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INTERACTION OF DRUGS WITH A MODEL MEMBRANE PROTEIN

EFFECT OF DIBUCAINE ON CYTOCHROME OXIDASE PROTEOLIPOSOMES

MICHAEL A. SINGER
Department of Medicine, Queen's University, Kingston, Ontario, Canada K7L 3N6

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Abstract—Cytochrome oxidase is a mitochondrial trans-membrane protein which catalyzes the vectorial transfer of electrons from cytochrome *c* to molecular oxygen. When the oxidase was incorporated into liposomes composed of saturated phospholipids, enzymatic activity was reduced as compared to the activity of either the isolated enzyme or the enzyme incorporated into soy bean phospholipid (asolectin) liposomes. This reduced activity probably resulted from partial replacement of retained oxidase boundary lipid with exogenously added lipid and an unfavourable orientation of a portion of the oxidase molecules for reaction with externally added substrate. On the other hand, substrate binding at the low affinity site was enhanced by incorporation of the oxidase into vesicles composed of either saturated phospholipids or asolectin. At pH 7.4 the local anesthetic dibucaine behaved as an uncompetitive inhibitor of the enzyme, while at pH 6.0 the inhibition pattern became mixed in type. Dibucaine had similar effects on both the isolated and incorporated enzyme except that, in general, the anesthetic caused less inhibition of the incorporated oxidase. It is postulated that positively charged anesthetic molecules act predominantly by competing with substrate for binding while non-charged anesthetic molecules interact with the oxidase boundary lipid to form non-productive complexes.

Many drugs interact with a cellular membrane at some stage of their pharmacologic action. Consequently an understanding of drug-membrane interactions is important to a definition of the mechanism of action of these drugs. The study of such interactions is made difficult by the enormous complexity of cellular membranes. However, model membranes can be constructed with incorporated proteins, thus permitting the study of functional interactions between protein and lipid and the effects of drugs on these interactions. Since the protein molecule(s) that constitutes the target for a given set of drugs is usually not available for such studies, we have chosen the enzyme cytochrome oxidase as a suitable model membrane protein. This molecule is a well-defined mitochondrial trans-membrane protein that catalyzes the vectorial transfer of electrons from cytochrome *c* to molecular oxygen [1].

In a previous study [2], we examined the effects of three cationic local anesthetics on the activity of solubilized cytochrome oxidase. The three anesthetics caused a mixed-type inhibition of oxidase activity with the order of potency being dibucaine > tetracaine > procaine. Since the substrate cytochrome *c* is positively charged and binds at a negative site on the oxidase, it was postulated that the positively charged end of the anesthetic competed with cytochrome *c* for binding while the non-polar end interacted with oxidase-associated phospholipids to produce non-productive complexes.

In the present study we have extended these experiments to examine the interaction of one of these local anesthetics with liposomes containing incorporated cytochrome oxidase. Preliminary experiments had demonstrated that the three cationic local anesthetics inhibited the activity of these proteoliposomes, with dibucaine being the most potent. Since the three anesthetics appear to differ quantitatively rather than qualitatively, dibucaine alone was used in the experiments reported in this communication.

MATERIALS AND METHODS

Materials. Cytochrome *c* (type VI from horse heart), sodium ascorbate, sodium cholate and L-α-dimyristoyl phosphatidylcholine (DMPC) were obtained from the Sigma Chemical Co., St. Louis, MO. Dibucaine was purchased from ICN Pharmaceuticals, Plainview, NY. Asolectin (soybean phospholipids) was obtained from Associated Concentrates, Woodside, Long Island, NY, while L-α-dimyristoyl phosphatidylethanolamine (DMPE) was purchased from CalBiochem, San Diego, CA. Both DMPC and DMPE showed single spots on thin-layer chromatography.

Cytochrome oxidase. Cytochrome oxidase was extracted from beef heart and purified according to the method of Kuboyama *et al.* [3]. Following this procedure, the oxidase was dialyzed for 72 hr against 2% sodium cholate, 100 mM phosphate, 25% ammonium sulfate (pH 8.0). The mixture was centrifuged and the supernatant fraction was dialyzed overnight against 100 mM phosphate buffer (pH 7.4). The final preparation was stored in 100 mM sodium phosphate buffer (pH 7.4) at −80°. Cytochrome aa_3 concentration was measured spectrophotometrically using a millimolar extinction coef-

ficient of 24 at 605 nm (reduced-oxidized) [4]. Protein concentration was measured using the biuret reaction [5]. The oxidase preparations obtained had heme to protein ratios of 8–9 nmoles heme a/mg protein.

Proteoliposome preparation. Cytochrome-oxidase-containing proteoliposomes were prepared as follows [6]. Twenty-five milligrams of lipid (dissolved in chloroform) was dried under vacuum in a glass tube. Three milliliters of buffer (100 mM potassium phosphate, pH 7.4 or 6.0) was then pipetted into the tube and the mixture was mechanically shaken on a vortex mixer to form multilamellar liposomes. Saturated lipids such as DMPE and DMPC do not form liposomes unless the temperature exceeds their phase transition temperature (DMPC 24°, DMPE 49°). Hence, for these two lipids the buffer was preheated to the appropriate temperature, and the tube, when not applied to the vortex, was immersed in a water bath also at this temperature. Following dispersion of the lipids, cytochrome oxidase (1.3 mg protein, equivalent to 5.6 nmoles aa_3) was added and the mixture was sonicated with the intermediate tip of an Artek sonic 300 dismembrator. The sonication step was carried out in a thick walled polycarbonate tube surrounded by ice water for 15 min at 120 W. The opalescent solution was centrifuged at 15,000 rpm in a Sorvall RC-5 centrifuge (Rotor SS-34) for 10 min. The supernatant fraction was transferred to a test tube and placed on ice.

The composition of the proteoliposomes was estimated as follows. The concentration of cytochrome oxidase in the proteoliposomes (supernatant fraction) was measured spectrophotometrically and expressed in terms of the aa_3 unit, using a millimolar extinction coefficient of 27 for the reduced–oxidized spectrum (605–630 nm) [6]. The pellet (following centrifugation) consisted almost entirely of polylamellar lipid. This was dried and weighed. The amount of lipid in the supernatant fraction was calculated as the difference between the amount of lipid originally used and the amount remaining in the pellet.

Using these measurements, we calculated that between 80 and 95% of cytochrome oxidase (as aa_3) and between 65 and 95% of lipid were incorporated. Generally we observed that asolectin gave the highest incorporation (80–95%) and DMPE the lowest (65–70%). It should be stressed that the amounts of cytochrome oxidase and lipid used were chosen somewhat arbitrarily. For any given lipid an occasional sample would show much less incorporation than usual. For example, although DMPC generally gave 70–75% incorporation, an occasional sample only gave 50%. The lipid to protein ratio between these samples would obviously be different. However, we did not observe any effect of these different protein:lipid ratios on enzymatic activity.*

The morphology of asolectin and DMPC proteoliposomes prepared in the above fashion was examined by transmission electron microscopy using potassium phosphotungstate negative staining. These preparations consisted almost entirely of single walled small vesicles.

Polarographic assay of oxidase activity. Rates of oxygen uptake were measured with a Yellow Springs Instruments oxygen electrode fitted to a conventional chart recorder. The assay mixture had a volume of 3 ml and contained cytochrome *c* (range 2–100 μM), 20 mM sodium ascorbate, 100 mM potassium phosphate (pH 7.4 or 6.0). After a stable baseline was achieved, cytochrome oxidase (isolated or as proteoliposomes) was added. Final concentration (as aa_3) was about 0.03 to 0.06 μM. After a steady state was achieved, aliquots of a stock solution of dibucaine were added to give final concentrations between 1.0 and 5.0 mM. The assay chamber was immersed in a thermostatted water bath to allow measurements at different temperatures (range 15–35°). Enzyme activity is expressed in terms of a turnover number (TN) that is calculated by multiplying the oxygen consumption by 4 and dividing by the oxidase concentration (μM aa_3). Four moles of cytochrome *c* are oxidized for each mole of oxygen consumed. The maximum turnover number (TN_{max}) is the value extrapolated to infinite cytochrome *c* concentration.

RESULTS

Figure 1 illustrates the temperature dependence of TN_{max} and K_m for solubilized and lipid incorporated cytochrome oxidase. Asolectin-oxidase proteoliposomes displayed about the same activity as the

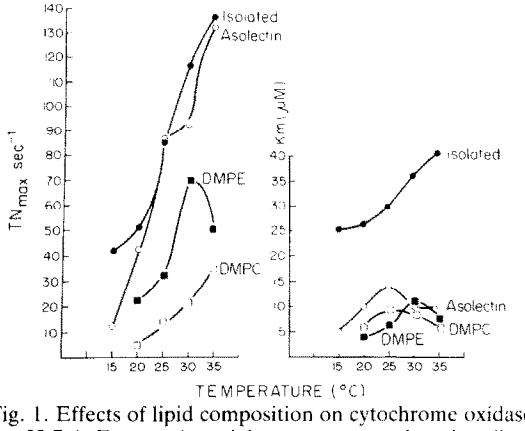

Fig. 1. Effects of lipid composition on cytochrome oxidase at pH 7.4. Enzymatic activity was measured as described in the text. The assay mixture had a volume of 3 ml and contained cytochrome *c*(2–100 μM), 20 mM sodium ascorbate, 100 mM potassium phosphate (pH 7.4) and cytochrome oxidase (0.03 to 0.06 μM, measured as aa_3). The left-hand side of the figure is a plot of TN_{max} versus temperature. Each experimental point was obtained as follows. At a given temperature, cytochrome oxidase activity was measured at different concentrations of cytochrome *c* (range 2–100 μM). For each series, a Lineweaver–Burk plot was constructed using the method of least squares and TN_{max} was calculated as the activity extrapolated to infinite substrate concentration. For each series the K_m was also calculated from the slope and *y*-intercept of the Lineweaver–Burk plot. The right-hand side of the figure is a plot of these K_m values versus temperature. Some representative Lineweaver–Burk plots at pH 7.4 are illustrated in Fig. 3.

* Assuming 75% lipid incorporation and 85% oxidase incorporation, one would obtain a lipid to protein ratio of 17:1 (mg/mg).

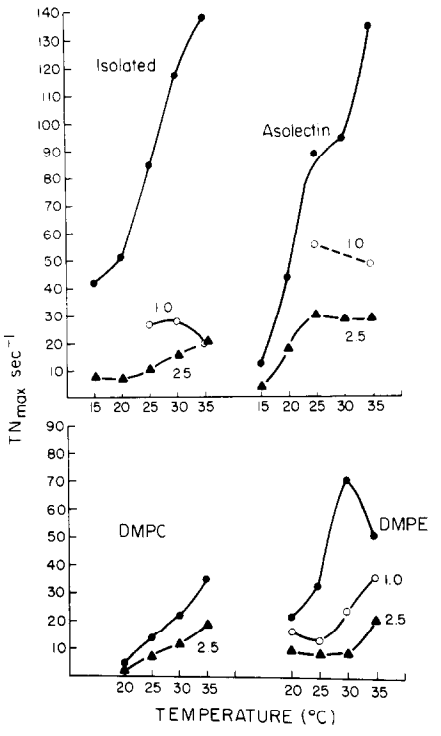

Fig. 2. Effect of dibucaine on cytochrome oxidase activity at pH 7.4. Proteoliposomes were made as described in Materials and Methods. The experimental points at a given temperature were derived from the same series as follows. At a given temperature, cytochrome oxidase activity was measured at different concentrations of cytochrome *c* (range 2–100 μM). For each cytochrome *c* concentration, activity was measured before and following addition of dibucaine (final concentrations 1.0 and 2.5 mM). A Lineweaver–Burk plot was constructed for each series by the method of least squares, and TN_{max} was calculated as the activity extrapolated to infinite cytochrome *c* concentration. The assay mixture had a volume of 3 ml and contained cytochrome *c* (2–100 μM), 20 mM sodium ascorbate, 100 mM potassium phosphate, (pH 7.4) and cytochrome oxidase (0.03 to 0.06 μM, measured as aa_3). Open circles and closed triangles represent enzyme activity in the presence of 1.0 and 2.5 mM dibucaine respectively.

isolated enzyme except at the lowest temperature (15°) examined where the isolated enzyme was more active. In comparison, DMPE and DMPC-oxidase proteoliposomes had less enzymatic activity throughout the whole temperature range. In summary, the activity of these cytochrome oxidase preparations was in the order: isolated $\geqslant$ asolectin > DMPE > DMPC.

The K_m of the isolated enzyme showed a progressive rise with temperature, whereas the K_m values of the three types of proteoliposomes did not display any consistent temperature dependence. However, the most striking difference was the significant decrease in K_m brought about by incorporating the enzyme into a lipid environment. In contrast to the clear-cut separation of the different types of proteoliposomes in terms of TN_{max}, there was no consistent separation with respect to their K_m values. For example, at 25° the K_m values appeared to differ, but at 30° they became quite similar.

Figure 2 illustrates the effect of dibucaine on the activity of solubilized cytochrome oxidase and oxidase incorporated into liposomes. Dibucaine caused a dose-dependent decrease in TN_{max} in all cases.

The observation that dibucaine reduced the TN_{max} of cytochrome oxidase (both isolated and incorporated) excludes purely competitive inhibition. However, a reduction in TN_{max} is consistent with a number of inhibitor patterns. For the sake of simplicity, we can consider three main types [7]. A non-competitive inhibitor decreases TN_{max} but does not alter the K_m of the enzyme. An uncompetitive inhibitor causes reductions in both TN_{max} and K_m to the same extent. Finally, a mixed type of inhibition is characterized by a decrease in TN_{max} but an increase in the apparent K_m of the enzyme.

Table 1 summarizes the effects of dibucaine on TN_{max} and K_m of both isolated and lipid-incorporated cytochrome oxidase. The results have been tabulated as follows. The numbers refer to the ratio of TN_{max} and K_m in the presence and absence of dibucaine. In all cases the "TN_{max} ratio" was less than 1, i.e. the TN_{max} was reduced by the presence of dibucaine. At pH 7.4, dibucaine also caused a reduction in K_m

Table 1. Effects of dibucaine on cytochrome oxidase*

	Preparation							
	Isolated		Asolectin		DMPC		DMPE	
Temperature	T_r	K_r	T_r	K_r	T_r	K_r	T_r	K_r
(A) pH 7.4								
15°	0.19	0.16	0.30	0.19				
20°	0.13	0.31	0.43	0.64	0.66	0.56	0.44	0.47
25°	0.12	0.13	0.34	0.37	0.49	0.60	0.27	0.72
30°	0.13	0.24	0.29	0.50	0.50	0.84	0.11	0.11
35°	0.15	0.13	0.22	0.48	0.51	0.90	0.40	0.55
(B) pH 6.0								
25°	0.64	1.42	0.77	1.34				
30°	0.41	1.35	0.73	4.9	0.44	3.6		

* Experiments in section A were performed at pH 7.4 while those in section B were done at pH 6.0. T_r refers to the ratio of TN_{max} in the presence of 2.5 mM dibucaine over TN_{max} in the absence of anesthetic. Similarly, K_r refers to the ratio of K_m in the presence of 2.5 mM dibucaine over K_m in the absence of anesthetic. Ratios below 1.0 indicate a reduction in TN_{max} or K_m by the anesthetic. For section A the values for TN_{max} with and without dibucaine are illustrated in Fig. 2. The K_m values for the uninhibited enzyme at pH 7.4 are given in Fig. 1.

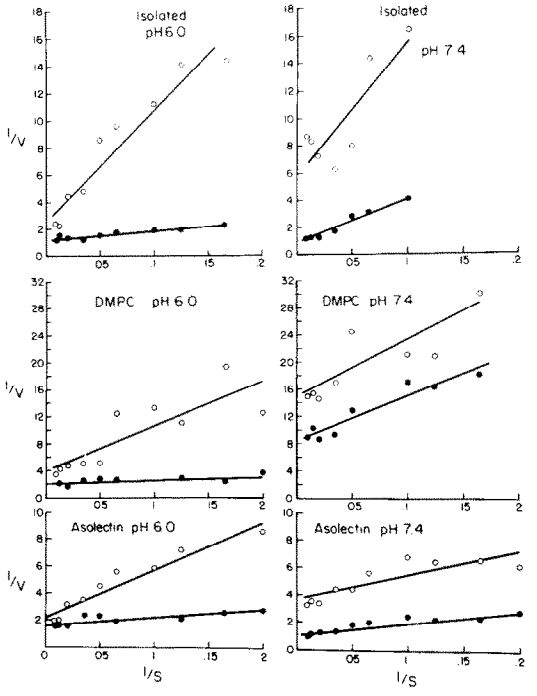

Fig. 3. Effect of dibucaine on cytochrome oxidase activity at pH 7.4 and 6.0 (Lineweaver–Burk plots). Enzymatic activity was measured as described in the text. The assay mixture had a volume of 3 ml and contained cytochrome *c* (2–100 μM), 20 mM sodium ascorbate, 100 mM potassium phosphate (pH 6.0 and 7.4) and cytochrome oxidase (0.03 to 0.06 μM, measured as aa_3). The final concentration of dibucaine was 2.5 mM. The ordinate ($1/V$) is the reciprocal of the turnover number (sec^{-1}) multiplied by 10^2. The abscissa ($1/S$) is the reciprocal of the cytochrome *c* concentration (μM). All experiments were performed at 30°. Lines were drawn by the method of least squares. Open circles represent enzyme activity in the presence of 2.5 mM dibucaine.

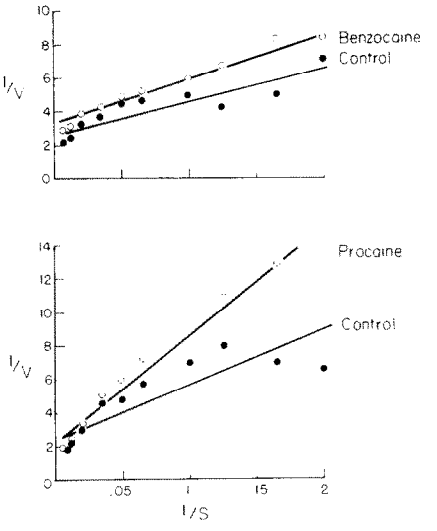

Fig. 4. Effect of procaine and benzocaine on cytochrome oxidase activity in asolectin proteoliposomes, pH 6.0 (Lineweaver–Burk plots). Enzymatic activity was measured as described in the text. The assay mixture had a volume of 3 ml and contained cytochrome *c* (2–100 μM), 20 mM sodium ascorbate, 100 mM potassium phosphate (pH 6.0) and cytochrome oxidase (0.03 to 0.06 M, measured as aa_3). The final concentration of procaine and benzocaine was 10 mM. Benzocaine was added from a concentrated stock ethanol solution. An equal volume of ethanol alone had no effect on oxidase activity. The ordinate ($1/V$) is the reciprocal of the turnover number (sec^{-1}) multiplied by 10^2. The abscissa ($1/S$) is the reciprocal of the cytochrome *c* concentration (μM). All experiments were performed at 30°. Lines were drawn by the method of least squares.

since the "K_m ratio" was less than 1. Although dibucaine reduced the apparent K_m of the enzyme, the extent of the reduction was not always of the same magnitude as the reduction in TN_{max}. (A decrease in TN_{max} and K_m of the same magnitude would be indicated by an equality of the two ratios.) Thus, at pH 7.4, dibucaine behaved predominantly as an uncompetitive inhibitor.* In contrast, at pH 6.0 dibucaine became a mixed type of inhibitor since at this pH the "K_m ratio" became greater than 1. This difference in the kinetic pattern of inhibition between pH 7.4 and 6.0 is illustrated in Fig. 3. In this figure, experimental results of enzyme activity (in the presence and absence of dibucaine) for the isolated oxidase and DMPC and asolectin-proteoliposomes are presented in the form of Lineweaver–Burk plots. At pH 6.0 the control and plus inhibitor lines converged above the abscissa, whereas at pH 7.4 these lines were either parallel or converged below the abscissa.

One explanation consistent with these pH experiments is that the charged and uncharged forms of the anesthetic are responsible for the different inhibitor patterns. Since dibucaine has a pK of 8.5 [8], more than 99% will be ionized and positively charged at pH 6.0. At pH 7.4 approximately 10% of the anesthetic molecules will be uncharged. At higher pH dibucaine becomes much less water soluble. For example, a pH of 10.5 would be required to have over 99% of the molecules unionized. Furthermore, at this pH enzyme activity is reduced markedly (see last footnote of text). To examine the differential role of charged and uncharged species, the following experiment was performed. Procaine and benzocaine are two local anesthetics with similar structures except that procaine possesses a tertiary amine group with a pK of 8.9 [8], while benzocaine lacks such a group and is electrically neutral over a wide pH range. Asolectin-proteoliposomes were prepared and enzyme activity was measured in the presence and absence of either 10 mM procaine or benzocaine at pH 6.0.This higher concentration was used since these local anesthetics are much less potent than dibucaine in inhibiting the isolated enzyme [2, †].

* Clearly, the classification of inhibitors as either uncompetitive, noncompetitive or mixed is too simplistic. An inhibitor that reduces K_m but not to the same extent as TN_{max} is not purely uncompetitive but in truth lies somewhere between uncompetitive and non-competitive.

† In a previous publication [2], it appeared that benzocaine had no effect on the isolated enzyme. A careful look at Fig. 1 of that reference indicates that benzocaine did cause a small and probably uncompetitive type of inhibition.

At pH 6.0 procaine will be almost 100% ionized while benzocaine will be uncharged at this same pH. The results are illustrated in Fig. 4. Procaine caused a competitive pattern of inhibition while benzocaine caused a modest degree of inhibition with a predominantly uncompetitive pattern.* For uncompetitive and mixed type inhibitors, a plot of the reciprocal of the enzyme TN_{max} versus inhibitor concentration can be used to determine the dissociation constant (K_i) of the enzyme–substrate–inhibitor complex [7]. These plots are illustrated in Fig. 5 and K_i values are summarized in Table 2. In general, dibucaine bound more strongly to the isolated than to the incorporated enzyme–substrate complex. Binding increased with increasing temperature and was also greater at pH 7.4 than at pH 6.0. Finally, there was a correspondence between the extent of inhibition (reduction in TN_{max}) and the strength of dibucaine binding to the enzyme–substrate complex.

DISCUSSION

Cytochrome oxidase is a protein of monomer molecular weight close to 140,000, containing two heme a groups and two copper atoms as electron acceptors. Current evidence indicates that this enzyme spans the mitochondrial inner membrane [9]. Cytochrome oxidase preparations, as used in this study, possess a phospholipid component in the order of 20% (w/w) lipid to protein. The retained phospholipid contains cardiolipin and other lipids such as phosphatidylcholine and phosphatidylethanolamine. Approximately three-fourths of this retained lipid (boundary layer) can easily exchange with nondenaturing detergents [10]. This phospholipid-depleted enzyme is inactive but can be activated by the re-addition of either phospholipids or detergents. The remaining one-fourth of the boundary layer which does not readily exchange is composed predominantly of cardiolipin [10]. The exact role of this strongly bound cardiolipin component is unknown. Yu *et al.* [11] have suggested that the phospholipid is necessary to accelerate the internal transfer of electrons between the two heme moieties within the oxidase complex. Recently, at least two binding sites for cytochrome *c* on isolated cytochrome oxidase have been described. One is of high affinity and the second is of low affinity. Bisson *et al.* [9] have presented evidence to indicate that lipid may represent the low affinity site whereas the high affinity site is probably a straight protein–protein interaction.†

In this study, since cytochrome oxidase was not specifically delipidated, the model membrane would structurally consist of the enzyme, with its retained phospholipid shell, spanning a bilayer of either asolectin, DMPE or DMPC. The data in Fig. 1 indicate that changing the composition of the bulk bilayer did alter the activity of the incorporated oxidase. The two saturated phospholipids reduced TN_{max}, whereas isolated oxidase and asolectin proteoliposomes had about the same enzymatic activity. Comparing the two saturated lipids suggests that there was some head group specificity since DMPE proteoliposomes had more activity than DMPC proteoliposomes. By contrast, all three phospholipids significantly reduced the Michaelis constant (K_m)

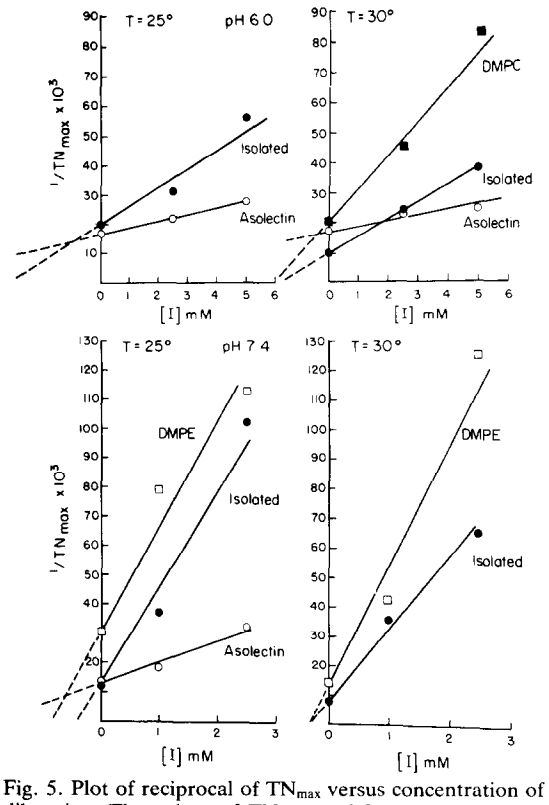

Fig. 5. Plot of reciprocal of TN_{max} versus concentration of dibucaine. The values of TN_{max} used for the plots at pH 7.4 were taken from Fig. 2. Similar experiments to those illustrated in Fig. 2 were performed at pH 6.0, and the values of TN_{max} so obtained were used in the plots illustrated in this figure. At pH 7.4, the anesthetic concentrations used were 1.0 and 2.5 mM. At pH 6.0, dibucaine is more water soluble so that higher concentrations were employed (2.5 and 5.0 mM). The intercept of a given line on the *x*-axis gives K_i.

Table 2. Dissociation constants of anesthetic–enzyme–substrate complexes*

	K_i (mM) 25°	30°
(A) pH 7.4		
Isolated	0.4	0.3
DMPE	0.8	0.3
Asolectin	2.0	
(B) pH 6.0		
Isolated	3.2	1.5
DMPC		1.7
Asolectin	7	7

* K_i is defined by the following relationship: $K_i = [ES][I]/[ESI]$. The values listed were obtained from the *x*-axis intercepts of the plots illustrated in Fig. 5.

* From Fig. 4, the ratios of TN_{max} (T_r) and K_m (K_r) in the presence and absence of either benzocaine or procaine are: benzocaine, $T_r = 0.76$ and $K_r = 0.85$; procaine, $T_r = 1.04$ and $K_r = 2.14$.

† In the present study, only the low affinity site was examined.

indicating that the substrate cytochrome *c* was bound more strongly to the proteoliposomes than to the isolated preparation.*

There are several possible explanations for these "lipid" effects. Nicholls *et al.* [13] have demonstrated that in asolectin proteoliposomes approximately 50% of the oxidase molecules are oriented in the wrong direction to react with externally added cytochrome *c*. This being the case the asolectin proteoliposomes in the present experiments would actually have had a higher enzymatic activity than the isolated preparation if this "orientation effect" were taken into account. It is possible that in DMPE and DMPC proteoliposomes an even larger fraction of oxidase molecules are unavailable for reaction with substrate because of an unfavourable orientation. There is certainly no *a priori* reason for this to be true, but at the moment this possibility cannot be excluded.

The physical state of the phospholipid could possibly have been playing a role. DMPE and DMPC are both saturated lipids while asolectin contains unsaturated fatty acyl chains. This possibility seems unlikely though. DMPC has a transition temperature (Tc) at 24°, yet there was no significant increase in oxidase activity at this temperature. DMPE has a Tc of 49° and would have been in the gel state at all temperatures examined. Yet oxidase incorporated into this lipid had a higher activity than when in an environment of DMPC.

The temperature dependence of cytochrome oxidase activity has been reported previously in the literature [14, 15]. Arrhenius plots revealed definite changes (breaks) in slope for both the isolated and lipid reconstituted enzyme, although the temperatures at which these breaks occurred were not necessarily the same in different studies. Suffice it to say that the mechanism responsible for these breaks is unknown, but it is clear that there was no obvious relationship between these breaks and the main gel to liquid crystal transition of the reconstituting lipids. If Arrhenius plots are constructed using the TN_{max} values of Fig. 1, definite changes in slope are observed at 20° and 25° for the isolated enzyme and at 25° (DMPC, asolectin) and 30° (DMPE) for proteoliposomes. Although the significance of these breaks is unclear, the important observation is that they bear no obvious relationship to the transition temperature of the dispersing lipids.

Another possibility is that exchange was occurring between the exogeneously added bulk bilayer and some or all of the oxidase-associated phospholipids. As already discussed, approximately three-fourths of the cytochrome oxidase boundary lipid can exchange with detergents. The remaining fourth (mostly cardiolipin) is more strongly bound. Vik and Capaldi [16] used this detergent exchange method to deplete beef heart cytochrome oxidase of most of its endogenous phospholipid. This delipidated enzyme was inactive but could be activated by the addition of exogeneous lipids. They observed that optimal cytochrome oxidase activity was obtained with lipids containing unsaturated fatty acyl chains (e.g. asolectin, mitochondrial lipids, dioleoylphosphatidylcholine, or dioleoylphosphatidylethanolamine). Saturated phospholipids restored only about 50% of oxidase activity and among these saturated lipids they did not detect any head group specificity. However, there did appear to be less restoration of activity with lengthening of the hydrocarbon chains. In the model system used in the present study, exchange most likely occurred between the bulk bilayer and part of the oxidase boundary lipid. The differences between DMPC and DMPE may just have been due to a differential ability of these two lipids to exchange with the endogenous lipid of the enzyme.

In summary, the "lipid" effects on enzymatic activity illustrated in Fig. 1 probably resulted from partial replacement of oxidase boundary lipid with the lipid species in the bulk bilayer and an unfavourable orientation of a portion of the oxidase molecules for reaction with externally added substrate.

In addition to these effects of changes in lipid composition on oxidase activity, the protein itself can alter the physical state of the surrounding bilayer. Griffith and Jost [17] and Knowles *et al.* [18], using the spin label technique, have measured an immobilization of lipid molecules induced by the presence of cytochrome oxidase. This immobilization appears to extend out to approximately six shells from the protein. By contrast Oldfield *et al.* [19] and Seelig and Seelig [20], using nuclear magnetic resonance spectroscopy, found evidence for a disordering effect of cytochrome oxidase on the surrounding bilayer rather than the creation of an immobilized layer. Whether these divergent results simply reflect the use of different techniques which measure molecular motions on different time scales and the functional significance of such alterations in lipid structure are unclear at the moment.

Figure 1 also illustrates that all three lipids reduced the Michaelis constant (K_m) to about the same extent. This effect on K_m, then, appears to be independent of the nature of the lipid species in the bulk layer. The simplest explanation would be that merely orienting the oxidase molecule in an anisotropic environment (a bilayer) somehow enhanced substrate binding at the low affinity site.

Dibucaine inhibited both the isolated enzyme and the proteoliposomes in a dose-dependent fashion. This local anesthetic has a pK value of 8.5 [8]. At pH 7.4 approximately 10% of the molecules will be electrically neutral, while at pH 6.0 this fraction falls to less than 1%. The pattern of inhibition also varies between these pH values. At pH 7.4 dibucaine behaved predominantly as an uncompetitive inhibitor of both the isolated and incorporated enzyme. TN_{max} and the apparent K_m of the enzyme were both reduced. The simplest scheme consistent with this pattern is that dibucaine combined with the enzyme–substrate (ES) complex to form non-productive or dead-end enzyme–substrate–inhibitor (ESI) complexes [7]. At pH 6.0 the inhibition pattern became more consistent with a mixed-type since at this pH, although TN_{max} was reduced, the apparent K_m of the enzyme was also increased. As described in a previous publication, the simplest scheme is one

* Ferguson-Miller *et al.* [12] have demonstrated that the Michaelis constant (K_m) is a good measure of the dissociation constant for cytochrome *c*.

in which the enzyme–inhibitor (EI) complex has a lower affinity than the free enzyme for the substrate and the enzyme–substrate–inhibitor (ESI) complex is non-productive or dead-end [2, 7].

It is also apparent that the effects of dibucaine on the isolated and incorporated enzyme were very similar. The only significant difference between these preparations was in the magnitude of the inhibition. At pH 7.4 dibucaine caused a greater reduction in TN_{max} of the isolated enzyme than of the proteoliposomes. The only exception was at 30° were equal inhibition was observed for both the isolated enzyme and DMPE proteoliposomes. At pH 6.0 dibucaine caused a smaller reduction of TN_{max} than at pH 7.4. Again, the magnitude of the inhibition was greatest for the isolated preparation and less for asolectin proteoliposomes. Enzyme incorporated into DMPC showed about the same inhibition as the isolated enzyme. These effects on TN_{max} were paralleled by the K_i values listed in Table 2. A greater reduction in TN_{max} was associated with stronger binding of dibucaine to the ES complex. At most, then, the presence of the "bulk" bilayer appears to have reduced the inhibitory effect of dibucaine.

The following is presented as a working model only. We will make the simplifying assumption that the pH effects on the pattern of inhibition relate to the state of ionization of the local anesthetic only.* Furthermore, since the effects of dibucaine on the isolated and incorporated enzyme were similar, the main interactions must have been between the local anesthetic and either the protein component of the oxidase or its associated phospholipids. In a previous publication [2], evidence was presented pointing to the phospholipids as the site of action. The dibucaine–lipid interaction could significantly alter the physical state of these phospholipids since, in model bilayers, dibucaine causes a marked increase in fatty acyl chain motion below the phase transition temperature of the lipid [23] as well as decreasing the co-operativity of the transition [24]. This conclusion is strengthened by the observation that the lipids may actually represent the low affinity site [9]. In this context, the "protective" influence of the bulk bilayer in reducing the inhibitory effect of dibucaine (and its binding to the ES complex) may simply be the result of a dilutional effect, i.e. binding of some of the local anesthetic by the bulk bilayer.

At pH 6.0 more than 99% of the dibucaine is ionized and positively charged. As previously described, the mixed-inhibitory picture could be the result of the charged end of the anesthetic molecule competing with the substrate for binding and the non-polar end interacting with the boundary lipid layer of the oxidase to form dead-end ESI complexes. An equally plausible and perhaps more realistic model would envisage the uncharged anesthetic molecules as interacting with the lipids and the charged molecules as competing with substrate for binding rather than both interactions occurring with the same molecule. At pH 7.4, the concentration of uncharged dibucaine molecules would now make up about 10% of the anesthetic population. Anesthetic lipid interactions would become more dominant forming more dead-end ESI complexes. The resulting inhibitory pattern would become more uncompetitive in character.

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* Actually pH does have an effect on oxidase activity. Nicholls and Chance [1] reported a pH optimum of 6.0. Smith *et al.* [21] reported a pH optimum also of 6 when activity was measured spectrophotometrically but a pH optimum of between 7.3 and 7.8 when the polarographic method was used. Wilms *et al.* [22] found no pH optimum but just a continuous increase in activity as the pH was lowered from 8.6 to 4.6. Due to these conflicting observations and because the model presented here is only a working one, this simplifying assumption was made.

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