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INHIBITION OF MITOCHONDRIAL AND *PARACOCCUS DENITRIFICANS* NADH–UBIQUINONE REDUCTASE BY OXACARBOCYANINE DYES

A STRUCTURE–ACTIVITY STUDY

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Abstract—In this study, we determined that three structurally related oxacarbocyanine dyes, 3,3′-diethyloxacarbocyanine (DiOC2(3)), 3,3′-dipentyloxacarbocyanine (DiOC5(3)), and 3,3′-dihexyloxacarbocyanine (DiOC6(3)), and one oxadicarbocyanine, 3,3′-diethyloxadicarbocyanine (DiOC2(4)), inhibit bovine heart mitochondrial NADH oxidase activity and one of them, DiOC6(3), inhibits *Paracoccus denitrificans* NADH oxidase activity. The mitochondrial I_{50} values were 9 μM (DiOC2(3)), ~1 μM (DiOC5(3)) and DiOC6(3)), and ~3 μM (DiOC2(4)), whereas the I_{50} value for *P. denitrificans* was ~2 μM (DiOC6(3)). Neither succinate nor cytochrome oxidase (EC 1.9.3.1) activity was inhibited significantly by any of the compounds in either electron transport chain, localizing the inhibitory site of the oxacarbocyanine dyes to the respiratory chain segment between NADH and ubiquinone. With submitochondrial particles (SMP), NADH-dependent reduction of duroquinone and coenzyme Q_1 was inhibited markedly by all four compounds with DiOC6(3) being the most potent inhibitor, and the reduction of menadione was inhibited substantially by DiOC6(3). When purified complex I was used, NADH-dependent reduction of ferricyanide was inhibited by DiOC5(3) and coenzyme Q_1 reduction was inhibited by all oxacarbocyanines. With *P. denitrificans* membrane vesicles, DiOC6(3) substantially inhibited NADH-dependent reduction of coenzyme Q_1. All the oxacarbocyanines were more effective inhibitors with membrane preparations than with complex I, suggesting that membrane interactions play a role in inhibition. The mechanism of inhibition of the oxacarbocyanines appears to be similar to that of rotenone since (a) essentially only electron acceptors affected by rotenone were affected by the compounds, (b) inhibition of menadione reduction was diminished drastically with rotenone-saturated SMP, and (c) inhibition of coenzyme Q_1 was largely eliminated with rotenone-insensitive complex I, and *P. denitrificans* membrane vesicles.

Mitochondrial and *Paracoccus denitrificans* NADH–ubiquinone reductase is subject to inhibition by a number of compounds, many of which are lipophilic-cationic substances. Among these lipophilic-cationic inhibitors are 1-methyl-1-phenylpyridinium (MPP^+)† [1–7], dequalinium chloride [8, 9], several indolecarbocyanine dyes [10, 11], and recently several thiacarbocyanine dyes [12, 13]. Dequalinium chloride has been shown to inhibit NADH–ubiquinone reductase (EC 1.6.99.3) in both mitochondrial and *P. denitrificans* electron transport chains by binding to both a rotenone binding site and another site [9]. MPP^+ inhibition of this respiratory chain activity in mitochondria occurs by this lipophilic-cation binding to the rotenone binding site [2]. The indolecarbocyanine and thiacarbocyanine compounds examined thus far also appear to affect NADH–ubiquinone reductase activity by interacting with the rotenone binding site on the enzyme [10–13].

Oxacarbocyanine dyes have been used in a number of biological studies of membrane potential [14–16], neuronal transport [17], amino acid transport in renal tissue [18], ion transport in enterocytes [19], visualization of vasculature [20], visualization of endoplasmic reticulum in living cells [21], and monitoring of mitochondrial membrane potential in living cells [22]. Only one study has examined the possible inhibitory or cytotoxic effects of these compounds [23], in which 3,3′-dihexyloxacarbocyanine at micromolar concentrations was found to act as an uncoupler of succinate-supported metabolism in rat liver mitochondria. In the present study, we report that several of the oxacarbocyanine dyes inhibited mitochondrial NADH–ubiquinone reductase activity and one of them inhibited *P. denitrificans* NADH–ubiquinone reductase activity. Thus, there would appear to be a potential for cytotoxicity with some of the oxacarbocyanine dyes and care should be taken in using them to examine biological processes.

MATERIALS AND METHODS

The oxacarbocyanines 3,3′-diethyloxacarbo-

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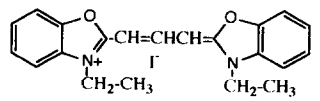
† Abbreviations: MPP^+, 1-methyl-1-phenylpyridinium; DiOC2(3), 3,3′-diethyloxacarbocyanine; DiOC5(3), 3,3′-dipentyloxacarbocyanine; DiOC6(3), 3,3′-dihexyloxacarbocyanine; DiOC2(4), 3,3′-diethyloxadicarbocyanine; DMSO, dimethyl sulfoxide; and SMP, submitochondrial particles.

cyanine (DiOC2(3)), 3,3'-dipentyloxacarbocyanine (DiOC5(3)), and 3,3'-dihexyloxacarbocyanine (DiOC6(3)) were obtained from Molecular Probes, Inc. (Eugene, OR, U.S.A.) and 3,3'-diethyloxadicarbocyanine (DiOC2(4)) was purchased from the Eastman Kodak Co. (Rochester, NY, U.S.A.). NADH, potassium ferricyanide, menadione, juglone, duroquinone, antimycin A, bovine serum albumin (crystallized and lyophilized), *N,N,N',N'*-tetramethylphenylenediamine, sodium ascorbate, rotenone, and dimethyl sulfoxide (DMSO) were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Coenzyme Q_1 was a gift of the Eisai Co. (Tokyo, Japan). Stock solutions of the oxacarbocyanines (10 mM) were prepared in DMSO, and could be stored at −20° for at least 1 month. Light-sensitive dilute solutions were protected by wrapping in aluminum foil. All other chemicals were of reagent grade quality.

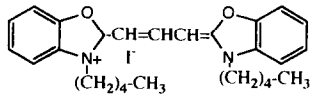
Preparation of mitochondria, submitochondrial particles and complex I. Mitochondria were prepared from fresh bovine hearts obtained from a slaughterhouse by the method of Hatefi *et al.* [24]. Nonphosphorylating submitochondrial particles (SMP) were prepared from either fresh or frozen mitochondria by the procedure of Löw and Vallin [25]. Binary complex I–III (NADH–cytochrome *c* reductase) was prepared from mitochondria by the procedure of Hatefi *et al.* [26], and complex I was purified from this preparation by the method of Hatefi *et al.* [24, 26]. Rotenone-insensitive complex I was prepared by treating complex I with chymotrypsin according to the procedure of Crowder and Ragan [27].

Preparation of P. denitrificans *membrane vesicles and rotenone-insensitive membrane vesicles. P. denitrificans*, ATCC 13543, cells were grown microaerophilically on 1% (w/v) nutrient broth, containing 0.5% (w/v) glucose and 10% (w/v) sodium nitrate, to late log phase. Cells were harvested by centrifugation, and membrane vesicles were prepared by sonication and stored in 100 mM Tris–acetate, pH 7.3, containing 1 mM $MgCl_2$ at −80°. Rotenone-insensitive *P. denitrificans* cells were obtained by the aerobic chemostat method of Meijer *et al.* [28] using the chemically defined medium of Burnell *et al.* [29] in the presence of 100 μM rotenone. Membrane vesicles were prepared by the procedure of Burnell *et al.* [29], and stored in 100 mM Tris–acetate, pH 7.3, containing 1 mM $MgCl_2$ at −80°.

Assays. NADH oxidase, succinate oxidase, and cytochrome oxidase activities of both submitochondrial particles and *P. denitrificans* membrane vesicles were determined as described previously [11]. Enzymatic activities of SMP, complex I and *P. denitrificans* membrane vesicles using the artificial electron acceptors ferricyanide (final concentration 1 mM) and menadione (final concentration 0.2 mM) were determined by the method of Galante and Hatefi [30]. NADH–juglone reductase (final concentration 0.3 mM juglone) and NADH–duroquinone reductase (final concentration 0.3 mM duroquinone) were determined spectrophotometrically in 120 mM sodium phosphate, pH 8.0, by the method of Ruzicka and Crane [31, 32]. NADH–coenzyme Q_1 reductase activity (final concentration 50 μM coenzyme Q_1) was determined by the method of Hatefi *et al.* [26]. Antimycin A (final concentration 3.3 μM) and KCN (final concentration 10 mM) were added for the determination of reduction of artificial electron acceptors and coenzyme Q_1 when either SMP or *P. denitrificans* membrane vesicles were used as the enzyme source. For the spectrophotometric assays, a unit of activity is defined as the amount of enzyme that oxidizes 1 μmol of NADH/min at 25°. For the oxygen electrode assays (succinate and cytochrome oxidase), a unit of activity is defined as the amount of enzyme that reduces 1 ng atom of oxygen/min at 30°. Protein concentration was determined using the biuret method [33] with bovine serum albumin as a standard.



DiOC2(3)



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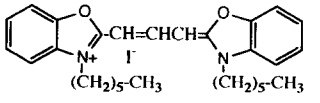
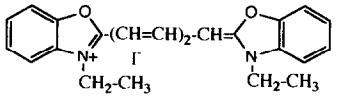


DiOC6(3)



DiOC2(4)

Fig. 1. Structures of the oxacarbocyanine dyes.

RESULTS

The structures of the oxacarbocyanines are shown in Fig. 1. Three of the compounds [DiOC2(3), DiOC5(3), and DiOC6(3)] differ only in the side chain attached to the ring nitrogen, with DiOC2(3) containing ethyl side chains, DiOC5(3) containing pentyl side chains and DiOC6(3) containing hexyl side chains. The other compound in the series examined in this study, DiOC2(4), is an oxadicarbocyanine, with a bridging group two carbons longer than the other three compounds, but it, like DiOC2(3), contains ethyl side chains attached to the ring nitrogens.

The concentration–response curves for bovine heart mitochondrial NADH oxidase, succinate

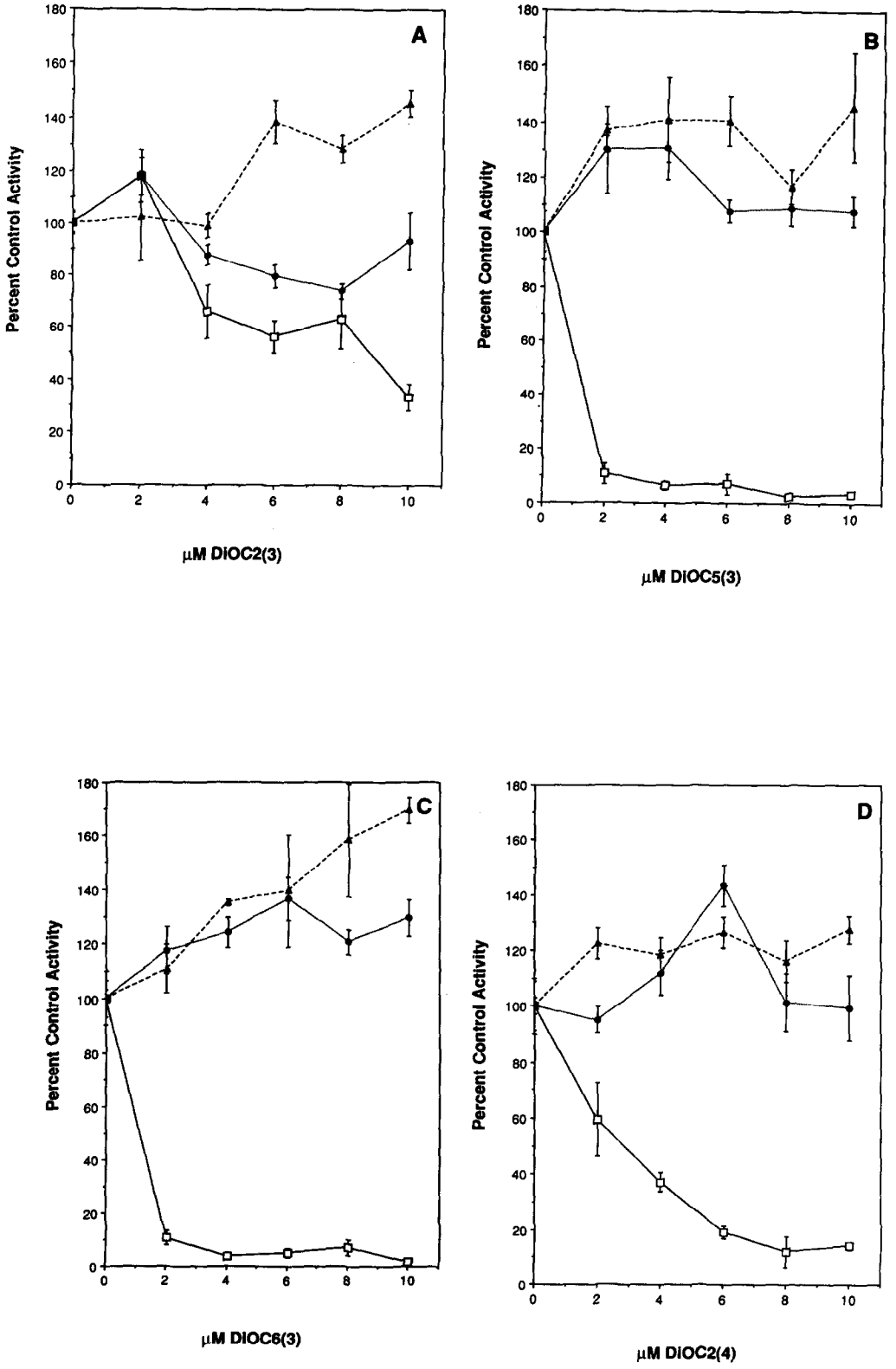


Fig. 2. Effects of increasing concentrations of oxacarbocyanines on mitochondrial NADH, succinate, and cytochrome oxidase activities. Assays were performed in triplicate and values are means ± SEM. Assays for NADH oxidase (1 mL) contained 0.05 mg of SMP protein. Assays for succinate or cytochrome oxidase (3 mL) contained 0.05 mg/mL of SMP protein. Key: (□) NADH oxidase; (▲) succinate oxidase; and (●) cytochrome oxidase. Control specific activities (units/mg protein) for NADH oxidase, succinate oxidase and cytochrome oxidase were 1543.2, 524.6 and 492.7, respectively.

oxidase and cytochrome oxidase using submitochondrial particles are shown in Fig. 2, A–D.

With respect to mitochondrial electron transport activity, DiOC2(3) (Fig. 2A) inhibited NADH oxidase activity with an I_{50} value of ~9 μM. It had no inhibitory effect on cytochrome oxidase activity but succinate oxidase activity was somewhat stimulated. DiOC5(3) (Fig. 2B) dramatically inhibited NADH oxidase activity with an I_{50} value of ~1 μM. Cytochrome oxidase activity was basically unaffected, whereas succinate oxidase activity was slightly stimulated. DiOC6(3) (Fig. 2C) also dramatically inhibited NADH oxidase activity with an I_{50} value of ~1 μM. Cytochrome oxidase activity was slightly activated, and succinate oxidase activity was substantially activated. The oxadicarbocyanine, DiOC2(4) (Fig. 2D), also inhibited NADH oxidase activity with an I_{50} value of ~3 μM. There was a very slight activation of both succinate and cytochrome oxidase activities. It is interesting that DiOC2(3) was inhibitory only at higher concentrations, whereas DiOC2(4), which is identical to DiOC2(3) except for the longer bridging group, was a more effective NADH oxidase activity inhibitor. DiOC5(3) and DiOC6(3), both of which have the same bridging distance as DiOC2(3), inhibited NADH oxidase activity at almost a 10-fold lower concentration when compared to DiOC2(3). This indicates that, with respect to the mitochondrial electron transport chain, the length of the side chains plays a primary role in the inhibitory capacity of the oxacarbocyanine compounds. The distance between the two ring systems modulates this inhibitory capacity such that compounds with longer bridging distances can be inhibitory even with shorter length side chains, e.g. DiOC2(4). A shorter bridging distance appears to require a longer length side chain to be an effective inhibitor, e.g. DiOC5(3) and DiOC6(3).

The stimulatory effects of DiOC2(3) (Fig. 2A), DiOC5(3) (Fig. 2B), DiOC6(3) (Fig. 2C), and DiOC2(4) (Fig. 2D) on succinate oxidase activity and of DiOC6(3) (Fig. 2C) and DiOC2(4) (Fig. 2D) on cytochrome oxidase activity could largely be eliminated by the inclusion of superoxide dismutase (100 U) in the assay system (data not shown). Thus, as we previously observed with several of the thiacarbocyanines [13], the oxacarbocyanines can interact with certain substrates in the presence of SMP to produce superoxide which interferes with the ability of the oxygen electrode to measure oxygen consumption. As with the thiacarbocyanines [13], attempts to determine cytochrome oxidase activity spectrophotometrically in the presence of the oxacarbocyanines were unsuccessful due to the formation of a precipitate in the assay system.

The concentration–response curves for *P. denitrificans* membrane vesicles NADH oxidase, succinate oxidase and cytochrome oxidase activities are shown in Fig. 3. DiOC2(3), DiOC5(3), and DiOC2(4) were ineffective as inhibitors of *P. denitrificans* NADH, succinate, or cytochrome oxidase activities (data not shown). However, DiOC6(3) (Fig. 3) significantly inhibited NADH oxidase activity with little effect on the other two oxidase activities. The

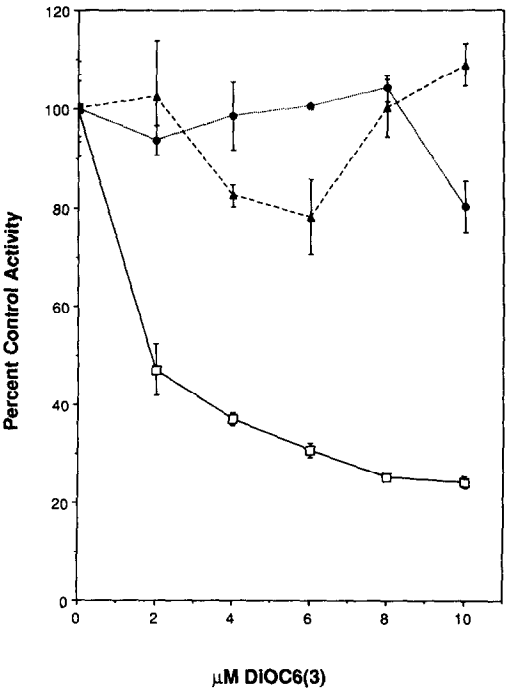


Fig. 3. Effect of increasing concentrations of DiOC6(3) on NADH, succinate, and cytochrome oxidase activities in *P. denitrificans* membrane vesicles. Assays were performed in triplicate and values are means ± SEM. Assays for NADH oxidase (1 mL) contained 0.1 mg of *P. denitrificans* membrane vesicles protein. Assays for succinate or cytochrome oxidase (3 mL) contained 0.1 mg/mL *P. denitrificans* membrane vesicles protein. Key: (□) NADH oxidase; (▲) succinate oxidase; (●) cytochrome oxidase. Control specific activities (units/mg protein) for NADH oxidase, succinate oxidase and cytochrome oxidase were 184.3, 511.1 and 907.6, respectively.

I_{50} value for DiOC6(3) with respect to *P. denitrificans* NADH oxidase activity was ~2 μM.

These results contrast with those of the mitochondrial electron transport system (see Fig. 2) where all four compounds tested were inhibitors of electron transport between NADH and ubiquinone. Only the oxacarbocyanine dye with the long side chains, DiOC6(3), had any significant effect on this segment of the *P. denitrificans* electron transport chain. These results would argue that there are some large differences between the two electron transport chains with respect to the structure–activity relationship of the oxacarbocyanines.

The locus of action of the oxacarbocyanines which inhibit mitochondrial and *P. denitrificans* electron transport chain activity appears to be the segment between NADH and ubiquinone, since they inhibited NADH oxidase activity without any significant inhibitory effect on either succinate or cytochrome oxidase activities.

Flux through the NADH to ubiquinone segment of the two electron transport chains may be examined by determining the rate of reduction of a number of different artificial electron acceptors and coenzyme

Table 1. Effect of 10 μM oxacarbocyanine on activities of bovine heart SMP and complex I (NADH–ubiquinone reductase)

Electron acceptor	Inhibition (% of control activity)			
	DiOC2(3)	DiOC5(3)	DiOC6(3)	DiOC2(4)
A. Bovine heart SMP				
Ferricyanide	90.5 ± 3.0	72.7 ± 8.3	74.5 ± 10.8	76.9 ± 9.8
Menadione	102.8 ± 4.3	96.9 ± 6.4	24.0 ± 3.0	97.5 ± 5.1
Juglone	92.5 ± 6.8	78.1 ± 0.9	83.9 ± 10.1	92.5 ± 7.0
Duroquinone	66.0 ± 3.4	48.1 ± 1.7	22.6 ± 1.5	61.5 ± 3.5
Coenzyme Q_1	48.5 ± 3.4	26.6 ± 1.2	4.8 ± 1.5	41.1 ± 3.9
B. Complex I				
Ferricyanide	97.5 ± 0.8	68.7 ± 7.1	80.0 ± 13.2	91.7 ± 15.6
Menadione	184.6 ± 18.0	130.5 ± 8.2	112.4 ± 6.9	147.5 ± 19.2
Juglone	86.6 ± 1.3	110.3 ± 9.2	85.3 ± 1.3	93.4 ± 4.0
Duroquinone	118.8 ± 15.4	101.2 ± 4.1	85.3 ± 1.0	116.2 ± 10.7
Coenzyme Q_1	67.3 ± 7.3	57.4 ± 3.1	55.1 ± 5.8	77.7 ± 5.8

All assays were performed as described in Materials and Methods in the absence and presence of 10 μM oxacarbocyanine. Activities with SMP contained 3.3 μM antimycin A and 10 mM KCN. Assays contained either 0.05 mg of SMP protein or 0.01 mg of complex I protein. Assays were performed in triplicate and values are means ± SEM. Control specific activities (units/mg protein) for SMP were: NADH–ferricyanide, 11.19; NADH–menadione, 0.39; NADH–juglone, 0.58; NADH–duroquinone, 0.56; NADH–Q_1, 0.92; and for complex I: NADH–ferricyanide, 63.18; NADH–menadione, 0.58; NADH–juglone, 1.07; NADH–duroquinone, 0.54; and NADH–Q_1, 0.56.

Q derivatives. Reduction of electron acceptors such as the duroquinone and coenzyme Q derivatives is sensitive to classical inhibitors of this segment of the two electron transport chains such as rotenone or piericidin A, indicating that their reduction occurs after the block in electron transport by these two inhibitors. Reduction of artificial electron acceptors such as ferricyanide, menadione, and juglone is insensitive to these classical inhibitors, indicating that their reduction occurs before the block in electron transport by rotenone or piericidin A.

The effect of a 10 μM concentration of oxacarbocyanine dyes on reduction of ferricyanide, menadione, juglone, duroquinone and coenzyme Q_1 was determined using both submitochondrial particles and purified complex I (Table 1). Using SMP, NADH-dependent menadione reduction was inhibited by DiOC6(3), whereas duroquinone and coenzyme Q_1 reductions were inhibited by all four. Reduction of ferricyanide and juglone was relatively unaffected by any of the four compounds. The pattern of inhibition of NADH-dependent reduction of coenzyme Q_1 somewhat resembled that of the effect of the oxacarbocyanines on NADH oxidase activity (see Fig. 2). Thus, DiOC5(3) and DiOC6(3) were the most potent inhibitors while DiOC2(4) and DiOC2(3) were less inhibitory.

When purified complex I was used as the enzyme source (Table 1), only coenzyme Q_1 reduction was inhibited by all four compounds, with DiOC2(4) being only slightly inhibitory. Reduction of all the rest of the electron acceptors tested was largely unaffected. In all cases, the degree of inhibition of coenzyme Q_1 reduction was less with the purified enzyme than with the membrane bound form. Taken together, these data indicate that (1) the membrane environment of the enzyme must modulate the inhibitory action of the oxacarbocyanines, and (2) the primary inhibitory effect of these compounds is on those substrates whose reduction is also affected by rotenone and piericidin A. Thus, the oxacarbocyanines, like the indolecarbocyanines [11]

Table 2. Effect of 10 μM DiOC6(3) on activities of *Paracoccus denitrificans* membrane vesicle NADH–ubiquinone reductase

Electron acceptor	Inhibition (% of control activity)
Ferricyanide	84.4 ± 3.6
Menadione	82.8 ± 1.8
Juglone	222.0 ± 18.7
Duroquinone	84.3 ± 2.6
Coenzyme Q_1	11.5 ± 1.4

All assays were performed as described in Materials and Methods in the absence and presence of 10 μM DiOC6(3). Assays contained 3.3 μM antimycin A, 10 mM KCN, and 0.1 mg of *P. denitrificans* membrane vesicle protein. Assays were performed in triplicate and values are means ± SEM. Control specific activities (units/mg protein) were: NADH–ferricyanide, 4.21; NADH–menadione, 0.42; NADH–juglone, 2.46; NADH–duroquinone, 0.39; and NADH–Q_1, 0.79.

and thiacarbocyanines [12, 13], may be inhibiting NADH–ubiquinone reductase activity by interacting at or close to the rotenone site of this enzyme. Even though menadione reduction is not affected by either rotenone or piericidin A, we have shown previously that rotenone binding induces a conformational change in the enzyme structure as shown by an altered cross-linking pattern [34]. Rotenone-induced conformational changes in complex I have also been suggested by other workers using a different technique [35]. Thus, oxacarbocyanine DiOC6(3) binding to this site could also result in an altered conformation which could affect menadione reduction.

These types of experiments were repeated with *P. denitrificans* membrane vesicles using 10 μM DiOC6(3) (Table 2). In this case, only coenzyme Q_1 reduction was inhibited significantly by this oxacarbocyanine dye. Curiously, NADH-dependent reduction of juglone was stimulated dramatically by DiOC6(3). We have no explanation for this phenomenon, but it was highly reproducible. These results suggest that the effect of this oxacarbocyanine compound on *P. denitrificans* NADH–ubiquinone reductase activity is similar to the effects of the four oxacarbocyanines on the mitochondrial enzyme, i.e. in a manner similar to rotenone.

To test the hypothesis that the oxacarbocyanines are acting similar to rotenone, three studies were performed. First, the ability of DiOC6(3) to inhibit reduction of menadione using rotenone-saturated SMP was determined. This study revealed that DiOC6(3) inhibition of the reduction of menadione was largely eliminated in the presence of saturating rotenone [71.6% control activity with saturating rotenone vs 24.0% control activity in the absence of saturating rotenone (see Table 1)]. Second, reduction of coenzyme Q_1 was determined in the presence of each of the four oxacarbocyanines using rotenone-insensitive complex I. The results of this study indicated that inhibition of reduction of coenzyme Q_1 by each of the four dyes was largely abolished by eliminating the rotenone binding site [78.3 to 96.4% control activity using rotenone-insensitive complex I vs 55.1 to 67.3% control activity using rotenone-sensitive complex I (see Table 1)]. Third, reduction of coenzyme Q_1 was determined in the presence of DiOC6(3) using rotenone-insensitive *P. denitrificans* membrane vesicles. This study also showed that inhibition of coenzyme Q_1 reduction by DiOC6(3) was substantially eliminated by abolishing the rotenone binding site [75.9% control activity vs 11.5% control activity using rotenone-sensitive *P. denitrificans* membrane vesicles (see Table 2)]. These data, taken together, support the hypothesis that with both mitochondrial and *P. denitrificans* NADH–ubiquinone reductase, the inhibition caused by oxacarbocyanine dyes is due to these compounds interacting at or close to the rotenone site.

DISCUSSION

We have demonstrated previously that two indolecarbocyanine dyes (HIDC and HITC) are rather potent inhibitors of both mitochondrial and *P. denitrificans* NADH–ubiquinone reductase activity and appear to act by binding to the rotenone site of this electron transport complex in both electron transport chains [10, 11]. In the case of the indolecarbocyanines, the shorter the distance between the two ring systems, the stronger the inhibition. Thiacarbocyanines are also potent inhibitors of mitochondrial NADH–ubiquinone reductase activity [12, 13] and the structure–activity relationships of this class of carbocyanine dyes reveal that longer side chains and shorter bridging distances appear to result in greater inhibitory capacity. These compounds, like the indolecarbocyanines, also interact with the rotenone site on mitochondrial NADH–ubiquinone reductase. Quinolinium compounds, which we have also demonstrated to be rather potent inhibitors of NADH–ubiquinone reductase activity in both mitochondrial and *P. denitrificans* electron transport chain systems, exhibit just the opposite structure–activity characteristics, i.e. the longer the distance between the two ring systems the more potent the inhibition [9].

With respect to the mitochondrial NADH–ubiquinone reductase activity, both the distance between the two ring systems and the length of the side chains are important for inhibitory capacity of the oxacarbocyanine dyes examined in this study. Thus, a shorter distance between the two rings coupled with long aliphatic side chains such as pentyl or hexyl groups, e.g. DiOC5(3) and DiOC6(3), results in potent inhibition whereas shortening the side chains to ethyl groups, e.g. DiOC2(3), lowers the inhibitory capacity. Lengthening the distance between the two ring systems by two methylene groups can potentiate inhibition when short (ethyl) side chains are present. It would be interesting to determine the effect of the longer side chains coupled with the longer bridging distance upon the inhibitory capacity with the mitochondrial electron transport chain. The above structure–activity relationships are apparently not as operative with the *P. dentrificans* electron transport chain, since only DiOC6(3) exhibited significant inhibitory capacity in this system. Structure–activity differences were also noted between these two electron transport chains with both the quinolinium compounds [9] and the indolecarbocyanine dyes [10, 11].

As with the indolecarbocyanines and thiacarbocyanines, the oxacarbocyanine compounds target mitochondrial NADH–ubiquinone reductase as their site of inhibitory action. The fact that the oxacarbocyanines were more inhibitory with the membrane bound form of NADH–ubiquinone reductase as compared with the purified soluble complex I indicates that the environment of the enzyme is a contributory factor to inhibitory capacity, which was also true for the indolecarbocyanine dyes [10, 11] and the thiacarbocyanine dyes [12, 13]. Even though the membrane environment is a factor in the inhibitory capacity of the oxacarbocyanines, the fact that neither succinate nor cytochrome oxidase activity was inhibited by any of the four dyes makes it unlikely that the integration of the dye into the lipid bilayer results in a generalized type of membrane damage. Also, the fact that the dyes only inhibited certain of the activities of complex I argues against

a specific damaging effect of the compounds upon this respiratory chain enzyme. Since all four of the oxacarbocyanines significantly inhibited mitochondrial electron transport chain activity with an I_{50} in the micromolar range, there could be cytotoxic effects of these compounds which may affect their use in biological systems as fluorescent labeling agents or as sensors of membrane potential.

It is curious that of the four oxacarbocyanines examined in this study, all were inhibitors of mitochondrial NADH–ubiquinone reductase, while only one oxacarbocyanine, DiOC6(3), exerted significant inhibition of *P. denitrificans* NADH oxidase activity. This may be an indication of differences between the two enzymes, but may also reflect differences in the membrane environment between the mammalian and *P. denitrificans* NADH–ubiquinone reductase. In the case of the two indolecarbocyanines previously examined, both compounds inhibited mitochondrial and *P. denitrificans* NADH–ubiquinone reductase activity [10, 11].

As with the indolecarbocyanine [10, 11] and the thiacarbocyanine dyes [12, 13], the oxacarbocyanine dyes affect NADH–ubiquinone reductase activity by interacting at or close to the rotenone binding site of the enzyme. Thus, one is tempted to generalize that carbocyanine compounds that inhibit NADH–ubiquinone reductase activity act in a manner similar to rotenone. The subunit which binds rotenone has been identified in the bovine mitochondrial enzyme as a 33 kDa hydrophobic fraction polypeptide [36] encoded by the mitochondrial DNA. The rotenone-binding subunit has not yet been identified in the *P. denitrificans* enzyme, but one would assume that it would also be a rather hydrophobic polypeptide. Either because of its hydrophobic nature or because of its position in the enzyme structure, it appears to interact with a wide variety of hydrophobic compounds, including rotenone, which is nonionic, and an array of lipophilic-cationic compounds like MPP^+ [1–7], and the various types of carbocyanines [10–13]. Work is currently underway to devise a photoaffinity derivative of one or more of the carbocyanine-type compounds, such as DiOC5(3), in order to elucidate the binding site on this hydrophobic subunit.

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