Biochemical Pharmacology, Vol. 45, No. 3, pp. 691–696, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

CYTOTOXIC EFFECT OF THIACARBOCYANINE DYES ON HUMAN COLON CARCINOMA CELLS AND INHIBITION OF BOVINE HEART MITOCHONDRIAL NADH–UBIQUINONE REDUCTASE ACTIVITY VIA A ROTENONE-TYPE MECHANISM BY TWO OF THE DYES

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(*Received* 18 *July* 1992; *accepted* 20 *October* 1992)

Abstract—Five lipophilic-cationic thiacarbocyanine compounds differing in the side chains (methyl-S13, ethyl-S23, propyl-S33, butyl-S43, and pentyl-S53) and a related thiadicarbocyanine compound with ethyl side chains (S25) exhibited a selective cytotoxic effect on human colon carcinoma cells compared to green monkey kidney epithelial cells. The inhibitory concentration for 50% inhibition of growth (IC_{50}) for the carcinoma cells ranged from 13 nM for S13 and S23 to 160 nM for S25. The carcinoma cells were 4- to 100-fold more sensitive than the normal cells. Two of the five compounds, S13 and S23, selectively inhibited NADH oxidase activity with bovine heart submitochondrial particles (SMP). There was no discernable inhibitory effect by the other three thiacarbocyanine compounds on electron transport chain activity. The primary site of inhibition within the respiratory chain for S13 and S23 appeared to be the NADH to coenzyme Q portion of the mitochondrial electron transport chain. Artificial electron acceptors for this segment of respiratory chain were used to localize the inhibitory site. Using SMP, both S13 and S23 inhibited reduction of menadione, duroquinone, and coenzyme Q. Using purified complex I (NADH–ubiquinone reductase) (EC 1.6.99.3), S13 slightly inhibited reduction of juglone, duroquinone, and coenzyme Q, whereas S23 had no effect on any of the substrates. When rotenone-saturated SMP were used, the inhibitory effects of S13, but not S23, on the reduction of menadione were abolished, as was the inhibitory effect of S13 on coenzyme Q reduction when rotenone-insensitive complex I was used as the source of the enzyme. These results suggest that (1) S13 and S23 inhibition of NADH–ubiquinone reductase activity is enhanced by the membrane environment of the enzyme, and (2) the inhibition appears to be in part akin to the inhibiting mode of rotenone.

Carcinoma cells are known to accumulate and retain lipophilic cationic compounds such as rhodamine 123 [1–3] and dequalinium chloride [4]. These compounds localize to the mitochondria of the cell, due to the mitochondrial membrane potential [2, 4]. At high doses, rhodamine 123 is toxic to carcinoma cells both *in vitro* [5] and *in vivo* [6]. Dequalinium, at low doses, exhibits a high degree of anticarcinoma activity *in vivo* and *in vitro* [4]. This compound has been used for a number of years as an antimicrobial agent in several commercial products, and its cytotoxicity is due, at least in part, to inhibition of electron transport activity. One of the inhibitory effects of dequalinium has been localized to the NADH–ubiquinone reductase portion of the electron transport chain of both the mammalian mitochondria and *Paracoccus denitrificans* [7, 8], a bacterium with an electron transport chain possessing many characteristics of the mitochondrial respiratory chain. In both cases, this lipophilic cationic compound appears to exert its inhibitory effect through a non-rotenone type of mechanism. Two other lipophilic–cationic compounds, both indocarbocyanines, have also been shown to inhibit markedly both mitochondrial and *P. denitrificans* NADH–ubiquinone reductase activity at the rotenone binding site [9]. The cytotoxicity and anticarcinoma activity of these indocarbocyanine compounds have yet to be evaluated completely.

In this study we investigated the cytotoxic effects of a series of structurally related thiacarbocyanines on normal epithelial and carcinoma cells, as well as their inhibitory effects on mitochondrial electron transport. Evidence is presented that these thiacarbocyanines are more cytotoxic to carcinoma cells *in vitro* than to normal cells and that using bovine heart mitochondria as a model system, two of the compounds inhibit mitochondrial electron transport primarily between NADH and ubiquinone. The results indicate that this inhibition is due in part to an interaction of the thiacarbocyanines with NADH–ubiquinone reductase similar to that of rotenone.

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MATERIALS AND METHODS

Materials. Thiacarbocyanine compounds were

obtained from Eastman Kodak, Rochester, NY. Stock solutions of the thiacarbocyanines of either 1 or 10 mM were prepared in dimethyl sulfoxide (DMSO*), protected from light, and could be stored at −20° for several weeks. 3-Acetylpyridine adenine dinucleotide ($AcPyAD^+$), antimycin A, sodium ascorbate, bovine serum albumin, duroquinone, juglone, menadione, NADH, rotenone, and *N, N, N', N'* - tetramethyl - *p* - phenylenediamine (TMPD) were purchased from the Sigma Chemical Co., St. Louis, MO. Coenzyme Q_1 was a gift of the Eisai Co., Tokyo, Japan. All other chemicals were of reagent grade quality.

Cell lines and reagents. All cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) calf serum in 5% CO_2/95% O_2 and 100% humidity. CV-1, an African green monkey kidney epithelial cell line, was purchased from the American Type Culture Collection. CX-1, a human colon carcinoma cell line, was obtained from S. D. Bernal (Harvard Medical School).

Clonogenic assays. About 300 cells were seeded in 60-mm dishes and incubated overnight. The following day, unattached cells were washed off and fresh medium was added. The thiacarbocyanine compound was added to the growth medium at various concentrations. After a 3-hr incubation, the thiacarbocyanine compound was removed by washing the cells and adding drug-free medium. Colony-forming units were determined ~2 weeks later by staining with 0.2% methylene blue. Controls consisted of the two cell lines treated in the same manner without the addition of a thiacarbocyanine compound. The plating efficiency was 80% for CV-1 and 50% for CX-1.

Preparation of mitochondria and submitochondrial particles. Mitochondria were prepared from fresh bovine hearts as described by Hatefi *et al.* [10], and submitochondrial particles were prepared by the procedure of Löw and Vallin [11]. Complex I–III was prepared according to the method of Hatefi *et al.* [12], and complex I (NADH–ubiquinone reductase) was further purified from this binary complex by the procedure of Hatefi *et al.* [10, 12]. Rotenone-insensitive complex I was prepared by treatment with chymotrypsin as described by Crowder and Ragan [13].

Enzymatic assays. NADH oxidase activity of submitochondrial particles (SMP) was determined spectrophotometrically at room temperature in 120 mM sodium phosphate, pH 8.0. Succinate oxidase activity was determined with an oxygen electrode (Yellow Springs Instrument Co.) at 30° in the presence of 10 mM sodium succinate in the above sodium phosphate buffer. With the preparations used in this study, there was negligible increase in succinate oxidase activity of SMP when preincubated with 50 mM succinate at 30°, so this step was omitted. Cytochrome oxidase (EC 1.9.3.1) activity was also determined with an oxygen electrode at 30° in the presence of the above buffer containing 5 mM sodium ascorbate, and 0.1 mM TMPD and 3.3 μM antimycin A. Enzymatic activities using the artificial electron acceptors $AcPyAD^+$ and menadione were determined spectrophotometrically as described by Galante and Hatefi [14] in the above buffer at 25°. Reduction of the artificial electron acceptors juglone and duroquinone were determined as described by Ruzicka and Crane [15, 16]. Reduction of coenzyme Q_1 was measured by the method of Hatefi *et al.* [12]. Specific activities for spectrophotometric assays are in $\mu mol \cdot min^{-1} \cdot mg^{-1}$ and for oxygen electrode assays are in ng atom oxygen $\cdot min^{-1} \cdot mg^{-1}$. Protein concentration was estimated by the biuret method [17] using bovine serum albumin as a standard.

RESULTS

Cytotoxic effects of thiacarbocyanine compounds. The following lipophilic–cationic thiacarbocyanine

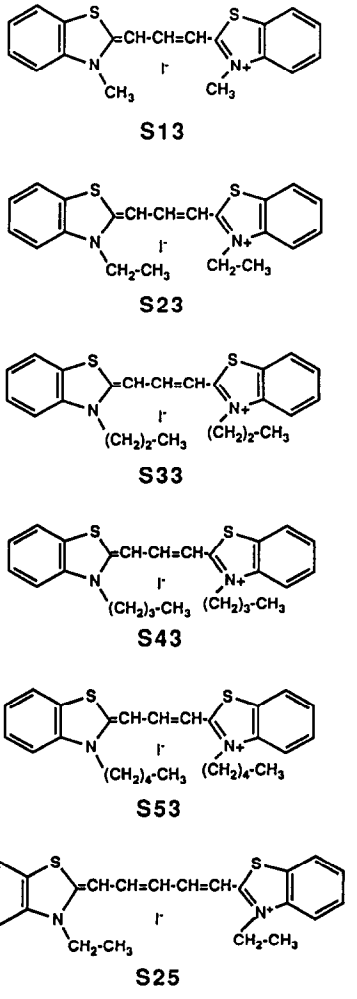

Fig. 1. Structures of the thiocarbocyanine compounds.

* Abbreviations: DMSO, dimethyl sulfoxide; $AcPyAD^+$, 3-acetylpyridine adenine dinucleotide; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; S13, 3,3'-dimethylthiacarbocyanine iodide; S23, 3,3'-diethylthiacarbocyanine iodide; S33, 3,3'-dipropylthiacarbocyanine iodide; S43, 3,3'-dibutylthiacarbocyanine iodide; S53, 3,3'-dipentylthiacarbocyanine iodide; S25, 3,3'-diethylthiadicarbocyanine iodide; and SMP, submitochondrial particles.

Table 1. Cytotoxic effect of thiacarbocyanines on CV-1 and CX-1 cells as determined by clonogenic assay

Thiacarbocyanine	IC_{50}*	
	CV-1	CX-1
S13	0.5 (1.33)	0.005 (0.013)
S23	0.4 (1.00)	0.005 (0.013)
S25	0.3 (0.70)	0.07 (0.16)
S33	0.2 (0.46)	0.009 (0.021)
S43	0.15 (0.33)	0.012 (0.03)
S53	0.3 (0.62)	0.03 (0.06)

* The IC_{50} values are expressed in μg/mL followed in parentheses by the μM value.

Values are ±10%. The clonogenic assay was performed as described in Materials and Methods.

compounds were used in this study: 3,3'-dimethylthiacarbocyanine iodide (S13), 3,3'-diethylthiacarbocyanine iodide (S23), 3,3'-dipropylthiacarbocyanine iodide (S33), 3,3'-dibutylthiacarbocyanine iodide (S43) and 3,3'-dipentylthiacarbocyanine iodide (S53). These compounds differ only in the alkyl group attached to the nitrogen of the thiazine ring. An additional compound, 3,3'-diethylthiadicarbocyanine iodide (S25), is a thiadicarbocyanine with ethyl side chains. Structures of all the compounds are shown in Fig. 1. Clonogenic assays with two cell types, CV-1 and CX-1, shown in Table 1, demonstrated that all the compounds tested were selectively cytotoxic to the human carcinoma cell line, when compared to a monkey normal epithelial cell line. These findings are in line with previous studies which showed that lipophilic–cationic compounds such as rhodamine 123 and dequalinium chloride are selectively toxic to carcinoma cells [1–5]. The IC_{50} ratio of carcinoma to normal cells ranged from 4 with S25 to 100 with S13 and the order of potency with respect to the CX-1 cell line was S13 = S23 ≥ S33 > S43 = S53 > S25.

The major contributory factor to the cytotoxicity with CX-1 cells appeared to be the size (length) of the side chain attached to the two ring systems. Cytotoxicity was inversely proportional to the length of these side chains, e.g. S13 with methyl side chains was more cytotoxic than S33 with propyl side chains. Another contributory factor may be the bridging distance between the two ring systems, i.e. the cytotoxicity for CX-1 cells was inversely proportional to the length of this bridging group. Thus, S23 (which has a shorter bridging group than S25) was 12-fold more cytotoxic to CX-1 cells than S25.

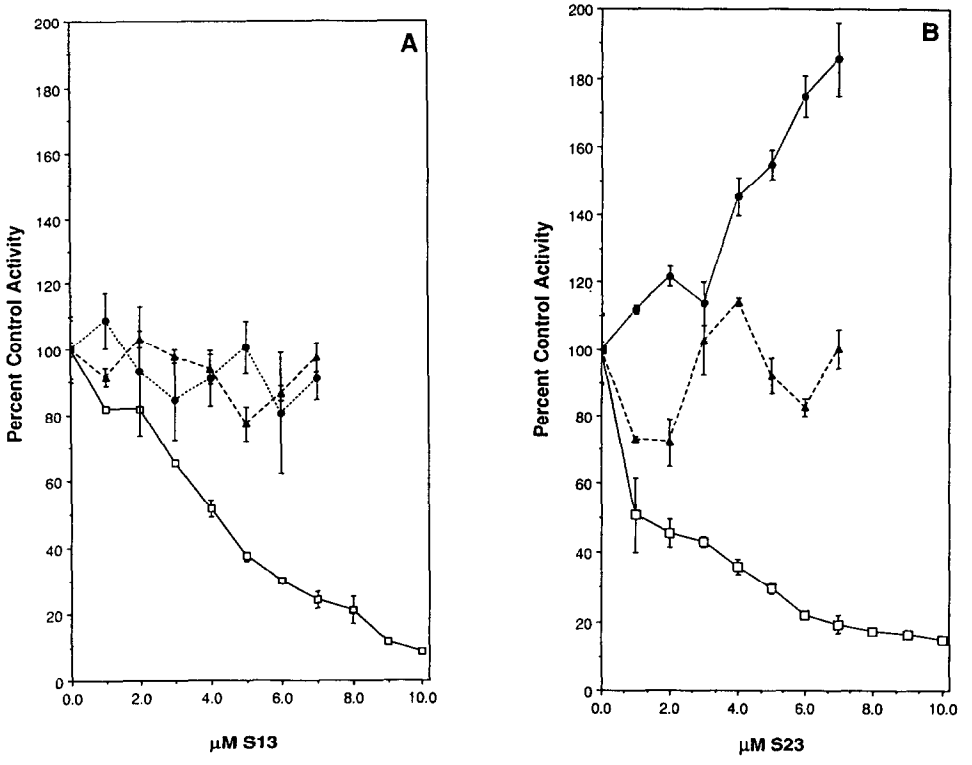

Fig. 2. Effects of thiacarbocyanines on bovine heart submitochondrial particle NADH oxidase, succinate oxidase, and cytochrome oxidase activities. Assays were performed as described in Materials and Methods in triplicate and data represent means ± SEM. Assays contained 0.05 mg of submitochondrial particle protein. Control specific activities were: NADH oxidase, 0.51 μmol·min^{-1}·mg^{-1}; succinate oxidase, 370 ng atom oxygen·min^{-1}·mg^{-1}; and cytochrome oxidase, 237 ng atom oxygen·min^{-1}·mg^{-1}. Key: NADH oxidase (□), succinate oxidase (▲), and cytochrome oxidase (●).

Effects of thiacarbocyanine compounds on mitochondrial electron transport. Titrations of electron transport activity (NADH, succinate, and cytochrome oxidases) with bovine heart SMP are shown in Fig. 2. Titrations with S13, S23, S25 and S33 were conducted between 2 and 10 μM, but titrations with S43 and S53 were stopped at 7 μM, since precipitation occurred above this concentration in the assay system. This study revealed that two of the thiacarbocyanine compounds, S13 and S23, were preferentially inhibitory to NADH oxidase activity. There was no general trend with S25, S33, S43 and S53 (data not shown); therefore, no concrete conclusions can be drawn about them. These data, taken together, indicate that of the three respiratory chain activities examined two of the thiacarbocyanine dyes, S13 and S23, inhibited within the region of the mitochondrial electron transport chain between NADH and ubiquinone, which is spanned by NADH–ubiquinone reductase (complex I).

The data presented in Fig. 2B indicate that S23 had a stimulatory effect on cytochrome oxidase activity. This stimulatory effect was largely negated by the inclusion of superoxide dismutase (100 U) in the assay system (data not shown). Thus it would appear that S23 is interacting with the artificial electron donors (ascorbate and TMPD) in the presence of SMP to produce superoxide which is interfering with the ability of the oxygen electrode to measure oxygen consumption. Attempts to alleviate this effect by using reduced cytochrome *c* as the electron donor were largely unsuccessful, since in the presence of S23 a precipitate rapidly formed in the assay system, thus invalidating the results. The sine wave pattern of the S23 effect on succinate oxidase seen in Fig. 2B was interpreted as noise, and thus no activation or inhibitory effect on mitochondrial succinate–ubiquinone reductase can be attributed to S23.

Effects of thiacarbocyanine compounds on mitochondrial NADH–ubiquinone reductase activities. Attempts were made to localize further the site of inhibition within the NADH–ubiquinone reductase portion of the respiratory chain. One way in which this can be accomplished is to determine the effects of the thiacarbocyanines on reduction of coenzyme Q_1 and other artificial electron acceptors. These electron acceptors can be divided into two categories. The first category includes the artificial electron acceptors $AcPyAD^+$, potassium ferricyanide, menadione, and juglone. Reduction of these compounds is insensitive to inhibition by classical inhibitors of NADH–ubiquinone reductase such as rotenone and piericidin A [15]. The second category includes such acceptors as duroquinone and coenzyme Q_1, whose reduction is blocked by rotenone and piericidin A [12, 16]. For this study, two forms of NADH–ubiquinone reductase, a membrane bound form (SMP) and a soluble form, purified complex I, were used to determine if the association of the thiacarbocyanine with the membrane contributed to the inhibitory effects. The concentration of thiacarbocyanine compound chosen for use in this study was 10 μM. This was the maximal concentration used for the titration of electron transport activities.

Table 2. Effects of 10 μM thiacarbocyanines on activities of bovine heart SMP and complex I NADH–ubiquinone reductase

Activity*	Inhibition (% control activity) S13	S23
	A. Bovine heart SMP	
NADH–$AcPyAD^+$	122.3 ± 3.8	135.6 ± 4.7
NADH–menadione	64.7 ± 4.6	55.6 ± 4.4
NADH–juglone	96.1 ± 7.4	94.5 ± 10.9
NADH–duroquinone	62.2 ± 5.3	28.7 ± 9.0
NADH–Q_1	32.4 ± 2.3	4.6 ± 0.1
	B. Complex I	
NADH–$AcPyAD^+$	124.1 ± 12.1	105.8 ± 8.8
NADH–menadione	97.0 ± 3.5	118.1 ± 12.4
NADH–juglone	61.5 ± 12.8	88.4 ± 7.4
NADH–duroquinone	82.9 ± 7.0	94.1 ± 8.1
NADH–Q_1	63.9 ± 12.5	93.0 ± 1.5

* All assays were performed as described in Materials and Methods in the absence and presence of either S13 of S23. Assays with SMP contained 3.3 μM antimycin A and 10 mM KCN. Assays contained either 0.05 mg of SMP or 0.01 mg of complex I protein. Values are means ± SEM of assays performed in triplicate. Control specific activities (U/mg protein) for SMP were: $AcPyAD^+$, 2.05; menadione, 0.29; juglone, 0.61; duroquinone, 0.32; and coenzyme Q_1, 0.80; and for complex I: $AcPyAD^+$, 0.94; menadione, 0.50; juglone, 1.44; duroquinone, 0.51; and coenzyme Q_1, 0.72.

The effects of 10 μM S13 and S23 on NADH-dependent reduction of the artificial electron acceptors and coenzyme Q_1 using SMP as the source of NADH–ubiquinone reductase are shown in Table 2. Reduction of $AcPyAD^+$ and juglone was unaffected by either thiacarbocyanine compound. Reduction of ferricyanide, another frequently used artificial electron acceptor, could not be determined in the presence of any of the thiacarbocyanines due to the formation of a precipitate in the reaction mixture. Menadione, duroquinone and coenzyme Q_1 reduction was inhibited by both of the thiacarbocyanine compounds with S23 being a slightly stronger inhibitor.

These results indicate that, although these two thiacarbocyanines inhibited reduction of three of the electron acceptors of mitochondrial NADH–ubiquinone reductase, duroquinone and coenzyme Q_1 reduction were the most severely affected by S23. As mentioned above, reduction of these two electron acceptors was susceptible to rotenone action.

This study was repeated using the soluble purified mammalian mitochondrial NADH–ubiquinone reductase, complex I. These results are also presented in Table 2. S13 demonstrated only slight inhibition of juglone, duroquinone and coenzyme Q_1 and S23 was without significant effect. A comparison of the data for SMP and complex I revealed that, in general, the thiacarbocyanines were less inhibitory with the soluble electron transport component segment (complex I) than with the membrane

Table 3. Effects of 10 μM thiacarbocyanines on activities of rotenone-saturated bovine heart SMP and rotenone-insensitive complex I NADH–ubiquinone reductase

Activity*	Inhibition (% control activity) S13	S23
A. Rotenone-saturated bovine heart SMP		
NADH–menadione	123.6 ± 9.6	54.2 ± 2.6
B. Rotenone-insensitive complex I		
NADH–juglone	102.4 ± 3.2	100.3 ± 4.1
NADH–duroquinone	103.0 ± 3.7	103.0 ± 3.8
NADH–Q_1	98.2 ± 1.8	98.2 ± 1.7

* All assays (1 mL) were performed as described in Materials and Methods in the absence and presence of either S13 or S23. Assays with SMP contained 40 μM rotenone, 3.3 μM antimycin A and 10 mM KCN. Assays contained either 0.05 mg of SMP or 0.01 mg of rotenone-insensitive complex I protein. Values are means ± SEM of assays performed in triplicate. Control specific activities (U/mg protein) for SMP were: menadione, 0.27; and for rotenone-insensitive complex I: juglone, 1.44; duroquinone, 1.15; and coenzyme Q_1, 0.60.

bound segment. This indicates that the membrane environment of the enzyme must play some role in modulating the overall inhibitory capacity of the two thiacarbocyanine dyes. Also, menadione reduction by SMP, effected by both thiacarbocyanines, was not inhibited when complex I was used as the source of the enzyme. This may again represent the contribution of membrane interaction to the role of thiacarbocyanines as inhibitors, but this is not possible to elucidate further from these data.

Taken together the data suggest that the thiacarbocyanines may be exerting their inhibitory effects by interacting primarily in a manner similar to the manner in which rotenone inhibits mitochondrial NADH–ubiquinone reductase. This interpretation, however, does not explain how the thiacarbocyanines inhibit reduction of menadione (by SMP) and reduction of juglone (by complex I), since reduction of these two electron acceptors is known to be rotenone insensitive. We have demonstrated previously that binding of rotenone induces a marked conformational change in the structure of complex I [18]. Ahmed and Krishnamoorthy [19] have also obtained evidence of a rotenone-induced conformational change in complex I. Thus, the thiacarbocyanines may be inducing a conformational change upon binding to the respiratory enzyme which results in inhibition of menadione and juglone reduction.

One way to test the hypothesis is to determine the effects of the two thiacarbocyanines on menadione reduction using rotenone-saturated SMP and on juglone, duroquinone, and coenzyme Q_1 reduction using rotenone-insensitive complex I. The results of this study are presented in Table 3. Except for the S23 effect on menadione reduction with rotenone-saturated SMP, which was unaffected by the presence of saturating rotenone, thiacarbocyanine inhibition was abolished. These data suggest that these two thiacarbocyanine dyes inhibit mitochondrial NADH–ubiquinone reductase in a manner similar to rotenone.

DISCUSSION

The lipophilic–cationic thiacarbocyanines exhibit a wide range of cytotoxicity with respect to human colon carcinoma cells. The order of diminishing toxicity indicates that compounds with shorter alkyl side chains (S13, S23, and S33) are more toxic than those with longer side chains (S43 and S53). All the compounds containing a conjugated propyldiene bridge were more cytotoxic than the thiadicarbocyanine, S25 (which contains a conjugated pentyltriene bridge). This indicates that the distance between the two charged ring systems plays an important role in the cytotoxic mechanism.

The observation that lipophilic–cationic compounds are selectively cytotoxic to neoplastic cells has also been observed with dequalinium chloride [4]. This phenomenon was found to be related to a membrane potential driven concentration of the compound within the mitochondria of the neoplastic cell. Further studies will have to be performed to ascertain if this is also case with the thiacarbocyanines.

Montecucco *et al.* [20] have shown that a thiadicarbocyanine, 3,3′-dipropylthiadicarbocyanine iodide, blocks lymphocyte capping and also causes depletion of ATP stores and inhibits oxygen uptake in isolated rat liver mitochondria. The authors concluded from studies with isolated mitochondria that this compound blocks electron transport somewhere within the NADH to ubiquinone region of the respiratory chain. S25, also known as dithiazanine, had been used as an antihelminthic drug [21], but its exact toxic properties were not investigated completely.

In contrast to the order of cytotoxicity, there does not appear to be any definite pattern to the inhibitory capacity with respect to the mitochondrial electron transport chain. Thus, no definite conclusions can be drawn about the effect of either side chain length or distance between the two charged ring systems. Since non-phosphorylating, inside out, submitochondrial particles were used as the source of the electron transport chain, the inhibitory capacity cannot reflect the ability to concentrate the thiacarbocyanine compounds within the mitochondrial matrix, but must reflect interaction of the respective thiacarbocyanine compound with both the respiratory chain and its membrane environment. That the membrane environment plays an important role in inhibition is well demonstrated by the fact that both S13 and S23 were less inhibitory with soluble complex I than with the membrane bound form of the enzyme.

Although the results of this investigation suggest that S13 and S23 may be inhibiting mitochondrial NADH–ubiquinone reductase in a manner akin to that of rotenone, we cannot determine from the present data whether these compounds are actually binding to the rotenone site of the enzyme or perhaps interact at another site which mimics the action of

rotenone. Further studies involving EPR and affinity labeling should shed more light on their exact mode of action.

Acknowledgements—This work was supported by a grant from N.I.H. (HL32829) and a grant from the Indiana Heart Association to W. M. A. The authors wish to thank Dr. B. Beatrice Chambers for a critical reading of the manuscript.

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