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QUINONE INTERACTION WITH THE RESPIRATORY CHAIN-LINKED NADH DEHYDROGENASE OF BEEF HEART MITOCHONDRIA

I. JUGLONE REDUCTASE ACTIVITY

F. J. RUZICKA AND F. L. CRANE

Department of Biological Sciences, Purdue University, Lafayette, Ind. 47907 (U.S.A.)

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SUMMARY

1. Substituted benzoquinones and napthoquinones can function as electron acceptors for the NADH dehydrogenase segment of the mitochondrial electron transport system. Anthraquinones and several hydroxylated quinones are not reduced by the enzyme complex.

2. Piericidin A treatment at concentrations known to inhibit electron transport causes varying degrees of inhibition of quinone reductase activities. The pattern of piericidin A inhibition suggests that certain quinones are reduced at sites either before or after the piericidin A inhibition site. Reduction of quinones such as 5-hydroxy-1,4-napthoquinone (juglone) and 1,2-napthoquinone is inhibited only slightly. Reduction of ubiquinone 1 and 2 and 2,3,5,6-tetramethyl-1,4-benzoquinone (duroquinone) is almost completely inhibited following piericidin A treatment.

3. Comparison of juglone reductase activity with ferricyanide reductase activity suggests that these acceptors are reduced at nonequivalent sites in the NADH dehydrogenase. Juglone reductase activity is stimulated following mercurial treatment while ferricyanide reductase activity is slightly inhibited. Preincubation of NADH dehydrogenase with NADH followed by mercurial treatment causes almost complete inhibition of ferricyanide reductase activity. Activation of juglone reductase activity still occurs under these conditions. The activation is specific for preparations of NADH dehydrogenase, for mercurials, and for napthoquinone reductase activity.

INTRODUCTION

Attempts to resolve the respiratory chain-linked NADH dehydrogenase have been well documented[1,2]. The complexity and the general insolubility of the complex enzyme system do not allow for traditional enzymatic purification and characterization. Extraction procedures have given multiple products which differ from each other by a high degree of chemical and physical heterogeneity. Such heterogeneity has led to disagreement between investigators as to the true nature of the enzyme.

The enzyme is associated with phospholipid[3] and other insoluble proteins[4]

Abbreviation: PCMB, *p*-chloromercuribenzoate.

which make up the inner mitochondrial membrane. The association of the enzyme with these membranous components is substantiated by the fact that rigorous chemical and enzymatic treatments have been required for release and solubilization of the enzyme. These treatments in turn have resulted in profound changes in the catalytic properties of the NADH dehydrogenase[5,6]. Treatment of particulate NADH dehydrogenase with heat–acid–ethanol[7–10] or phospholipase A at 37° (ref. 11) produces soluble flavoproteins with characteristic antimycin A-insensitive cytochrome *c* reductase and dye reductase activities. Antimycin A-insensitive cytochrome *c* reductase activity is present in particulate preparations of the enzyme only in trace levels.

A recurring problem has been the lack of suitable parameters for which to judge the NADH dehydrogenase. The complexity of the enzyme and its association with other components of the cristal membrane have made direct photometric and fluorometric analysis difficult[12]. Severe limitations also result by the lack of information as to the identity of the electron acceptor for each of the components of the enzyme complex. The NADH dehydrogenase is known to contain flavin and non-heme iron components[13,14] as well as sulfhydryl groups[15–17] and ubiquinone 10 (ref. 14). The application of natural components of the NADH dehydrogenase as electron acceptors awaits their purification and identification. However, these components are largely membrane-bound proteins which are difficult to solubilize and purify without irreversible change of their enzymatic properties. The quinone endogenous to heart mitochondria, ubiquinone 10, has been utilized as an electron acceptor in a specific assay system[10], although its application in spectrophotometric studies is difficult and not always reliable because of its extreme insolubility in aqueous systems.

The use of artificial electron acceptors has been strengthened by the successful use of ferricyanide reduction for NADH dehydrogenase[18] and phenazine methosulfate reduction for succinate dehydrogenase[19]. The complex electron transport sequence in liver aldehyde oxidase has been examined by RAJOGOPALAN AND HANDLER[20]. They utilized artificial electron acceptors such as dichloroindophenol, ferricyanide, and nitro blue and showed their reduction to have precise inhibitor specificities.

We have found that certain classes of quinones can act as electron acceptors for the NADH dehydrogenase. Furthermore, several of these quinones show a unique specificity in their ability to be reduced at different sites in the enzyme complex as defined by selective inhibitor effects on the intact system or of fractions derived from the NADH dehydrogenase. The combined application of these quinones has led to a novel approach to the study and the further resolution of the respiratory chain-linked NADH dehydrogenase.

MATERIALS AND METHODS

Materials

Beef heart mitochondria were prepared according to the method of LÖW AND VALLIN[21]. Electron transport particles were obtained by sonic disruption of mitochondria suspended in 0.25 M sucrose–0.05 M Tris–HCl (pH 7.4) at a protein concentration of 30 mg/ml. Sonic disruption was accomplished with a Branson Sonifier set at 6–7 A for two 3-min intervals in a vessel submerged in a salt–ice bath. The supernatant obtained by centrifugation at 27000 × *g* for 15 min was recentrifuged at 105000 × *g*

for 45 min. The pellet (electron transport particles) was washed twice with 0.25 M sucrose–0.05 M Tris–HCl (pH 7.4) by homogenization with a glass homogenizer fitted with a teflon pestle and centrifugation at 105000 × *g* for 45 min. The electron transport particles were resuspended in the same buffer and stored at −20°.

NADH: cytochrome *c* reductase (Complex I + III) and NADH: coenzyme Q reductase (Complex I) were prepared according to published procedures[22,14]. NADH dehydrogenase was prepared by phospholipase A treatment by the revised method of CREMONA AND KEARNEY[23]. The enzyme was used at the Sephadex G-200 stage of purification. NADH dehydrogenase from Complex I was extracted by the urea treatment of HATEFI AND STEMPEL[24]. NADH: ubiquinone reductase was prepared according to the method of PHARO *et al.*[10].

The lipoamide reductase-containing supernatant was obtained by sonic disruption of beef heart mitochondria. Beef heart mitochondria were added to an equal volume of 0.25 M sucrose–0.05 M Tris–HCl (pH 7.4) and centrifuged at 27000 × *g* for 20 min. The supernatant was discarded and the pellet was resuspended in the same buffer to a concentration of 20 mg/ml. The suspension was subjected to sonic disruption with a Branson Sonifier set at 6–7 A for two 3-min intervals in a vessel submerged in an ice-salt bath. The sonicated mitochondria were centrifuged at 144000 × *g* for 60 min. The supernatant was used for subsequent assays.

Methods

Assays of quinone reductases were carried out at 30° by following the oxidation of NADH at 340 mμ after the addition of quinone. All assays unless specified done in the presence of antimycin A and KCN. The assay system (3 ml) contained 30 μmoles Tris–HCl (pH 7.4), 0.5 μmole NADH, 0.5 μg antimycin A, and 0.3 μmole KCN. 0.3 μmole of quinone (10μl in absolute ethanol) was added to start the reaction. 0.12 nmole piericidin A was added when applicable. NADH: ferricyanide reductase assays were carried out at fixed ferricyanide concentration (1.7 mM) under assay conditions described by MINAKAMI *et al.*[18]. Triethanolamine buffer was substituted for phosphate buffer. All assays were done on a Unicam SP 800 spectrophotometer with a multispeed Heath external recorder. 0.2–0.3 mg of electron transport particles was routinely used for quinone reductase assays to obtain a ΔA/min of 0.05–0.1.

p-Chloromercuribenzoate (PCMB), sodium mersalyl, and *N*-ethylmaleimide were dissolved in 0.05 M Tris–HCl (pH 7.4). The sulfhydryl agents were incubated with enzyme in the same buffer. Juglone (5-hydroxy-1,4-napthoquinone) was recrystallized twice from 95% ethanol. Quinones, piericidin A, and antimycin A were dissolved in absolute ethanol and added in 10-μl amounts.

Chemicals

Ubiquinone 1 and 2 were kindly provided by Dr. A. F. Wagner of Merck, Sharp, and Dohme. Piericidin A and substituted benzoquinones not commercially available were kindly provided by Dr. K. Folkers. Duroquinone, benzoquinone, trimethylbenzoquinone, and 2-methyl-1,4-benzoquinone, 1,2-napthoquinone, and juglone were obtained from K & K Laboratories, Inc. 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone was obtained from Arapahoe Chemicals, Inc. β-NADH, antimycin A, PCMB, sodium mersalyl, and *N*-ethylmaleimide were purchased from Sigma.

RESULTS

Several classes of substituted quinones were found to function as electron acceptors in the NADH dehydrogenase segment of the electron transport system. When electron transfer to O_2 was blocked by the addition of antimycin A and KCN, the quinones could serve as effective electron acceptors resulting in the oxidation of NADH. The activity as measured by the ability of the quinone to oxidize NADH enzymatically by electron transport particles was determined for three general classes of quinones (Table I). Generally benzoquinones and napthoquinones were found to function as electron acceptors. Anthraquinones, on the other hand, were found to be completely unreactive.

The rate of NADH oxidation varied greatly when equivalent concentrations of benzoquinones or napthoquinones were used. Methylated benzoquinones displayed

TABLE I

ACTIVITIES OF QUINONES AS ELECTRON ACCEPTORS WITH ELECTRON TRANSFER PARTICLES

The following quinones were inactive as electron acceptors: 2-hydroxy-1,4-napthoquinone, 2-hydroxy-3-methyl-1,4-napthoquinone, anthraquinone, anthraquinone-1,5-disulfonic acid, 1,4-diaminoanthraquinone, 2,6-diaminoanthraquinone, 1,8-diamino-4,5-dihydroxyanthraquinone, 1-aminoanthraquinone, 1-hydroxyanthraquinone.

Quinones	*Specific activity (μmoles NADH/min per mg protein)*	*Percent inhibition by $1 \cdot 10^{-6}$ M piericidin A*	*Concentration of piericidin A for 50% inhibition (mM)*
Benzoquinones			
1,4-Benzoquinone	0.23	7	$1 \cdot 10^{-5}$
2-Methyl-1,4-benzoquinone	0.34	15	$1 \cdot 10^{-5}$
2,3-Dimethyl-1,4-benzoquinone	0.22	40	$1 \cdot 10^{-5}$
2,5-Dimethyl-1,4-benzoquinone	0.23		
2,6-Dimethyl-1,4-benzoquinone	0.29		
2,3-Dimethyl-5-butyl-1,4-benzoquinone	0.23		
2,3,5-Trimethyl-1,4-benzoquinone	0.27	85	$1 \cdot 10^{-5}$
2,3,5,6-Tetramethyl-1,4-benzoquinone	0.25	93	$1 \cdot 10^{-5}$
2,5-Dimethoxy-3,6-dimethyl-1,4-benzoquinone	0.38	90	$1 \cdot 10^{-5}$
2,6-Dimethoxy-3,5-dimethyl-1,4-benzoquinone	0.27	82	$1 \cdot 10^{-5}$
2,3-Dimethoxy-5-methyl-6-chloro-1,4-benzoquinone	0.93	57	$2 \cdot 10^{-5}$
2,3-Dimethoxy-5-methyl-6-bromo-1,4-benzoquinone	1.03	50	$2 \cdot 10^{-5}$
2-Methoxy-3-hydroxy-5-methyl-6-chloro-1,4-benzoquinone	0.02	25	$2 \cdot 10^{-5}$
2,3-Dichloro-5-methyl-1,4-benzoquinone	2.72	34	$2 \cdot 10^{-5}$
2,3-Dichloro-5,6-dicyano-1,4-benzoquinone	0.23	0	
2,5-Dihydroxy-1,4-benzoquinone	0		
Ubiquinone 0	0.31	68	$1 \cdot 10^{-5}$
Ubiquinone 1	0.62	92	$1 \cdot 10^{-5}$
Ubiquinone 2	0.23	97	$5 \cdot 10^{-5}$
Napthoquinones			
1,4-Napthoquinone	0.29	37	$1 \cdot 10^{-5}$
1,2-Napthoquinone	1.97	2	$1 \cdot 10^{-5}$
2-Methyl-1,4-napthoquinone	0.16	73	$1 \cdot 10^{-5}$
5-Hydroxy-1,4-napthoquinone	0.43	21	$1 \cdot 10^{-5}$

moderate activity. Some of the halogenated benzoquinones (*e.g.* 2,3-dimethoxy-5-methyl-6-chloro or bromo-1,4-benzoquinone) were able to oxidize NADH at rates comparable to NADH oxidase rates. Moderate activities were obtained from napthoquinones such as 2-methyl-1,4-napthoquinone and 5-hydroxy-1,4-napthoquinone. 1,2-Napthoquinone, on the other hand, displayed high reactivity. It is interesting to note that both benzoquinones and napthoquinones with hydroxyl groups *ortho* to the carbonyl groups of the quinone (*e.g.* 2,5-dihydroxy-1,4-benzoquinone and 2-hydroxy-1,4-napthoquinone) did not function as electron acceptors.

The previous observation that ubiquinone 1 and 2 reduction could be inhibited by amytal or rotenone, while ferricyanide reduction was not affected by the same inhibitors[14], suggests the existence of multiple reduction sites for these electron acceptors. Recently piericidin A has been found to be an effective inhibitor of electron transport in the NADH oxidase system[25,26]. The proposed site(s) of interaction of piericidin A as well as amytal and rotenone appear(s) to be on the O_2 side of the non-heme iron of NADH dehydrogenase[27]. It was of interest to determine the effect of piericidin A on the rate of reduction of the quinones tested previously. When electron transport particles were treated with piericidin A at a concentration known to inhibit electron transport completely, quinone reductase activities were found to be inhibited to varying degrees. Concentrations of piericidin A greater than that necessary to inhibit NADH oxidase completely did not increase the degree of quinone reductase inhibition. Such a pattern of inhibition may be explained as selective reactivity of the quinones at sites before and after the piericidin A inhibition site and before the antimycin inhibition site.

The extent of piericidin A inhibition of methylated quinone reductase activities was found to increase as the number of methyl groups on the quinone ring increased. 1,4-Benzoquinone reduction and 2-methyl-1,4-benzoquinone reduction could be inhibited only to a limited extent. 2,3-Dimethyl-1,4-benzoquinone reduction was inhibited approx. 50%. 2,3,5-Trimethyl-1,4-benzoquinone and 2,3,5,6-tetramethyl-1,4-benzoquinone (duroquinone) reduction were almost completely inhibited by piericidin A. The location of the methyl groups on the quinone ring of dimethylbenzoquinones changed the degree of piericidin A inhibition only slightly. The inhibition of 2,5-dimethyl-1,4-benzoquinone, 2,3-dimethyl-1,4-benzoquinone, and 2,6-dimethyl-1,4-benzoquinone reduction was no greater than 55, 40, and 32% respectively.

Piericidin A inhibition of the reduction of other substituted benzoquinones is shown in Table I. Reduction of 2,5-dimethoxy-3,6-dimethyl-1,4-benzoquinone and 2,6-dimethoxy-3,5-dimethyl-1,4-benzoquinone was almost completely inhibited. When a halogen group was substituted for a methyl group as in the 6 ring position of 2,3-dimethoxy-5-methyl-1,4-benzoquinone, activities were inhibited approx. 50%. Further halogen or cyanide substitution decreased the inhibition. 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone reduction was completely insensitive to piericidin A treatment.

Variation in the degree of piericidin A inhibition also occurred when the ubiquinone and napthoquinone analogues were tested. In the ubiquinone series, ubiquinone 0 (no terpene side chain) reductase activity was inhibited approx. 70%. Ubiquinone 1 and 2 activities, on the other hand, were inhibited almost completely by piericidin A. The pattern of inhibition of the napthoquinone reductases showed 2-methyl-1,4-napthoquinone (vitamin K_3, menadione) reduction to be inhibited al-

most 75%. 1,4-Napthoquinone and 5-hydroxy-1,4-napthoquinone (juglone) activities were moderately inhibited. 1,2-Napthoquinone activity was almost completely insensitive to piericidin A.

Within the range of enzymes used routinely, the rate of NADH oxidation by these quinones was found to be proportional to the amount of enzyme added. All reactions were zero order with respect to the oxidation of NADH during the first 2 min of the assay. Study of the effect of quinone concentration at a fixed NADH concentration indicated apparent simple enzyme–quinone complex formation according to Michaelis. Addition of KCN or antimycin A at levels which inhibit NADH oxidase did not inhibit reduction of any of the quinones tested.

The pattern of piericidin A inhibition exhibited by the quinone reductases suggests that certain quinones such as 1,2-napthoquinone, 5-hydroxy-1,4-napthoquinone, and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone prefer to react at sites before the piericidin A inhibition site. Quinones such as ubiquinone 1 and 2 and 2,3,5,6-tetramethyl-1,4-benzoquinone interact primarily at sites after the piericidin A inhibition site but before the antimycin A inhibition site. The observation that these quinones show greater specificity to sites either before or after the piericidin A inhibition site allows for their selection as model quinones representative of interactions with particular sites in the NADH dehydrogenase. As a consequence, 5-hydroxy-1,4-napthoquinone (juglone) and 2,3,5,6-tetramethyl-1,4-benzoquinone (duroquinone) were chosen to be representative of their class of quinone and used in conjunction with ubiquinone 1 and 2 and ferricyanide in order to delineate four sites of quinone reduction in the enzyme complex. The remainder of this paper will concern the napthoquinone reduction site as represented by juglone reduction and the ferricyanide reduction site. The following paper will concern the remaining two sites as represented by the interaction of NADH dehydrogenase with ubiquinone 1 and 2 and duroquinone.

Enzyme preparations other than electron transport particles which are known to have NADH dehydrogenase activity were also examined in regard to their ability to reduce juglone and ferricyanide (Table II). All preparations of NADH dehydrogenase examined exhibited both juglone and ferricyanide reductase activities. Prepara-

TABLE II

JUGLONE AND FERRICYANIDE ACTIVITIES OF VARIOUS NADH DEHYDROGENASE PREPARATIONS

	Specific activity (μmoles NADH/min per mg protein)	
	Juglone	*Ferricyanide*
Beef heart mitochondria	0.24	4.7
Electron transport particles	0.42	14.7
NADH: cytochrome *c* reductase (Complex I + III)	4.00	221
NADH: coenzyme Q reductase (Complex I)	4.39	270
NADH dehydrogenase (electron transport particles + phospholipase A at 30°)	5.28	190
NADH dehydrogenase (Complex I + urea)	11.4	21.6
NADH: ubiquinone reductase (electron transport particles + heat–acid–ethanol)	28.0	91.7
NADH: quinone reductase (supernatant from sonicated beef heart mitochondria)	0.16	0.53

tion of electron transport particles from beef heart mitochondria increased the specific activity of both acceptors. Preparations such as NADH: cytochrome *c* reductase (Complex I + III), NADH: coenzyme Q reductase (Complex I), and NADH dehydrogenase (phospholipase A at 30°) exhibited moderate juglone reductase activity and high ferricyanide reductase activity. NADH dehydrogenase (Complex I + urea) and NADH ubiquinone reductase (heat–acid–ethanol) showed high juglone reductase activity and moderate ferricyanide reductase activity. The supernatant from sonicated beef heart mitochondria had low but detectable activities for both acceptors.

The presence of sulfhydryl groups in the NADH dehydrogenase has been demonstrated by CREMONA AND KEARNEY[16] and TYLER *et al.*[28]. Utilizing different incubation conditions, they were able to show differences in reactivity of the sulfhydryl groups in the enzymes towards mercurials such as PCMB and sodium mersalyl. The differences in reactivity of sulfhydryl groups can be utilized to show differences in the response of juglone and ferricyanide activities to mercurial treatment. When electron transport particles were incubated with PCMB (1 mM) at 0°, ferricyanide activity diminished slightly through the course of a 1-h incubation (Fig. 1). Juglone reduction was found to increase during the same incubation. If the temperature was increased to 30°, a greater activation of juglone reduction was observed (Fig. 2) than at 0°. Ferricyanide reductase activity decreased by approximately the same extent as at 0°. The 500% increase of juglone reductase activity at 30° was not due to heat treatment alone. The activation of juglone reductase occurred between the narrow concentration range of 0.1 and 1.0 mM PCMB (Fig. 3). The activation was greatest at approx. 0.8 mM PCMB at which concentration juglone reduction was increased over 10-fold. Ferricyanide reduction under identical conditions decreased slightly throughout the concentration range used. The low PCMB inhibition of ferri-

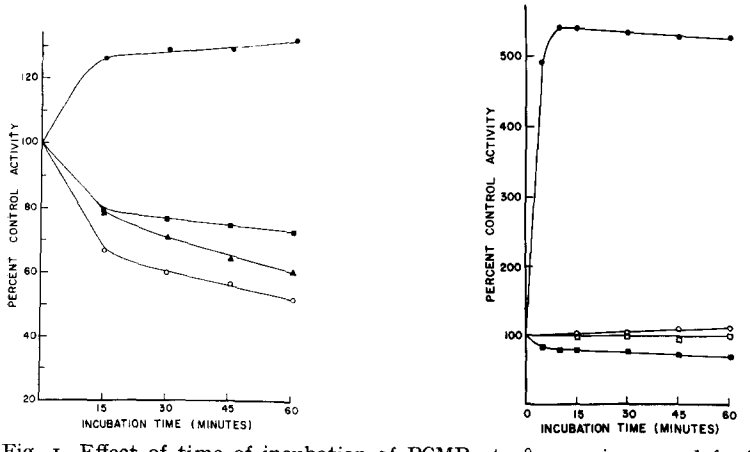


Fig. 1. Effect of time of incubation of PCMB at 0° on quinone and ferricyanide activities of electron transport particles. The enzyme was incubated with 1 mM PCMB in 0.05 M Tris–HCl (pH 7.4). ●—●, juglone activity; ■—■, ferricyanide activity; ▲—▲, ubiquinone 2 activity; ○—○, duroquinone activity.

Fig. 2. Effect of time of incubation of PCMB at 30° on juglone and ferricyanide activities of electron transport particles. The enzyme was incubated with 1 mM PCMB in 0.05 M Tris–HCl (pH 7.4). ●—●, juglone activity + PCMB + heat; ○—○, juglone activity + heat; ■—■ ferricyanide activity + PCMB + heat; □—□, ferricyanide activity + heat.

cyanide reductase activity in electron transport particles is in agreement with the previous findings of CREMONA AND KEARNEY[16].

The activation of quinone reduction was found to be unique to mercurial treatment and napthoquinone reduction. When sodium mersalyl and *N*-ethylmaleimide were used as sulfhydryl agents, only the former was found to activate juglone reduction (Table III). *N*-Ethylmaleimide, on the other hand, inhibited juglone reductase activity approx. 60% when incubated with electron transport particles in the same manner as the mercurials. TYLER *et al.*[28] found that when electron transport particles are preincubated with NADH, ferricyanide activity was inhibited almost completely by subsequent mersalyl treatment. Without NADH preincubation, ferricyanide reduction was only slightly decreased. Table III shows that NADH preincubation followed by PCMB treatment also causes an increased loss of ferricyanide activity from the control PCMB treatment. On the other hand, the PCMB activation of juglone reduction still occurred with an average 3–4-fold increase. However, the extent of

TABLE III

EFFECT OF SULFHYDRYL AGENTS ON JUGLONE AND FERRICYANIDE ACTIVITIES OF ELECTRON TRANSPORT PARTICLES

	Specific activity (μmoles NADH/min per mg protein)			
	*NADH: juglone reductase**		*NADH: ferricyanide reductase*	
	15 min	*30 min*	*15 min*	*30 min*
Control	0.27	0.26	12.8	12.6
PCMB (1 mM)	1.40	1.10	10.3	9.5
Mersalyl (1 mM)	0.93	1.11		
N-Ethylmaleimide (1 mM)	0.13	0.11		
NADH preincubation – PCMB (1 mM)	0.86	0.86	4.7	2.9

* NADH: juglone reductase measured in the presence of piericidin A (0.12 nmole) at 30°.

TABLE IV

EFFECT OF PCMB ON BENZOQUINONE AND NAPTHOQUINONE ACTIVITIES OF ELECTRON TRANSPORT PARTICLES

Quinone activity was measured in the presence of piericidin A (0.12 nmole).

Quinone acceptor	*Specific activity (μmoles NADH/min per mg protein)*	
	Electron transport particles	*Electron transport particles + PCMB**
2,3-Dichloro-5,6-dicyano-1,4-benzoquinone	0.21	0.16
2,5-Dimethyl-1,4-benzoquinone	0.09	0.07
1,4-Napthoquinone	0.17	0.65
5-Hydroxy-1,4-napthoquinone	0.26	1.12
2-Methyl-1,4-napthoquinone	0.03	0.17

* PCMB concentration, 1 mM; incubation at 30° for 1 h.

PCMB activation of juglone reductase was not as great with NADH preincubation as with PCMB treatment alone. Since quinones other than juglone showed piericidin A-insensitive activity, the effect of PCMB on the activities of these quinones was also examined (Table IV). PCMB was found to stimulate reduction of napthoquinones such as 1,4-napthoquinone and 2-methyl-1,4-napthoquinone approx. 5-fold. The reduction of benzoquinones such as 2,3-dichloro-5,6-dicyano-1,4-benzoquinone and 2,5-dimethyl-1,4-benzoquinone was decreased by PCMB treatment.

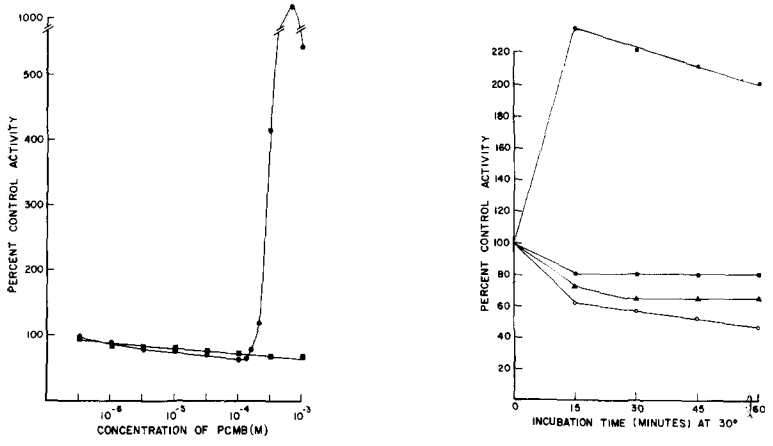


Fig. 3. Effect of PCMB concentration on juglone and ferricyanide activities of electron transport particles. The enzyme was incubated with PCMB in 0.05 M Tris–HCl (pH 7.4) at 30° for 30 min. ●—●, juglone activity; ■—■, ferricyanide activity.

Fig. 4. Effect of time of incubation of PCMB at 30° on juglone and ferricyanide activities of Complex I + III. The enzyme was incubated with 1 mM PCMB in 0.05 M Tris–HCl (pH 7.4). ●—●, juglone activity + PCMB + heat; ■—■, juglone activity + heat; ○—○, ferricyanide activity + PCMB + heat; ■—■, ferricyanide activity + heat.

PCMB activation of juglone reduction was not confined to electron transport particles preparations. NADH: cytochrome *c* reductase (Complex I + III) was also activated by PCMB treatment when juglone reductase activity was measured (Fig. 4). When ferricyanide reduction was examined with the same enzyme under identical conditions, the activity decreased slightly through the course of the mercurial treatment. The activation of juglone reduction and the slight decrease in ferricyanide reduction was quite similar to that response found with electron transport particles preparations. The NADH dehydrogenase preparation of RINGLER *et al.*[13] which retains high NADH: ferricyanide reductase activity and almost no ubiquinone reductase activity was also examined for mercurial activation. Fig. 5 shows that the 1 mM PCMB concentration used in previous experiments inhibits both juglone and ferricyanide reduction almost equally when compared to control activities. The almost complete loss of ferricyanide reductase activity with mercurial treatment of this enzyme is in agreement with the findings of CREMONA AND KEARNEY[16]. However, when the levels of PCMB are decreased, juglone reduction was found to be activated at lower concentrations of mercurial and inhibited at higher concentrations (Fig. 6). Maximal activation of juglone reductase activity occurred at 0.01 mM PCMB. Ferricyanide reduction, on the other hand, was inhibited throughout the concentra-

tion range of PCMB used. At the concentration of PCMB yielding maximal activation of juglone reduction (0.01 mM), ferricyanide reduction was inhibited approx. 65%.

The low molecular weight class of NADH dehydrogenase extracted from mitochondria by several chemical and physical treatments was also examined for the PCMB effect on juglone reductase activity. The NADH: ubiquinone reductase[10], which is representative of the class of dehydrogenases isolated from mitochondria following heat–acid–ethanol treatment, has been shown to have high quinone reductase activity.

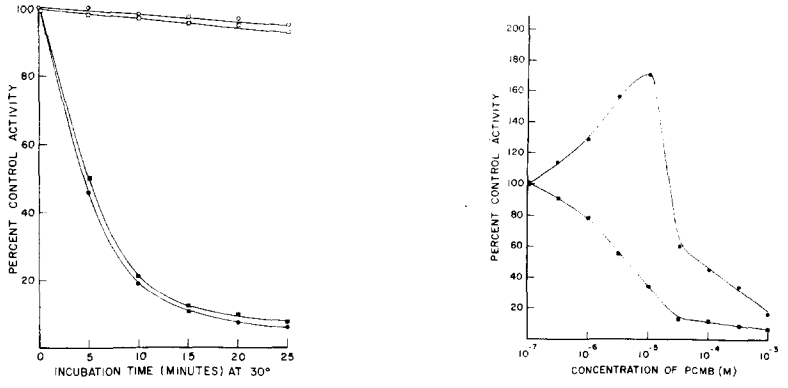


Fig. 5. Effect of time of incubation of PCMB at 30° on juglone and ferricyanide activities of Singer's enzyme. The enzyme was incubated with 1 mM PCMB in 0.05 M Tris–HCl (pH 7.4). ●—●, juglone activity + PCMB + heat; ○—○, juglone activity + heat; ■—■, ferricyanide activity + PCMB + heat; □—□, ferricyanide activity + heat.

Fig. 6. Effect of PCMB concentration on juglone and ferricyanide activities of Singer's enzyme. The enzyme was incubated with PCMB in 0.05 M Tris–HCl (pH 7.4) at 30° for 30 min. ●—●, juglone activity; ■—■, ferricyanide activity.

The enzyme has been proposed to be the primary NADH dehydrogenase of the respiratory chain[29] although recent evidence suggests that the enzyme has been modified following the heat–acid–ethanol treatment[6]. Fig. 7 shows that the juglone reductase activity of the enzyme was inhibited throughout the concentration range of the mercurial used. There was no evidence of activation at any concentration of PCMB. Furthermore, inhibition was immediate at 0° and did not require prior preincubation with PCMB as was necessary with the other preparations of NADH dehydrogenase. Ferricyanide reduction by heat–acid–ethanol preparations of NADH dehydrogenase has been previously shown to be inhibited by this mercurial[30].

A class of enzymes which have been frequently confused with the respiratory chain-linked NADH dehydrogenase is the lipoamide reductase (diaphorase) found in the soluble matrix of mitochondria. This enzyme is easily confused with the respiratory chain-linked enzyme due to its ability to reduce dyes and quinones with NADH. Therefore, the effect of PCMB on the ability of enzymes extracted by sonic disruption of beef heart mitochondria to catalyze the reduction of juglone was examined. At 1 mM PCMB, juglone reductase activity of these diaphorases was almost completely inhibited when the incubation was done at 30 or at 23° (Fig. 8). However when the diaphorase-containing fraction was incubated at 0° with PCMB, inhibition was only partial. When the concentration of PCMB was varied, inactivation occurred throughout the concentration range used when the enzyme was incubated with PCMB

at either 30 or 23° (Fig. 9). The extent of inhibition of juglone reductase activity increased as the temperature of incubation was increased. At 30° maximal inhibition was evident at approx. 0.05 mM PCMB, whereas at 23° maximal inhibition took place at 1 mM PCMB. Only at 0° was PCMB treatment found to be ineffective. The slight activation of juglone reduction at 0° did not exceed 10% of the control activity and did not occur at elevated incubation temperatures.

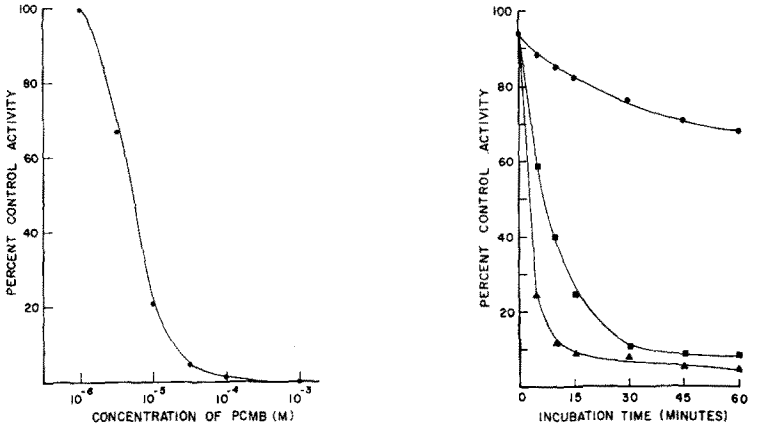


Fig. 7. Effect of PCMB concentration on juglone activity of supernatant derived from heat–acid–ethanol treatment of electron transport particles. The enzyme was incubated in 0.05 M Tris–HCl (pH 7.4) at 0° for 5 min.

Fig. 8. Effect of time of incubation of PCMB on juglone activity of diaphorase-containing supernatant. The enzyme was incubated with 1 mM PCMB in 0.05 M Tris–HCl (pH 7.4). ●—●, juglone activity + PCMB at 0°; ■—■, juglone activity + PCMB at 23°; ▲—▲, juglone activity + PCMB at 30°.

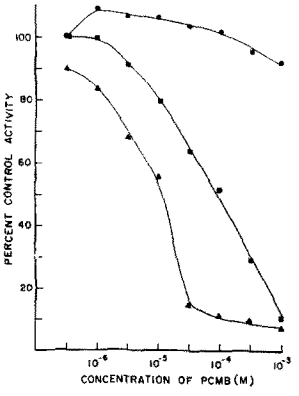


Fig. 9. Effect of PCMB concentration on juglone activity of diaphorase-containing supernatant. The enzyme was incubated with PCMB in 0.05 M Tris–HCl (pH 7.4) for 30 min. ●—●, juglone activity + PCMB at 0°; ■—■, juglone activity + PCMB at 23°; ▲—▲, juglone activity + PCMB at 30°.

DISCUSSION

When the reactivity of three general classes of quinones was investigated, benzoquinones and napthoquinones were found to be reduced by the NADH dehydro-

genase. Anthraquinones, on the other hand, were found to be completely unreactive. In general, the class of quinone (benzoquinone or napthoquinone) did not determine its ability to be reduced by the enzyme. In both series, activities varied from zero to values approaching maximum NADH oxidase rates. Furthermore, initial evidence indicated that the quinones were reduced at different sites in the NADH dehydrogenase by the observed differences in the extent of piericidin A inhibition. While some of the quinones in each class were reduced predominantly at sites before the piericidin A inhibition site, others were more specific for sites after the piericidin A inhibition site.

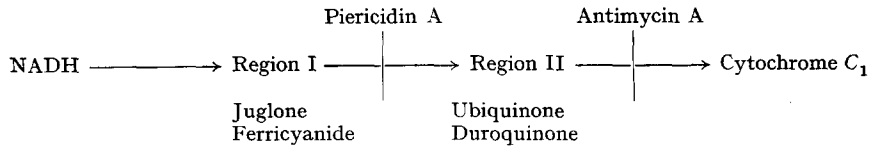
Enzyme activity and site specificity appear to involve several factors. For example, a change in site specificity was observed by changing the number of methyl groups on benzoquinone. As the number of methyl groups increased, sites after the piericidin A inhibition site were preferred. Increasing the number of methyl groups, which are electron releasing groups, lowers the redox potential of the quinone. The observed site specificity was opposite to what would be expected since electron flow in the NADH dehydrogenase system may go from NADH to carriers with successively higher redox potential. CHANCE *et al.*[31] have reported that the primary dehydrogenase is a low potential flavoprotein. The factor which may govern the specificity change in this case is the increased lipid solubility of benzoquinones with increased number of methyl groups.

Other substituted benzoquinones were found to vary widely in both activity and site specificity. Benzoquinones with methoxy and methyl groups favored sites after the piericidin A block. Addition of halogen groups in place of a methyl group on these compounds increased activity 3-fold and decreased piericidin A inhibition by over 50%. Further addition of halogen groups in place of the dimethoxy groups further increased activity and decreased piericidin A sensitivity.

When the napthoquinone series is considered, the position of the carbonyl groups significantly changes the activity and the piericidin A sensitivity. 1,2-Napthoquinone was over 5-fold as active as 1,4-napthoquinone. Furthermore, the activity of 1,2-napthoquinone was almost completely insensitive to piericidin A. Contrary to the findings with benzoquinones, these results would suggest that redox potential can play an important role in determining specificity and activity of some quinones. However, addition of a hydroxyl group in the 5 position of 1,4-napthoquinone causes a slight decrease in activity and an increase in piericidin A sensitivity. Thus, lipid solubility could again be a factor in the determination of site specificity.

The unreactivity of the anthraquinones is surprising since these quinones have favorable redox potentials for this segment of the electron transport system but they are generally water soluble. Also, the unreactivity of quinones containing hydroxyl groups *ortho* to the carbonyl groups is difficult to explain. 2-Hydroxy-3-methyl-1,4-napthoquinone which has a favorable redox potential for the primary dehydrogenase is not reduced by NADH dehydrogenase. Hydroxylation in the 5 position (juglone) gives a quinone which is active for sites before the piericidin A inhibition site.

The effect of piericidin A on the reduction of the various quinones provides for a convenient method for study of different sites of the NADH dehydrogenase. Inhibition varies according to the quinone utilized. A few of the quinones have the tendency to react almost exclusively at sites either before or after the piericidin A inhibition site as depicted in the following scheme.



By utilization of these quinones or ferricyanide, a further delineation of the NADH dehydrogenase could be made. A question which arose was whether the acceptors in Region I were reduced at a site common to both acceptors or at multiple sites. A similar question arose as to the ubiquinone and duroquinone reduction in Region II. Evidence presented in this paper and in the following paper suggest the presence of more than one site of reduction in each region.

The piericidin A-insensitive juglone and ferricyanide reduction of various preparations of NADH dehydrogenase were subjected to treatment with several sulfhydryl agents. MINAKAMI *et al.*[15] have shown that different sulfhydryl groups are present in the NADH dehydrogenase. Whereas reduction of externally added cytochrome *c* and ubiquinone by electron transport particles was progessively inhibited by mercurials such as PCMB, ferricyanide reduction was affected only partially by the same treatment. However, TYLER *et al.*[28] have shown that pretreatment of NADH dehydrogenase preparations with NADH followed by mercurial treatment caused almost complete inhibition of ferricyanide reduction. These observations led them to propose the presence of two different sulfhydryl groups, a fast reacting group located in the region of the primary dehydrogenase whose modification caused a loss of ferricyanide reduction and the disappearance of the electron paramagnetic signal for iron at $g = 1.94$ and a slow reacting group located at a higher redox potential of the electron transport system. During the same period, HORGAN *et al.*[26] presented evidence that the ferricyanide reductase activity of their purified NADH dehydrogenase could be almost completely inhibited by PCMB if the incubation took place in the absence of phosphate. The ferricyanide reductase activity of electron transport particles preparations, on the other hand, was inhibited only slightly by PCMB treatment as had been observed by others. They concluded that the site of ferricyanide reduction was spatially blocked in electron transport particles preparations and exposed upon extraction and purification of their enzyme.

The introduction of a new assay system involving juglone reduction in Region I has allowed for further examination and assessment of the mercurial treatment phenomenon. An important result of the application of this procedure was the finding that juglone reductase activity of NADH dehydrogenase preparations was activated upon mercurial treatment. Furthermore, the activation was quite significant in preparations such as electron transport particles in which over a 10-fold activation was observed. Ferricyanide reductase in such preparations responded to mercurial treatment by minor inactivation.

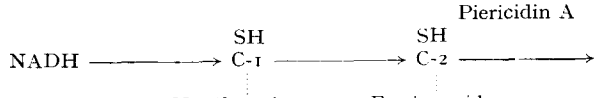
This mercurial activation suggests the possibility that napthoquinones such as juglone and ferricyanide are reduced by NADH dehydrogenase at different sites. The state of these sites in turn is influenced by sulfhydryl groups which respond differently to mercurial agents. An alternate possibility is that the activation phenomenon is the result of an opening effect which allows for greater interaction of larger molecules such as quinones with a reduction site which is normally accessible to small

molecules such as ferricyanide. However, this suggestion is difficult to explain in light of the observed mercurial inhibition of piericidin A-insensitive reduction of benzoquinones which is of the same order of magnitude as the mercurial inhibition of ferricyanide reduction. Furthermore, mercurial treatment preceded by NADH incubation did not abolish the activation of juglone reduction even though inhibition of ferricyanide reduction was almost complete. Also, the suggestion that juglone as a hydroxylated napthoquinone in combination with mercurial agent could cause the activation phenomenon had to be ruled out as activation occurred when 1,4-napthoquinone and 2-methyl-1,4-napthoquinone were used as electron acceptors.

The occurrence of mercurial activation of juglone reduction in various preparations of NADH dehydrogenase was investigated. The possibility that juglone reduction and mercurial activation of this activity was due to a component foreign to the NADH dehydrogenase was considered. The observed activation of juglone reduction in purified preparations of NADH dehydrogenase such as NADH: cytochrome *c* reductase (Complex I + III) and NADH dehydrogenase (phospholipase A at 30°) rules out the likelihood that a contaminant was responsible. Furthermore, the response of mercurial treatment of an isolated fraction containing the matrix protein NADH: lipoamide reductase showed no evidence of mercurial activation under conditions favorable for activation in NADH dehydrogenase-containing preparations.

Mercurial activation of juglone reduction did not occur in all preparations which were derived from the NADH dehydrogenase. NADH ubiquinone reductase displayed high juglone reductase activity. However, this activity was completely inhibited by mercurial at low incubation temperatures. This observation is in keeping with previous reports of mercurial inactivation of ferricyanide and cytochrome *c* reduction of heat–acid–ethanol preparations of NADH dehydrogenase[30] and is in agreement with the reported transformation which occurs as a result of this treatment[5]. Thus in this case there is no evidence to suggest that the site of juglone reduction is different from the site of ferricyanide reduction.

Thus the data would support the idea that the two sites of reduction are present before the piericidin A inhibition site. The sites differ in their ability to catalyze the reduction of napthoquinones such as juglone on the one hand, and ferricyanide on the other. The sites can be delineated by the response these activities have towards mercurial treatment. The reduction of juglone is activated as a result of mercurial treatment, is not lost with NADH preincubation and mercurial treatment, and does not occur in mitochondrial preparations which do not contain the respiratory chain enzyme or which contain modified preparations of the NADH dehydrogenase. The reduction of ferricyanide, on the other hand, is slightly diminished by mercurial treatment and lost completely upon preincubation with NADH followed by mercurial treatment. The results may be summarized as follows with C-1 and C-2 representing the respective electron transport carriers or sites on an enzyme complex.



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