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The inhibition of a membrane-bound enzyme as a model for anaesthetic action and drug toxicity

Brian B. HASINOFF* and John P. DAVEY†
Memorial University of Newfoundland, Department of Chemistry and Faculty of Medicine, St. John's, Newfoundland A1B 3X7, Canada

The inhibition of the membrane-bound enzyme cytochrome *c* oxidase by aliphatic n-alcohols and other neutral organic compounds was studied as a model for anaesthetic action and drug toxicity. The n-alcohols (C_1 to C_{14}) displayed a variation in inhibition constant of over 500000-fold. The inhibition constants correlated well with the number of carbon atoms in the n-alcohols and also their n-octanol/water partition coefficients. General anaesthetic potency is known to be similarily well correlated with octanol/water partition coefficients. The free-energy change for transferring a methylene group of the n-alcohol to the more hydrophobic environment bound to the enzyme is similar to that for transferring a methylene group from water to pure alcohol. These results are consistent with the n-alcohols inhibiting by binding to an octanol-like environment on the enzyme or the protein/phospholipid interface. Neither negatively charged carboxylates nor positively charged amine analogues were observed to cause any inhibition, indicating that this postulated binding site may be uncharged. Inhibition of cytochrome *c* oxidase by n-alcohols was also demonstrated in both bovine heart and rat liver sonicated submitochondrial fragments.

INTRODUCTION

There is new experimental support for the theory that the molecular mechanism of anaesthetic action comes about from an interaction with specific receptor sites (Evers *et al.*, 1987; Franks & Lieb, 1978, 1982, 1984, 1985, 1986) rather than a non-specific interaction with membrane lipids (Miller, 1985). A ^{19}F-n.m.r. study (Evers *et al.*, 1987) has demonstrated that inspired halothane in rat brain tissue exists in two distinct environments, one of which is saturable and highly immobilized and the other of which is not. The former site possibly represents binding to many membrane proteins or a specific membrane lipid, whereas the latter environment was consistent with non-specific lipid interaction. Kinetic inhibition studies have also shown that competitive inhibition of a soluble protein (firefly luciferase) by n-alcohols, n-alkanes and other neutral organic compounds mirrored the anaesthetic effects of these compounds (Franks & Lieb, 1984, 1985, 1986). The levelling out and subsequent decrease of activity with increasing chain length ('the cut-off effect') on inhibition and of anaesthetic action as well was thought to be due to binding to an amphiphilic protein pocket of circumscribed dimensions.

Cytochrome *c* oxidase has been previously used as a model for drug–membrane enzyme interactions (Singer, 1980, 1982; Chazotte & Vanderkooi, 1981; Vanderkooi & Chazotte, 1982; Casanovas *et al.*, 1983, 1985*a*,*b*). A variety of amine local anaesthetics (11 in all) with a significant range of lipophilicities (as measured by their octanol/water partition coefficients) demonstrated both competitive and mixed-type inhibition. Inhibition constants ranged from 40 mM for lidocaine (Casanovas *et al.*, 1983) to 0.4 mM for dibucaine (Singer, 1982). It has been postulated both by Singer (1982) and by Casanovas *et al.* (1985*a*,*b*) that the polar end of the anaesthetic molecule competes with substrate for binding whereas the non-polar end interacts with phospholipids bound to cytochrome *c* oxidase. Quinacrine (Casanovas *et al.*, 1985*a*) was also observed to bind to cytochrome *c* oxidase. A variety of alcohols (butan-1-ol, pentan-1-ol, hexan-1-ol and benzyl alcohol) as well as several amine local anaesthetics were observed to inhibit at multiple sites along the mitochondrial electron-transport chain, including the cytochrome *c* oxidase site (Chazotte & Vanderkooi, 1981; Vanderkooi & Chazotte, 1982). Six fully reduced flavin analogues have also been observed to bind to cytochrome *c* oxidase, with dissociation constants ranging from 5 to 8 μM (Ahmad *et al.*, 1982). Galactosyl-sphingosine as well was observed (Igisu & Nakamura, 1986) to inhibit cytochrome *c* oxidase strongly ($K_i \sim 5$ μM). In previous studies cytochrome *c* oxidase kinetics exhibited weak inhibition by pentan-1-ol, propan-1-ol, propan-2-ol, methanol, glycerol and sucrose (Hasinoff & Davey, 1987, 1988). It has been shown by Orii & Yoshikawa (1973) that hydroxylamine, hydrazine, semicarbazide, salicylaldoxime and ethylxanthate inhibit cytochrome *c* oxidase with inhibition constants as low as 0.5 μM. Thus this diverse range of compounds that have been reported either to bind or to inhibit cytochrome *c* oxidase indicates that the enzyme has a relatively non-specific and possibly hydrophobic inhibitor-binding site. The present study extends the previous work to much longer-chain alcohols as well as a variety of other neutral organic compounds and demonstrates that the n-alcohols bind to a largely hydrophobic site on cytochrome *c* oxidase. The binding of a drug to a hydrophobic site on

* To whom correspondence should be addressed.
† Present address: University Chemical Laboratory, University of Kent at Canterbury, Canterbury, Kent CT2 7NH, U.K.

an enzyme may, in some cases, result in either reversible inhibition or irreversible enzyme inactivation. As an example, cytochrome *c* oxidase has been observed to be inactivated (Goormaghtigh *et al.*, 1982; Demant, 1983) by the widely used cardiotoxic antitumour drug adriamycin. Thus the present investigation with a well-defined series of n-alcohols is a useful model of both drug toxicity and anaesthetic action.

The inhibition exhibited by aliphatic n-alcohols has previously been used as a probe of hydrophobic binding sites on membrane-bound $Na^+ + K^+$-dependent ATPase (Hegyvary, 1973), lipoxygenase (Mitsuda *et al.*, 1967), pepsin (Tang, 1965) and pancreatic lipase (Mattson *et al.*, 1970). The association of hydrocarbons and amphiphiles with hydrophobic sites on proteins such as β-lactoglobulin, bovine serum albumin and serum lipoproteins has been summarized by Tanford (1980).

MATERIALS AND METHODS

Horse heart ferrocytochrome *c* was reduced by dithionite (> 98 %) and then stripped of excess dithionite by passing it down a Sephadex G-25 column (all under N_2) as described by Yonetani & Ray (1965). Bovine heart cytochrome *c* oxidase (Sigma Chemical Co.) was dissolved in sodium phosphate buffer, pH 7.4, containing 0.4 % Tween 80, giving a final Tween 80 concentration of 0.002 %. The enzyme used was prepared according to the method of Yonetani (1967), which results in a preparation that is about 10 % lipid. The stock enzyme concentration was determined spectrophotometrically at 605 nm by using a millimolar absorption coefficient (reduced minus oxidized) of 24.0 $mM^{-1} \cdot cm^{-1}$ per aa_3 haem unit (Van Gelder, 1966). A turnover number (per haem unit) was calculated from $V_{max.}/[E]$ to be 59 s^{-1}. This compares with a value of 60 s^{-1} reported by Yonetani & Ray (1965) under identical conditions. Turnover numbers ranging from 30 to 300 s^{-1} have been variously reported (Vik & Capaldi, 1980). However, apart from the preparation used, these values are highly variable as they depend upon a number of factors such as temperature, pH, buffer, the presence of added lipids, the presence of detergent and even the method of measurement (whether spectrophotometric or polarographic). The experiments were not conducted under a set of 'optimal' assay conditions because of the need to work under a set of conditions that give simple well-defined Michaelis–Menten kinetics.The reaction was studied in phosphate buffer, pH 7.0, of ionic strength 0.1 at 25 °C. At high ionic strengths cytochrome *c* oxidase exhibits only simple Lineweaver–Burk kinetics (Yonetani & Ray, 1965; Malmström & Andréasson, 1985), consistent with a single kinetically significant low-affinity binding site. Thus the results obtained in the present study are concerned only with the low-affinity site.

The initial velocities, *v*, for the cytochrome *c* oxidase-catalysed aerobic oxidation of ferrocytochrome *c* were measured at 550 nm on a Shimadzu UV-260 spectrophotometer by using the following millimolar absorption coefficients (Yonetani & Ray, 1965): $\epsilon_{red.}$ 27.7 $mM^{-1} \cdot cm^{-1}$ and $\Delta(\epsilon_{red.} - \epsilon_{ox.})$ 18.5 $mM^{-1} \cdot cm^{-1}$. The initial velocities, *v*, were estimated from the spectrophotometer-calculated first derivatives graphically extrapolated back to time zero. The reaction was started by stirring the enzyme into the reaction cell containing buffer, inhibitor and ferrocytochrome *c*. This procedure ensured that the inhibitor was present with the enzyme for 10–15 s at most. The Lineweaver–Burk plots, the Dixon plots and their respective secondary plots were analysed by weighted (assuming a constant percentage error in *v*) linear least-squares analyses.

The C_9 to C_{16} n-alcohols (Sigma Chemical Co.) were added as ethanol solutions such that the final concentration of ethanol did not exceed 5 % of its inhibition constant. The final concentration of the n-alcohol in the phosphate buffer of ionic strength 0.1 was kept below 60 % of its aqueous solubility (Franks & Lieb, 1986) to ensure that all of the alcohol fully dissolved.

Both the rat liver (Johnson & Lardy, 1967) and the bovine heart (Azzone *et al.*, 1979) whole mitochondria were prepared by differential centrifugation. Sonicated submitochondrial fragments were prepared by sonicating (tip-type sonicator) whole mitochondria at 0 °C in phosphate buffer with 12 × 5 s bursts interspersed with 10 s cooling periods.

In order to test if the alcohol inhibition was an artifact of the detergent, the initial velocities in the presence of inhibitory concentrations of octan-1-ol were measured at concentrations of Tween 80 up to 0.08 %, which was 40-fold higher than that routinely used. The lack of any dependence on detergent concentration rules out detergent effects and indicates that at the detergent concentrations used the detergent does not significantly compete for octan-1-ol. That alcohol inhibition of cytochrome *c* oxidase on the sonicated submitochondrial fragments was also observed also rules out detergent effects.

A couple of tests for reversibility of alcohol inhibition were carried out. In one test the initial velocities were unchanged after the enzyme was incubated in 100 mM-pentan-1-ol and subsequently diluted 5-fold. In another test the initial velocities were unchanged whether or not the enzyme was incubated with octan-1-ol for several minutes before rate measurements. The fact that the removal of phospholipid from cytochrome *c* oxidase in high concentrations of detergent is carried out in the presence of high concentrations of glycerol (10–30 %, v/v, 1–3.3 M) without irreversible damage (Fry & Green, 1980; Vik *et al.*, 1981) is good evidence that the effects of moderately high concentrations of alcohols are reversible. There was no spectral evidence seen for alcohol binding to ferrocytochrome *c*, as the visible-region spectrum of ferrocytochrome *c* was unchanged in 24 mM-pentan-1-ol.

RESULTS

Inhibition with detergent-solubilized enzyme

The inhibition kinetics were, except where noted, interpreted within a general linear mixed inhibition (Cornish-Bowden, 1979) scheme, as both K_m and $V_{max.}$ values were observed to vary with inhibitor concentration:

$$\begin{array}{ccc} E + S \rightleftharpoons ES \rightarrow E + P & & (1) \\ + & + & \\ I & I & \\ \Big\| K_i & \Big\| K_i' & \\ EI & ESI & \end{array}$$

The reciprocal initial-velocity equation for reaction

scheme (1) is given in its Dixon-plot form (Cornish-Bowden, 1979) by:

$$v^{-1} = \left(\frac{K_m}{V_{max.}K_i[S]} + \frac{1}{K_i'V_{max.}}\right)[I] + \frac{K_m}{V_{max.}[S]} + \frac{1}{V_{max.}} \quad (2)$$

The initial-velocity data were usually analysed in a weighted Dixon-plot form (Fig. 1) holding [S] constant, or occasionally in a weighted Lineweaver–Burk plot form holding [I] constant (Fig. 2). Secondary plots of the slopes of Dixon plots versus 1/[S] from eqn. (2) gave the inhibition constants. Similarly secondary plots of $K_m/V_{max.}$ and $1/V_{max.}$ from the Lineweaver–Burk plots against [I] also gave the inhibition constants. In general the parameter K_i' was not as well determined as was K_i. The Dixon plots of the n-alcohols below heptan-1-ol displayed some positive curvature at the highest inhibitor concentrations used. Non-linear Dixon plots can arise if the ESI complex of reaction scheme (1) also can turn over to produce product, or, alternatively, if more than one inhibitor molecule can bind to E or ES (Cornish-Bowden, 1979). Thus in order to simplify the analyses only the initial linear portions of the Dixon plots were used in calculating K_i.

The inhibition constants measured for the n-alcohols are plotted in Fig. 3 versus the number of carbon atoms in the alcohol. The low solubility of the n-alcohols beyond tetradecan-1-ol prevented a determination of their inhibition constants. No inhibition was observed for the C_{16} alcohol haxadecan-1-ol up to its solubility limit of 0.07 μM. On this basis it is estimated that K_i is greater than 0.7 μM for hexadecan-1-ol. The data of Fig. 3 indicate that if there is a 'cut-off' as the number of carbon atoms is increased it occurs at a chain length of 15 carbon atoms or greater. Franks & Lieb (1985) observed a cut-off after about C_{12} for firefly luciferase inhibition. The $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ fatty acids comprise the major fatty acids (some 91%) of bovine heart mitochondrial membrane cardiolipin (Hostetler, 1982). It is also known that the phospholipid cardiolipin is required for optimal cytochrome *c* oxidase activity (Fry & Green, 1980; Vik *et al.*, 1981). Thus the lack of a cut-off below 15 carbon atoms may be a consequence of the fatty acid composition of the cardiolipin bound to cytochrome *c* oxidase. The low-affinity cytochrome *c* binding site is known to be close to the phospholipid (Bisson *et al.*, 1980), and the lack of a cut-off may be consistent with the n-alcohols binding at a site adjacent to the enzyme-bound lipid. It is possible that molecules of dimensions of the same order as bound cardiolipin could potentially be accommodated at this binding site. As shown in Fig. 3, the pK_i is linearly related to the number of carbon atoms, N, in the n-alcohol. A linear-regression analysis of the data for the 14 n-alcohols of Fig. 3 gave:

$$pK_i = (0.43 \pm 0.02)N - (0.70 \pm 0.19)$$
$$r^2 = 0.968 \qquad s = 0.340$$

Thus a high fraction of the variation in the experimental data can be accounted for by the model. It can be calculated from the slope of this plot that there is a free-energy change of -2427 ± 126 J/mol (-580 ± 30 cal/mol) for transferring a methylene unit of the n-alcohol from the aqueous environment to the more hydrophobic environment bound to the enzyme. This value compares with a value of -3435 J/mol (-821 cal/mol) (Tanford,

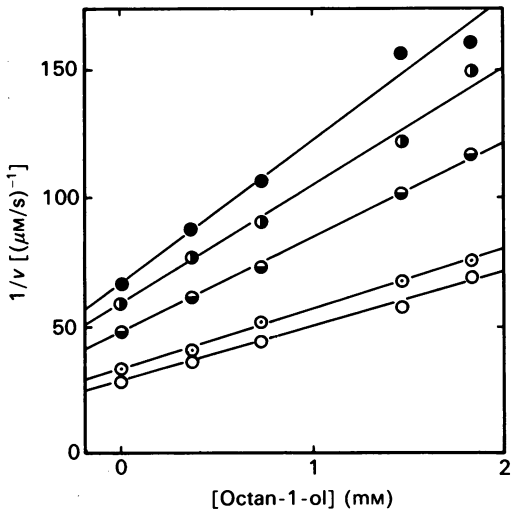

Fig. 1. Dixon plots showing the inhibition of cytochrome *c* oxidase by octan-1-ol at 25.0 °C in phosphate buffer, pH 7.0, of ionic strength 0.1

The straight lines are weighted linear-least-squares calculated. The ferrocytochrome *c* concentration was maintained constant at 19 μM (○), 10 μM (⊙), 4.1 μM (◒), 2.4 μM (◑) and 1.7 μM (●) from the bottom plot to the top plot respectively. The enzyme concentration was 0.8 nM on a haem aa_3 basis.

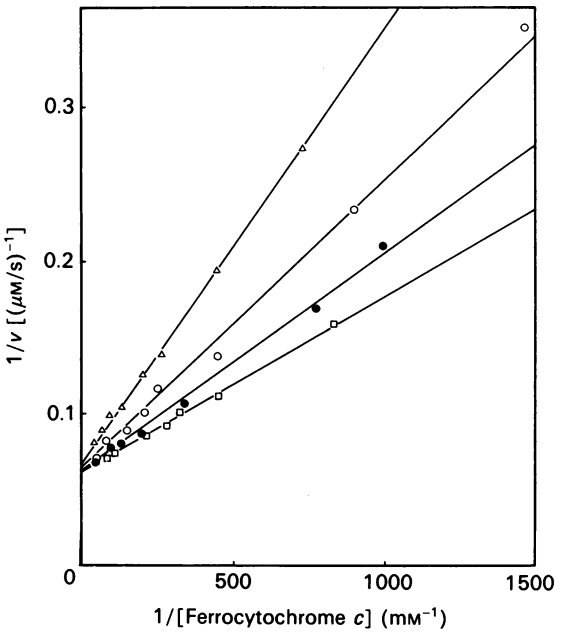

Fig. 2. Lineweaver–Burk plots for the cytochrome *c* oxidase-catalysed oxidation of ferrocytochrome *c* at 25.0 °C in phosphate buffer, pH 7.0, of ionic strength 0.1 showing inhibition due to added triacetin

The straight lines are weighted linear-least-squares calculated. □, No inhibitor; ●, 5.3 mM-triacetin; ○, 10.6 mM-triacetin; △, 16.0 mM-triacetin. The enzyme concentration was 1.0 mM on a haem aa_3 basis.

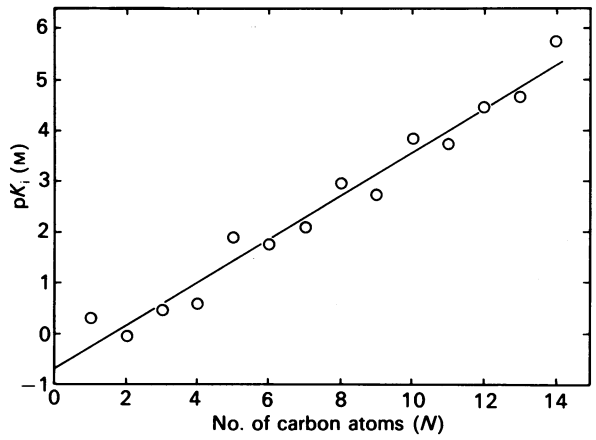

Fig. 3. Regression of pK_i versus N, the number of carbon atoms in the aliphatic n-alcohol.

The straight line is linear-least-squares calculated.

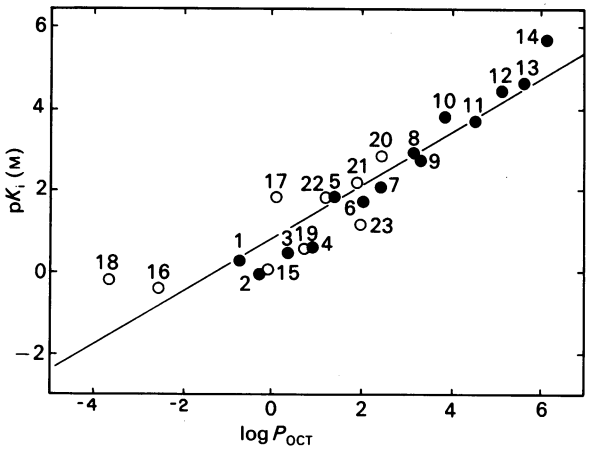

Fig. 4. Plot of pK_i versus $\log P_{OCT}$ for 14 aliphatic n-alcohols (C_1 to C_{14}) (●) and several other compounds (○).

The straight line is the least-squares-calculated best-fit line for the whole data set of 23 compounds. The P_{OCT} values were from Leo *et al.* (1971) with the following exceptions. The nonan-1-ol and decan-1-ol P_{OCT} values were obtained from Roth & Seeman (1972), and those for undecan-1-ol, tridecan-1-ol, tetradecan-1-ol, octan-2-one and heptan-3-one were extrapolations. Because of the increased viscosities (Hasinoff & Davey, 1987) in the glycerol and sucrose solutions a correction to the initial velocities of a maximum of 18% for sucrose and 33% for glycerol was made in computing their K_i values. The compounds are identified by number. Numbers 1 to 14 identify the normal aliphatic alcohols from methanol (1) to tetradecan-1-ol (14). The other compounds are propan-2-ol (15), glycerol (16), triacetin (17), sucrose (18), ethyl acetate (19), octan-2-one (20), heptan-3-one (21), n-butyraldehyde (22) and chloroform (23).

1980) for the transfer of a methylene group from water to the pure alcohol. Similar free-energy changes have been found for the alcohol inhibition of other enzymes. Thus a value of -2510 J/mol (-600 cal/mol) was found for $Na^+ + K^+$-dependent ATPase (Hegvary, 1973), a value of -2720 J/mol (-650 cal/mol) for lipoxygenase (Mitsuda *et al.*, 1967), a value of -2343 J/mol (-560 cal/mol) for pepsin and a value of -3431 J/mol (-820 cal/mol) for pancreatic lipase (Mattson *et al.*, 1970). Thus it can be concluded that the measured free-energy change from the slope of Fig. 3 indicates that the n-alcohols bind to a largely hydrophobic site on cytochrome *c* oxidase.

The octan-1-ol/water partition coefficient, P_{OCT}, of a substance is often taken as a measure of its lipophilicity. The data of Fig. 4 show that the pK_i for the n-alcohols is also linearly related to $\log P_{OCT}$. Linear-regression analysis of the 14 n-alcohols gave:

$$pK_i = (0.81 \pm 0.04)\log P_{OCT} + (0.33 \pm 0.14)$$
$$r^2 = 0.969 \qquad s = 0.335$$

It is not surprising that this regression gives a similar fit to the first one, as $\log P_{OCT}$ and N are themselves highly correlated ($r = 0.999$). Similarly, these results show that the inhibitors bind to a largely hydrophobic site on the enzyme. Also plotted on Fig. 4 are pK_i values of a number of other neutral organic compounds. When the whole data set is considered (a total of 23 compounds), it can be seen that pK_i is still linearly related to $\log P_{OCT}$. Linear-regression analysis for this extended data set gave:

$$pK_i = (0.65 \pm 0.05)\log P_{OCT} + (0.83 \pm 0.16)$$
$$r^2 = 0.873 \qquad s = 0.624$$

The r^2 is decreased compared with that found for the homologous series of n-alcohols but is still sufficiently high to show the hydrophobic character of the binding of these inhibitors to cytochrome *c* oxidase. The decrease in r^2 can be taken to indicate that the hydrophobic model cannot explain all of the variation in the experimental data. The variety of different shapes, sizes and functional groups of the inhibitors are not accommodated by the simple hydrophobic model. The fact that compounds with such a variety of functional groups inhibit cytochrome *c* oxidase indicates both that the binding site is not specific for n-alcohols and that it is relatively spacious.

Several other compounds were tested to test whether they inhibited cytochrome *c* oxidase. No inhibition was seen for n-heptane up to its solubility limit of 30 μM (Franks & Lieb, 1985). Hence the K_i of n-heptane was estimated to be greater than 0.3 mM. This limit compares with the K_i of 8.2 mM for heptan-1-ol. By comparison, the inhibitor-binding site on luciferase (Franks & Lieb, 1985) shows an approx. 24-fold increase in affinity for n-hexane (K_i of 0.06 mM) over that shown by hexan-1-ol (K_i of 1.4 mM) and thus has a hydrophobic binding site. It should be noted that the cytochrome *c* oxidase K_i values are high compared with the anaesthetic potencies (Miller, 1985). The anaesthetic potency of heptan-1-ol is, by comparison, 0.35 mM (Miller, 1985).

Several positively and negatively charged analogues of the alcohols were tested to test whether they could inhibit cytochrome *c* oxidase. None of the charged analogues tested was observed to inhibit the enzyme. Thus for the positively charged compounds a minimum K_i for n-butylamine and n-hexylamine of greater than 130 mM and a minimum K_i for hexamethylenediamine of greater than 50 mM can be estimated. Similarly, for the negatively charged analogues, n-pentanoic acid and n-octanoic acid, K_i is estimated to be greater than 260 mM. All of these limits were determined by the need to keep the ionic strength constant to within 10%. Since neither the positively charged amines nor the negatively charged

carboxylates were observed to inhibit cytochrome *c* oxidase, it may be that the inhibitor-binding site is uncharged. Similarly Casanovas *et al.* (1985*b*) concluded from their studies on the inhibition of cytochrome *c* oxidase by amine local anaesthetics that it is the neutral form of the anaesthetic that is the most potent and is responsible for enzyme inhibition. The inhibitor-binding site on cytochrome *c* oxidase probably contains a polar region that can interact with the oxygen atom of the inhibitor. The polar binding group on the enzyme could be a hydrogen-bonding donor group. Even though ethyl acetate and triacetin have similar octanol/water partition coefficients, triacetin was observed to inhibit 20 times more strongly than ethyl acetate. As triacetin has two more acyl groups than ethyl acetate a possible interpretation of this result is that there is more than one polar binding group in the inhibitor-binding site. The variety of type and size of inhibitor molecules that inhibit indicates a spacious non-specific inhibitor-binding site on the enzyme. In support of this conclusion it has also been shown that quinacrine (Casanovas *et al.*, 1985*a*) and several fully reduced flavin analogues ($K_{diss.}$ of 5–8 μM) (Ahmad *et al.*, 1982) bind to cytochrome *c* oxidase.

Inhibition of the cytochrome *c* oxidase activity of submitochondrial fragments

The cytochrome *c* oxidase activity of sonicated submitochondrial fragments was also shown to be inhibited by the presence of n-alcohols. These experiments were conducted in the absence of any solubilizing detergent, and thus the alcohol inhibition observed on the detergent-solubilized purified enzyme cannot be explained as an artifact of the detergent or the removal of the enzyme complex from the mitochondrial membrane.

The alcohol inhibition kinetics of the enzyme activity of the sonicated submitochondrial fragments were consistent with an uncompetitive kinetic scheme (Cornish-Bowden, 1979). This is a special case of the more general reaction scheme (1) where K_i is very large. It was observed that at low ferrocytochrome *c* concentration ($[S] \ll K_m$), $1/v$ in the Dixon plot showed little dependence on n-alcohol inhibitor concentration. This result indicated that K_i was too large to measure accurately and is thus larger than K_i'. Hence the K_i' values for n-alcohol inhibition on submitochondrial fragments were determined at a constant [S] approximately equal to K_m from plots of $1/v$ versus [I] but with the modification of eqn. (2) that K_i is very large. The values of K_i' measured on the submitochondrial fragments were slightly larger than the values of K_i' measured for the detergent-solubilized purified enzyme (Table 1). The 50% increase in the octan-1-ol K_i' in going from the detergent-solubilized bovine heart enzyme to the submitochondrial-fragment enzyme preparation can be compared with the analogous 3-fold increase that occurs in K_m under the same conditions. It is worth noting that Singer (1980, 1982) also observed at pH 7.4 a change from mixed-type to uncompetitive inhibition of cytochrome *c* oxidase by the amine local anaesthetic dibucaine when cytochrome *c* oxidase was incorporated into several types of phospholipid liposomes. Singer (1982) also observed a similar relative increase in K_i' to that shown in Table 1 when the enzyme was incorporated into liposomes. It has been postulated (Casanovas *et al.*, 1985*b*) that the neutral forms of the amine local anaesthetics interact with the cytochrome *c* oxidase boundary lipid. It has been established that there is lipid adjacent to the low-affinity substrate-binding site (Bisson *et al.*, 1980). Attempts were made to measure the inhibition of the submitochondrial cytochrome *c* oxidase activity by two other large alcohols, decan-1-ol and dodecan-1-ol. A slight amount of inhibition was noted for decan-1-ol at concentrations up to 0.15 mM, but no inhibition for dodecan-1-ol up to 10 μM was seen.

DISCUSSION

Although it is generally agreed that the ultimate effects of general anaesthetics are probably on proteins (Franks & Lieb, 1982), the question that has not been answered is whether or not anaesthetics act directly on proteins or the protein/lipid interface or indirectly through membrane lipids. This question arises as well on the inhibition of cytochrome *c* oxidase by n-alcohols. The fact that the inhibition follows normal inhibition patterns can be considered support for the n-alcohols interacting directly with enzyme or the boundary lipid rather than through a general perturbation of the lipid environment. Franks & Lieb (1978, 1982) have demonstrated that, although there is a good correlation of general anaesthetic potencies with the octanol/water partition coefficients, the correlation with hydrocarbon partition coefficients is not so good. It was argued that, because the slope of the double-logarithmic plot of potency versus the octanol/water partition coefficient is unity, this implies that there is but a single site of action and that this site provides an

Table 1. Inhibition of purified and submitochondrial cytochrome *c* oxidase

Measurements were made at 25.0 °C in phosphate buffer, pH 7.0, of ionic strength 0.1.

Michaelis and inhibition constants	Rat liver submitochondrial-fragment preparation	Bovine heart submitochondrial-fragment preparation	Bovine heart purified preparation
K_m (μM)	25*	12*	3.9
K_i' (octan-1-ol (mM)	3.1	2.1	1.4
K_i (pentan-1-ol) (mM)	110	–	13†

* Compares with a K_m value of 17 μM previously determined (Yonetani & Ray, 1965).
† This is a K_i value, as the K_i' for pentan-1-ol was not well determined.

octanol-like environment. The observation that pK_i for the n-alcohol inhibition of cytochrome *c* oxidase is similarly highly linearly correlated with $\log P_{OCT}$ with a slope also not greatly different from unity can also be considered evidence that the n-alcohols also bind to a single octanol-like site on cytochrome *c* oxidase. Also, the fact that the value of the free-energy change for transferring a methylene group of the n-alcohol to the more hydrophobic environment of the enzyme is of comparable size with that for transferring a methylene group from water to pure alcohol is also evidence that the n-alcohols inhibit by binding to an octanol-like environment on the enzyme rather than by perturbing the lipid environment. The protein/phospholipid interface might be expected to provide an octanol-type environment.

The inhibition of luciferase by n-alcohols yields a slope of the double-logarithmic plot of luciferase potency versus animal potency that is close to 1 (Franks & Lieb, 1984). However, Casanova *et al.* (1985*b*) found a slope of 0.51 for a plot of pK_i versus $\log P_{OCT}$ for the inhibition of cytochrome *c* oxidase by eight amine local anaesthetics. A reasonable interpretation of a slope significantly different than unity might be that the inhibitor-binding site is not purely octanol-like.

Several other lines of evidence support the conclusion that the site of interaction of the n-alcohols is directly on the protein or the protein/lipid interface rather than through a general perturbation of the lipid environment. Chazotte & Vanderkooi (1981) also came to the same conclusion on the basis of the effects of n-alcohols on various sites of the mitochondrial respiratory chain. The fact that different components of the respiratory chain were much more sensitive than others argued against general perturbation of the lipid bilayer. For example, NADH:cytochrome *c* oxidoreductase in bovine heart submitochondrial particles underwent 50% inhibition at a hexan-1-ol concentration 150 times smaller (1.5 mM compared with 220 mM) than that observed for cytochrome *c* oxidase. Also, the fact that inhibition is occurring at such low concentrations, in both the present study and that by Chazotte & Vanderkooi (1981), may also be taken as evidence that the inhibition is not occurring as the result of some general perturbation of the lipid environment.

It is known that a photochemically activated arylazido-cytochrome *c* derivative modified at lysine-13 cross-links with both lipid and subunit II of cytochrome *c* oxidase (Bisson *et al.*, 1980). However, the lysine-22 derivative cross-linked to only bound lipid and thus this group is closer to the lipid. These results place this binding site close to the phospholipid. Thus a plausible binding site for the mainly hydrophobic inhibitors examined in the present study also is adjacent to the bound phospholipid. The usual interpretation of linear mixed inhibition is that the inhibitor binds to a site close to the substrate-binding site, thus diminishing access to the substrate-binding site. If there is a mainly hydrophobic inhibitor-binding site on cytochrome *c* oxidase, then this could imply that, in addition to the well-known strong electrostatic component of ferrocytochrome *c* binding to cytochrome *c* oxidase (Margoliash, 1982; Matthew, 1985), there may be, in addition, a hydrophobic component as well. It thus might be reasonable to expect a complementary hydrophobic patch on the surface of the cytochrome *c* molecule as well. Such a hydrophobic interaction for either the 'left' or 'right' hydrophobic channel on ferrocytochrome *c* was initially suggested from the X-ray structure (Dickerson *et al.*, 1971). Both of these channels were noted to be surrounded on the surface of the molecule by a ring of positively charged lysine residues, and it was speculated that one of the channels may be involved in recognition and binding to cytochrome *c* oxidase. It was also noted from the results of molecular-modelling experiments on hypothetical three-dimensional models of the yeast cytochrome *c* peroxidase–tuna cytochrome *c* complex that a particularly good set of hydrophobic contacts is formed at the highly conserved isoleucine-81 (Poulos & Kraut, 1980; Poulos & Finzel, 1984) of cytochrome *c*. It was suggested that this region acted as a hydrophobic anchor in forming electron-transfer complexes.

In conclusion, the normal aliphatic alcohols up to tetradecan-1-ol are observed to inhibit cytochrome *c* oxidase, both the purified detergent-solubilized enzyme and the enzyme in sonicated submitochondrial fragments. The binding of these n-alcohols to a membrane-bound enzyme provides a model for specific anaesthetic–protein or anaesthetic–protein/lipid interface interactions and a model for the toxic action of drugs on enzymes as well.

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