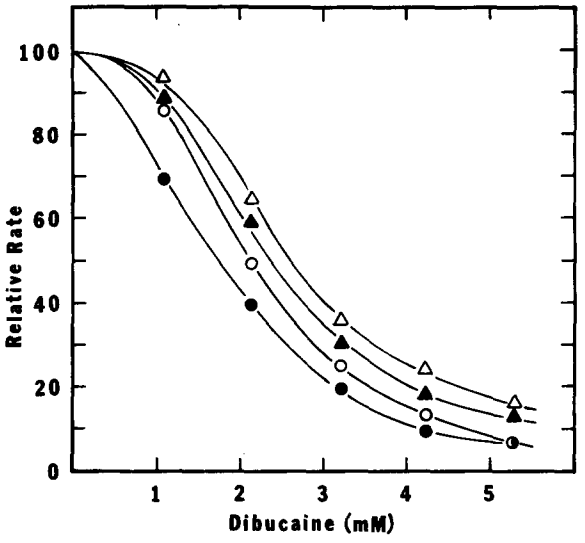

Fig. 7. The temperature dependence of cytochrome *c* oxidase inhibition in the rat liver submitochondrial particles by dibucaine. △, 20°C; ▲, 35°C; ○, 39°C; ●, 43°C.

e.g., tetracaine on rat liver cytochrome *c* oxidase, the potency was also observed to increase slightly at low temperature (below 25°C), giving an intermediate temperature range (25–35°C) of minimum inhibitory potency.

The 50% inhibitory concentrations for each of the four tertiary amine anesthetics are given on a semilogarithmic plot as a function of temperature in Fig. 8,

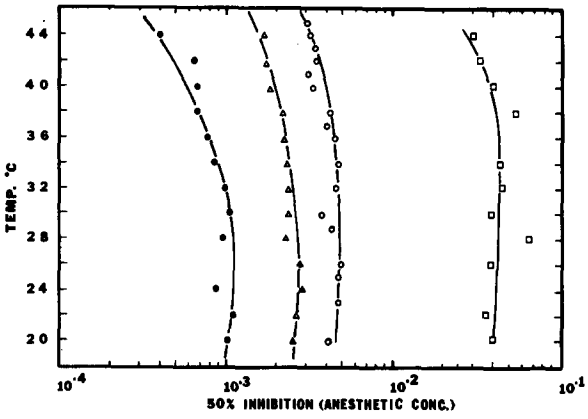

Fig. 8. A semi-logarithmic plot showing the concentrations of tertiary amine anesthetics required for 50% inhibition of cytochrome *c* oxidase activity in the rat liver submitochondrial particles, as a function of temperature. •, chlorpromazine; △, dibucaine; ○, tetracaine; □, procaine.

for rat liver cytochrome *c* oxidase. The temperature dependence of chlorpromazine is clearly much greater than is that of the other three agents shown here.

Homologous series effects on inhibitory potency. The concentrations of the various agents required for 50% inhibition of the respective enzyme activities are spread over three orders of magnitude. Within the homologous series of normal alcohols, it is seen in Table I that the 50% inhibitory concentrations are inversely proportional to chain length and water solubility. A similar statement can be made for the tertiary amines; procaine is the most water-soluble of these, and for all enzyme systems studied the concentration of procaine required for inhibition is much higher than that of tetracaine or dibucaine. These observations suggest that hydrophobic interactions may be of considerable importance in determining the strength of interaction of the various agents with the enzyme systems.

Discussion

The results presented here indicate that there are several sites or regions of the mitochondrial electron transport chain which are affected by alcohols and tertiary amine local anesthetics. First, there are sites of inhibition at Complex II and Complex IV, which carry out the succinate dehydrogenase and cytochrome *c* oxidase reactions, respectively. Since succinate oxidase and durohydroquinone oxidase are both more sensitive to inhibition than is cytochrome *c* oxidase, there must be another site between Complex II and cytochrome *c*. Finally, since NADH oxidase and NADH-cytochrome *c* oxidoreductase are inhibited at conisderably lower concentrations of anesthetic than either the succinate or durohydroquinone oxidations, the site which has the greatest sensitivity to inhibition must be in or near Complex I.

Local anesthetics evidently have a broad specificity for interaction with membrane enzymes. The alcohols are well-known nonspecific conformational perturbants of protein structure [25–27]; it appears that the tertiary amine local anesthetics may likewise be nonspecific or weakly specific conformational perturbants. The opposite effects of tetracaine and dibucaine on membrane-bound acetylcholine receptor were recently reported [28]. We have also shown that

water-soluble, lipid-free F_1-ATPase isolated from beef heart mitochondria is inhibited by tetracaine as well as by *n*-butanol [29]. Thus, the effects of these agents are not restricted to enzymes associated with lipids.

We cannot say with certainty whether the anesthetic effects on electron transport are primarily due to interactions with the proteins, with the bilayer lipid, or with the boundary lipid, since all are present. Considering the low concentrations of some of the agents required for inhibition, however, it would seem to be most unlikely that this could result from a general perturbation of the lipid bilayer. Also, the difference in relative sensitivity of the succinate oxidation reactions to inhibition by tetracaine and dibucaine (Figs. 3 and 4) implies a degree of specificity which cannot be accounted for in terms of lipid bilayer perturbation. Hence, we conclude that the effects must either be due to direct interactions with the protein or else with the lipid molecules intimately associated with the proteins.

There is some precedent in the literature for a biphasic response of proteins to alcohols as a function of concentration. It has been shown, for example, that low concentrations of the normal alcohols protect hemoglobin from denaturation, but that higher concentrations of the same agents cause denaturation [30]. Thus the electron transport activation which we observed in several instances at low concentrations of alcohols and tertiary amines may be due to a protein structure stabilizing effect of these compounds.

It is not the purpose of this paper to attempt to show how anesthetics affect nerves. Considering the widespread interest in that subject, however, it is only fair to point out that the concentrations of local anesthetics required to give 50% inhibition of beef heart NADH oxidase at 25°C are lower than the reported concentrations required to block axonal transmission in frog sciatic nerve [31–33]. This implies two things. First, the clinical anesthesiologist must be concerned about inhibiting other cellular processes (e.g., mitochondrial) when he is administering a nerve-blocking concentration of local anesthetic; and second, the fundamental molecular mechanisms involved in affecting the neural proteins may be similar to those involved in the inhibition of mitochondrial and other enzymes.

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