Biochemical Pharmacology, Vol. 32, No. 10, pp. 1619–1625, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + .00

INTERACTION OF DRUGS WITH A MODEL MEMBRANE PROTEIN

EFFECTS OF LOCAL ANESTHETICS ON ELECTRON TRANSFER AND HYDROGEN ION UPTAKE IN IONOPHORE STIMULATED CYTOCHROME OXIDASE PROTEOLIPOSOMES*

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(*Received* 3 *July* 1982; *accepted* 10 *November* 1982)

Abstract—Cytochrome oxidase extracted from beef heart was incorporated into vesicles composed of soy bean phospholipids (asolectin). The oxidation of externally added cytochrome *c* by such vesicles is associated with proton uptake from the external medium. The rates of both cytochrome *c* oxidation and proton uptake were stimulated by addition of ionophores such as trifluoromethoxy carbonyl cyanide phenylhydrazone (FCCP), nigericin and valinomycin. These agents probably dissipate pH and/or electrical potential gradients which develop as a result of enzyme activity and which have a "restraining" influence on the turnover of the oxidase. Local anesthetics inhibited oxidase activity but had a much greater effect on the stimulated (ionophore-treated) than the unstimulated enzyme. In addition, pretreating proteoliposomes with local anesthetics completely prevented the stimulating effects of these ionophores. Based on this and previous studies, a model was developed in which local anesthetics interacted with the phospholipid component of the oxidase complex resulting in reduced internal electron transfer and dissociation of the oxidase from the regulatory role of the proton gradient.

Since many drugs interact with a cellular membrane as part of their pharmacologic effects, the characterization of drug–membane interactions becomes important for understanding mechanisms of action. In two previous publications [1, 2] we have examined the effects of local anesthetics in a model system (viz. lipid vesicles containing an incorporated mitochondrial membrane protein, cytochrome oxidase) in order to study such interactions. This protein is part of the mitochondrial respiratory chain and catalyses the transfer of electrons from cytochrome *c* to molecular oxygen. Enzyme turnover number is reduced by cationic local anesthetics which, at pH 7.4, act predominantly by interacting with the oxidase, probably with its boundary lipid, to form non-productive complexes [2].

The oxidation of externally added cytochrome *c* by cytochrome oxidase proteoliposomes is associated with the intravesicular consumption of hydrogen ions as a result of water formation. In addition, there is evidence that the reconstituted enzyme also operates as a redox-linked proton pump since net hydrogen ion extrusion has been observed for up to eleven turnovers of the enzyme [3, 4]. Hence, the oxidation of cytochrome *c* leads to both a trans-membrane electrical potential gradient (inside negative) and a pH gradient [4, 5]. Both of these gradients will promote back diffusion of hydrogen ions into the vesicle interior.

The rate of oxidation of cytochrome *c* by cytochrome oxidase proteoliposomes can be stimulated by the addition of ionophores such as trifluoromethoxy carbonyl cyanide phenylhydrazone (FCCP) and valinomycin [6, 7], which render the vesicle membrane permeable to hydrogen ions and alkali metal cations respectively. It is believed that these agents dissipate the pH and/or electrical potential gradients resulting from enzyme activity [7, 8]. Presumably these gradients have a restraining influence on the turnover of the oxidase. In this report we describe the effects of local anesthetics on electron transfer (cytochrome *c* oxidation) and hydrogen ion movement in ionophore-stimulated cytochrome oxidase proteoliposomes.

MATERIALS AND METHODS

Cytochrome *c* (type VI from horse heart), sodium ascorbate, sodium cholate and *N,N,N*^{1},*N*^{1}-tetramethyl *p*-phenylene diamine dihydrochloride (TMPD) were obtained from the Sigma Chemical Co., St. Louis, MO. The four local anesthetics (dibucaine, tetracaine, procaine and benzocaine) were purchased from ICN Pharmaceuticals, Plainview, NY. Asolectin (soybean phospholipids) was obtained from Associated Concentrates, Woodside, Long Island, NY. Valinomycin was purchased from Calbiochem, San Diego, CA. Nigericin and FCCP were gifts of the Eli Lilly & Co. and the Dupont Company (Dr. P. G. Heytler) respectively. All other chemicals were of reagent grade wherever possible. Twice distilled water was used for all experiments.

* This research was supported by the Medical Research Council of Canada.

Cytochrome oxidase. Cytochrome oxidase was extracted from beef heart and purified according to the method of Kuboyama *et al.* [9]. Following this procedure, the oxidase was dialysed 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 dialysed 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 coefficient of 24 at 605 nm (reduced–oxidized) [1]. Protein concentration was measured using the biuret reaction [1]. Two oxidase preparations were used for the experiments in this study. They had aa_3 concentrations and heme to protein ratios (nmoles heme *a*/mg protein) of 48.5 μM, 10 and 85 μM, 12.8 respectively.

Proteoliposome preparations. Cytochrome oxidase containing proteoliposomes were prepared according to the method of Hinkle *et al.* [6] and Racker [10]. To 2 ml of buffer containing 30 mg asolectin suspension and 40 mg sodium cholate, oxidase aa_3 (3.4 nmoles) was added. These amounts of enzyme and lipid were chosen somewhat arbitrarily but were comparable (within a factor of 2) to the ratios used in Ref. 2. The dispersion was dialysed at 4° against buffer (1000 ml) lacking sodium cholate. The initial dialysis step was carried out for 4 hr followed by dialysis overnight against new external solution. Buffer composition is given in the figure and table legends.

The dialysis steps remove approximately 90% of the cholate [11]. In addition, the sample is diluted 80-fold when added to the medium for assay of cytochrome oxidase activity. The latter step itself may be sufficient since Racker [10] has described successful reconstitution of cytochrome oxidase vesicles by dilution of the cholate alone without an initial dialysis procedure.

As discussed by Nicholls *et al.* [12], this reconstitution process forms vesicles with up to 75% of the oxidase molecules oriented with their cytochrome *c* binding site facing outward. We examined oxidase "sidedness" in our preparation using the technique described in Refs. 8 and 11 and also found that 70% of the enzyme molecules were oriented so as to expose their cytochrome *c* reaction sites to the external medium. In all of the assays described in this study, cytochrome *c* was added to the outside of the vesicles and hence would only "react" with these favourably oriented oxidase molecules. Finally, the observation that the activity of the incorporated enzyme could be stimulated (3- to 5-fold) by ionophores indicates that the vesicles were intact.

Electron transfer. Rates of oxygen uptake were measured with a Yellow Springs Instruments oxygen electrode immersed in a sealed, thermostatted glass cell and fitted to a conventional chart recorder. Stirring was accomplished with a magnetic flea. The assay mixture had a volume of 4 ml and contained buffer, cytochrome *c*, sodium ascorbate (5 mM) and TMPD (0.18 mM). Buffer compositions are given in the figure and table legends. An enzyme turnover number (TN) O_2/aa_3 was calculated by dividing oxygen consumption in a nmoles/per ml/per sec by the oxidase concentration (nmoles aa_3/ml). An electron transfer turnover number can be obtained by multiplying the above TN by 4 since four electrons are transferred for each mole of oxygen consumed. All assays were performed at 30°.

Hydrogen uptake. pH changes associated with the function of the cytochrome oxidase vesicles were measured with an Orion pH combination electrode in conjunction with an Orion digital ionalyzer meter connected to a chart recorder. The glass cell, in this case, however, was open rather than sealed. pH changes were calibrated with standard HCl. An enzyme turnover number (H^+/aa_3) was calculated by dividing the change in hydrogen ion concentration (nmoles/per ml/per sec) by the oxidase concentration (nmoles aa_3/ml). All assays were performed at 30°.

Stock methanol solutions of the ionophores were prepared as follows: nigericin 0.27 mM, valinomycin 0.18 mM, and FCCP 0.20 mM. Preliminary experiments indicated that these ionophores had direct inhibitory effects on oxygen consumption by the *isolated* enzyme if added in sufficient amount. It was found that 10 μl aliquots of the above stock solutions, giving final concentrations of nigericin 0.68 μM, valinomycin 0.45 μM and FCCP 0.50 μM, had maximal stimulatory effects on proteoliposomes and minimal inhibitory effects on the isolated enzyme. Methanol, alone in equal volume had no effect on either proteoliposomes or the isolated enzyme.

Since the main purpose of this study was to investigate the effects of local anesthetics on ionophore-stimulated proteoliposomes, the concentrations of ionophores used were kept constant at the values determined in the preliminary experiments as described above.

RESULTS

Figure 1 illustrates representative tracings of oxygen consumption and pH changes when proteoliposomes are respiring on external cytochrome *c*. Oxidation of substrate is associated with alkalinization of the external medium. The stimulatory effects of valinomycin and FCCP on oxygen consumption were accompanied by corresponding increases in the extent of the external alkalinization. Since the external alkalinization was enhanced by agents such as FCCP and valinomycin which induce specific permeability changes in lipid bilayers, it seems reasonable to assume that this alkalinization represented proton uptake by the proteoliposomes.

Figure 2 illustrates the kinetic pattern for cytochrome *c* oxidation by proteoliposomes exposed to ionophores as well as the effect of the local anesthetic dibucaine on this pattern. The combination of FCCP plus valinomycin increased TN_{max} from 17.5 to 49 sec^{-1} with little change in K_m (4.0 to 5.8 μM). It would thus appear that these ionophores increase enzyme turnover without altering the apparent binding of substrate, a conclusion similar to that reached by Hansen *et al.* [7]. Dibucaine (2.5 mM) reduced the TN_{max} of ionophore-treated proteoliposomes from 49 to 11.6 sec^{-1} while causing only a small and insignificant increase in K_m (5.8 to 7.2 μM). This

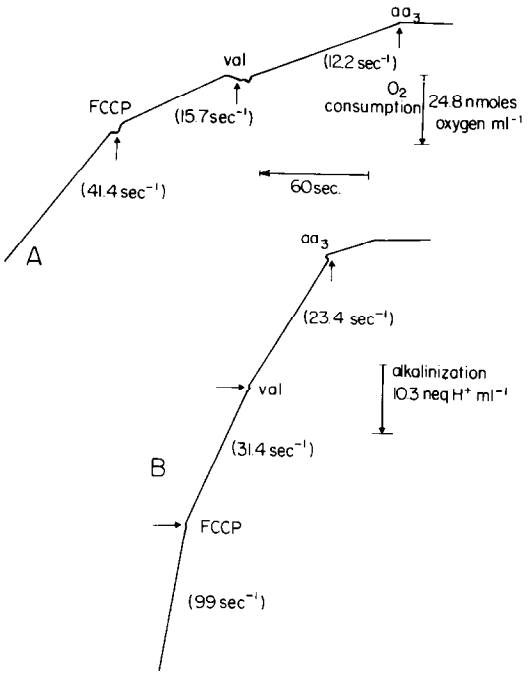

Fig. 1. Tracings of oxygen consumption and hydrogen ion uptake by proteoliposomes. Proteoliposomes were prepared in 95 mM KCl, 5 mM Tris–HCl, pH 7.4. The assay medium (volume 4 ml) contained 100 mM KCl, 1 mM Tris–HCl, 5 mM sodium ascorbate, 0.18 mM TMPD, and 75 μM cytochrome *c*, pH 7.4. The reaction was initiated by adding proteoliposomes, final concentration 0.02 μM (indicated by the arrow opposite aa_3). Final concentrations of valinomycin and FCCP were 0.45 and 0.5 μM respectively. Trace A represents oxygen consumption; trace B, hydrogen ion uptake. Values in parentheses are turnover numbers. The stoichiometry linking electron transfer to hydrogen ion uptake can be calculated as follows. Four electrons are transferred for each mole of oxygen consumed. The turnover number 12.2 sec^{-1} becomes 48.3 sec^{-1} in terms of electron transfer. The H^-/e^- stoichiometry is then 23.4/48.4 = 0.48. However, since 0.5 H^+ is released per electron removed from ascorbate as it is oxidized to dehydroascorbate [13], the actual H^+e^- for hydrogen ion uptake per electron transferred is 0.48 + 0.5 = 0.98. This is close to the stoichiometry of 1.0 expected on the basis of the overall chemical reaction.

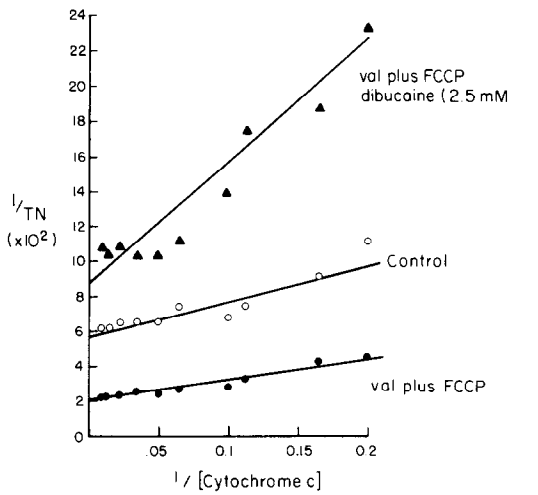

Fig. 2. Oxygen consumption by proteoliposomes in the presence of valinomycin + FCCP and dibucaine (Lineweaver–Burk plots). Proteoliposomes were prepared in 90 mM KCl, 10 mM Tris–HCl, pH 7.4. The assay medium (volume 4 ml) contained 90 mM KCl, 10 mM Tris–HCl, 5 mM sodium ascorbate, 0.18 mM TMPD, and cytochrome *c* (5–100 μM), pH 7.4. The ordinate is the reciprocal of the turnover number (sec^{-1}) multiplied by 10^2. The abscissa is the reciprocal of the cytochrome *c* concentration (μM). For a given cytochrome *c* concentration oxygen consumption was measured prior to and following the addition of first valinomycin (0.45 μM) + FCCP (0.5 μM) and then dibucaine at two concentrations, 1.0 and 2.5 mM. The data points for 1.0 mM dibucaine have not been included. The TN_{max} and K_m values were as follows: control 17.5 sec^{-1}, 4 μM; valinomycin + FCCP 49 sec^{-1}, 5.8 μM; dibucaine (1.0 mM) 21.5 sec^{-1}, 5.7 μM and dibucaine (2.5 mM) 11.6 sec^{-1}, 7.2 μM. Lines were drawn by the method of least squares.

pattern is most consistent with non-competitive inhibition.

The data summarized in Table 1 illustrate the relative potencies of three different ionophores (valinomycin, FCCP and nigericin) in terms of their capacities to stimulate electron transfer (cytochrome *c* oxidation) and hydrogen ion uptake by proteoliposomes in the presence of externally added cytochrome *c*. These ionophores have different mechanisms of action. FCCP [14] and valinomycin [15] render the bilayer selectively permeable to hydrogen and potassium *ions*, respectively, while nigericin promotes *electrically neutral* hydrogen/potassium exchange [15]. The order of potencies of these agents in terms of both electron transfer and hydrogen ion uptake was FCCP > nigericin > valinomycin. The combinations valinomycin plus FCCP and valinomycin plus nigericin had about the same effects and were even *more potent* than the individual ionophores. Thus, maximum stimulation was achieved when the membrane was made permeable to both potassium ions and hydrogen, irrespective of whether the latter crosses in the charged or uncharged form.

If the ability of valinomycin to stimulate both electron transfer and hydrogen ion uptake is the result of its known effects on alkali metal ion permeability, then the cation selectivity pattern with respect to its action on oxidase proteoliposomes should be the same as the cation permeability pattern it induces in membranes.

Proteoliposomes were prepared with Tris as the internal cation and either Cs, Rb, K, Na or Li as the external cation. FCCP was added to the assay medium so that bilayer permeability to hydrogen ions would not be "rate-limiting" to the action of valinomycin. The subsequent addition of valinomycin further augmented both electron transfer and hydrogen ion uptake, and this effect was clearly cation selective, as summarized in Table 2. The observed selectivity pattern was Rb > Cs > K > Na > Li which is similar to the pattern obtained for the effects of this ionophore on the ionic permeability of red cell ghosts [16] although quantitatively the discrimination was much less.

Table 1. Effect of different ionophores on oxygen consumption and hydrogen ion uptake by proteoliposomes*

Additions	Turnover number (sec^{-1}) (O_2/aa_3)	(H^+/aa_3)
Valinomycin	(14.3) 16.6 (1.16)	(29.7) 39 (1.3)
Nigericin	(14.6) 28.7 (2.0)	(29.7) 41.2 (1.4)
FCCP	(11.6) 37.8 (2.7)	(29.7) 62 (2.1)
Nigericin + valinomycin	(14.4) 58.6 (4.1)	(29.7) 106 (3.6)
FCCP + valinomycin	(11.6) 59.5 (5.1)	(29.7) 106 (3.6)

* Proteoliposomes were prepared in 95 mM KCl, 5 mM Tris–HCl, pH 7.4. The assay medium (volume 4 ml) contained 100 mM KCl, 1 mM Tris–HCl, 75 μM cytochrome *c*, 5 mM sodium ascorbate, and 0.18 mM TMPD, pH 7.4. The reaction was initiated by adding proteoliposomes (final concentration 0.02 μM aa_3). Oxygen consumption and hydrogen ion uptake were measured prior to and following the addition of an individual ionophore or an ionophore combination as listed above. The final concentrations were valinomycin 0.45 μM, nigericin 0.68 μM, and FCCP 0.50 μM. The numbers given in parentheses are the turnover number prior to any addition and the ratio of the turnover numbers after and before the addition. Although the results of only one series of assays are presented, the order of potencies of these ionophores was always FCCP > nigericin > valinomycin, though the actual stimulatory ratios varied for different proteoliposome preparations. As discussed under Materials and Methods, these ionophore concentrations were ones that gave maximal stimulatory effects in proteoliposomes and minimal inhibitory effects on the isolated enzyme, as determined in preliminary experiments.

In another experiment, similar in design to that summarized in Table 2, the cation selectivity of the valinomycin effect on oxygen consumption only was tested. The assay medium differed in that it contained 10 mM Tris–HCl and 90 mM (Rb, Cs, K, Na or Li) Cl, pH 7.4, in addition to ascorbate, TMPD and FCCP. For each cation, assays were performed over a range of cytochrome *c* concentrations from 5 to 100 μM. A TN_{max} (O_2/aa_3) was obtained by extrapolation to infinite cytochrome *c* concentration. The selectivity pattern was Rb (1.7) > Cs (1.22) = K (1.21) > Na (1.0) > Li (1.0). The values in parentheses refer to the ratio of the TN_{max} values after and before the addition of valinomycin. The cation selectivity sequence is the same as listed in Table 2, but the actual "stimulatory" ratios were different.

Figure 3 illustrates the effects of four local anesthetics on cytochrome *c* oxidation by proteoliposomes in the presence and absence of ionophores. With respect to the unstimulated enzyme, there was little discrimination among these four anesthetics (Fig. 3B), whereas there were quite marked differences among these agents when tested in proteoliposomes exposed to ionophores (Fig. 3A). The order of potencies in the latter case was dibucaine > tetracaine > procaine > benzocaine. Furthermore the local anesthetics caused a much greater reduction in the turnover number of ionophore-treated as compared to untreated proteoliposomes. For example, dibucaine at a concentration of 2.5 mM inhibited the untreated enzyme by about 25% but the treated enzyme by 70–75%. The effects of these anesthetics on hydrogen ion uptake in ionophore-treated proteoliposomes were comparable to their effects on electron transfer. Table 3 summarizes experiments illustrating this observation.

Table 2. Cation selectivity of valinomycin action*

Cation	Oxygen consumption	Hydrogen ion uptake
Rb	(30.8) 1.80	(22) 1.64
Cs	(32) 1.72	(26) 1.46
K	(33.2) 1.66	(23) 1.49
Na	(26) 1.45	(22) 1.05
Li	(31) 1.17	(26) 1.0

* Proteoliposomes were prepared in 100 mM Tris–HCl, pH 7.4. The assay mixture (volume 4 ml) consisted of 100 mM (Rb, Cs, K, Na, or Li) Cl, 1 mM Tris–HCl, 75 μM cytochrome *c*, 5 mM sodium ascorbate, 0.18 mM TMPD, and FCCP (0.5 μM), pH 7.4. The reaction was initiated by adding proteoliposomes (final concentration 0.02 μM aa_3). For each cation, oxygen consumption and hydrogen ion uptake were measured before and after the addition of valinomycin (0.45 μM). The numbers listed are the ratios of the turnover numbers after and before this addition. The turnover number (sec^{-1}) prior to valinomycin is given in parentheses.

The striking differences in the potencies of anesthetics when tested in the presence, as opposed to the absence, of ionophores, were *not* characteristic of all oxidase inhibitors. Azide, for example, a known heme ligand [17], caused a comparable degree of inhibition of both the stimulated and unstimulated enzyme (Fig. 4). Azide also reduced hydrogen ion uptake to the same extent as electron transfer (data not shown).

Finally, as illustrated in Fig. 5, pretreating proteoliposomes with a local anesthetic can prevent ionophore stimulation of oxidase activity despite the very limited effect of the anesthetic on enzyme turnover in the absence of ionophores. By contrast, although azide caused a profound inhibition of oxidase activity in the absence of ionophores, the enzyme still retained its responsiveness to the stimulating effects of FCCP plus valinomycin.

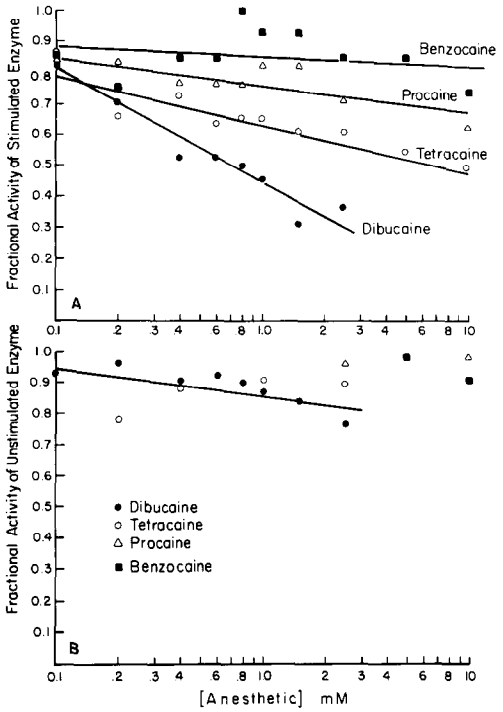

Fig. 3. Effects of local anesthetics on oxygen consumption by proteoliposomes. Proteoliposomes were prepared in 90 mM KCl, 10 mM Tris–HCl, pH 7.4. The assay medium (volume 4 ml) contained 90 mM KCl, 10 mM Tris–HCl, 5 mM sodium ascorbate, 0.18 mM TMPD, and 75 μM cytochrome *c*, pH 7.4. (A) Oxygen consumption was measured prior to and following the addition of first valinomycin (0.45 μM) + FCCP (0.5 μM) and then a given concentration of one of the local anesthetics. The ordinate represents the ratio of the turnover numbers after and before the addition of the anesthetic. In these assays, as already indicated, the anesthetic was added after the enzyme was stimulated with valinomycin + FCCP. The abscissa is the anesthetic concentration (mM) and is plotted on a log scale because of the wide range of concentrations used. (B) Oxygen consumption was measured prior to and following the addition of a local anesthetic. The ordinate represents the ratio of the turnover numbers after and before the addition. Ionophores were *not* used in these assays. The abscissa is the anesthetic concentration (mM). A line has been drawn for the dibucaine data points only. Since procaine and benzocaine caused little inhibition, these agents were tested only at higher concentrations.

Table 3. Effect of different anesthetics on oxygen consumption and hydrogen ion uptake by proteoliposomes*

Anesthetic	Turnover number (sec^{-1}) (O_2/aa_3)	(H^+/aa_3)
Dibucaine	(31.8) 12.9 (0.40)	(51.5) 23.8 (0.46)
Tetracaine	(37.6) 25.8 (0.69)	(47) 34.4 (0.73)
Procaine	(37.6) 29.5 (0.78)	(47) 39.7 (0.84)
Benzocaine	(37.6) 34.5 (0.92)	(51.5) 51.5 (1.0)

* Proteoliposomes were prepared in 95 mM KCl, 5 mM Tris–HCl, pH 7.4. The assay medium (volume 4 ml) consisted of 100 mM KCl, 1 mM Tris–HCl, 75 μM cytochrome *c*, 5 mM sodium ascorbate, and 0.18 mM TMPD, pH 7.4. The reaction was initiated by adding proteoliposomes (final concentration 0.02 μM aa_3). Oxygen consumption and hydrogen ion uptake were measured prior to and following first the addition of valinomycin (0.45 μM) + FCCP (0.5 μM) and then the addition of one of the anesthetics (2.5 mM). In these assays, then the anesthetic was added to the stimulated enzyme. Control turnover numbers (means plus range of values) for oxygen consumption and hydrogen ion uptake were 15.1 sec^{-1} (14.6–15.9) and 18.8 sec^{-1} (18.2–19.4) respectively. The numbers given in parentheses in the table are the turnover numbers following addition of FCCP + valinomycin and the ratio of the turnover numbers after and before the addition of the local anesthetic.

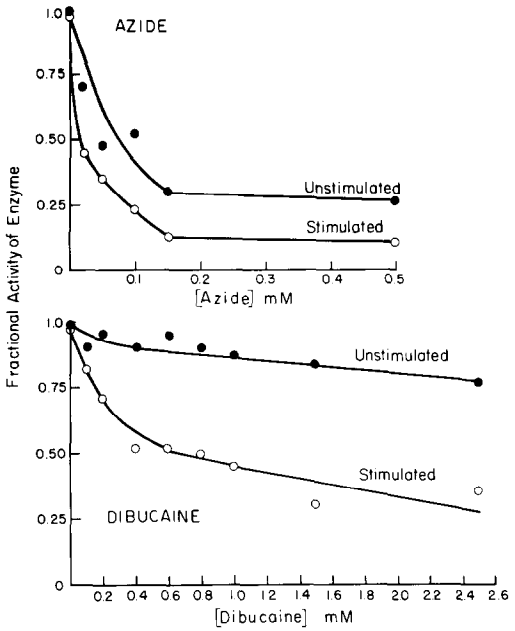

Fig. 4. Comparative effects of sodium azide and dibucaine on oxygen consumption by proteoliposomes. Proteoliposomes were prepared in 90 mM KCl, 10 mM Tris–HCl, pH 7.4. The assay medium (volume 4 ml) contained 90 mM KCl, 10 mM Tris–HCl, 5 mM sodium ascorbate, 0.18 mM TMPD, and 75 μM cytochrome *c*, pH 7.4. The ordinate represents the ratio of the turnover numbers after and before the addition of sodium azide or dibucaine. The abscissa is the concentration (mM) of sodium azide or dibucaine added. The "stimulated" curves refer to assays in which valinomycin (0.45 μM) + FCCP (0.5 μM) were added prior to the azide or dibucaine. The "unstimulated" curves represent data from assays in which ionophores were not used.

DISCUSSION

The transfer of electrons by oxidase proteoliposomes from externally added cytochrome *c* to molecular oxygen is associated with the intravesicular consumption of hydrogen ions as a result of water formation as well as the extrusion of this ion into the external medium. The proton extrusion phase is best observed when the enzyme is turning over slowly and intravesicular buffer capacity is high [4], since under these conditions the back leak of protons develops more slowly. The assay conditions used in these experiments were not favourable for detecting proton extrusion. Oxidase turnover was high (10–20/sec) and intravesicular buffer capacity was relatively low. Hence, with initiation of the reaction, the

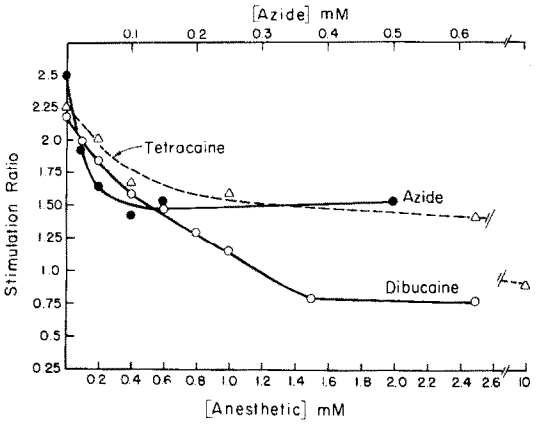

Fig. 5. Comparative effects of anesthetics and sodium azide on oxygen consumption by ionophore-stimulated proteoliposomes. Proteoliposomes were prepared in 90 mM KCl, 10 mM Tris–HCl, pH 7.4. The assay medium (volume 4 ml) contained 90 mM KCl, 10 mM Tris–HCl, 5 mM sodium ascorbate, 0.18 mM TMPD, and 75 μM cytochrome *c*, pH 7.4. Oxygen consumption was measured prior to and following the addition of first sodium azide or an anesthetic (dibucaine or tetracaine) and then valinomycin (0.45 μM) + FCCP (0.5 μM). The ordinate represents the ratio of the turnover numbers after and before the addition of valinomycin + FCCP. It should be noted that the "before" turnover number is the value measured after the addition of the azide or anesthetic. The abscissa is the concentration of azide or anesthetic.

back leak of protons would have occurred quickly due to the development of a transmembrane electrochemical potential hydrogen ion gradient and rapidly approached the stoichiometry of one hydrogen taken up per electron transferred. This was, in fact, the case. As illustrated in Fig. 1, only an alkalinization of the external medium was observed following addition of proteoliposomes to the assay mixture used in these experiments with the stoichiometry of this alkalinization being one H^+/electron, as noted in the legend to Fig. 1.

Considering their mechanisms of action, we can infer from the effects of different ionophores that the electrochemical potential gradient of hydrogen across the vesicle membrane controls the turnover of oxidase. The observation that nigericin was a more potent stimulator of oxidase activity than valinomycin suggests that the chemical potential (pH) gradient plays the more dominant "regulatory" role. Although valinomycin displayed a similar cation selectivity pattern in terms of ionic permeability and oxidase stimulation, the observation that the discrimination was much larger for the former property would support this conclusion. However, it is clear from the data in Table 1 that maximum stimulation occured only when both pH and electrical gradients were dissipated.

The mechanism by which the electrochemical potential gradient of hydrogen regulates oxidase turnover is unknown. Since the ionophores increased the TN_{max} values without altering the K_m values an effect of this gradient on cytochrome *c* binding, at least at the low affinity site;* can probably be excluded. Wikstrom and coworkers [3, 18] have suggested that the electrochemical gradient of protons across the membrane controls the conformation and redox properties of the oxidase heme centres and thereby the rate of oxygen consumption.

Local anesthetics, particularly the more potent ones, had a greater inhibitory effect on the ionophore-stimulated, as compared to the unstimulated, enzyme. In addition, pretreating proteoliposomes with dibucaine and tetracaine prevented ionophore stimulation. These effects differ from the behaviour of azide, a more traditional oxidase inhibitor and a known heme ligand. This agent caused a comparable degree of inhibition of the oxidase, both in the presence and absence of ionophores, and did not eliminate the responsiveness of the enzyme to the stimulating effects of FCCP plus valinomycin.

As previously described [2], at pH 7.5 dibucaine behaves as an uncompetitive inhibitor of both the isolated and unstimulated reconstituted enzyme. From Fig. 2 it is evident that dibucaine caused a non-competitive pattern of inhibition with respect to the ionophore-stimulated oxidase. Central to both of these patterns of inhibition is the formation of inactive enzyme–substrate–inhibitor (ESI) complexes [19]. They differ in that an uncompetitive inhibitor only reacts with the ES complex whereas a non-competitive inhibitor can combine with both ES and the free enzyme (E). To develop a working model of anesthetic action in this model system, we will make the simplifying assumption that the resulting ESI complex is the same whether the oxidase is isolated or incorporated into a lipid vesicle (± ionophores). Cytochrome oxidase preparations contain a protein and phospholipid component. The anesthetic could combine with the protein moiety of the complex although the site of interaction would not be the heme centres since dibucaine and azide behave differently. However, in two previous studies [1, 2] we have presented evidence that local anesthetics interact with the phospholipids (composed predominantly of cardiolipin), which are associated with the oxidase, the so-called boundary lipid. Certainly such a lipid site of action could account for the differential effects of these local anesthetics since their capacities to alter the permeability and phase properties of lipid bilayers are in the order dibucaine > tetracaine > benzocaine > procaine [20].

Could these phospholipids serve as a link to the previously discussed regulating effect of the proton electrochemical gradient? Yu *et al.* [21] have proposed that phospholipids are necessary to accelerate the internal transfer of electrons between the two heme moieties (a and a_3). Since Wikstrom and coworkers [3, 18] have suggested that the proton gradient controls the conformation and redox properties of these heme centres, one could speculate that this control is accomplished through some alteration in the (physical) state of the phospholipids. This model could be extended to include the action of the local anesthetics in that the proposed anesthetic–lipid interaction could reduce electron

* The concentration range of cytochrome *c* used in the experiments illustrated in Fig. 2 allows one to examine only the low affinity cytochrome *c* binding site.

transfer between a and a_3 as well as "dissociating" the oxidase from the regulatory role of the proton gradient.

Acknowledgement—I would like to thank Mrs. M. Dinda for expert technical assistance.

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