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CARBOCYANINE DYES WITH LONG ALKYL SIDE-CHAINS: BROAD SPECTRUM INHIBITORS OF MITOCHONDRIAL ELECTRON TRANSPORT CHAIN ACTIVITY

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Abstract—Certain indocarbocyanine, thiacarbocyanine, and oxacarbocyanine dyes possessing short alkyl side-chains (one to five carbons) are potent inhibitors of mammalian mitochondrial NADH-ubiquinone reductase (EC 1.6.99.3) activity (Anderson *et al.*, *Biochem Pharmacol* **41**: 677–684, 1991; Anderson *et al.*, *Biochem Pharmacol* **45**: 691–696, 1993; Anderson *et al.*, *Biochem Pharmacol* **45**: 2115–2122, 1993), and act similarly to rotenone. This study examines the inhibitory capacities of twelve other carbocyanine dyes (six indocarbocyanines, four oxacarbocyanines, and two thiacarbocyanines) possessing long alkyl side-chains (seven to eighteen carbons with both saturated and unsaturated side-chains) on mitochondrial NADH, succinate and cytochrome *c* oxidase activities. Three of the indocarbocyanines inhibited electron transport chain activity, while three were non-inhibitory. Two of the oxacarbocyanines also inhibited electron transport chain activity, while the other two were without effect. Both the thiacarbocyanines were non-inhibitory. In contrast to previous studies, the long alkyl side-chain carbocyanines exhibited a broad spectrum of inhibition of respiratory chain activity, affecting either oxidation of all three substrates or of NADH and cytochrome *c*, rather than specific inhibition of mitochondrial NADH-ubiquinone reductase activity, indicating that there could be multiple binding sites for these compounds. The five inhibitory long side-chain carbocyanines also inhibited reduction of ferricyanide and coenzyme Q_1 by NADH, using submitochondrial particles, but not when tested with purified complex I, indicating that the mitochondrial inner membrane was an integral component in their inhibitory capacity. No general correlation of side-chain length or degree of unsaturation and inhibitory capacity was discernible.

Key words: carbocyanine; inhibition; electron transport chain; mitochondria

There has been much interest recently in the inhibitory characteristics of various compounds on mammalian mitochondrial electron transport chain activity. A number of new inhibitors have been described that affect the NADH-ubiquinone reductase portion of the electron transport chain [1–10], as well as new interest in the classical inhibitory compounds [11–13]. Several inhibitors have been implicated in human disease processes [14–19]. We have reported that carbocyanine derivatives specifically inhibit mitochondrial NADH-ubiquinone reductase activity in a fashion similar to rotenone [20–22] with no inhibitory effect on succinate or cytochrome *c* oxidation. In the previous studies, the inhibitory compounds possessed alkyl side-chains ranging from one to five carbons, except for the thiacarbocyanines, where inhibition was negligible with compounds containing side-chains longer than two carbons.

In this study we examined twelve carbocyanine dyes with saturated and unsaturated alkyl side-chains ranging from seven to eighteen carbons, for their ability to inhibit oxidation of NADH, succinate, or reduced cytochrome *c* by the mammalian mitochondrial electron transport chain. Those demonstrating inhibition of NADH oxidation were tested further for their ability to inhibit NADH reduction of an artificial electron acceptor and coenzyme Q with both SMP† and purified NADH-ubiquinone reductase (complex I).

MATERIALS AND METHODS

Carbocyanines DiIC12(3), DiIC16(3), DiIC18(3),

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† Abbreviations: SMP, submitochondrial particles; DiIC12(3), 1,1′-didodecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; DiIC16(3), 1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; DiIC18(3), 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo- carbocyanine perchlorate; DiIC18(5), 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate; Δ^9-DiI, 1,1′-dioleyl-3,3,3′,3′-tetramethylindocarbocyanine methanesulfonate; DiI$\Delta^{9,12}$-C18(3), 1,1′-dilinoleyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; DiOC7(3), 3,3′-diheptyloxacarbocyanine perchlorate; DiOC16(3), 3,3′-dihexadecyloxacarbocyanine perchlorate; DiOC18(3), 3,3′-dioctadecyloxacarbocyanine perchlorate; DiO$\Delta^{9,12}$-C18(3), 3,3′-dilinoleyloxacarbocyanine perchlorate; DiSC16(3), 3,3′-dihexadecylthiacarbocyanine perchlorate; and DiSC18(3), 3,3′-dioctadecylthiacarbocyanine perchlorate.

DiIC18(5), Δ^9-DiI, DiI$\Delta^{9,12}$-C18(3), DiOC7(3), DiOC16(3), DiOC18(3), DiO$\Delta^{9,12}$-C18(3), DiSC16(3), and DiSC18(3) were purchased from Molecular Probes, Inc. (Eugene, OR). The purity of all the dyes was judged as 95% or greater by HPLC. NADH, potassium ferricyanide, antimycin A, bovine serum albumin (crystallized and lyophilized), cytochrome *c*, sodium ascorbate and DMSO were purchased from the Sigma Chemical Co. (St. Louis, MO). Coenzyme Q_1 was a gift of the Eisai Co. (Tokyo, Japan). DiO$\Delta^{9,12}$-C18(3), DiI$\Delta^{9,12}$-C18(3), and Δ^9-DiI were dissolved in methanol, whereas the other compounds were dissolved in DMSO. All solutions were protected from light and could be stored at −20° for periods of up to 1 week.

Preparation of mitochondria, submitochondrial particles and complex I. Mitochondria were prepared from fresh bovine hearts, obtained from a local slaughterhouse, by the method of Hatefi *et al.* [23]. Non-phosphorylating SMP were prepared from either fresh or frozen mitochondria as described by Löw and Vallin [24]. Binary complex I-III (NADH-cytochrome *c* reductase) was prepared from mitochondria according to the procedure of Hatefi *et al.* [25], and complex I was purified from this preparation by the method of Hatefi *et al.* [23, 25].

Assays. NADH oxidase and succinate oxidase activities of submitochondrial particles were determined as described previously [20]. Cytochrome oxidase (EC 1.9.3.1) activity was determined spectrophotometrically following a decrease in absorbance at 550 nm at room temperature using reduced cytochrome *c* as a substrate. The assay contained, in 1 mL, 120 mM sodium phosphate (pH 8.0), 0.5 mg of reduced cytochrome *c*, 3.3 μM antimycin A, and 0.05 mg of SMP protein. Enzymatic activities of SMP and complex I using the artificial electron acceptor ferricyanide (final concentration 1 mM) were determined by the method of Galante and Hatefi [26]. NADH-coenzyme Q_1 reductase activity (final concentration 50 μM coenzyme Q_1) was determined by the method of Hatefi *et al.* [25] Antimycin A (final concentration 3.3 μM) and KCN (final concentration 10 mM) were added for the determination of reduction of ferricyanide and coenzyme Q_1 when SMP was 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° (NADH oxidase) to 1 μmol of reduced cytochrome *c*/min at 25° (cytochrome oxidase). For the oxygen electrode assays (succinate oxidase), a unit of activity is defined as the amount of enzyme that reduces 1 ng atom of oxygen/min at 30°. Cytochrome *c* was reduced by treatment with sodium ascorbate, followed by dialysis against 180 nM sodium phosphate (pH 8.0). Protein concentration was determined using the biuret method [27] with bovine serum albumin as a standard.

RESULTS

Effects of long alkyl side-chain carbocyanine dyes on NADH, succinate and cytochrome oxidase activities. The structures of the carbocyanine dyes used in this study are shown in Fig. 1. Δ^9-DiI (Fig. 2) inhibited all three oxidase activities: NADH oxidase, succinate oxidase, and cytochrome oxidase. The IC_{50} for NADH oxidase was 20 μM, 30 μM for succinate oxidase, and 20 μM for cytochrome oxidase. The rise in NADH oxidase activity between 40 and 80 μM Δ^9-DiI, followed by a steep drop between 90 and 100 μM Δ^9-DiI is unexplainable at present. Tests of other concentrations within this range confirmed that this was indeed a real rise and fall. This finding suggests that Δ^9-DiI has different effects upon NADH oxidase activity between 40 and 80 μM than it does at lower or higher concentrations.

DiIC18(3) (Fig. 3) inhibited cytochrome oxidase with an IC_{50} of 17 μM. NADH oxidase was inhibited moderately, but maximum inhibition was not much more than 50% over the range of concentrations of DiIC18(3) used in this study. Succinate oxidase was not inhibited at any concentration of DiIC18(3) tested.

DiIC18(5) (Fig. 4) inhibited both NADH oxidase and cytochrome oxidase activities. The IC_{50} for cytochrome oxidase was 60 μM, while the inhibition pattern for NADH oxidase had a definite biphasic characteristic, with inhibition up to 60 μM followed by reactivation almost to control activity. Succinate oxidase activity was unaffected up to 80 μM DiIC18(5), followed by dramatic activation above this concentration of DiIC18(5).

The other three indocarbocyanines, DiIC12(3), DiIC16(3), and DiI$\Delta^{9,12}$-C18(3), did not inhibit any of the three electron transport chain activities at any concentration tested (data not shown). No further examination was done with these three compounds.

DiO$\Delta^{9,12}$-C18(3) (Fig. 5) inhibited NADH oxidase with an IC_{50} of 38 μM, moderately inhibited cytochrome oxidase, and slightly inhibited succinate oxidase activity at higher (80–100 μM) concentrations. Both NADH oxidase and cytochrome oxidase appeared to be inhibited in a biphasic manner.

DiOC7(3) (Fig. 6) inhibited all three oxidase activities. Both NADH oxidase and cytochrome oxidase activities were abolished completely between 40 and 60 μM DiOC7(3), while succinate oxidase activity was about 15% at 60 μM DiOC7(3). The IC_{50} for NADH oxidase was 12 μM, 13 μM for cytochrome oxidase, and 25 μM for succinate oxidase activity. The other two oxacarbocyanines tested, DiOC16(3) and DiOC18(3), were non-inhibitory to all three electron transport chain activities (data not shown). The two thiacarbocyanines tested were also non-inhibitory to all three electron transport chain activities (data not shown). Table 1 summarizes the inhibitory efficiencies of the five long alkyl side-chain carbocyanine dyes that affected electron transport chain activity.

Effects of the five inhibitory carbocyanines on NADH-ubiquinone reductase activities. To determine if the five inhibitory carbocyanines might have multiple sites of inhibition, these compounds were tested for their effects on reduction of ferricyanide and coenzyme Q_1 by the NADH-ubiquinone reductase portion of the mitochondrial electron transport chain, using both SMP and purified

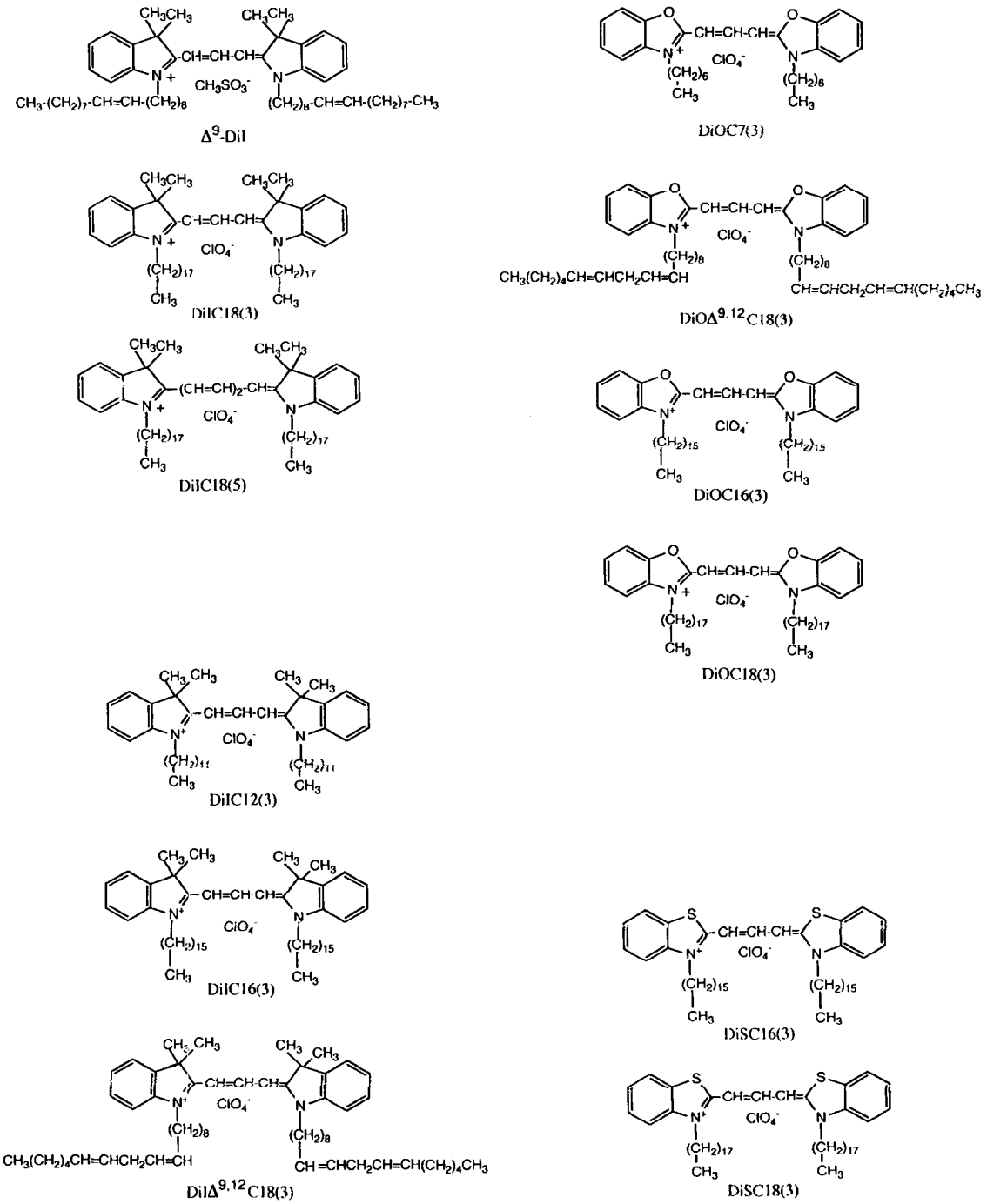

Fig. 1. Structures of the long alkyl side-chain carbocyanine dyes.

complex I as the source of the enzyme. A concentration of carbocyanine yielding ~50% inhibition of NADH oxidase was used in this study. All five carbocyanines inhibited both the reduction of ferricyanide and coenzyme Q_1 when SMP was used as the enzyme source. Inhibition ranged from 39% of control activity (control equals 100%) for DiIC18(3) to 48.3% of control activity for Δ^9-DiI. Reduction of coenzyme Q_1 was also inhibited by all five carbocyanines. Inhibition ranged from 30% for DiIC18(3) to 37.3% of control activity with DiOC7(3). Using purified complex I as the source

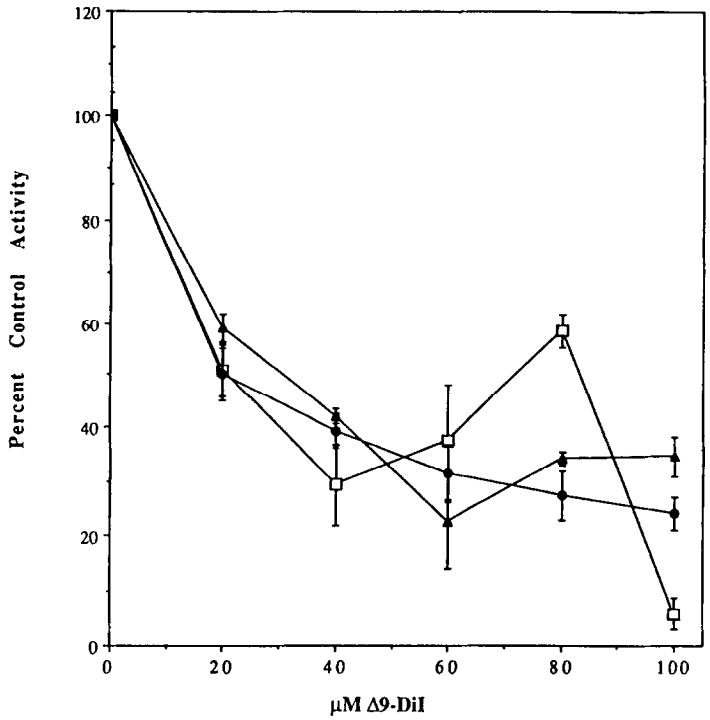

Fig. 2. Effect of increasing concentrations of Δ^9-DiI on mitochondrial NADH, succinate, and cytochrome oxidase activities. Assays were performed in triplicate, and values are means ± SEM. Assays for NADH oxidase (1 mL) contained 0.05 mg of SMP protein. Assays for succinate or cytochrome oxidase (3 mL) contained 0.05 mg/mL of SMP protein. Key: (□) NADH oxidase; (▲) succinate oxidase; and (●) cytochrome oxidase. Control specific activities (U/mg protein) for NADH oxidase were 1561.7; for succinate oxidase, 507.9; and for cytochrome oxidase, 523.6.

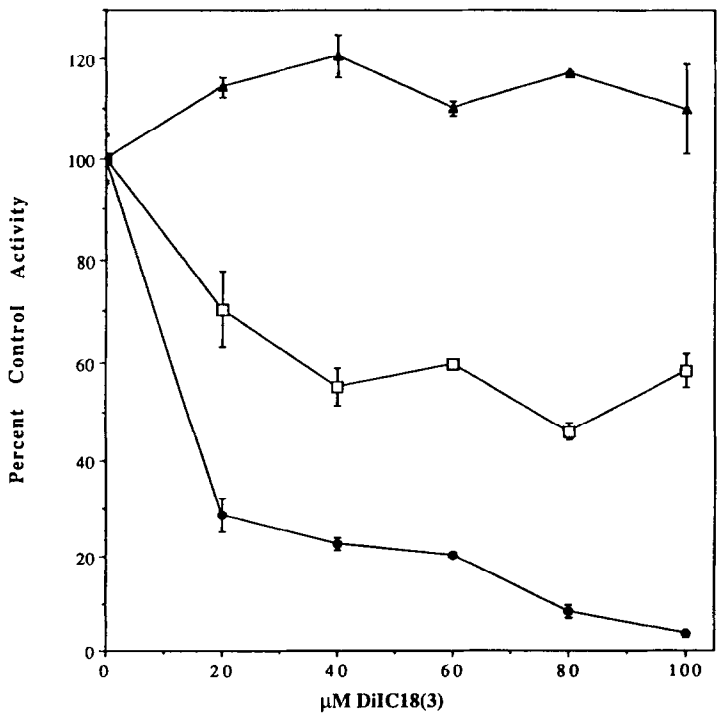

Fig. 3. Effect of increasing concentrations of DiIC18(3) on mitochondrial NADH, succinate, and cytochrome oxidase activities. For details of assay conditions and control specific activities, see the legend of Fig. 2. Key: (□) NADH oxidase; (▲) succinate oxidase; and (●) cytochrome oxidase. Assays were performed in triplicate, and values are means ± SEM.

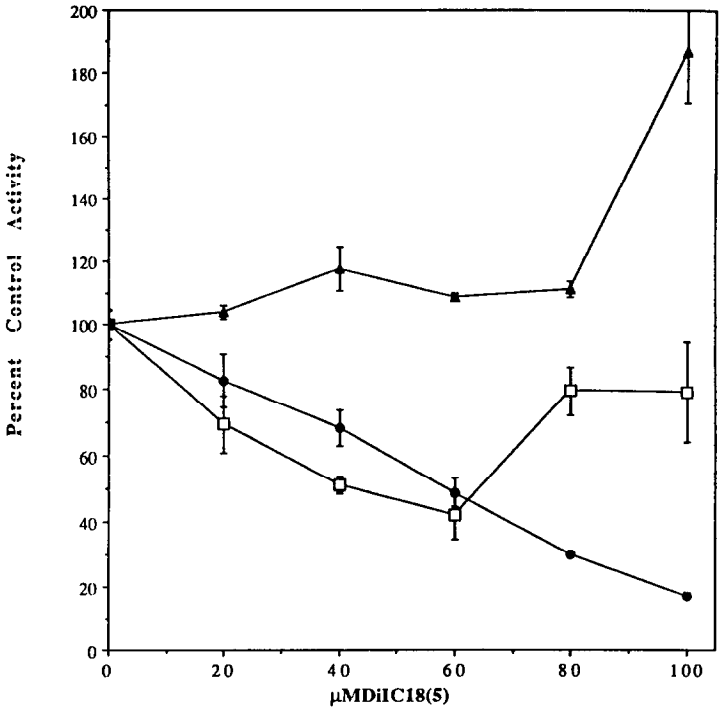

Fig. 4. Effect of increasing concentrations of DiIC18(5) on mitochondrial NADH, succinate, and cytochrome oxidase activities. For details of assay conditions and control specific activities, see the legend of Fig. 2. Key: (□) NADH oxidase; (▲) succinate oxidase; and (●) cytochrome oxidase. Assays were performed in triplicate, and values are means ± SEM.

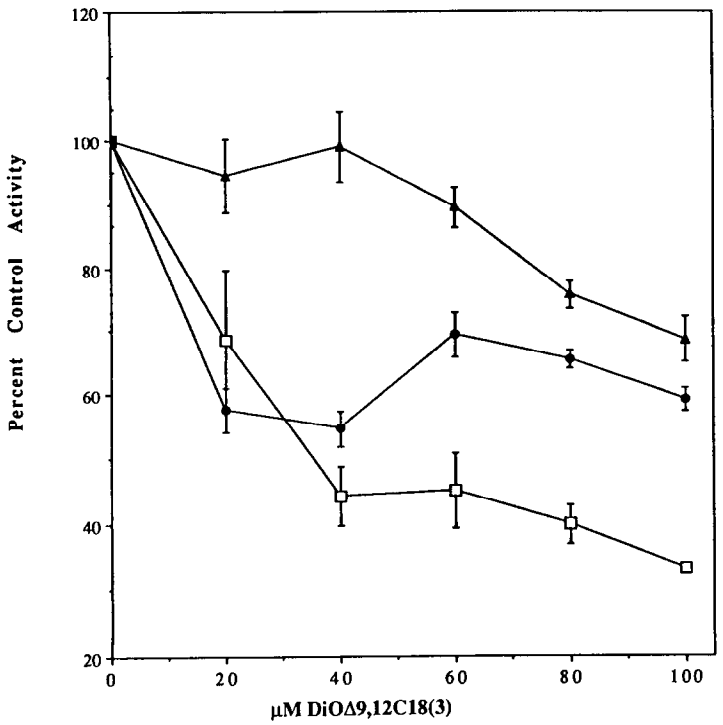

Fig. 5. Effect of increasing concentrations of DiO$\Delta^{9,12}$-C18(3) on mitochondrial NADH, succinate, and cytochrome oxidase activities. For details of assay conditions and control specific activities, see the legend of Fig. 2. Key: (□) NADH oxidase; (▲) succinate oxidase; and (●) cytochrome oxidase. Assays were performed in triplicate, and values are means ± SEM.

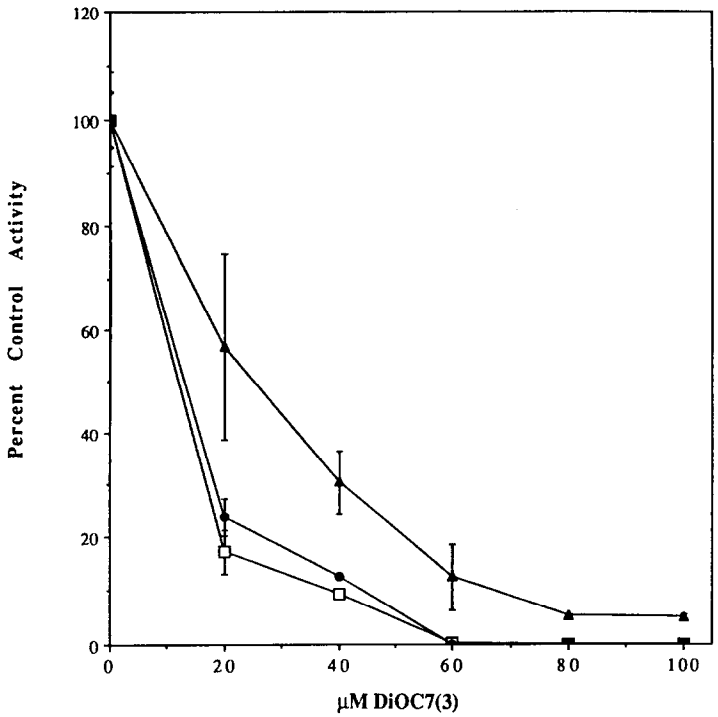

Fig. 6. Effect of increasing concentrations of DiOC7(3) on mitochondrial NADH, succinate, and cytochrome oxidase activities. For details of assay conditions and control specific activities, see the legend of Fig. 2. Key: (□) NADH oxidase; (▲) succinate oxidase; and (●) cytochrome oxidase. Assays were performed in triplicate, and values are means ± SEM.

Table 1. Inhibitory efficiency of long alkyl side-chain carbocyanine dyes

	Inhibitory efficiency* (IC_{50}, μM)		
Dye	NADH oxidase	Succinate oxidase	Cytochrome oxidase
Δ^9-DiI	20	30	20
DiIC18(3)	Moderate inhibition	No inhibition	17
DiIC18(5)	Biphasic pattern	No inhibition	60
DiO$\Delta^{9,12}$C18(3)	38	No inhibition	Moderate inhibition
DiOC7(3)	12	25	13

* DiIC12(3), DiIC16(3), DiI$\Delta^{9,12}$-C18(3), DiOC16(3), DiOC18(3), DiSC16(3), and DiSC18(3) were non-inhibitory to any of the three oxidase activities at any concentration tested.

of the enzymatic activity, none of the five carbocyanines significantly inhibited reduction of either ferricyanide or coenzyme Q_1. These results indicate that (1) there appear to be multiple inhibitory sites for these five long aliphatic side-chain carbocyanines within the mitochondrial electron transport chain, but (2) the membrane environment must play a necessary role in these binding sites.

DISCUSSION

Carbocyanine dyes with long alkyl side-chains have been used in a wide variety of studies in recent years. The two most commonly used compounds are DiIC18(3), also referred to as DiI, and DiOC18(3), also referred to as DiO. DiIC18(3) has been used for staining neurons [28–30] and in the study of neuronal development [31, 32]. Both DiIC18(3) and DiOC18(3) have been used to study membranous structure in general [33] and endoplasmic reticulum membranes in particular in living cells [34], while DiIC16(3) has been used to study membrane fusion induced by an electric field [35]. DiIC18(3) has been an important tool in the investigation of proliferation of smooth muscle cells expressing retrovirally

introduced human genes after carotid artery injury [36] and in examining muscle regeneration in general after injury [37]. Malarial parasite invasion of erythrocytes has been studied using DiIC16(3) [38], and DiIC18(3)-labeled low-density lipoprotein (LDL) was used to elucidate LDL binding and subsequent metabolism by the human parasite *Schistosoma mansoni* [39]. DiIC18(3) has also been used to detect tumor-specific cytotoxic T lymphocyte clones [40]. Chazotte *et al.* [41–43] used low concentrations of both DiIC16(3) and DiIC18(3) and a technique termed fluorescence recovery after photobleaching (FRAP) to determine the lateral diffusion of redox components in the mitochondrial inner membrane in support of the random collision model of mitochondrial electron transport.

Five of the six indocarbocyanines examined in this study have a bridging group of three carbons, while DiIC18(5) has a five-carbon bridging group. Alkyl side-chains range from twelve carbons [DiIC12(3)] to eighteen carbons [DiI$\Delta^{9,12}$-C18(3), Δ^{9}-DiI, DiIC18(3) and DiIC18(5)]. Two of the indocarbocyanines have unsaturated alkyl side-chains, Δ^{9}-DiI with one double bond and DiI$\Delta^{9,12}$-C18(3) with two double bonds. All of the four oxacarbocyanines examined have a three-carbon bridging group with alkyl side-chains ranging from seven carbons [DiOC7(3)] to eighteen carbons [DiOC18(3)]. DiO$\Delta^{9,12}$-C18(3) contains alkyl side-chains with two double bonds. The two thiacarbocyanines studied have a three-carbon bridging group, and saturated alkyl side-chains ranging from sixteen carbons [DiSC16(3)] to eighteen carbons [DiSC18(3)].

Our previous studies with indocarbocyanines [20], thiacarbocyanines [21], and oxacarbocyanines [22], all possessing relatively short alkyl side-chains, revealed that these compounds preferentially inhibit the NADH-ubiquinone reductase portion of the mitochondrial electron transport chain. The length of the side-chain did not play a role in the indocarbocyanine inhibition study, since both compounds, 1,1′,3,3,3′,3′-hexamethylindodicarbocyanine iodide (HIDC) and 1,1′,3,3,3′,3′-hexamethylindotricarbocyanine iodide (HITC), contain methyl side-chains. Several of the thiacarbocyanines with side-chains longer than two carbons were non-inhibitory, but with the oxacarbocyanines, the longer side-chains, DiOC5(3) and DiOC6(3), were the most potent inhibitors of NADH-ubiquinone reductase activity.

In the present study, lengthening the side-chain beyond six carbons had some interesting effects upon its inhibitory capacity for mitochondrial electron transport chain activities. Unlike the shorter side-chain carbocyanines, these longer side-chain compounds affected NADH oxidase, succinate oxidase, and cytochrome oxidase activities [Δ^{9}-DiI, DiOC7(3), and to some extent DiO$\Delta^{9,12}$-C18(3)], or inhibited both NADH oxidase and cytochrome oxidase activities. The length of the side-chain did not appear to play any significant role in the degree or inhibition or type of activities affected. For example, an intermediate length side-chain and a long chain oxacarbocyanine, DiOC7(3) and DiO$\Delta^{9,12}$-C18(3), respectively, were inhibitory, whereas two long-chain oxacarbocyanines, DiOC16(3) and DiOC18(3), were not inhibitory. The same held true for the indocarbocyanines, with three long-chain compounds, Δ^{9}-DiI, DiIC18(3), and DiIC18(5), all with eighteen carbon side-chains, being inhibitory, whereas a twelve-, sixteen- and eighteen-carbon indocarbocyanine [DiIC12(3), DiIC16(3), and DiI$\Delta^{9,12}$-C18(3)] demonstrated no inhibitory effect on mitochondrial electron transport chain activities. The one exception was the thiacarbocyanines. Previously, we demonstrated that thiacarbocyanines with side-chains longer than two carbons were non-inhibitory to mitochondrial respiratory chain activities [21]. This appears to hold true, since neither DiSC16(3) nor DiSC18(3) showed any effect on NADH oxidase, succinate oxidase, or cytochrome oxidase activities in this study. Two of the three carbocyanines containing unsaturated side-chains, Δ^{9}-DiI and DiO$\Delta^{9,12}$-C18(3), were inhibitory, whereas DiI$\Delta^{9,12}$-C18(3) was without effect. Thus, there is no general correlation of either length of side-chain or degree of unsaturation with mitochondrial electron transport chain inhibitory capacity.

Previous studies with all three classes of carbocyanine dyes [20–22] indicated that the membrane environment of the electron transport chain played an important role on the inhibitory capacity of these dyes. The present study indicates that with respect to the activities of NADH-ubiquinone reductase, as determined using both SMP and isolated complex I, significant inhibition is observed only when the enzyme is in its native membrane environment. Thus, with the long side-chain carbocyanines, the membrane provides a necessary component for inhibition. This is supported by studies on the partitioning of carbocyanine with long alkyl chains into membranes. Axelrod [44] observed that DiIC18(3) partitioned into the red cell membrane with the chromophore parallel to the surface of the cell and the alkyl side-chain in the bilayer parallel to the phospholipid alkyl chains. Spink *et al.* [45] studied partitioning of carbocyanine with alkyl chains ranging from eight to twenty-two carbons in model membranes and concluded that compounds with alkyl side-chains from eight to twelve carbons partitioned in the fluid phase, while compounds with alkyl side-chains of twenty and greater partitioned in the gel phase. Since all the dyes used in this study, with the exception of DiOC7(3), fall into the range of eight to twenty-two carbons, it is logical to assume that they would all (a) have the same relative hydrophobicity and (b) partition into the fluid phase of the inner mitochondrial membrane. However, the inhibitory long alkyl side-chain carbocyanines appear to have a specific rather than a general membrane perturbation effect on mitochondrial electron transport. This was especially evident for DiIC18(3) (see Fig. 3) which dramatically inhibited cytochrome oxidase, moderately inhibited NADH oxidase, and had no effect on succinate oxidase activity. Also, only five of the twelve long alkyl side-chain carbocyanines tested were inhibitory, although the seven non-inhibitory carbocyanines must partition

into the fluid phase of the mitochondrial inner membrane.

There appears to be only one report on the toxicity of carbocyanines with long alkyl side-chains. St. John [46], studying the effects of DiIC18(3) on embryonic rat motorneurons, found that this compound was toxic to these neurons, whereas DiIC12(3), which stained the neurons to the same extent, appeared to be non-toxic. Our present findings support this conclusion, since DiIC18(3) was found to inhibit cytochrome oxidase with an IC_{50} of 20 μM, exhibited moderate inhibition on NADH oxidase, and was the most potent of the five carbocyanine inhibitors of NADH reduction of coenzyme Q_1 and ferricyanide. DiIC12(3), however, was non-inhibitory to the three oxidase activities, in our hands. Should this correlation hold true, our results would suggest that DiIC18(3), Δ^9-DiI, DiIC18(5), DiOC7(3), and DiO$\Delta^{9,12}$-C18(3) would be cytotoxic, whereas DiIC12(3), DiIC16(3), DiI$\Delta^{9,12}$-C18(3), DiOC16(3), DiOC18(3), DiSC16(3), and DiSC18(3) would be non-cytotoxic. Cytotoxicity was determined in our study of the thiacarbocyanines [21] where we observed that for compounds with short bridging groups the cytotoxicity to tumor cells decreased with increased length of the alkyl side-chain. Further studies will be necessary to clarify this point with respect to carbocyanines with long alkyl side-chains.

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