Bioenergetics (1971) **2**, 317–326

A Durohydroquinone Oxidation Site in the Mitochondrial Transport Chain

J. F. Hare and F. L. Crane

Department of Biological Sciences, Purdue University, W. Lafayette, Indiana 47907

Received 2 September 1971

Abstract

Although duroquinone had little effect upon NADH oxidation in neutral lipid depleted mitochondria, durohydroquinone was oxidized by ETP at a rate sensitive to antimycin A. Fractionation of mitochondria into purified enzyme systems showed durohydroquinone: cytochrome *c* reductase to be concentrated in NADH: cytochrome *c* reductase, absent in succinate: cytochrome *c* reductase, and decreased in reduced coenzyme Q: cytochrome *c* reductase. Durohydroquinone oxidation could be restored by recombining reduced coenzyme Q: cytochrome *c* reductase with NADH: coenzyme Q reductase. Pentane extraction had no effect upon either durohydroquinone or reduced coenzyme Q_{10} oxidation, indicating lack of a quinone requirement between cytochromes *b* and *c*. Both chloroquine diphosphate and acetone (96%) treatment irreversibly inhibited NADH but not succinate oxidation. Neither reagents had any effect upon durohydroquinone oxidation but both inhibited reduced coenzyme Q_{10} oxidation 50%, indicating a site of action between Q_{10} and duroquinone sites. Loss of chloroquine sensitive reduced coenzyme Q_{10} oxidation after acetone extraction suggests two sites for Q_{10} before cytochrome *b*.

Introduction

Although ubiquinone is required for both succinate and NADH oxidation,[1, 2] its site of interaction with the mitochondrial electron transport chain has been obscured by its presence in most detergent derived fragments and lack of inhibitors which act near the quinone site. Suggested functions for coenzyme Q_{10} in the electron transport chain include that of a mobile carrier between complex I (NADH: coenzyme Q reductase), complex II (succinate: coenzyme Q reductase), and complex III (reduced coenzyme Q: cytochrome *c* reductase;[3] that of a specific carrier between cytochrome *b* and c_1;[4] and that of a specific oxidant for each primary dehydrogenase.[5, 6] Ruzicka and Crane[7, 8] used a variety of quinone analogues as oxidants for NADH to determine the specificity associated with chemical structure. By both fractionation and inhibitor studies, four sites of reduction were defined: those corresponding to naphthoquinone, ferricyanide, coenzyme Q_2, and duroquinone. The latter two sites were distinguished by chloroquine diphosphate, poly-L-lysine, and phospholipase *c* sensitivity.

The present study treats the oxidation of durohydroquinone and reduced coenzyme Q_{10} in intact and neutral lipid-depleted submitochondrial particles and isolated

fragments of the electron transport chain. Evidence will be presented for two non-identical reduced Q_{10} oxidation sites before cytochrome *b* and a separate oxidation site for durohydroquinone in complex I before cytochrome *b*.

Materials and Methods

Beef heart mitochondria were prepared by the method of Low and Vallin.[9] Electron transport particles (ETP) were prepared essentially as described by Ruzicka and Crane[7] except the isolation medium was 0·01 M rather than 0·05 M in Tris and the particles were washed once after sedimentation at 105,000 × *g*. Purified NADH:cytochrome *c* reductase, NADH:coenzyme Q reductase, succinate:cytochrome *c* reductase, and reduced coenzyme Q:cytochrome *c* reductase were prepared from published procedures.[10–13]

96% acetone extraction of ETP was by the method of Fleischer, Fleischer and, Stoekenius[14] while pentane extraction of mitochondria was according to Szarkowska.[2] Duroquinone was reduced by adding $NaBH_4$ and 0·5 volumes of 0·1 M sodium phosphate, pH 7·4, to a 0·5 mg/ml ethanolic solution of duroquinone. The reduced quinone was then extracted three times with diethyl ether, washed once with phosphte buffer, evaporated to dryness, and taken up in ethanol to a concentration of 5 mg/ml. Q_{10} was reduced in a similar manner except that extraction was with cyclohexane and the wash was omitted. NADH oxidase, succinoxidase, NADH:cytochrome *c* reductase, succinate:cytochrome *c* reductase, and NADH:ferricyanide reductase assays were performed at 30° according to published procedures.[15–19] NADH:quinone reductase was assayed as described by Ruzicka and Crane.[7] Hydroquinone:cytochrome *c* reductase assays were conducted at 0° on a Unicam SP800 spectrophotometer with a multispeed Heath external recorder by following the reduction of cytochrome *c* at 550 mμ. The assay system (1 ml) contained 29 μM NaN_3, 0·144 μmoles EDTA, 57·5 μmoles phosphate (pH 7·4), 0·05–0·005 mg enzyme, and 0·05 mg reduced quinone in ethanol. 0·5 mg cytochrome *c* in 0·05 ml H_2O was added to start the reaction. Reduction of cytochrome *c* was followed at 550 mμ against a reaction mixture blank containing no enzyme. 2-heptyl-4-hydroxy-quinoline-N-oxide in ethanol and chloroquine diphosphate in H_2O were added to the assay medium to a final concentration of 10^{-6} and 5×10^{-5} M respectively. Antimycin A in ethanol was preincubated 10 min. at 0° with undiluted enzyme at a level of 1 μg/mg protein and then diluted before addition. Mitochondrial phospholipid micelles were prepared as described by Sun and Crane.[20] Protein was determined by the method of Lowry, Rosenbrough, and Farr.[21]

Deoxycholic acid and poly-L-lysine (62,000 MW) were obtained from Mann and duroquinone from K and K Laboratories. Antimycin A, 2-heptyl-4-hydroxy-quinoline-N-oxide, chloroquine diphosphate, cytochrome *c* (type III), β-NADH, and coenzyme Q_{10} were purchased from Sigma.

Results

Both durohydroquinone (DQH_2) and reduced coenzyme Q_{10} (H_2Q_{10}) reduced cytochrome *c* in the presence of ETP at a rate which was zero order with respect to substrate. Rates were not stimulated by phospholipid but were inhibited 79% by heptyl-hydroxy-quinoline-N-oxide and 100% by antimycin A. Since H_2Q_{10} is a more lipophillic sub-

strate than DQH_2, deoxycholate was used at low and high levels in comparing rates of hydroquinone oxidation in particulate ETP (Table I). Previous work has determined that low or 0·2 mg deoxycholate mg protein result in ruptured vesicles whereas higher levels or 0·4 mg deoxycholate mg protein results in partially solubilized ETP[22].

Deoxycholate stimulated DQH_2 oxidation at low levels but further additions of detergent did not increase activity. H_2Q_{10} oxidation by ETP was significantly increased by detergent to a level of 0·4 mg/mg protein giving a maximal activity which was 8·7 times greater than that shown by DQH_2.

TABLE I. Effect of deoxycholate on hydroquinone oxidation in ETP

mg potassium deoxycholate added/mg protein	Specific activity (μmoles cytochrome *c*-reduced/min/mg protein)	
	DQH_2	H_2Q_{10}
0	0·04	0·27
0·2	0·14	0·58
0·4	0·12	1·24

Fractionation of mitochondria into four complexes resulted in rates of hydroquinone oxidation which varied upon the reductant used (Table II). DQH_2 oxidation was enriched 6·6 times in complex I + III but was absent in complex II + III. Activity in complex III was 50% that of complex I + III. Although complex I showed no

TABLE II. Hydroquinone oxidation in isolated complexes of the mitochondrial electron transport chain.*

Complex	Specific activity (μmoles cytochrome *c*-reduced/min/mg protein)	
	DQH_2	H_2Q_{10}
ETP (partially soluble)	0·14	1·24
I + III	0·93	3·22
II + III	0·02	0·38 (3·08)*
III	0·46	5·41 (19·0)*
I	0	0

* Figures in parentheses indicate values obtained when assayed in presence of mitochondrial phospholipid micelles (200 μg phosphorous/ml).

activity, when increasing quantities of complex I protein were dialyzed against complex III 12 hours at 0°, both DQH_2 and NADH oxidation with cytochrome *c* as an acceptor was restored (Fig. 1). Both complex I + III and complex II + III with added phospholipid oxidized H_2Q_{10} at only three times the rate of ETP. Complex III oxidized

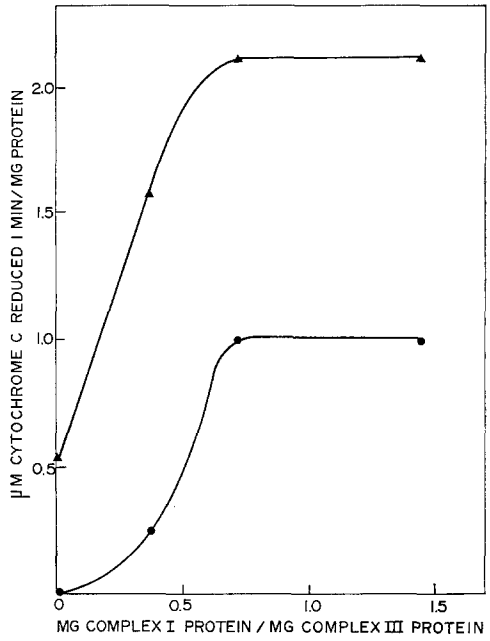

Figure 1. Restoration of DQH_2 and NADH: cytochrome *c* reductase from dialyzed complex I and complex III. 0 to 1·0 ml of complex I (16·5 mg protein/ml) was added to 1ml complex III (11·4 mg protein/ml); dialyzed 12 hrs against 0.01 M Tris-Cl (pH 8·0); centrifuged at 144,800 × *g* 30 min.; resuspended in 0·67 M sucrose, 0·001 M histidine, 0·05 M Tris-Cl (pH 8·0); and assayed as described in Methods. ● NADH: cytochrome *c* reductase; ▲ DQH_2: cytochrome *c* reductase. Specific activity based on complex III protein.

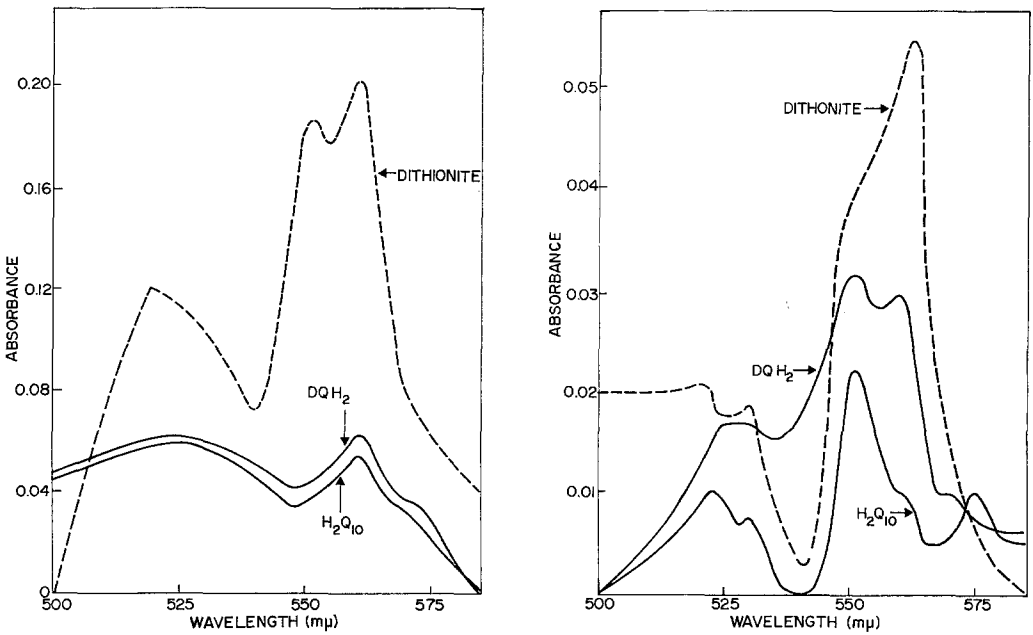

Figure 2. A: Difference spectra of dithionite, DQH_2, and H_2Q_{10} reduced ETP. 15 μl of reduced quinone was added to 22·5 mg ETP protein in 3·0 ml of assay medium described in Methods. B: Difference spectra of dithionite, DQH_2 and H_2Q_{10} reduced complex I + III. 5 μl of reduced quinone was added to 2·99 mg complex I + III protein in 1·0 ml of assay medium.

H_2Q_{10} at 12 times greater rate without and more than 40 times greater rate with phospholipid present than DQH_2 with or without phospholipid.

Although rates of DQH_2 oxidation were inhibited by both antimycin *A* and heptyl-hydroxy-quinoline-N-oxide, it was of interest to know if this quinone was reducing cytochrome *b*, and if so, if the same amount of cytochrome *b* was being reduced as with H_2Q_{10} as a reductant. Spectra (Fig. 2) show that in ETP both DQH_2 and H_2Q_{10} reduce 36% of cytochrome *b* but no cytochrome c_1. In complex I + III DQH_2 reduced 43% and H_2Q_{10} reduced 20% of cytochrome *b* while both quinones reduced all of cytochrome c_1.

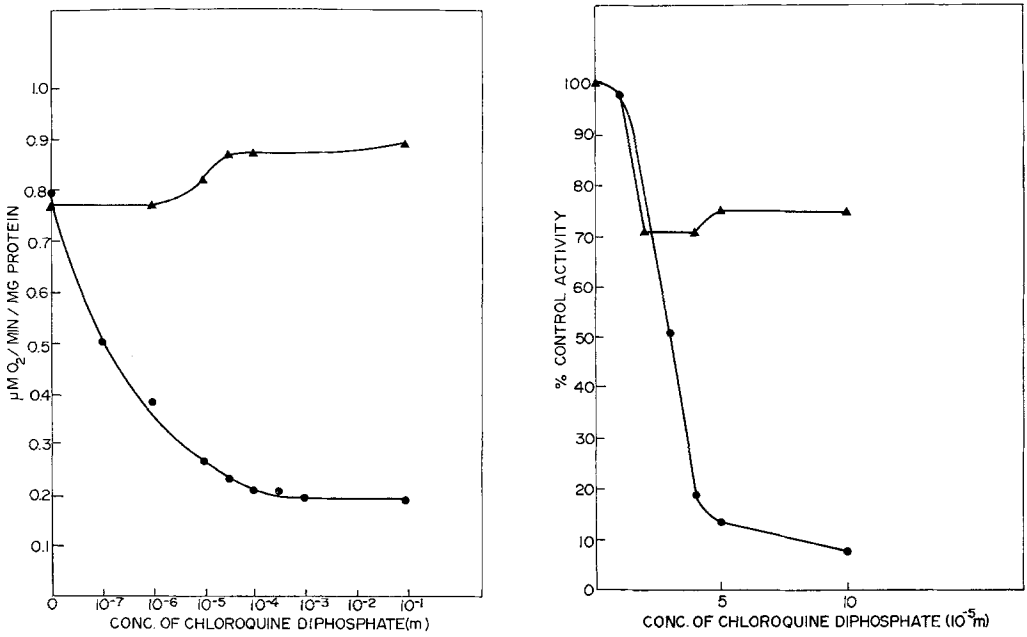

Figure 3. A: Effect of chloroquine diphosphate upon NADH and succinoxidase in ETP. ▲ succinoxidase; ● NADH oxidase. B: Effect of chloroquine diphosphate on NADH (6·75 μM cytochrome *c*-reduced/min/mg protein) and succinate (6·43 μM cytochrome *c*-reduced/min/mg protein) cytochrome *c* reductase in partially soluble ETP. ▲ succinate:cytochrome *c* reductase; ● NADH:cytochrome *c* reductase.

Although Skelton *et al.*[23] noted an inhibition by chloroquinone diphosphate on NADH oxidase but not succinoxidase in *Plasmodium*, no work has been reported concerning the inhibitory effect of this compound on mammalian electron transport. Succinoxidase is stimulated to 114% of control activity at 10^{-1} M chloroquine while NADH oxidase is inhibited to 23% of control activity (Fig. 3A). Figure 3B shows the inhibitory effect of chloroquine on NADH but not succinate-cytochrome *c* reductase in the presence of 0·4 mg deoxycholate/mg protein.

Chloroquine inhibited DQH_2 oxidation only in complex III (Table III). H_2Q_{10} oxidation was nearly completely inhibited in complex I + III but not inhibited in complex II + III. Complex III showed chloroquine sensitivity only in the presence of phospholipid.

Although poly-L-lysine inhibits NADH:cytochrome *c* reductase at very low concentration, this polycation also inhibits NADH:DQ reductase at higher concentrations.[8]

TABLE III. Inhibition of hydroquinone oxidation by chloroquine in isolated complexes

	Percent inhibition by 5×10^{-5} M chloroquine diphosphate (control activities are those noted in Tables I and II)	
	H_2Q_{10}	DQH_2
ETP	55	0
II + III	0	0
I + III	97	0
III	85	100

Both DQH_2 and H_2Q_{10} oxidation are sensitive to poly-L-lysine (Fig. 4) but H_2Q_{10} is inhibited 90% by 0·04 mg poly-L-lysine/ml while DQH_2 oxidation is not inhibited 90% until 0·08 mg/ml.

To determine if DQH_2 oxidation depended on endogenous Q_{10}, we looked at NADH, succinate, H_2Q_{10}, and DQH_2 oxidation in pentane and acetone extracted ETP and mitochondria.

In accord with previous studies, acetone extraction irreversibly destroyed NADH: cytochrome *c* reductase activity in ETP, whereas succinate:cytochrome *c* reductase activity could be restored by addition of Q_{10} and phospholipid (Table IV). After pentane extraction, reduction of cytochrome *c* by both NADH and succinate could be reconstituted by addition of Q_{10} and phospholipid. Substitution of DQ for Q_{10} after pentane

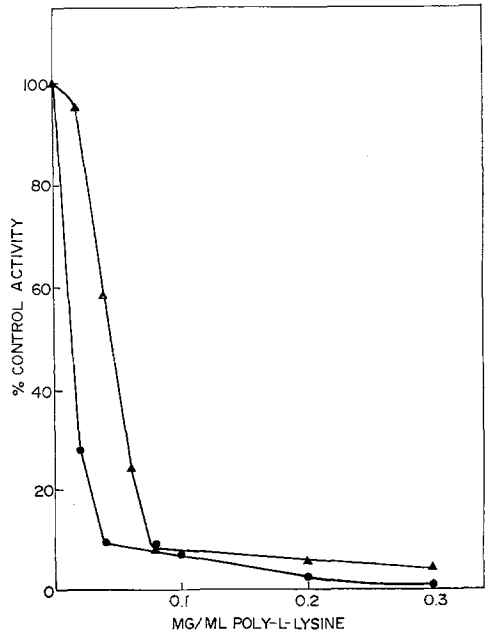

Figure 4. Inhibition of hydroquinone:cytochrome *c* reductase by poly-L-lysine. Percent based upon control activities noted in Table 1. ▲ DQH_2:cytochrome *c* reductase; ● H_2Q_{10}:cytochrome *c* reductase.

TABLE IV. Succinate and NADH:cytochrome *c* reductase in acetone and pentane extracted ETP and mitochondria

		Specific activity (μmoles cyt *c*-reduced/min/mg protein at 30°C)	
	Additions*	Succinate	NADH
ETP	Q_{10} + MPL	3·89	6·16
acetone ETP	MPL	0·63	0
acetone ETP	Q_{10} + MPL	3·10	0·28
Lyo Mito.	Q_{10} + MPL	3·56	7·68
Pentane LM	MPL	0	0
Pentane LM	Q_{10} + MPL	3·61	6·22
Pentane LM	DQ + MPL	0·32	0·11

* 0·01 ml of a mitochondrial phospholipid micellar suspension (MPL), 200 μg phosphorous/ml, or quinone were added directly to assay medium and preincubated 10 min at 30° before assay.

extraction resulted in activities 0·09 times that of Q_{10} restored activity with succinate as a donor and less than 0·02 times with NADH as a donor.

Removal of neutral lipids from ETP by extraction with 96% acetone (Table V) decreased the rate of H_2Q_{10} oxidation in presence of high levels of deoxycholate to half that of the unextracted control. Remaining activity was insensitive to chloroquine.

TABLE V. Hydroquinone oxidation in neutral lipid extracted ETP

	Specific activity (μmoles cytochrome *c*-reduced/min/mg protein)		
Reductant	H_2Q_{10}	H_2Q_{10} + 5×10^{-5}M chloroquine	DQH_2
ETP	1·24	0·68	0·12
acetone extracted ETP	0·65	0·65	0·14
lyophillized mitochondria	1·57	0·74	0·16
pentane extracted, lyophillized mitochondria	1·45	0·74	0·15

Acetone extraction did not effect DQH_2 oxidation. Milder pentane extraction had little effect upon oxidation of H_2Q_{10} or DQH_2 by lyophillized mitochondria. Catalytic amounts of Q_{10} (5 μg) added to the reaction cell did not stimulate DQH_2 oxidation in pentane extracted mitochondria or acetone extracted ETP.

Table VI summarizes the activities of a number of enzymes involving complex I components before and after acetone extraction. DQH_2 oxidation remains after extraction and although H_2Q_{10} oxidation persists, activity is reduced by 50%. Loss of chloroquine sensitivity indicates that only the Q_{10} site between NADH and cytochrome *b* is

TABLE VI. Effects of acetone treatment of ETP on quinone oxidases and reductases

	Specific activity (μmoles cytochrome *c*-reduced at 0°C or μmoles NADH oxidized at 30°C/min/mg protein)	
	ETP	Acetone treated ETP
DQH_2: cytochrome *c* reductase	0·14	0·14
H_2Q_{10}: cytochrome *c* reductase	1·24	0·65
plus chloroquine	0·62	0·49
NADH: $Fe(CN)_6^{-3}$ reductase*	27·4	24·5
NADH: DQ reductase	0·194	0
NADH: Q_{10} reductase	0·012	0·007
NADH: juglone reductase	0·357	0·241

* Specific activity expressed as V_{max}.

sensitive to acetone. NADH: ferricyanide reductase, the second feedout site defined by Ruzicka and Crane, has similar V_{max} values before and after extraction although the K_m value increased from ·071 M to 0·159 M. NADH: DQ reductase activity is removed by acetone treatment while NADH: Q_{10} reductase and NADH: juglone reductase appear to remain after extraction.

Discussion

The comparatively low restoration of NADH: cytochrome *c* reductase activity by duroquinone compared with Q_{10} after pentane extraction demonstrates that DQH_2 is feeding directly into the transport chain rather than functioning as a quinone in the main pathway. Ruzicka and Crane have distinguished the site of reduction of DQ from the Q_2 site by sensitivity of duroquinone reductase but not Q_2 reductase to chloroquine, poly-L-lysine, and phospholipase C. The site of H_2Q_{10} oxidation also can be distinguished from DQH_2 oxidation by partial sensitivity of the former to chloroquine. In addition acetone treatment mimics the effect of chloroquine in eliminating half of H_2Q_{10} oxidation and all of duroquinone reductase but having no effect upon DQH_2 oxidation. Although there appears to be another site for poly-L-lysine inhibition between cytochromes *b* and *c*, loss of H_2Q_{10} oxidation at a lower titer of this inhibitor than DQH_2 oxidation also indicates a site of action between the two quinones. Thus the concept of separate and non-identical points of quinone interaction with the respiratory chain appears possible from both oxidation and reduction standpoints.

The site of DQH_2 oxidation and reduction can be distinguished from the primary NADH dehydrogenase, believed to be identical to NADH: ferricyanide reductase. Neither acetone nor chloroquine greatly alter the rate of ferricyanide or juglone reduction but eliminate or decrease duroquinone reduction and H_2Q_{10} oxidation

The same site of DQH_2 oxidation can also be distinguished from cytochrome *b*. Although H_2Q_2, H_2Q_{10}, and DQH_2 have been used as reductants for the cytochrome system, there is no evidence to indicate that these feed directly into cytochrome *b*.

Fractionation studies reported here indicate that DQH_2 feeds into a component in complex I which is devoid of cytochrome *b*. Complex II + III showed little activity with DQH_2 and complex III showed only 50% of complex I + III activity. Fractionation of complex I + III does not seem to have modified the DQH_2 oxidizing component since activity is restored upon recombining complex I with complex III. Only in complex III where DQH_2 oxidation becomes sensitive to chloroquine does a fraction deviate from the main pathway of electrons in the intact system as evidenced by lack of sensitivity of DQH_2 oxidation to chloroquine in ETP and all other fractions. DQH_2 remains sensitive to antimycin A and reduces cytochrome *b* in all fractions assayed.

Evidence here indicates two sites of interaction of Q_{10} with the respiratory chain before cytochrome *b*. Recent extraction studies have indicated there to be a quinone site associated with both succinate and NADH dehydrogenases.[5, 6] Sensitivity of half of H_2Q_{10} oxidation to chloroquine, loss of half of H_2Q_{10} oxidation after acetone extraction, and only a three-fold enrichment of H_2Q_{10} activity from ETP to complexes I + III and II + III over that of ETP compared to a six-fold enrichment for DQH_2 oxidation in complex I + III indicate sites for Q_{10} association with both succinate and NADH dehydrogenase. Thus the QH_2:cytochrome *c* reductase must be reconsidered as representing a functional unit in electron transport.

In accord with the above results we have modified and refined the scheme of quinone reduction proposed originally by Ruzicka and Crane in Fig. 5 to include additional site determining reagents and the durohydroquinone oxidation site.

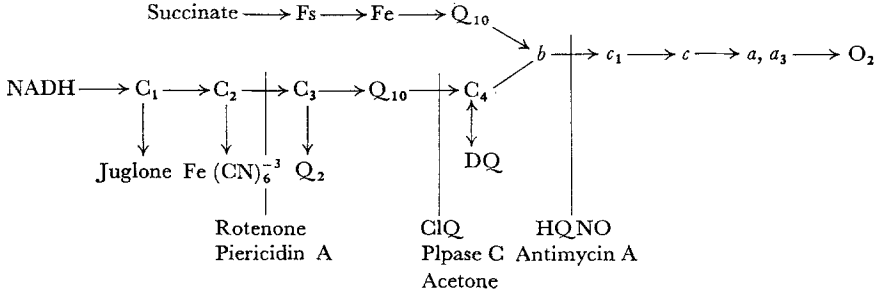

Figure 5.

Although loss of chloroquine-sensitive H_2Q_{10} oxidation with acetone extraction defines the site of susceptibility of NADH oxidation to acetone, this phenomenon remains unexplained. Since DQH_2 and succinate oxidation can be restored after extraction, the denaturing effect of acetone upon the enzyme system must occur at a point before the duroquinone site. Although NADH:juglone reductase activity and NADH:ferricyanide reductase V_{max} remain, a change in K_m value of the latter enzyme suggests either a conformational change or loss of a lipid component which causes the ferricyanide to bind differently. Alternatively acetone may cause the irreversible denaturation of a component necessary for H_2Q_{10} oxidation.

Acknowledgements

Supported under grants from the National Institutes of Health, AMO4663 and Career Grant (F.L.C.) GM K6, 21, 839.

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