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

II. DUROQUINONE REDUCTASE ACTIVITY

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SUMMARY

1. Enzymatic reduction of 2,3,5,6-tetramethyl-1,4-benzoquinone (duroquinone) by NADH can be used in an assay procedure for the NADH dehydrogenase. The reduction of this quinone occurs in the region of the electron transport system between the primary dehydrogenase and the cytochrome system as defined by the almost complete loss of reductase activity following piericidin A treatment.

2. Duroquinone reduction can be distinguished from ubiquinone 2 reduction by the marked inhibition of the former following phospholipase C, poly-L-lysine, or chloroquine diphosphate treatment. In addition, duroquinone reduction requires the presence of endogenous ubiquinone 10 specifically whereas ubiquinone 2 reduction does not require the presence of endogenous quinone. These observations are consistent with the nonequivalency of the reduction sites of duroquinone and ubiquinone 2.

3. Duroquinol can be utilized as an electron donor for the energy-linked reduction, of NAD^+. Duroquinol reduction of NAD^+ is dependent upon the presence of ATP, is inhibited by oligomycin, carbonyl cyanide *p*-trifluoro methoxyphenylhydrazone and piericidin A, and is not inhibited by antimycin A at levels which inhibit electron transport.

4. Duroquinone reduction as well as ubiquinone 2 reduction are inhibited almost completely by phospholipase A, *p*-chloromercuribenzoate, *o*-phenanthroline, and Triton X100 treatments.

INTRODUCTION

The extent of ubiquinone 10 function in the mitochondrial electron transport system has been debated for a number of years. The presence of ubiquinone in electron transport systems has been known since the early studies in GREEN's and MORTON's laboratories (see refs. 1 and 2). However, some question has remained concerning a primary[3] or secondary[4] role of the quinone in the electron transport pathway. Recently LENAZ *et al.*[5] have shown a specific requirement for ubiquinone 10 in the NADH

Abbreviations: Duroquinone, 2,3,5,6-tetramethyl-1,4-benzoquinone; FCCP, carbonyl cyanide *p*-trifluoro methoxyphenylhydrazone; PCMB, *p*-chloromercuribenzoate; ETP, electron transport particles; TTFA, thenoyl-trifluoroacetone.

oxidase system following extraction of the quinone from mitochondria with dry pentane. Ubiquinone 2 could not replace ubiquinone 10 for efficient restoration of NADH oxidase activities in these deficient particles.

The difference of opinion as to ubiquinone 10 function in the NADH oxidase system has been further complicated by the lack of information as to the identity of the other NADH dehydrogenase components located in the ubiquinone 10 region. This region is known to be sensitive to treatment with polar solvents[6], detergents[7], and snake venom phospholipase A (refs. 8 and 9). Ubiquinone 10 reduction, on the other hand, is inhibited by electron transport inhibitors such as Amytal, rotenone and piericidin A (refs. 10–12).

Assays in the ubiquinone 10 region of the NADH dehydrogenase complex have relied almost exclusively on the direct spectrophotometric technique[3,4] or to the reduction of externally added ubiquinone. Ubiquinone 10 is difficult to apply to assay procedures due to its insolubility in aqueous systems. However, assay of the reduction of externally added ubiquinone 10 has been developed by PHARO *et al.*[13] and utilized with their preparation of NADH dehydrogenase. The lower isoprenologues of ubiquinone 1 and 2, have been used as electron acceptors for the purification of NADH coenzyme Q reductase (Complex I)[14]. However, the reduction of these lower isoprenologues by NADH dehydrogenase has not been completely understood. MACHINIST AND SINGER[8] have suggested that the reduction of externally added ubiquinone requires the presence of endogenous ubiquinone 10 as well as phospholipid.

We have observed that various quinones can function as electron acceptors in the region of the NADH dehydrogenase (see previous paper). Furthermore, the reduction of one of these quinones, 2,3,5,6-tetramethyl-1,4-benzoquinone (duroquinone) is inhibited by piericidin A and thus resembles the reduction of ubiquinone in this regard. The present study will examine the relation between duroquinone and ubiquinone 2 reduction.

MATERIALS AND METHODS

Materials

Beef heart mitochondria, electron transport particles (ETP), NADH cytochrome *c* reductase (Complex I + III), NADH coenzyme Q reductase (Complex I), NADH dehydrogenase (phospholipase A at 30°), NADH dehydrogenase (Complex I + urea), NADH ubiquinone reductase (ETP + heat–acid–ethanol), and NADH diaphorase were prepared as described in the preceding communication[15]. Phosphorylating ETP were obtained by the method of HANSEN AND SMITH[16]. Phospholipase A was purified from *Naja naja* venom according to the method of CREMONA AND KEARNEY[17]. After acid precipitation, the solution was applied to a Sephadex G75 column and 5-ml fractions were collected. The highest activity fractions (manometric assay) were combined, lyophilized, and reapplied to another Sephadex G75 column. 5-ml fraction were collected and the highest activity fractions were used in the phospholipase A treatment.

Methods

Assay of NADH ubiquinone 2 reductase and NADH duroquinone reductase was the same as described in the preceding communication[15]. NADH cytochrome *c*

reductase was carried out under identical conditions as the NADH quinone reductases except that 1.0 mg of cyrochrome *c* was substituted for the quinone and antimycin A was omitted. Succinate cytochrome *c* reductase was assayed according to the method of TISDALE[18] at 30°.

Energy-linked reduction of NAD^+ was assayed according to ERNSTER AND LEE[19]. 0.6 μmole of reduced quinone in 0.02 ml of absolute ethanol was substituted for succinate when quinone was used as electron donor. Quinone reduction was accomplished by addition of potassium borohydride to a 0.05 M ethanolic solution of quinone. The pH was lowered to neutrality by the addition of 1 M hydrochloric acid. The neutralized solution was extracted with diethyl ether and the ether layer was washed three times with distilled water. The ether was aspirated on a rotary aspirator and the quinol kept under an atmosphere of nitrogen until the time of assay when it was dissolved in absolute ethanol and used immediately.

The rate of hydrolysis of phospholipids by phospholipase A was measured by quantitative thin-layer chromatography on silica gel g in the solvent system chloroform–methanol–water (100:40:6, by vol.). Aliquots of the digests containing at least 20 mg lipids were extracted (Folch procedure) under a pure nitrogen atmosphere and the solvent removed below 20°. The dried samples were redissolved in a known volume of solvent and 1 to 1.5 mg of lipid (based on phosphorus determination) were spotted on plates and phosphorus analyses were carried out on well-resolved spots. Purified lipids were taken to have a phosphorus content of 4 % (ref. 20). The rate of hydrolysis of phospholipids by phospholipase C was measured by estimating phosphorus release in the aqueous phase and by thin-layer chromatography.

Extraction of ubiquinone 10 was done by the method of SZARKOWSKA[21]. ETP were lyophilized, extracted 3 times with pentane for 30-min intervals, filtered, aspirated under vacuum and washed with 0.25 M sucrose–0.01 M Tris–HCl pH 7.4 buffer. Assays were performed immediately after the extraction procedure. Restoration was accomplished by addition of ubiquinone in ethanol in 0.01 ml amounts directly to the cuvette. The system was allowed to stabilize approximately 30 sec before the assay was initiated.

Polyacrylamide gel electrophoresis was accomplished according to the method of TAKAYAMA *et al.*[22]. The acetone-extracted proteins (residual acetone was removed by vacuum) were dissolved in phenol–acetic acid to a concentration of 20 mg/ml. 0.01, 0.02, and 0.03 ml amounts were applied to the columns. 0.02 ml was found to give the best concentration for most fractions. The electrophoresis was run at 1.5 mA per tube for 10 min in order to stack the protein against the gel. The current was then adjusted to 5 mA per tube and the electrophoresis run an additional 80 min. The gels were stained in 0.5 % amido black instead of the suggested 1 %. Microdensitometer tracings of the gels were made by placing the gels in a glass holder and scanning them on a Joyce–Loebl microdensitometer.

Phosphorus was estimated by the method of CHEN *et al.*[23] and protein was assayed by the modified biuret method of YONOTANI[24].

Chemicals

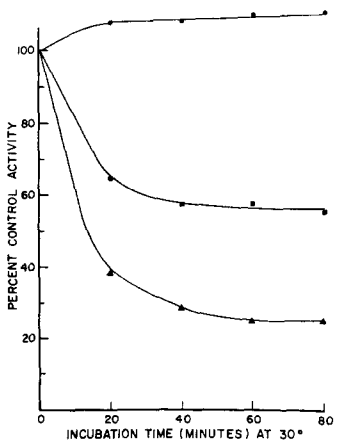
Ubiquinone 2 was kindly provided by Dr. A. F. Wagner of Merck, Sharp, and Dohme. Piericidin A was kindly provided by Dr. K. Folkers. 2,3,5,6-tetramethyl-1,4-benzoquinone (duroquinone) and 2-methyl-1,4-benzoquinone were obtained from

Aldrich. *Naja naja* venom, phospholipase C type I, *p*-chloromercuribenzoate (PCMB), chloroquine and primaquine diphosphate, cytochrome *c* type III, β-NADH, and antimycin A were purchased from Sigma. Poly-L-lysine (mol. wt., 62000) was obtained from Miles Laboratory.

RESULTS

When ETP were treated with piericidin A in order to block electron transport in the NADH oxidase system, it was found that quinone reductase activities of this preparation were inhibited to varying degrees depending on which quinone was used as the electron acceptor[15]. In addition, the reduction of ubiquinone 2 and duroquinone was almost completely inhibited by piericidin A suggesting that these acceptors were reduced by sites almost exclusively in region 2 after the piericidin A inhibition site but before the antimycin A inhibition site.

The question which arose previously in regard to the mode of juglone and ferricyanide reduction by NADH dehydrogenase in the region before the piericidin A inhibition site also applied to the reduction of ubiquinone 2 and duroquinone. It was important to determine whether the reduction of these quinones was occurring at one and the same site or at different sites. That the latter may be the case can be seen by the effects of phospholipase C, poly-L-lysine, and the chloroquinone diphosphate on the reduction of ubiquinone 2 and duroquinone. When ETP were incubated with 0.05 mg phospholipase C/mg ETP protein at 30°, duroquinone activity was lost to a greater extent than ubiquinone 2 activity (Fig. 1). After 60 min incubation, 40 % of the ubiquinone 2 activity was lost while over 75 % of the duroquinone activity was lost. During this digestion, 90 % of the lecithin and only 10 % of the phosphatidylethanolamine were hydrolyzed by phospholipase C. Cardiolipin was not hydrolyzed at this concentration of phospholipase C.



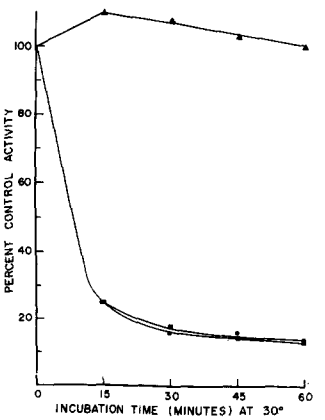


Fig. 1. Effect of phospholipase C (*Clostridium welchii*) treatment of ETP on quinone activities. ETP were treated with 0.05 mg phospholipase C/mg protein at 30°. ●—●, juglone activity; ■—■, ubiquinone-2 activity; ▲—▲, duroquinone activity.

Fig. 2. Effect of phospholipase A (*Naja naja*) treatment of ETP on quinone activities. ETP were incubated with 0.05 μg phospholipase A/mg ETP at 30°. ▲—▲, juglone activity; ●—●, ubiquinone-2 activity; ■—■, duroquinone activity.

Phospholipase A from *Naja naja* venom (0.05 μg/mg ETP protein) caused equal inhibition of both ubiquinone 2 and duroquinone reductase activities during the digestion (Fig. 2). Both activities decreased over 80% from control values. Phospholipase A digestion caused the hydrolysis of 69% of the lecithin and almost 100% of the phosphatidylethanolamine during the 60-min digestion period[34]. No cardiolipin breakdown occurred at the level of phospholipase A used.

The greater loss of duroquinone reductase activity *versus* ubiquinone 2 reductase activity was also observed following treatment of ETP with poly-L-lysine (Fig. 3).

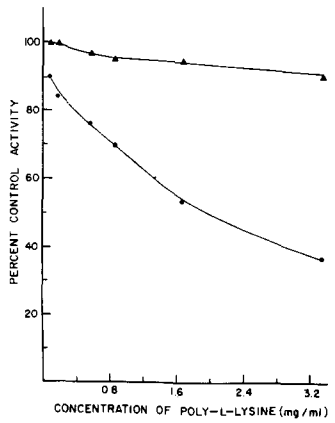


Fig. 3. Effect of poly-L-lysine concentration on quinone activities of ETP. ▲—▲, ubiquinone-2 activity; ●—●, duroquinone activity.

At 3.2 mg poly-L-lysine/ml, ubiquinone 2 reduction decreased only slightly from control values (<6%). Duroquinone reduction, on the other hand, was inhibited over 60% at this concentration of poly-L-lysine. Others[25,35] have previously shown the potent inhibitory effect of several basic proteins to electron transport function. Table I shows the effect of poly-L-lysine on electron transport function as expressed by NADH cytochrome *c* reductase activity. In this case, the basic protein inhibited NADH cytochrome *c* reductase almost completely at very low levels ($1 \cdot 10^{-3}$ mg poly-L-lysine/ml). Thus it is apparent that poly-L-lysine acts at several sites in the NADH oxidase system. Significantly, the loss of duroquinone reductase activity

TABLE I

EFFECT OF POLY-L-LYSINE CONCENTRATION ON NADH CYTOCHROME *c* REDUCTASE ACTIVITY OF ETP

Poly-L-lysine concentration (mg/ml)	*Specific activity (μmoles NADH/min per mg protein)*	*% of control*
Control	0.80	100
$1.4 \cdot 10^{-4}$	0.70	87
$4.2 \cdot 10^{-4}$	0.31	39
$4.2 \cdot 10^{-3}$	0.03	3
$4.2 \cdot 10^{-2}$	0.03	3
0.33	0.03	3
1.67	0.04	5

without concomitant loss of ubiquinone 2 reductase activity by poly-L-lysine treatment supports the suggestion that these quinones are reduced at nonequivalent sites.

Ubiquinone 10 is said to function in the mitochondrial electron transport system in the region between the primary dehydrogenase and the cytochrome system[26]. As a consequence of its location in the region of the NADH dehydrogenase, it was of interest to study the relationship between ubiquinone 10 and the ability of NADH dehydrogenase to catalyze the reduction of ubiquinone 2 and duroquinone. Table II shows the effect of removing ubiquinone 10 from ETP by dry pentane extraction on quinone reductase activities. Duroquinone reductase activity was lost almost completely following extraction of ubiquinone 10. Ubiquinone 2 reductase activity, on the other hand, remained essentially constant whether or not ubiquinone 10 was present in the enzyme preparation. Juglone reduction decreased slightly as a result of loss of the piericidin A sensitive part of the juglone activity. When ubiquinone 10 was restored to the extracted particles, duroquinone reductase activity increased to approximately 70 % of the control nonextracted activity. The restored duroquinone reductase activity was piericidin A sensitive. Ubiquinone 2 activity remained constant following the addition of ubiquinone 10. Oxidation of NADH by ubiquinone 10 was negligible at the levels of quinone used in this experiment unless ubiquinone 2 or duroquinone were present under the specified assay conditions.

TABLE II

EFFECT OF REMOVAL OF UBIQUINONE 10 BY PENTANE EXTRACTION OF ETP

	Specific activity (μmoles NADH/min per mg protein)		
	Juglone	*Duroquinone*	*Ubiquinone 2*
Control (lyophilized ETP)	0.32	0.22	0.15
Control + piericidin A	0.26	0.02	0.02
Pentane-extracted ETP	0.25	0.02	0.16
Extracted ETP + ubiquinone 10	0.29	0.16	0.16
Extracted ETP + ubiquinone 10 + piericidin A	0.25	0.02	0.02

The specificity of restoration of duroquinone reductase activity by addition of ubiquinone to ubiquinone 10 deficient particles was also considered (Table III). LENAZ *et al.*[5] have shown that ubiquinone 10 was necessary for complete restoration of NADH oxidase activity of mitochondrial particles deficient in ubiquinone 10 following dry pentane extraction. They found that ubiquinone 2 could not substitute effectively for ubiquinone 10 in the restoration electron transport function in the NADH oxidase system. Table III shows that restoration of NADH duroquinone reductase activity as well as NADH cytochrome *c* reductase activity require the presence of ubiquinone 10. Addition of $5 \cdot 10^{-7}$ mole ubiquinone 10/mg protein restored 94 % of the cytochrome *c* reductase activity and 76 % of the duroquinone reductase activity. Addition of ubiquinone 2, on the other hand, caused only partial restoration of activity. Restoration was no greater than 40 % for cytochrome *c* reductase and 33 % for duroquinone reductase at comparable levels of quinone.

TABLE III

RESTORATION OF NADH CYTOCHROME *c* AND DUROQUINONE REDUCTASE ACTIVITIES BY UBIQUINONE 10 AND 2 FOLLOWING PENTANE EXTRACTION OF ETP

Amount of ubiquinone added (moles/mg protein)	*NADH cytochrome c reductase*		*NADH duroquinone reductase*	
	*Specific activity**	*% of control*	*Specific activity**	*% of control*
Control (lyophilized ETP)	0.64	100	0.19	100
Pentane-extracted ETP	0.02	3	0.02	9
Ubiquinone 10				
$1.7 \cdot 10^{-8}$	0.25	39	0.03	17
$3.4 \cdot 10^{-8}$	0.33	52	0.04	22
$1.7 \cdot 10^{-7}$	0.40	62	0.07	38
$3.4 \cdot 10^{-7}$	0.55	86	0.11	57
$5.1 \cdot 10^{-7}$	0.60	94	0.15	76
Ubiquinone 2				
$2.8 \cdot 10^{-8}$	0.08	12	0.05	27
$5.6 \cdot 10^{-8}$	0.20	32	0.06	33
$1.1 \cdot 10^{-7}$	0.25	40	0.06	33
$5.6 \cdot 10^{-7}$	0.22	35	0.05	24

* Specific activity = μmoles NADH/min per mg protein.

SKELTON *et al.*[27] have suggested that the antimalarial agents, chloroquine and primaquine diphosphate, which inhibit electron transport acted in the region of ubiquinone 10 function. Utilizing the quinone reductase assays, this suggestion was tested. Table IV shows the effect of chloroquine diphosphate treatment of ETP on quinone and ferricyanide reductase activities. Chloroquine was found to inhibit duroquinone reductase activity almost completely. Ubiquinone 2 reductase activity decreased only 35 % of its control activity. Juglone reductase activity decreased slightly to the extent of its piercidin A sensitive activity. Ferricyanide reductase, on the other hand, increased following chloroquine treatment by approximately 40 % of its control activity. Thus the nonequivalency of the ubiquinone 2 and duroquinone reduction sites was also observed following chloroquine treatment. That these antimalarial agents inhibit electron transport function can be seen in Table V. Chloroquine and primaquine diphosphate both inhibit cytochrome *c* reductase

TABLE IV

EFFECT OF CHLOROQUINE DIPHOSPHATE ON NADH QUINONE AND FERRICYANIDE REDUCTASE ACTIVITIES OF ETP

Concentration of chloroquine (M)	*Specific activity (μmoles NADH/min per mg protein)*			
	Juglone	*Ferricyanide*	*Ubiquinone 2*	*Duroquinone*
Control	0.31	12.4	0.20	0.19
$1.7 \cdot 10^{-6}$	0.30	12.4	0.18	0.14
$3.3 \cdot 10^{-6}$	0.29	13.3	0.18	0.12
$1.7 \cdot 10^{-5}$	0.29	15.8	0.16	0.13
$3.3 \cdot 10^{-5}$	0.29	17.2	0.13	0.04

TABLE V

EFFECT OF CHLOROQUINE AND PRIMAQUINE DIPHOSPHATE ON NADH CYTOCHROME *c* REDUCTASE ACTIVITY OF ETP

Concentration of inhibitor (M)	*Specific activity (μmoles NADH/min per mg protein)*	
	Chloroquine	*Primaquine*
Control	0.51	0.51
$3.3 \cdot 10^{-6}$	0.47	0.47
$1.7 \cdot 10^{-5}$	0.29	0.47
$3.3 \cdot 10^{-5}$	0.20	0.38
$1.7 \cdot 10^{-4}$	0.08	0.15
$3.3 \cdot 10^{-4}$	0.06	0.11

activity of ETP. Chloroquine appears to be a slightly more effective inhibitor than primaquine.

In light of the observations made above, the use of both ubiquinone 2 and duroquinone in assay procedures provides for a method to assess the capability of published preparations of solubilized NADH dehydrogenase to catalyze the reduction of these quinones. Table VI shows the distribution of ubiquinone 2 and duroquinone reductase activities in several preparations of NADH dehydrogenase. NADH cytochrome *c* reductase (Complex I + III) was able to reduce both ubiquinone 2 and duroquinone effectively. Both activities were inhibited almost completely by piericidin A. Further purification of this enzyme gives NADH coenzyme Q reductase (Complex I) which retains ubiquinone 2 reductase activity but shows diminished duroquinone reductase activity. The NADH dehydrogenase prepared by phospholipase A treatment at 30° displayed low levels of ubiquinone 2 and duroquinone reductase activities which were found to be insensitive to piericidin A treatment. Urea treatment of Complex I and heat–acid–ethanol treatment of ETP gave enzymes which had moderately high ubiquinone 2 and duroquinone reductase rates. However

TABLE VI

UBIQUINONE 2 AND DUROQUINONE ACTIVITIES OF VARIOUS NADH DEHYDROGENASE PREPARATIONS
BHM, beef heart mitochondria.

	Specific activity (μmoles NADH/min per mg protein)	
	Ubiquinone 2	*Duroquinone*
BHM	0.14	0.16
ETP	0.23	0.22
NADH cytochrome *c* reductase (Complex I + III)	1.6	1.5
NADH coenzyme Q reductase (Complex I)	2.1	0.38
NADH dehydrogenase (ETP + phospholipase A at 30°)	0.25*	0.32*
NADH dehydrogenase (Complex I + urea)	1.4*	2.3*
NADH ubiquinone reductase (ETP + heat–acid–ethanol)	4.8*	7.3*
NADH Quinone Reductase (supernatant from sonicated BHM)	0.03	0.13

* Quinone reduction not inhibited by piericidin A.

as before, the activities were not inhibited by piericidin A. The supernatant from sonically disrupted mitochondria catalyzed low levels of piericidin insensitive reduction of these quinones. Thus only the more complex enzyme preparations (*e.g.* ETP and Complex I + III) were observed to effectively catalyze the piericidin A sensitive reduction of both ubiquinone 2 and duroquinone.

CHANCE[28] and ERNSTER[29] have shown that succinate can be used as an electron donor for the reduction of NAD^+ if energy is supplied in the form of ATP. Since duroquinone and ubiquinone 2 activities of ETP are almost completely inhibited by piericidin A, this would suggest that the sites of their reduction could be at or after coupling site one which has been localized in the NADH dehydrogenase segment of the electron transport system[30]. Table VII shows that the reduced form of duroquinone, duroquinol, can be used as an electron donor for the energy-linked reduction of NAD^+ in ETPH (phosphorylating electron transport particles). The reduction of NAD^+ by duroquinol was completely dependent on ATP and inhibited by piericidin A and oligomycin and FCCP (carbonyl cyanide *p*-trifluoro-methoxyphenylhydrazone), inhibitors of oxidative phosphorylation. The activity was not affected by levels of antimycin A which inhibit electron transport to cytochrome *c*. Reduced ubiquinone 2 and 2-methyl-1,4-benzoquinone did not function as electron donors for the energy-linked reduction of NAD^+.

TABLE VII

DUROQUINONE AS SUBSTRATE FOR THE ENERGY-LINKED REDUCTION OF NAD^+ WITH PHOSPHORYLATING ETP

Additions	*Specific activity (μmoles NADH/min per mg protein)*			
	Succinate	*Duroquinol*	*Ubiquinol 2*	*2-Methyl-1,4-benzoquinol*
No ATP	0	0	0.02	0.02
+ ATP	0.17	0.09	0.02	0.01
+ Piericidin A*	0	0	0.01	0.02
+ Antimycin A**	0.17	0.08	0.02	0.02
+ Oligomycin***	0	0		
+ FCCP§	0	0		

* Piericidin A, 0.12 nmoles added.
** Antimycin A, 0.05 μg added.
*** Oligomycin, 1.2 μg added.
§ FCCP, 3.0 nmoles added.

MINAKAMI *et al.*[31] reported that NADH oxidase and the reduction of externally added ubiquinone, of endogenous ubiquinone 10, and of cytochrome *c* were inhibited by PCMB. They found that ferricyanide reduction was only slightly inhibited by this mercurial. The effect of PCMB treatment of ETP at 30° on NADH ubiquinone 2 and duroquinone reductase activities is shown in Fig. 4. Both ubiquinone 2 and duroquinone reduction were inhibited almost completely by 1 mM PCMB within the first 30 minutes of incubation at 30°. Also the rate of decrease for both activities was approximately the same. The effect of PCMB concentration on the reduction of ubiquinone 2 and duroquinone is given in Fig. 5. Mercurial inhibition occurred

between 0.05 mM and 1 mM PCMB. Maximum inhibition was apparent at PCMB concentrations of 0.5 mM or greater.

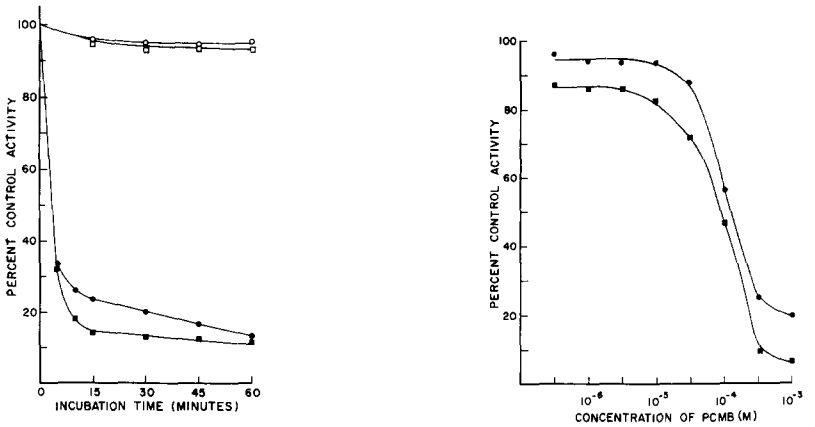


Fig. 4. Effect of time of incubation of PCMB at 30° on ubiquinone-2 and duroquinone activities of ETP. The enzyme was incubated with 1 mM PCMB in 0.05 M Tris–HCl, pH 7.4. ●—●, ubiquinone-2 activity + PCMB + heat; ○—○, ubiquinone-2 activity + heat; ■—■, duroquinone activity + PCMB + heat; □—□, duroquinone activity + heat.

Fig. 5. Effect of PCMB concentration on ubiquinone-2 and duroquinone activities of ETP. Th[e] enzyme was incubated with PCMB in 0.05 M Tris–HCl pH 7.4 at 30° for 30 min. ●—●, ubiquinone-2 activity; ■—■, duroquinone activity.

o-Phenanthroline, an iron chelator, has been reported to inhibit electron transport in the NADH oxidase system[33]. Addition of *o*-phenanthroline to ETP inhibited ubiquinone 2 and duroquinone reductase activity without affecting juglone and ferricyanide reductase activity (Fig. 6). When another iron chelator, thenoyl-

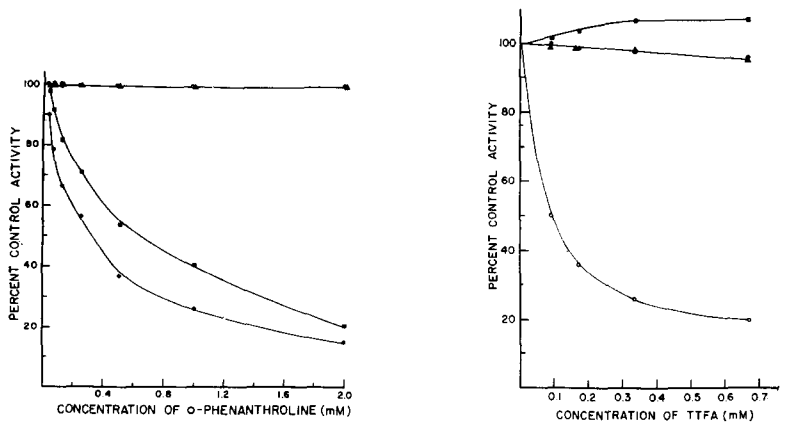


Fig. 6. Effect of *o*-phenanthroline concentration on quinone and ferricyanide activities of ETP. *o*-Phenanthroline was dissolved in absolute ethanol and added in 10 μl amounts. ○—○, juglone activity; ▲—▲, ferricyanide activity; ■—■, ubiquinone-2 activity; ●—●, duroquinone activity.

Fig. 7. Effect of TTFA (thenoyltrifluoroacetone) concentration on quinone and cytochrome *c* activities of ETP. TTFA was dissolved in absolute ethanol and added in 10 °1 amounts. ○—○, succinate cytochrome *c* reductase; ▲—▲, NADH cytochrome *c* reductase; ■—■, NADH ubiquinone-2 reductase; ●—●, NADH duroquinone reductase.

trifluoroacetone (TTFA), was used in place of *o*-phenanthroline, TTFA did not inhibit NADH quinone reductase activities of ETP (Fig. 7). However, TTFA was found to be an effective inhibitor of succinate cytochrome *c* reductase in agreement with previous published accounts[32] which suggest that the iron chelator inhibits a component of the succinate dehydrogenase complex.

Anionic detergents such as the bile salts and nonionic detergents such as Triton have been used for solubilization and purification of NADH dehydrogenase[14,33]. Their widespread use has prompted the study of their effect on quinone reductase activities of ETP preparations. Addition of potassium deoxycholate (10% stock solution, pH 8) to ETP caused a moderate decrease in NADH ubiquinone 2 and duroquinone reductase activities at higher levels of detergent (Fig. 8). Juglone and ferricyanide reduction were not affected by deoxycholate treatment while NADH cytochrome *c* reductase was activated over 300%. The use of the nonionic detergent, Triton X100 (10% stock solution) markedly affected ubiquinone 2 and duroquinone reduction (Fig. 9). Both activities decreased almost 80% at higher levels of detergent. Loss of cytochrome *c* reduction was also evident. Juglone and ferricyanide reduction changed only slightly, the former activity increased slightly at lower levels of Triton X100.

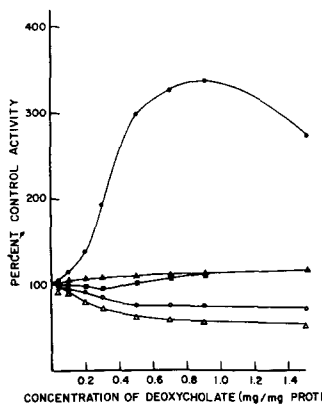


Fig. 8. Effect of deoxycholate concentration on quinone, ferricyanide, and cytochrome *c* activities of ETP. 10% (w/v) potassium deoxycholate, pH 9, was added to ETP and incubated 15 min at 0° before assay. ▲—▲, juglone activity; ■—■, ferricyanide activity; ○—○, ubiquinone-2 activity; △—△, duroquinone activity; ●—●, cytochrome *c* activity.

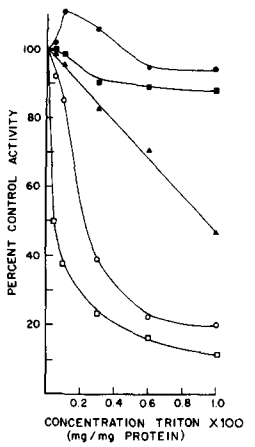


Fig. 9. Effect of triton X100 concentration on quinone, ferricyanide, and cytochrome *c* activiteis of ETP. 10% Triton X100 (w/v) was added to ETP and incubated 15 min at 0° before assay. ●—●, juglone activity, ■—■, ferricyanide activity; ○—○, ubiquinone-2 activity; □—□, duroquinone activity; ▲—▲, cytochrome *c* activity.

DISCUSSION

From these results and those of the preceeding paper[15] we conclude that four sites can be defined for interaction of external acceptors with the NADH dehydrogenase complex. The relation of these sites to acceptors and inhibitors is outlined in Fig. 10. Each site may be a separate electron carrier "C" or more than one site may be associated with a single protein. The selective effects of PCMB on juglone and

ferricyanide reduction have been discussed previously. Selective inhibition of the duroquinone site is evident in the effects of phospholipase C, polylysine and chloroquine diphosphate. Both ubiquinone and duroquinone reduction is inhibited by piericidin A and rotenone as well as by phospholipase A, *o*-phenanthroline, PCMB and Triton X-100.

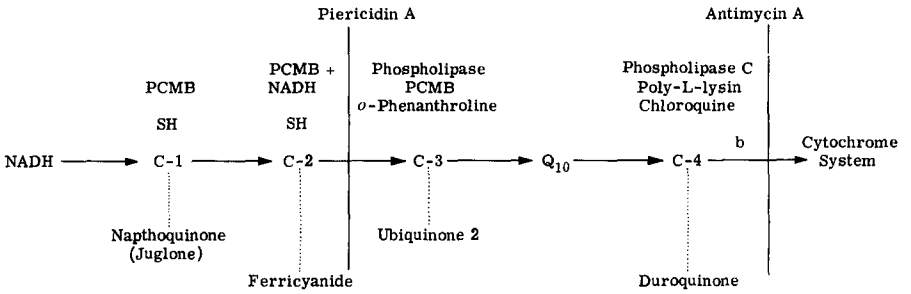


Fig. 10. Diagram of the quinone reduction sites in the NADH dehydrogenase complex. See text for discussion of these sites.

The loss of duroquinone reduction is correlated with hydrolysis of lecithin by phospholipase C which suggests that lecithin is required at this site. The effects of phospholipase A may not be directly dependent on loss of phospholipid since the products of hydrolysis act as inhibitors of both ubiquinone and duroquinone reduction.

SKELTON *et al.*[27] have suggested that chloroquine and primaquine diphosphate inhibit NADH oxidase in the region of ubiquinone function. Since duroquinone reduction is inhibited much more than ubiquinone by these agents we suggest that they inhibit the oxidation of ubiquinol rather than the reduction of ubiquinone.

The specific requirement for ubiquinone 10 for restoration of duroquinone reduction after pentane extraction also suggests a specific site for reoxidation of long chain ubiquinones and supports the idea that C4 may be a separate carrier site in the chain. The specificity for long chain ubiquinones has also been observed for restoration of NADH oxidase activity after pentane extraction[5].

Evidence for a separate carrier site for duroquinone reduction is also seen in the selective loss of duroquinone activity during the separation of Complex I from Complex III (ref. 14). Complex I retains good ubiquinone reductase activity and can be combined with Complex III to restore NADH cytochrome *c* reductase activity. Therefore, destruction of an essential element during fractionation is unlikely and a search for a duroquinone reduction factor in Complex III is suggested.

The loss of piericidin A sensitivity of ubiquinone and duroquinone reduction in other preparations of NADH dehydrogenase exposed to heat, acid, urea or ethanol is consistant with the view that changes occur in the complex during these procedures[36] and emphasizes that the native duroquinone reduction can only be evaluated during purification by use of suitable inhibitors to define the activity.

The use of duroquinol as an electron donor for the energy-linked reduction of NAD^+ introduces electrons at a site closer to the NADH dehydrogenase complex (site 1) than previously available. Since reduced ubiquinone 2 and 2-methyl-1,4-benzoquinol could not substitute for duroquinol it suggests that linkage of reversed electron flow to ATP hydrolysis must pass through the ubiquinone region and be introduced correctly to the complex.

Since numerous protein bands were found by gel electrophoresis of the various NADH dehydrogenase preparations the existance of four distinct carriers in the complex cannot be ruled out. Even the more purified preparations of the enzyme listed in Table VI showed a minimum of four major protein bands.

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