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A REDOX CYCLING MECHANISM OF ACTION FOR 2,3-DICHLORO-1,4-NAPHTHOQUINONE WITH MITOCHONDRIAL MEMBRANES AND THE ROLE OF SULFHYDRYL GROUPS*

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Abstract—The addition of 2,3-dichloro-1,4-naphthoquinone (CNQ) to substrate-depleted, GSH-supplemented rat liver mitochondria resulted in a dose-dependent depletion of reactable sulfhydryl groups and a concomitant increase in mitochondrial disulfide content at a ratio of 2 thiols depleted/disulfide generated. The molar ratio of thiol depleted/CNQ added approached 20 at low CNQ concentrations and was unity at higher doses. The addition of CNQ to substrate-depleted mitochondrial suspensions resulted in O_2 consumption which increased with increasing concentrations of mitochondria and was sensitive to *N*-ethylmaleimide (NEM) which establishes the ability of CNQ to interact with mitochondrial thiol redox centers. The CNQ-mediated large amplitude swelling of rat liver mitochondria was exacerbated by thiol oxidizing agents and depressed by disulfide reducing agents. A redox cycling mechanism between mitochondrial thiol groups, CNQ and oxygen was proposed to lower the matrix glutathione pool and make the mitochondria more susceptable to toxic oxygen radicals which induce swelling in isolated mitochondrial suspensions. In support of this mechanism, α-tocopherol was shown to prevent the CNQ-mediated swelling process. Beef heart mitochondrial NADH was oxidized by CNQ in a 1/1 molar ratio anaerobically and in a 3/1 molar ratio under aerobic conditions, whereas the fully reduced quinone, $CNQH_2$, oxidized NADH aerobically but not anaerobically. Thus, CNQ is capable of interacting with NADH of the mitochondrial electron transport chain in a redox cycling fashion.

The structural similarity between the fungicide 2,3-dichloro-1,4-naphthoquinone (CNQ§) and the antineoplastic agent 2,3-bis(chloromethyl)-1,4-naphthoquinone (CMNQ), as well as the recent report demonstrating antineoplastic activity of CNQ against Sarcoma 180 [1], prompted our interest in the interaction between CNQ and mitochondrial membrane systems. We have demonstrated that CNQ inhibits the beef heart mitochondrial respiratory chain at separate and distinct sites in complexes I and II [2]. The interaction between CNQ and the mitochondrial membranes caused an initial respiratory burst followed by irreversible inhibition of oxygen consumption. The respiratory burst comprised cyanide-sensitive and -insensitive portions. The cyanide-sensitive portion of the CNQ-induced respiratory burst was uncoupling of oxidative phosphorylation, whereas the cyanide-insensitive portion produced O_2^- and H_2O_2. Succinate was shown to be the source of reducing equivalents for the CNQ-mediated generation of O_2^- and H_2O_2. In addition, CNQ induced a dose-dependent, large amplitude mitochondrial swelling which was osmotic in nature, cation non-specific, non-energy linked, independent of substrate, and dependent on the presence of oxygen [2, 3]. Lipid peroxidation of the mitochondrial lipids accompanied the CNQ-induced respiratory burst and mitochondrial swelling [2]. The addition of cysteine in the suspension buffer prevented the CNQ-induced mitochondrial swelling which suggested that CNQ is capable of interacting with thiol containing amino acids.

Riley and Lehninger [4] have reported that mitochondrial swelling was initiated when 12% of the mitochondrial thiol groups, which react quickly with silver ions, was depleted. In addition, Kosower *et al.* [5] have concluded that perturbation of the thiol-disulfide status of cells causes a wide variety of biological responses, and Sikka *et al.* [6] have suggested that CNQ binds to erythrocyte membrane sulfhydryl groups.

These findings prompted further investigations of the interation of CNQ with mitochondrial thiol groups. The results from such investigations may be expanded to predict the reactivities of quinones with other selected redox active membranes.

MATERIALS AND METHODS

Beef heart mitochondria (BHM) isolated by dif-

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§ Abbreviations: CNQ, 2,3-dichloro-1,4-naphthoquinone; CMNQ, 2,3-bis(chloromethyl)-1,4-naphthoquinone; DTNB, 5,5'-dithiobis-(nitrobenzoic acid); NEM, *N*-ethylmaleimide; and pCMB, *p*-chloromercuribenzoate.

ferential centrifugation as previously described [7] and aged by repeated freezing and thawing were employed in studies not requiring tightly coupled mitochondria. Tightly coupled mitochondria were obtained from rat liver by differential centrifugation as described [8].

Mitochondrial respiration using BHM was measured polarographically on a Gilson Oxygraph equipped with a Clark electrode [9]. The reaction mixture consisted of 0.25 M sucrose, 7.5 mM $MgCl_2$, 15 mM Tris buffer (pH 7.4) and 15 mM phosphate buffer (pH 7.4). The reaction was initiated by the addition of substrate, 3.3 mM succinate, and the final volume of buffer was 2 ml. Tightly coupled rat liver mitochondria (RLM) were utilized to investigate mitochondrial swelling, which was monitored spectrophotometrically at 520 nm on an Aminco DW-2 UV-Visible spectrophotometer in the split beam mode in 1 cm cuvettes. RLM suspensions of 20–70 μl containing approximately 1 mg protein were diluted with suspension buffer (150 mM KCl, 20 mM Tris–HCl, pH 7.4) to give an initial absorbance of from 0.7 to 1.0 at 520 nm [10]. Swelling was initiated by the addition of CNQ as described in the figure legends. The final volume was 3 ml.

Thiol groups were determined by the procedure of Ellman [11], with certain modifications when using RLM. DTNB, 79.2 μg/assay, was added to 3 ml of 0.1 M Tris–acetate buffer, pH 7.6, containing an appropriate aliquot of the thiol to be determined. Following a 20-min reaction, the change in absorbance was recorded at 412 nm in the split beam mode of the Aminco DW-2 UV-Vis spectrophotometer ($E = 13{,}600\ M^{-1}\ cm^{-1}$). Thiol concentrations of intact RLM were measured by dual wavelength spectroscopy at 412–520 nm. Disulfide levels were estimated from the difference in thiol content following pretreatment and no treatment with dithiothreitol (DTT) as described by Zahler and Cleland [12]. CNQ did not interfere with the DTNB assay for thiol groups. CNQ absorbed slightly at 412 nm which was accounted for by running controls consisting of buffer and CNQ at appropriate concentrations and subtracting these values from the appropriate sample values.

Practical grade CNQ was recrystallized from hot ethanol so that the melting point range was between 192 and 193°. The recrystallized CNQ was dissolved in 100% ethanol at a concentration of 39.1 mg/100 ml and stored at 0–4°.

The hydroquinone form of CNQ ($CNQH_2$) was prepared from recrystallized CNQ by dissolving 0.1 g CNQ in 10–15 ml of anhydrous ethyl ether which was transferred to a small separatory funnel. Approximately 5 ml of a solution of sodium dithionite (1 g in 5 ml water) was added to the ether solution and mixed vigorously for a few minutes until the ether phase became colorless. The water phase was discarded, and the ether phase was washed twice with 2–3 ml of water. The ether phase was dried for 2 hr over dry granular sodium sulfate and then evaporated under a slow stream of nitrogen. During the drying process, an aluminium foil wrap protected the solution from exposure to light. Deoxygenated 100% ethanol was then added to the hydroquinone which was used within 4 hr after preparation.

RESULTS

The data in Fig. 1 show that CNQ caused a dose-dependent reduction in the concentration of the DTNB reactable thiol groups in rat liver mitochondria. At the higher concentrations, CNQ depleted 100% of the measurable sulfhydryl groups, indicating that membrane thiols as well as the matrix glutathione pool were susceptible to CNQ interaction. A replot of the data from Fig. 1 shows that the ratio of moles of thiol groups depleted/moles CNQ versus moles of CNQ added changed with decreasing concentration of CNQ (Fig. 2). At the lower CNQ doses, the ratio was greater than 1:1 and approached a ratio of 20:1 at the lowest CNQ concentration tested. A ratio of 2/1 (thiol depleted/CNQ added) is the highest predicted should thiol depletion result from direct Michael addition to CNQ at the 2,3 position as proposed by Nickerson *et al.* [13]. Since at low levels of CNQ the thiol/CNQ ratio approached 20/1, redox cycling (biological reduction of CNQ and its reoxidation by oxygen) is apparent. The data in Fig. 3 show that increasing con-

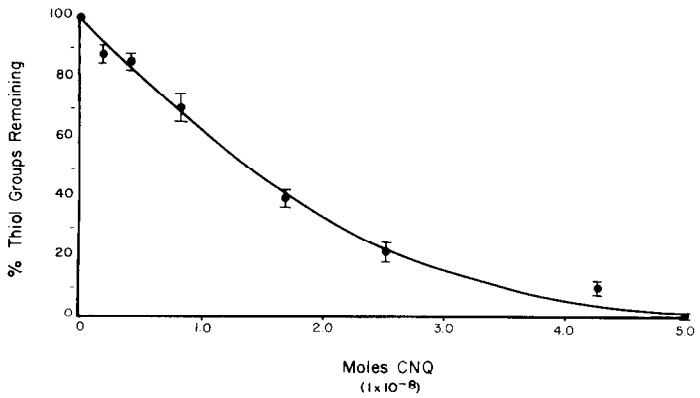

Fig. 1. Mitochondrial thiol group depletion by CNQ. Rat liver mitochondria (2 mg protein) were treated with various concentrations of CNQ for 10 min and were assayed for free sulfhydryl groups as described in Materials and Methods. Final volume was 3 ml. The actual 100% thiol concentrations varied between 25 and 60 nmoles/mg protein. Each value is an average of at least three determinations ± SD.

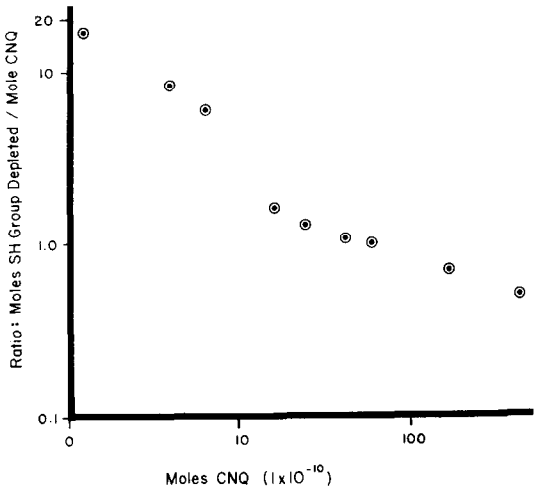

Fig. 2. Ratio of mitochondrial thiol group depletion per CNQ added. Rat liver mitochondria (2 mg protein) were treated with various concentrations of CNQ for 10 min and were assayed for free sulfhydryl groups as described in Materials and Methods. Final volume was 3 ml.

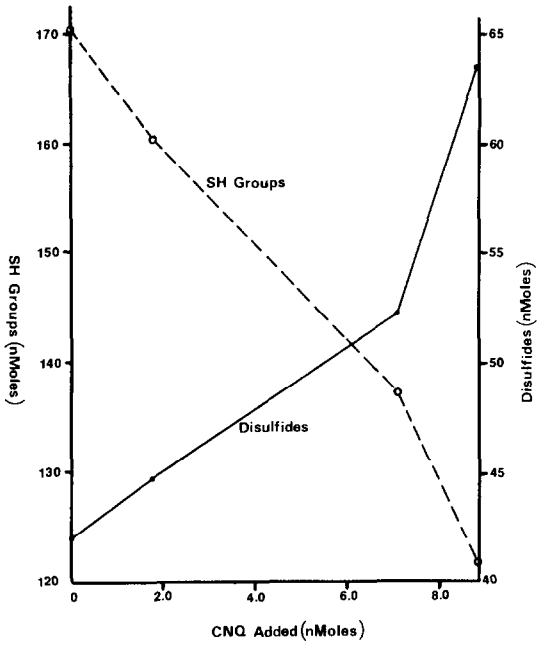

Fig. 3. CNQ-induced sulfhydryl group depletion and disulfide formation. Rat liver mitochondria were inhibited with 2 mg antimycin A. Reduced glutathione (0.22 mM) was added as an additional source of sulfhydryl groups. CNQ was added and allowed to react with the solution for 15 min. The samples were then assayed either for sulfhydryl or disulfide groups as described in Materials and Methods. Each value represents an average of three determinations. The dashed line represents sulfhydryl group values and the solid line represents disulfide group values.

centrations of CNQ caused sulfhydryl group depletion with corresponding quantitative disulfide group formation in substrate-depleted rat liver mitochondria supplemented with glutathione. The observed ratio of GSH depletion to GSSG formation was approximately 2/1, demonstrating that CNQ catalyzed a thiol–disulfide redox reaction. Under similar conditions, glutathione depletion but not disulfide formation occurred with CNQ in the absence of mitochondria (data not shown).

Increasing the concentration of mitochondrial protein from 1.3 to 5.1 mg/ml increased the rate of CN-insensitive oxygen consumption from 1.4 to 7.0 nmoles oxygen consumed/min respectively. This respiratory rate was absent in mitochondria preincubated with NEM which is capable of penetrating the mitochondria and reacting with the matrix glutathione pool [14] (data not shown).

To determine if CNQ was capable of reacting with thiol amino acids, we measured non-enzymatic oxygen consumption resulting from the interaction between CNQ and cysteine. The results in Table 1 demonstrate that the reaction of CNQ with cysteine consumed oxygen at a greater rate than the control in the absence of CNQ and amino acid, whereas the reaction with its disulfide, cystine, did not.

We then evaluated the effects of adding various sulfhydryl and disulfide reactive compounds to the

Table 1. Non-enzymatic CNQ-induced oxygen consumption with cysteine and cystine*

Compound added to buffer (2 mM)	CNQ added† (nmoles)	Total oxygen consumption‡ (nmoles/5 min)	Oxygen consumption due to CNQ (nmoles/5 min)
0	0	30.8 ± 2.4	
Cysteine	0	46.7 ± 3.6	
Cysteine	86	65.5 ± 5.9	18.8
Cystine	0	30.2 ± 2.7	
Cystine	86	33.3 ± 1.5	0

* Oxygen consumption was monitored polarographically as described in Materials and Methods. The amino acids were dissolved in 1.0 M Tris–HCl buffer, pH 8.1. This pH was selected to facilitate amino acid dissolution.

† CNQ was added in ethanol (50 μl). Samples receiving no CNQ had 50 μl of ethanol added as a control.

‡ Each value represents an average of at least five determinations ± S.D.

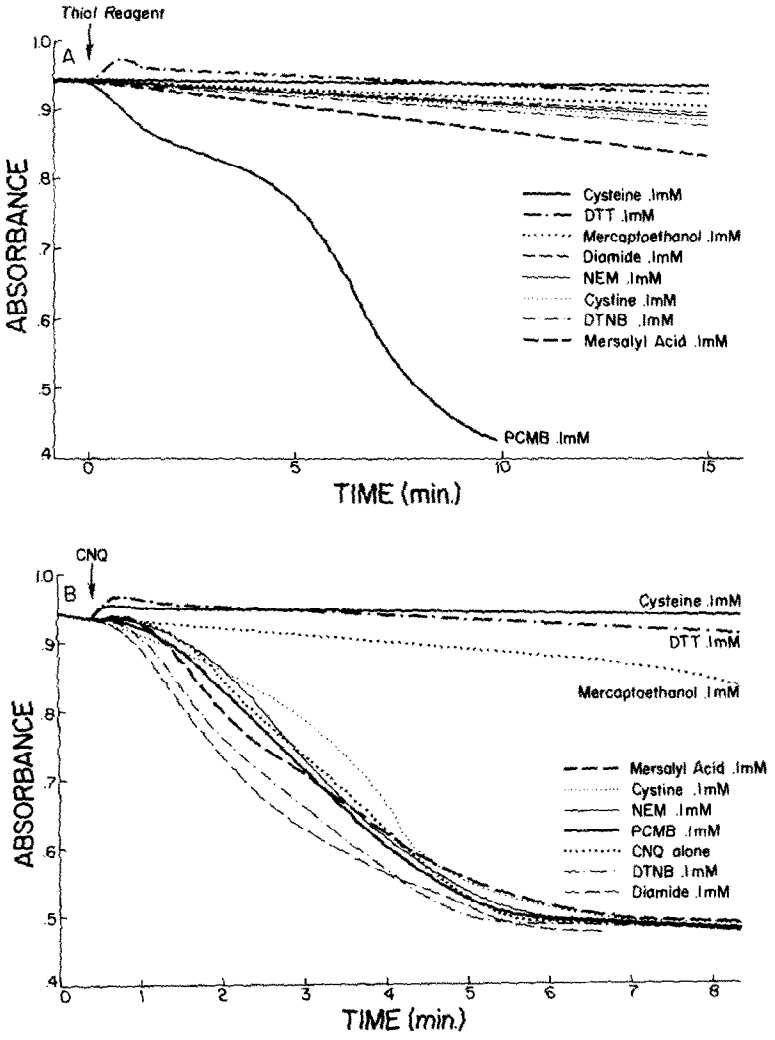

Fig. 4. (A) Thiol reagent effects on mitochondrial swelling. Rat liver mitochondria (1.3 mg protein) were added to 3 ml of suspension buffer. The spontaneous swelling rate was measured for 30 sec, then 0.1 mM concentrations of various thiol reagents were added in 20 μl of 0.05 M Tris–acetate buffer. pH 7.6, of DMSO depending on solubility, and swelling was monitored continously at 520 nm. (B). Thiol reagents effects on CNQ-induced mitochondrial swelling. Same as Figure 4A except 86 nmoles of CNQ was added in 50 μl ethanol 30 sec after the thiol reagent was added.

mitochondrial suspensions. The compounds tested included the sulfhydryl-alkylating reagents, pCMB, NEM and mersalyl acid, the disulfide-reducing compounds, mercaptoethanol, DTT and cysteine, a disulfide-forming compound, DTNB, a sulfhydryl-oxidizing reagent with high specificity for glutathione, diamide, and a disulfide amino acid, cystine. Each of these compounds was screened for the ability to induce mitochondrial swelling alone. The data in Fig. 4A show that only pCMB induced swelling although mersalyl acid, a known inducer of mitochondrial swelling at higher concentrations, caused minimal swelling at the concentrations employed. These data suggest that alkylation of the mitochondrial sulfhydryl groups on the surface of the inner membrane, as reported for pCMB and mersalyl acid [14, 15], initiated mitochondrial swelling. NEM, the other alkylating agent which did not cause swelling, has been reported to penetrate the mitochondrial membrane and react with the matrix pool of glutathione [14, 15]. The data in Fig. 4B demonstrate that disulfide-reducing agents inhibited swelling, with cysteine being the most potent inhibitor followed by DTT and mercaptoethanol. On the other hand, treatment with diamide, a glutathione oxidant [5], enhanced the rate of CNQ-induced swelling. DTNB, capable of undergoing a disulfide exchange reaction with mitochondrial thiol groups, also enhanced the rate of CNQ-induced swelling. The alkylating reagents and cysteine did not affect the rate of CNQ-induced swelling. These findings suggest that CNQ-induced mitochondrial swelling is associated with an oxidation of the mitochondrial sulfhydryl groups. These data, however, do not rule out the possibility that, in these studies, excess disulfide reductant reacted directly with the CNQ.

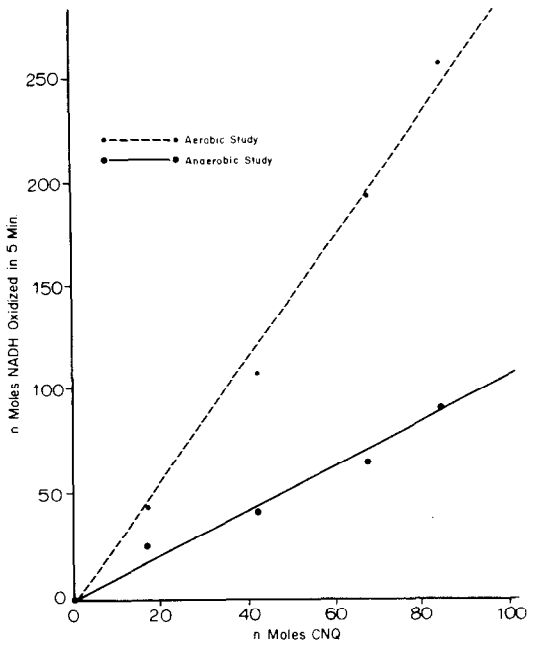

Fig. 5. Oxidation of NADH by CNQ in cyanide-inhibited beef heart mitochondria. To beef heart mitochondria (2.8 mg protein) in 3 ml of suspension buffer containing 1 mM potassium cyanide, NADH was added to a final concentration of 0.3 mM, and the NADH disappearance was monitored spectrophotometrically for 5 min at 340 nm. Each value represents an average of at least three determinations.

precluding its reaction with mitochondrial sulfhydryl groups. This possibility was minimized in these experiments by adding the thiol reagents first and CNQ 30 sec later. This is not a concern for the sulfhydryl-oxidizing compound diamide whose specificity is for glutathione [5], and for DTNB a disulfide reagent which has a high affinity for sulfhydryl groups and whose disulfide nature would preclude a direct interaction with CNQ. The rate of CNQ-induced mitochondrial swelling was enhanced by these two compounds.

At CNQ concentrations (8.6 μM) that did not induce mitochondrial swelling in the absence of respiratory substrate, the addition of 3.3 and 6.6 mM succinate resulted in swelling rates of 0.015 and 0.025 O.D. units at 520 nm in 5 min respectively. Under these conditions less than 12% of the thiol groups were directly depleted by CNQ (data not shown). A previous report [2], which demonstrated that the CNQ-ellicited production of O_2^- in mitochondria was up to 60-fold greater in the presence of succinate than in its absence (61 vs 0.9 nmoles of O_2/min/mg protein), implicates a CNQ/O_2 redox cycling mechanism and subsequent lipid peroxidation in the CNQ-swelling process.

We monitored the oxidation of NADH in CN^- inhibited mitochondria, under aerobic and anaerobic conditions as shown in Fig. 5. Under aerobic conditions an average of 3 nmoles of NADH were oxidized per nmole of CNQ added, whereas under anaerobic conditions the expected 1:1 ratio of NADH oxidized to CNQ added was found. CNQ did not oxidize NADH in the absence of enzyme or with heat-inactivated enzyme (data not shown). In

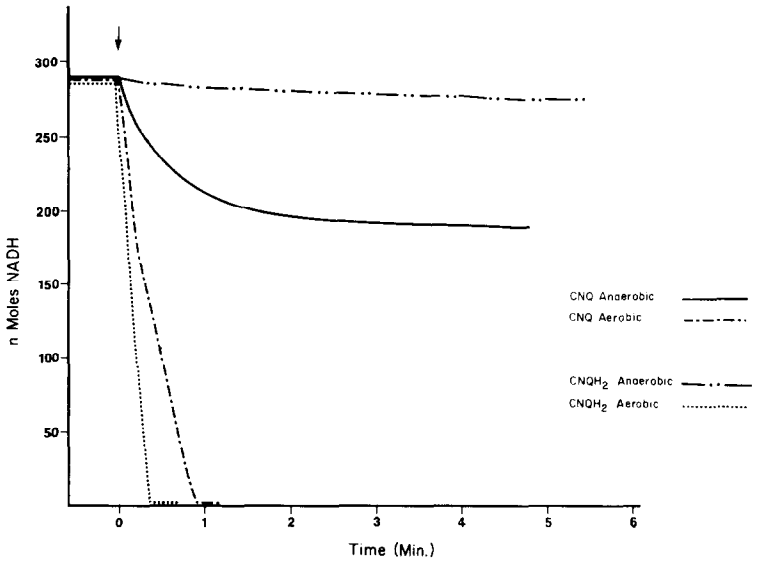

Fig. 6. Oxidation of NADH by CNQ at various oxidation states in cyanide-treated mitochondria. Beef heart mitochondria (2.8 mg protein) were added to 3 ml of suspension buffer containing 1 mM potassium cyanide, and 0.3 mM NADH was added. At the arrow, 43 nmoles CNQ at various oxidation states was added in 25 μl ethanol. NADH oxidation was monitored spectrophotometrically at 340 nm. In the anaerobic reactions, nitrogen was flushed through the cuvettes employing the anaerobic cell assembly in the Aminco DW-2 UV-visible spectrophotometer. Anaerobiosis was verified by monitoring cytochrome *c* reduction at 550 nm with excess substrate (NADH). Each tracing is a representative of at least three tracings. The tracings have been superimposed and the nmoles NADH scale expanded for clarity.

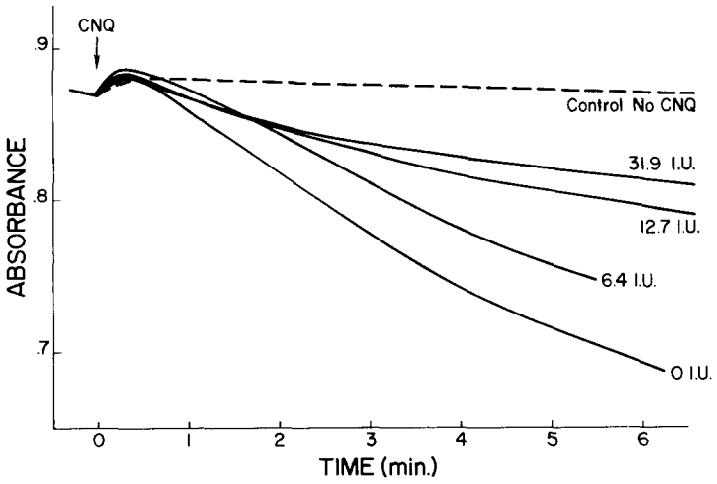

Fig. 7. Tocopherol effect on CNQ-induced rat liver mitochondrial swelling. Tocopherol (31.9 I.U./100μl ethanol) was added to 3 ml of suspension buffer and rat liver mitochondria (1.4 mg protein), and the solution was allowed to equilibrate for 3 min. CNQ (21.5 μM) was then added to initiate the swelling. Swelling was monitored as previously described. The curves shown are representative samples of six determinations.

addition, the oxidation of NADH by $CNQH_2$ in CN-inhibited mitochondria was assessed under aerobic and anaerobic conditions. The proposed redox cycling mechanism predicts that the addition of $CNQH_2$ to mitochondria would not oxidize NADH anaerobically but should cause a rapid oxidation of NADH aerobically. The data in Fig. 6 demonstrate that under aerobic conditions both CNQ and $CNQH_2$ were capable of oxidizing NADH at the 3:1 ratio previously described. Anaerobically, CNQ oxidized NADH at the predicted 1:1 ratio but $CNQH_2$ was unable to oxidize NADH. These findings establish a redox-cycling mechanism with NADH-oxidase as well as with succinoxidase [2].

The data in Fig. 7 demonstrate that α-tocopherol inhibited CNQ-induced swelling, a finding that further implicates a free radical mechanism of swelling.

DISCUSSION

CNQ has been reported to induce a respiratory burst in isolated mitochondria which generates O_2^-, H_2O_2 and lipid peroxides and which causes membrane perturbations leading to mitochondrial swelling [2, 3]. We now report that the addition of CNQ to isolated rat liver mitochondria devoid of substrate and supplemented with GSH resulted in the stoichiometric conversion of thiol to disulfide. In addition, when CNQ was added to substrate-depleted mitochondria,or cysteine (Table 1), oxygen consumption was observed, indicating that the CNQ thiol redox reaction was non-enzymatic. This finding is consistent with the findings of Gause *et al.* [16] who reported that the CNQH· radical was formed during the non-enzymatic reaction between CNQ and thiols. Semiquinone radicals [17–19] and thiols [20, 21] are capable of interacting with oxygen to form O_2^-. The data in Figs. 1 and 2 demonstrate that CNQ is able to undergo redox cycling in its interaction with thiols leading to the conclusion that CNQ oxidizes mitochondrial thiols, while in turn being reduced either to the semiquinone or hydroquinone [22]. The reduced form of the quinone in turn is oxidized by molecular oxygen to form superoxide. The data presented herein do not definitely establish stoichiometry of the reaction but, since superoxide has been observed during this interaction [2, 3], a one-electron oxidation–reduction reaction is involved. These findings are similar to reports on adriamycin [23, 24] and other anthracycline antibiotics [25] which have been shown to undergo redox cycling with mitochondrial NADH-dehydrogenase, with concomitant production of superoxide.

Thiol oxidation and superoxide production resulting from CNQ addition to mitochondrial suspensions account for the observed CNQ-promoted swelling and lipid peroxidation [2, 3]. Riley and Lehninger [4] reported that the disappearance of 12% or more of the fast reacting mitochondrial sulfhydryl groups caused mitochondrial swelling. In addition, Bindoli *et al.* [26] concluded that mitochondrial lipid peroxidation was dependent on a decrease of 15% of the membrane sulfhydryl groups.

Our data show that CNQ concentrations which deplete 12% or less of the total mitochondrial thiol content do not induce swelling. However, these same levels of CNQ induced mitochondrial swelling when respiratory substrate was added to the reaction medium (data not shown). The extent of swelling was proportional to the substrate concentration which was shown to be proportional to the amount of O_2^- produced [2]. That α-tocopherol, a known free radical scavenger, prevented CNQ-induced mitochondrial swelling (Fig. 7), and the observation that swelling required O_2 [2] supports the conclusion that oxygen radical formation is a necessary step in CNQ-mediated swelling. These findings are consistent with those of Schneider *et al.* [27] who reported a correlation between oxygen consumption, lipid peroxidation, and mitochondrial swelling and our previous report [2] that exogenously produced O_2^- promotes mitochondrial swelling.

The data in panels A and B of Fig. 4 demonstrate that well known thiol protecting agents (cysteine, DTT and mercaptoethanol) depressed, while the thiol oxidizing agents exacerbated, the CNQ-mediated mitochondrial swelling process. The addition of 0.1 mM diamide alone did not induce swelling whereas 0.1 mM pCMB did. In similar experiments (data not shown), lower concentrations of diamide (0.015 mM) or pCMB caused a depletion of 42 and 37% of the mitochondrial thiols respectively. That diamide reacts preferentially with the matrix glutathione pool and pCMB with membrane thiols accounts for the observation that pCMB preferentially induces mitochondrial swelling and diamide did not (Fig. 4A) at concentrations that deplete 40% of the mitochondrial thiol content.

Since CNQ was shown to cause 100% depletion of the mitochondrial thiol groups (Fig. 1) and to convert GSH to GSSG (Fig. 3), we conclude that CNQ interacts with mitochondria to oxidize the matrix glutathione pool which increases the susceptibility of the membrane thiols to the subsequent oxidative stress generated by the interaction of CNQ with the repiratory chain. The mitochondrial membrane thiol groups have been implicated in controlling permeability [28].

A recent preliminary report that CNQ inhibits glutathione reductase [29] provides a mechanism for the 100% depletion of mitochondrial sulfhydryl groups observed in the presence of high concentrations of CNQ (Fig. 1). Inhibition of glutathione reductase activity would preclude the reduction of glutathione disulfide resulting from CNQ action (Fig. 3) and allow the thiol content to approach zero. This finding is consistent with the observations of Babson *et al.* [30] that adriamycin treatment of isolated hepatocytes resulted in lipid peroxidation and decreased cell viability only when glutathione reductase activity was also inhibited. These observations exemplify the important role of glutathione reductase in maintaining cellular thiol levels.

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