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INHIBITORY EFFECTS OF TWO STRUCTURALLY RELATED CARBOCYANINE LASER DYES ON THE ACTIVITY OF BOVINE HEART MITOCHONDRIAL AND *PARACOCCUS DENITRIFICANS* NADH-UBIQUINONE REDUCTASE

EVIDENCE FOR A ROTENONE-TYPE MECHANISM

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Abstract—Two cationic, lipophilic laser dyes, 1,1′,3,3,3′,3′-hexamethylindodicarbocyanine iodide (HIDC) and 1,1′,3,3,3′,3′-hexamethylindotricarbocyanine iodide (HITC), inhibit bovine heart mitochondrial and *Paracoccus denitrificans* NADH oxidase activities. The mitochondrial I_{50} values were 0.5 μM (HIDC) and 1.2 μM (HITC), and the *P. denitrificans* I_{50} values 1.2 μM (HIDC) and 1.5 μM (HITC). Neither succinate nor cytochrome oxidase (EC 1.9.3.1) activities were inhibited significantly by either compound, localizing the site of inhibition to the segment of each electron transport chain between NADH and ubiquinone. With submitochondrial particles (SMP), NADH-dependent reduction of menadione, duroquinone and coenzyme Q_1 was inhibited markedly (HIDC was the more potent inhibitor). Using purified complex I, only NADH-dependent reduction of duroquinone and coenzyme Q_1 was inhibited markedly (HIDC was the more potent inhibitor) and reduction of menadione was inhibited slightly. With *P. denitrificans* membrane vesicles, NADH-dependent reduction of menadione, juglone, and coenzyme Q_1 was inhibited slightly and duroquinone reduction was inhibited markedly. Membrane-dependent interactions appear to be involved, since the compounds were more inhibitory with membrane preparations than with complex I. The mechanism of inhibition (except for the HIDC effect on coenzyme Q_1 reduction with *P. denitrificans*) appeared to be through the interaction of dye with the rotenone site on NADH-ubiquinone reductase (EC 1.6.99.3), since rotenone-insensitive preparations of complex I and *P. denitrificans* membrane vesicles were also insensitive to HIDC and HITC inhibition.

Several lipophilic-cationic compounds have been shown to be concentrated in mitochondria and to exhibit cytotoxic effects by inhibiting various mitochondrial functions [1–18]. The three most studied compounds in this respect are rhodamine 123 [1–6, 19–22], 1-methyl-4-phenylpyridinium (MPP^+) [8–13, 17) and dequalinium chloride [7, 15, 18, 23]. MPP^+ and dequalinium chloride inhibit mitochondrial NADH-ubiquinone reductase (EC 1.6.99.3) [7, 15, 18] and dequalinium also inhibits and photoinactivates the ATPase [23]. Recently, dequalinium was also shown to inhibit *Paracoccus denitrificans* NADH-ubiquinone reductase [15, 18]. The effects of rhodamine 123 are multiphasic. There are reports of rhodamine 123 inhibition of mitochondrial protein synthesis [5], ATPase activity [20], and import of cytoplasmically synthesized protein into mitochondria [19, 22].

Classical inhibitors of mitochondrial NADH-ubiquinone reductase reductase, such as rotenone and piericidin A, block electron transport between the final NADH-reducible iron–sulfur cluster, N-2, and ubiquinone [24–28]. MPP^+, a potent neurotoxin of dopamine-producing cells in the substantia nigra, acts by the same mechanism [9]. Dequalinium chloride, however, appears to block electron transport by binding to both the rotenone binding site and another site of NADH-ubiquinone reductase with mitochondria and *P. denitrificans* [15, 18].

1,1′,3,3,3′,3′-Hexamethylindicarbocyanine iodide (HIDC) and 1,1′,3,3,3′,3′-hexamethylindotricarbocyanine iodide (HITC), like rhodamine 123, are laser dyes [29, 30]. Those two compounds differ only in the length of the bridging group between the two indole rings. HIDC has two ethylene groups and HITC has three ethylene groups. HIDC has been used in several biological systems as a potentiometric dye [31–35], and HITC has been utilized in quantum counting fluorescent measurements with biological systems [36]. The present work describes the inhibitory effects of these two laser dyes on mitochondrial and *P. dentrificans* electron transport chains and their possible mechanism of action.

MATERIALS AND METHODS

HIDC, HITC, NADH, menadione, juglone, rotenone, antimycin A, bovine serum albumin

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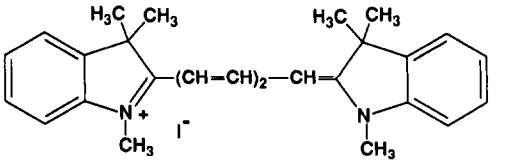
(crystallized and lyophilized), *N,N-N',N'*-tetramethylphenylenediamine (TMPD), sodium ascorbate and duroquinone were purchased from the Sigma Chemical Co. Coenzyme Q_1 was a gift of the Eisai Co., Tokyo, Japan. Stock solutions of HIDC and HITC (10 mM) in dimethyl sulfoxide (DMSO) could be stored at −20° for at least 1 week. Light-sensitive dilute solutions were wrapped with aluminium foil. All other chemicals were of reagent grade quality.

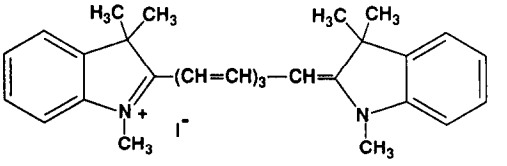
Preparation of mitochondria, submitochondrial particles and complex I. Mitochondria were prepared from fresh bovine hearts as described by Hatefi *et al.* [37]. Submitochondrial particles were prepared by the method of Low and Vallin [38]. Complex I–III (NADH-cytochrome *c* reductase) was prepared by the procedure of Hatefi *et al.* [39], and complex I (NADH-ubiquinone reductase) was resolved from this binary complex by the method of Hatefi *et al.* [37, 39]. Rotenone-insensitive complex I was prepared by treatment of complex I with chymotrypsin as described by Crowder and Ragan [40].

Preparation of P. denitrificans *membrane vesicles and rotenone-insensitive membrane vesicles. P denitrificans*, ATCC 13543, cells were grown microaerophilically on 1% nutrient broth, 0.5% glucose, 10% sodium nitrate to late exponential phase. The 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 prepared by the aerobic chemostat growth method as described by Meijer *et al.* [41] using the chemically defined medium of Burnell *et al.* [42], in the presence of 100 μM rotenone. Cells were harvested by centrifugation and washed with buffer containing 1% bovine serum albumin. Membrane vesicles were prepared as described by Burnell *et al.* [42], and stored at −80° in 100 mM Tris-acetate, pH 7.3, containing 1 mM $MgCl_2$.

Assays. NADH oxidase activities of both submitochondrial particles (SMP) and *P. denitrificans* membrane vesicles were determined by spectrophotometrically monitoring the disappearance of NADH (final concentration equals 150 μM) in 120 mM sodium phosphate, pH 8.0, at room temperature. Succinate oxidase activity was determined by monitoring the disappearance of oxygen with an oxygen electrode (Yellow Springs Instrument Co.), using 10 mM sodium succinate and 120 mM sodium phosphate, pH 8.0, at 30°. Succinate oxidase activity was unaffected by preincubation with 30 mM sodium succinate at 30°. Cytochrome oxidase (EC 1.9.3.1) activity was determined by following the disappearance of oxygen with an oxygen electrode in 120 mM sodium phosphate, pH 8.0, containing 5 mM sodium ascorbate plus 0.1 mM TMPD at 30° in the presence of 3.3 μM antimycin A. A unit of activity for the oxygen electrode assays is defined as the amount of enzyme that reduces 1 ng atom of oxygen/min at 30°. For the spectrophotometric assays, a unit is defined as the amount of enzyme that oxidizes 1 μmol of NADH/min at 25°. Enzymatic activities of complex I, SMP, and *P. denitrificans* membrane vesicles using the artificial electron acceptors ferricyanide (final concentration 1 mM) and menadione (final concentration 0.2 mM) were spectrophotometrically determined by the method of Galante and Hatefi [43]. NADH-juglone reductase (final concentration 0.3 mM juglone) and NADH-duroquinone reductase (final concentration 0.3 mM duroquinone) activities were spectrophotometrically determined according to Ruzicka and Crane [44, 45] in 120 mM sodium phosphate, pH 8.0. NADH-Q_1 reductase activity (final concentration 50 μM coenzyme Q_1) was spectrophotometrically determined by the method of Hatefi *et al.* [39] where a unit of enzyme activity is equivalent to the one-electron reduction of coenzyme Q_1. When activities with artificial electron acceptors or coenzyme Q_1 were determined using SMP or *P. denitrificans* membrane vesicles, 3.3 μM antimycin A and 10 mM KCN were added. Protein concentration was determined by the biuret method [46], using bovine serum albumin as a standard.



HIDC



HITC

Fig. 1. Structures of HIDC and HITC.

RESULTS

The structures of HIDC and HITC are shown in Fig. 1. Concentration–response curves for bovine heart mitochondrial and *P. denitrificans* NADH oxidase activities are shown in Fig. 2 A and B. Both compounds were highly inhibitory to electron transport. The I_{50} values were 0.5 μM (HIDC) and 1.2 μM (HITC) for mitochondrial NADH oxidase and 1.2 μM (HIDC) and 1.5 μM (HITC) for *P. denitrificans* NADH oxidase. In both cases, HIDC was a slightly more potent inhibitor than HITC, which may indicate the importance of the distance and/or the number of carbon—carbon double bonds between the two ring systems. With both compounds, 90% or greater inhibition of electron transport chain activity was achieved at approximately 7 μM; therefore, this concentration was chosen for all further dye inhibition studies.

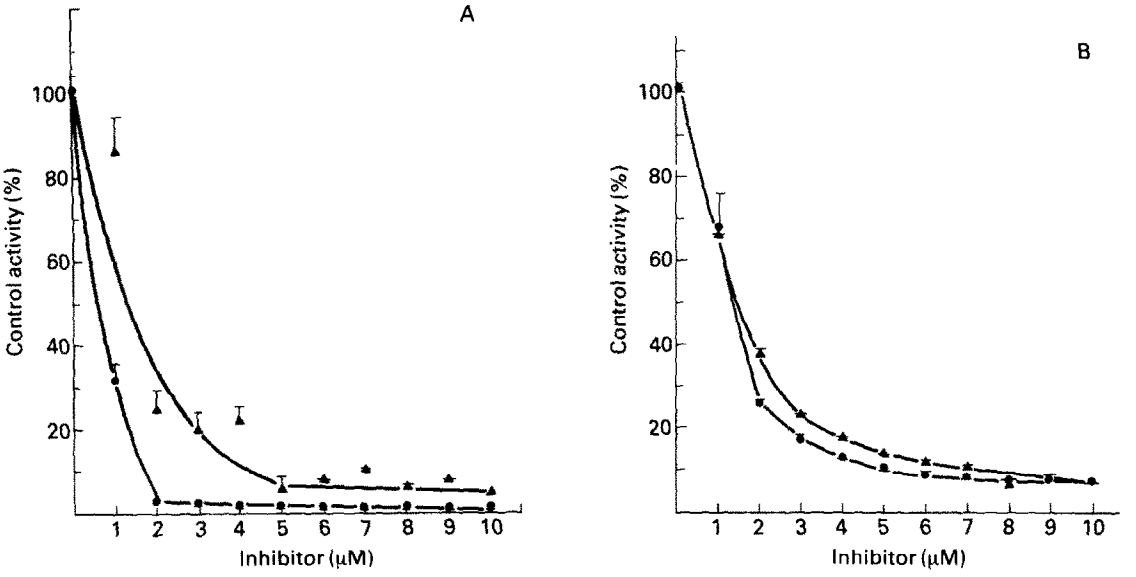


Fig. 2. Effect of increasing concentrations of HIDC and HITC on NADH oxidase activity. Assays were performed in triplicate, and values are means ± SEM. (A) Submitochondrial particles. Assays contained 0.05 mg of SMP protein. (B) *P. denitrificans* membrane vesicles. Assays contained 0.1 mg of *P. denitrificans* membrane vesicles protein. Key: (●) HIDC; and (▲) HITC. Control specific activity (units/mg protein) for SMP NADH oxidase was 1446.6 and for *P. denitrificans* membrane vesicles 181.56.

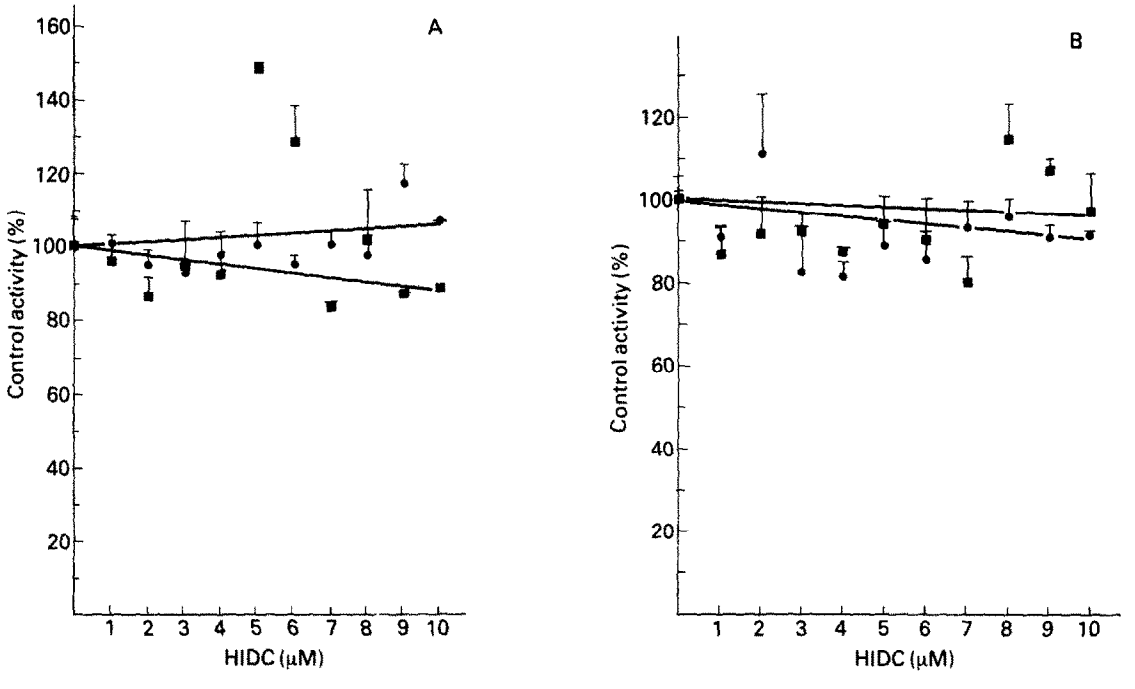


Fig. 3. Effect of increasing concentrations of HIDC on succinate oxidase and cytochrome oxidase activities. Assays were performed in triplicate, and values are means ± SEM. (A) Submitochondrial particles. Assays (3 mL) contained 0.05 mg/mL of SMP protein. (B) *P. denitrificans* membrane vesicles. Assays (3 mL) contained 0.1 mg/mL of *P. denitrificans* membrane vesicle protein. Key: (■) succinate oxidase activity; and (●) cytochrome oxidase activity. Control specific activities (units/mg protein): SMP succinate oxidase 544.13, and cytochrome oxidase 473.77; *P. denitrificans* membrane vesicle succinate oxidase 508.53, and cytochrome oxidase 914.67.

Since the NADH-dependent reduction of oxygen, NADH oxidase activity, represents flux of electrons through the entire electron transport chain and encompasses all three sites of action of classical electron transport inhibitors, more precise studies are necessary to localize the region of inhibition of these two compounds within the respective respiratory chains. Therefore, the effects of HIDC and HITC were determined on succinate oxidase and cytochrome oxidase activities, as shown in Figs. 3 and 4 respectively. For both electron transport chains, there was no significant inhibition of either succinate or cytochrome oxidase activity in the range of concentrations where inhibition of NADH oxidase activity occurred. The only exception may be in the effect of HITC on SMP cytochrome oxidase activity (Fig. 4A) where the scatter of the data precludes an unequivocal conclusion about inhibition. The maximum inhibition seen with cytochrome oxidase (approximately 40% inhibition at 10 μM) was insufficient to account for the dramatic effect on SMP NADH oxidase activity (90% inhibition at 5 μM HITC, see Fig. 2A) which leads to the conclusion that the major effect of the dyes is on the segment of the respiratory chain between NADH and ubiquinone. This is the region of the respiratory chain susceptible to the classical inhibitors rotenone and piericidin A. Unfortunately the spectrophotometric determination of the oxidation of reduced cytochrome *c*, which might clarify the question of the effect of HITC on SMP cytochrome oxidase, is not possible since these carbocyanine dyes absorb strongly in the region of the visible spectrum of reduced cytochrome *c*.

Electron transport by the NADH to ubiquinone segment of the respiratory chain can be assessed with either artificial electron acceptors or coenzyme Q analogs. Reduction of electron acceptors such as ferricyanide, menadione, and juglone by NADH with this segment of the chain was not subject to inhibition by rotenone and piericidin A, indicating that these compounds are reduced before the block by rotenone and piericidin. Reduction of the quinone, duroquinone, and coenzyme Q_1 was inhibited by rotenone and piericidin, indicating that they accept electrons after the rotenone block.

To localize further the site of action of HIDC and HITC within the NADH to ubiquinone segment of the two-electron transport chains, the effects of 7 μM HIDC and HITC on reduction of several artificial electron acceptors and coenzyme Q_1 were determined. These data are shown in Table 1. Using SMP as the source of NADH-ubiquinone reductase, neither HIDC nor HITC inhibited the reduction of ferricyanide or juglone. However, both compounds markedly inhibited the reduction of menadione, duroquinone and coenzyme Q_1. As is the case with the entire electron transport chain of SMP, HIDC was more potent than HITC.

Using purified complex I, again neither compound affected reduction of ferricyanide or juglone. Reduction of menadione was inhibited slightly by

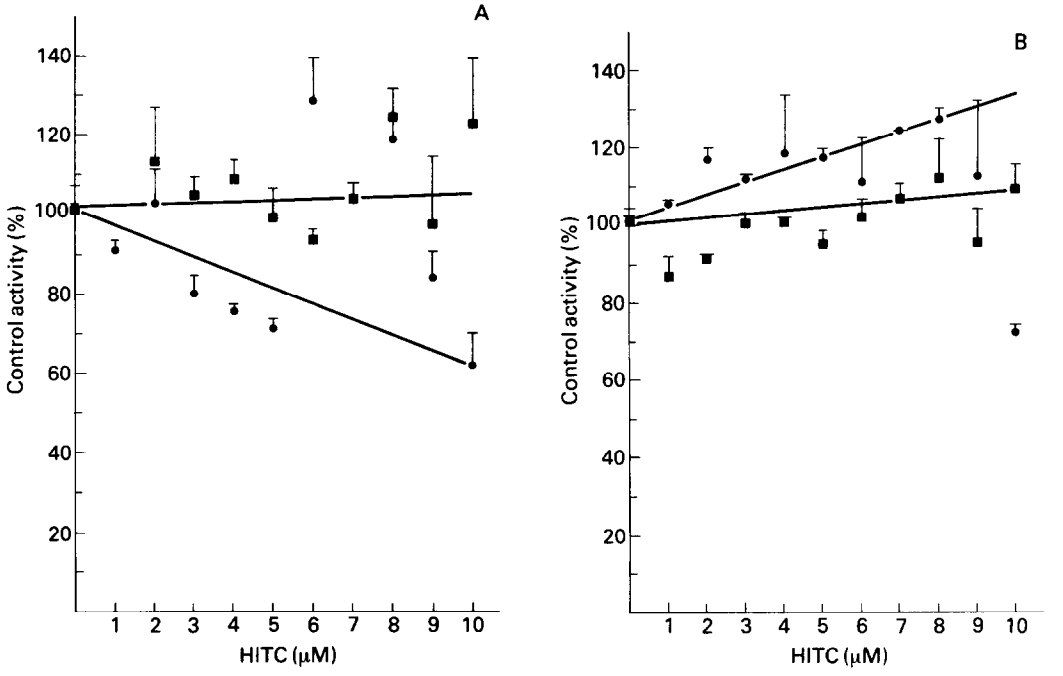


Fig. 4. Effect of increasing concentrations of HITC on succinate oxidase and cytochrome oxidase activities. Assays were performed in triplicate, and values are means ± SEM. (A) Submitochondrial particles. Assays (3 mL) contained 0.05 mg/mL of SMP protein. (B) *P. denitrificans* membrane vesicles. Assays (3 mL) contained 0.1 mg/mL of *P. denitrificans* membrane vesicle protein Key: (■) succinate oxidase activity; and (●) cytochrome oxidase activity. Control specific activities (units/mg protein): SMP succinate oxidase 544.13, and cytochrome oxidase 473.77; *P. denitrificans* membrane vesicle succinate oxidase 508.53, and cytochrome oxidase 914.67.

both compounds and reduction of duroquinone and coenzyme Q_1 was inhibited markedly. There appear to be membrane-dependent interactions associated with inhibition, since the two compounds are generally more inhibitory with SMP than with complex I.

The pattern of inhibition by HIDC and HITC on NADH-ubiquinone reductase of *P. denitrificans* membrane vesicles roughly follows that of SMP. Negligible inhibition was observed with reduction of ferricyanide, and slight inhibition was seen with menadione, juglone, and coenzyme Q_1, whereas, duroquinone reduction was inhibited markedly. As was seen with the effects on NADH oxidase activity, the two compounds were less inhibitory with *P. denitrificans* membrane vesicles than with SMP.

The two compounds markedly inhibited only the reduction of electron acceptors also affected by rotenone (except for slightly inhibiting the reduction of menadione and juglone). This suggests that the two laser dyes function by binding to the rotenone site of NADH-ubiquinone reductase, which has been identified with the 33 kD subunit of mammalian complex I [47], coded for by mitochondrial DNA [48]. this rotenone binding site can be destroyed by treating complex I with chymotrypsin [40] such that the complex still retains enzymatic activity including the ability to reduce coenzyme Q. *P. denitrificans* can be made rotenone insensitive by growing the bacteria in the presence of a constant concentration of rotenone in a chemostat. Membrane vesicles prepared from these cells, although lacking the EPR signal from iron–sulfur cluster N-2 [41], still retain electron transport activity, which is not sensitive to rotenone. Using these two preparations, it should be possible to assess the hypothesis that HIDC and HITC act through the rotenone site. These data are shown in Table 2. With rotenone-insensitive complex I, NADH-dependent reduction of menadione, duroquinone, and coenzyme Q_1 was no longer subject to inhibition by HIDC or HITC. These data suggest that both HIDC and HITC are acting through a rotenone-type mechanism in mammalian complex I, since abolishing the rotenone site eliminated their inhibition. The results with rotenone-insensitive *P. denitrificans* membrane vesicles were somewhat more

Table 1. Effect of 7 μM HIDC or HITC on activities of bovine heart SMP, complex I and *P. denitrificans* membrane vesicles NADH-ubiquinone reductase

Activity*	Inhibition (% control activity) HIDC	HITC
(A) Bovine heart SMP		
NADH-ferricyanide	99.3 ± 0.7	96.5 ± 1.2
NADH-menadione	34.8 ± 1.8	55.7 ± 1.8
NADH-juglone	104.3 ± 0.0	110.2 ± 5.8
NADH-duroquinone	9.1 ± 10.9	38.1 ± 14.7
NADH-Q_1	6.3 ± 1.2	30.0 ± 4.2
(B) Complex I		
NADH-ferricyanide	101.2 ± 1.6	109.8 ± 1.7
NADH-menadione	87.8 ± 0.0	86.6 ± 1.2
NADH-juglone	115.5 ± 0.0	92.2 ± 0.0
NADH-duroquinone	33.3 ± 0.6	37.5 ± 1.2
NADH-Q_1	55.7 ± 5.4	66.8 ± 4.8
(C) *P. denitrificans* Membrane vesicles		
NADH-ferricyanide	91.3 ± 3.2	89.3 ± 0.9
NADH-menadione	83.4 ± 9.1	87.5 ± 1.0
NADH-juglone	88.9 ± 5.5	83.4 ± 0.0
NADH-duroquinone	57.9 ± 2.6	72.9 ± 6.7
NADH-Q_1	77.9 ± 6.9	87.0 ± 1.1

* All assays were performed as described in Materials and Methods in the absence and presence of HIDC or HITC. Activities with SMP and *P. denitrificans* membrane vesicles contained 3.3 μM antimycin A and 10 mM KCN. Assays contained either 0.01 mg of complex I, 0.05 mg of SMP or 0.1 mg of *P. denitrificans* membrane vesicle protein. Assays were performed in triplicate; values are means ± SEM. Control specific activities (units/mg protein) for SMP were: NADH-ferricyanide, 11.45; NADH-menadione, 0.33; NADH-juglone, 0.60; NADH-duroquinone, 0.54; and NADH-Q_1, 0.88. Control specific activities (Units/mg protein) for complex I were; NADH-ferricyanide, 62.14; NADH-menadione, 0.54; NADH-juglone, 1.10; NADH-duroquinone, 0.51; and NADH-Q_1, 0.47. Control specific activities (units/mg protein) for *P. denitrificans* membrane vesicles were: NADH-ferricyanide, 4.10; NADH-menadione, 0.44; NADH-juglone, 2.40; NADH-duroquinone, 0.37; and NADH-Q_1, 0.83.

Table 2. Effect of 7 μM HIDC or HITC on NADH-ubiquinone reductase activities of rotenone-insensitive complex I and *P. denitrificans* membrane vesicles

Activity*	Inhibition (% control activity) HIDC	HITC
(A) Rotenone-insensitive complex I		
NADH-menadione	108.4 ± 0.0	101.8 ± 3.3
NADH-duroquinone	108.7 ± 7.0	111.7 ± 4.5
NADH-Q_1	100.3 ± 7.5	111.7 ± 4.0
(B) Rotenone-insensitive *P. denitrificans* membrane vesicles		
NADH-menadione	95.8 ± 4.6	93.8 ± 2.0
NADH-juglone	ND	103.7 ± 7.3
NADH-duroquinone	87.3 ± 3.5	141.5 ± 11.0
NADH-Q_1	86.0 ± 9.5	111.5 ± 9.3

* All assays were performed as described in Materials and Methods in the absence and presence of HIDC or HITC. Assays with rotenone-insensitive *P. denitrificans* membrane vesicles contained 3.3 μM antimycin A and 10 mM KCN. Assays contained either 0.01 mg of rotenone-insensitive complex I or 0.2 mg of rotenone-insensitive *P. denitrificans* membrane vesicle protein. Assays were performed in triplicate; values are means ± SEM. Control specific activities (units/mg protein) for rotenone-insensitive complex I were: NADH-menadione, 1.19; NADH-duroquinone, 1.15; and NADH-Q_1, 0.60. Control specific activities (units/mg protein) for rotenone-insensitive *P. denitrificans* membrane vesicles were: NADH-menadione, 0.07; NADH-juglone, 0.49; NADH-duroquinone 0.02; and NADH-Q_1 0.05. ND = not determined.

complicated. The slight inhibition of menadione reduction was little affected by removal of the rotenone site. Inhibition of juglone reduction by HITC was eliminated by removal of the rotenone site. Reduction of duroquinone and coenzyme Q_1 was inhibited slightly by HIDC and was not inhibited by HITC. Thus, with *P. denitrificans*, HITC appears to have a rotenone-type mechanism, but HIDC appears to have more than one site of action or binding site.

DISCUSSION

NADH-ubiquinone reductase activity is known to be inhibited by a number of substances [8–18, 49–53]. Rotenone and piericidin A are the most potent inhibitors found to date, with I_{50} values in the picomolar to nanomolar range [24–26]. The I_{50} of dequalinium chloride is 11 μM with the mammalian enzyme and 40 μM with the *P. denitrificans* enzyme [15, 18]. MPP^+ [8–10] and capsaicin [49] inhibit the mammalian enzyme in the millimolar range. The micromolar I_{50} values of both HIDC and HITC with both the mammalian and *P. denitrificans* systems demonstrate that they are rather potent inhibitors of electron transport.

Most inhibitors of the mammalian NADH-ubiquinone reductase act either at the rotenone site, as does MPP^+ [54], or as competitive inhibitors with respect to coenzyme Q, such as capsaicin [49]. HIDC and HITC appear to act on the rotenone site in the mammalian system, although one cannot rule out completely competitive inhibition with respect to coenzyme Q. This possibility is less likely however, since the degree of inhibition of reduction of coenzyme Q differed between complex I and SMP and a 3-fold increase in coenzyme Q concentration in the complex I assay (from 50 to 150 μM) only resulted in a 14% decrease in inhibition with either carbocyanine dye. Clarification of this problem will require rigorous kinetic analysis.

The large difference in inhibition of coenzyme Q reduction between complex I and SMP may indicate that dye interactions with the membrane must contribute to the inhibition. In this respect, it is interesting to speculate about the mechanism of inhibition of menadione reduction, particularly with SMP as the source of NADH-ubiquinone reductase. Since menadione reduction is not subject to rotenone inhibition, why would these two laser dyes, presumably acting through the rotenone site, have a slight inhibitory effect on menadione reduction? We have shown previously that rotenone binding to NADH-ubiquinone reductase induced a conformational change as detected by the cross-linking reagent dithiobis(succinimidylpropionate) [55]. One could envision that binding of either HIDC or HITC to the rotenone site induces a conformational change in the enzyme that affects menadione reduction. This does not mean, however, that rotenone induces the same conformational change that could result from the binding of HIDC or HITC.

It is interesting that in both electron transport chains, HIDC was a better inhibitor than HITC. Since the only difference in the two compounds is the spacing between the two ring systems, this spacing and/or the number of ethylene groups must be an important factor in the inhibitory capacity of the compound. Spacing between two ring systems has been found to be important with other compounds affecting the mammalian and *P. denitrificans* NADH-ubiquinone reductase, such as the quinolinium compounds [15, 18]. In the case of the quinoliniums, the longer the spacer bridge group, the more potent the inhibitor. It appears with these two laser cyanine dyes, however, that shorter distances between the two ring systems leads to more effective inhibition. We are currently investigating this hypothesis with other cyanine dyes.

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