Inhibition of Mitochondrial Electron Transport by Hydroxy-Substituted 1,4-Quinones†

Donna C. Phelps‡ and Frederick L. Crane*

ABSTRACT: Mitochondrial electron transport from NADH, succinate, or duroquinol to oxygen is inhibited by 5-ω-cyclohexyl-*n*-pentyl-6-hydroxy-2,3-dimethoxy-1,4-benzoquinone and 2-ω-cyclohexyl-*n*-pentyl-3-hydroxy-1,4-napthoquinone. Assays of partial electron transport activities indicate a site of inhibition in the region between the site of duroquinol oxidation and cytochrome *c* reduction. Effects of the inhibitors on cytochrome spectra indicate the principle site of inhibition is between cytochromes of the *b* group and cytochrome c_1. The quinones do not induce increased reduction of the b_{566} as does antimycin A. The benzoquinone can induce a partial antimycin insensitive bypass of the cytochromes b–c_1 region. Uncoupling agents do not affect the extent of inhibition as has been reported for analogous naphthoquinone inhibitors.

Previous work (Ball *et al.*, 1949; Estabrook, 1958; Tappel, 1960; Howland *et al.*, 1973) has shown that some 1,4-naphthoquinones inhibit mitochondrial electron transport in the cytochromes b–c_1 region as does antimycin A and HOQNO (Thorn, 1956; Thorn and Jackson, 1959; Low and Vallin, 1963; Lightbrown and Jackson, 1956). For example, these inhibitors inhibit NADH and succinate but not TMPD[1] oxidase activities. In spectra of reduced mitochondrial preparations these inhibitors caused the disappearance of the peaks for cytochromes c, c_1, and aa_3.

Certain dimethoxy-6-hydroxy-5-alkyl-1,4-benzoquinones have also been shown to inhibit succinate and NADH oxidase activity in mitochondrial preparations (Catlin *et al.*, 1968; Castelli *et al.*, 1971). Inhibition at the coenzyme Q level was indicated. However, the studies with the benzoquinones were not as complete as those with the naphthoquinones. Not eliminated was the possibility that these benzoquinones could also inhibit at the cytochrome b–c_1 level.

In this paper, a new benzoquinone ω-cyclohexylpentyl-2,3-dimethoxy-6-hydroxy-1,4-benzoquinone (C-OH-BQ) and a structurally very similar naphthoquinone 3-ω-cyclohexylpentyl-2-hydroxy-1,4-naphthoquinone (C-OH-NQ) are studied. The new benzoquinone differs structurally from the naphthoquinone only in that the benzoquinone has two methoxy groups in place of the four carbons of the naphthoquinone. The similarity of structure of these two new quinones suggests the possibility of similar inhibition patterns.

Methods and Materials

Heavy beef heart mitochondria were isolated by modified Hatefi and Lester procedures (Hatefi and Lester, 1958). Mitochondrially oriented particles (MOP) were isolated by a modified Hansen and Smith procedure (Hansen and Smith, 1964), 15 mM magnesium acetate in 0.5 M sucrose being used in place of 5 mM $MgCl_2$ and 10 mM $MnCl_2$ in 0.25 M sucrose. Protein concentration was determined by a modified biuret method (Yonetani, 1961).

Assay procedures were modifications of previously described procedures (Low and Vallin, 1963; Yonetani, 1961; King, 1967; King and Howard 1967; Tisdale, 1967; Ruzicka and Crane, 1970, 1971). Succinate oxidase rates represent maximal rather than initial rates. Maximal rates were usually obtained 3 min after the addition of succinate. Preincubation of the concentrated enzyme with succinate (25 mM) did not affect this lag. All activities were measured in 0.45 M sucrose–50 mM glycylglycine–12 mM MgO (pH 8.0) as were all enzyme spectra. All activities were measured at 30° after a 3-min incubation of the enzyme with any inhibitors. Other modification are given in the legends.

All chemicals were purchased from either Sigma, Calbiochem, or Alrich except for C-OH-BQ and C-OH-NQ which were synthesized according to previously described procedures (Fieser *et al.*, 1948; Bogentoft *et al.*, 1972) and heptyl coenzyme Q which were gifts from Dr. K. Folkers and Dr. T. H. Porter. Duroquinol (DQH_2) was made by reducing duroquinone (DQ) with borohydride in ethanol as described by Ruzicka (Ruzicka and Crane, 1971) and used as a methanol solution saturated at 0°.

C-OH-BQ changed color and inhibition properties upon prolonged storages in methanol even at −20°. Catlin *et al.* (1968) have noticed aging effects in other dimethoxy-6-hydroxy-5-alkylbenzoquinones. Therefore, only fresh solutions of C-OH-BQ were used.

Results

Inhibition of Forward and Reverse Electron Transport. Table I shows the inhibition patterns of four inhibitors, antimycin A, HOQNO, C-OH-BQ, and C-OH-NQ, on various oxidase activities. The inhibition patterns of oxidase activities by C-OH-BQ and C-OH-NQ are similar to those of antimycin A and HOQNO except that complete inhibitions of succinate and NADH oxidases with C-OH-BQ were not

† From Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907. *Received March 22, 1974.* Supported by Grant AM04663 from National Institute for Arthritis and Metabolic Diseases and Career Grant K6,21,839 from National Institute for General Medical Research.

‡ Predoctoral trainee supported by Training Grant TOQ-1195 from the National Institutes of Health.

[1] Abbreviations used are: MOP, mitochondrially oriented particle preparation; DQ, duroquinone; DQH_2 durohydroquinone; $HOQNO_2$, heptyl-4-hydroxyquinoline *N*-oxide; TMPD, tetramethylphenylenediamine; C-OH-BQ, 5-ω-cyclohexyl-*n*-pentyl-2,3-dimethoxy-6-hydroxy-1,4-benzoquinone; C-OH-NQ, 2-ω-cyclohexyl-*n*-pentyl-3-hydroxy-1,4-naphthoquinone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Q_6, coenzyme Q_6.

TABLE I: Inhibition of Oxidase Activities by Hydroxy Quinones.[a]

Inhibitor or Change	(μmol of 2e$^-$ per min per mg of Protein) NADH	Succinate	DQH_2	TMPD + Dithiothreitol
None	0.47	0.30	0.68	1.13
0.26 μg of antimycin A	0.02	0.02	0.16	1.16
20 μg of HOQNO	0.06	0.05	0.18	1.05
3.9 μg of C-OH-NQ	0	0.01	0.13	1.15
3.5 μg of C-OH-BQ	0.22	0.18	0.24	1.13
35.0 μg of C-OH-BQ	0.12 (74%)	0.09 (70%)	0.14 (79%)	1.06
Lower pH to 6.5 with HCl	0.55	0.12	0.15	0.79
pH 6.5 and 35.0 μg of C-OH-BQ	0.05 (90%)	0.01 (90%)	0.03 (80%)	0.80

[a] NADH and succinate oxidase activities were measured with 0.66 mg of protein in 1.8 ml of assay medium. The assays were started with either 0.72 μmol of NADH or 20 μmol of succinate. DQH_2 oxidase activities were measured with 0.40 mg of protein in 1.8 ml of assay medium and started with 25 μl of methanol saturated at 0° with DQH_2 (*ca.* 0.7 μmol). TMPD oxidase activities were measured with 0.26 mg of protein and 0.6 μmol of TMPD. The assays were started with 6 μmol of dithiothreitol. All assays contained 180 μg of cytochrome *c* and were measured on a Gilson oxygraph with a Clark electrode. Any CN^- insensitive blank rates were substracted. Such blanks were observed in all DQH_2 and TMPD oxidase assays. For NADH and succinate oxidase assay except at pH 6.5 in the presence of 35.0 μg of C-OH-BQ no CN^- insensitive blanks were observed.

obtained at pH 8.0. Stronger inhibitions of oxidase activities were obtained at pH 6.5. Both Ball (Ball *et al.*, 1949) and Howland (Howland, 1965) have found that other alkyl-1,4-hydroxynaphthoquinones were better inhibitors in acidic solutions than in basic solutions. No inhibition sites are beyond cytochrome *c* as indicated by the reduced TMPD oxidase activities.

Similar inhibition of NADH and succinate oxidase are observed if the assay medium is 83 μM EDTA–83 mM phosphate (pH 7.4), in which case the NADH oxidase rate is three times as fast (Table VIII). Although faster NADH oxidase rates were possible in EDTA–phosphate buffer, reverse electron transport activities were not observed in this buffer. The assay medium in which all activities could be observed was generally used. The faster rates of NADH oxidase in the hypotonic, magnesium-free EDTA–phosphate buffer as compared to the isotonic SGM8 buffer could be partially due to the breaking of mitochondrially oriented vesicles. That the cytochrome *c* side of the particles is exposed in SGM8 is indicated by the fact that the cytochrome *c* reductase rates are nearly as fast as the NADH and succinate oxidase rates in SGM8 (Tables I and II).

Table II shows the effects of C-OH-BQ, C-OH-NQ, and antimycin A on cytochrome *c* reductase activities. Since both succinate and NADH to cytochrome *c* reductases are inhibited by all three inhibitors, sites of inhibition by C-OH-BQ and C-OH-NQ between coenzyme Q and cytochrome *c* are indicated.

The DQ and $Fe(CN)_6^{3-}$ reductase activities given in Table III indicate no significant inhibition site for either C-OH-BQ or C-OH-NQ between the site of NADH dehydrogenation and the cytochromes *b*. The small amount of these activities inhibited by C-OH-NQ is equivalent to that per cent of the overall reductase activity which requires cytochrome *b*.

The study of reverse electron transport from succinate to NAD^+ (Table IV) shows that COOH-BQ does not uncouple coupling site 1 at concentrations sufficient to completely inhibit reverse electron transport through cytochrome c_1.

TABLE II: Cytochrome *c* Reductase Activities.[a]

Inhibitor	(μmol of Cytochrome *c* per min per mg of protein) NADH to Cytochrome *c* Reductase	Succinate to Cytochrome *c* Reductase
None	0.71 (av 3 runs)	0.50
0.26 μg of antimycin A	0.07	0.02
3.9 μg of C-OH-NQ	0.05	0.01
35.0 μg of C-OH-BQ	0.30	0.14

[a] All assays contained 4.8 μmol of NaCN, 1.8 mg of cytochrome *c*, and 0.13 mg of protein in 3 ml total volume. The succinate to cytochrome assays contained 20 μmol of succinate and were started with the addition of cytochrome *c*. The NADH to cytochrome *c* assays were started with the addition of 0.72 μmol of NADH. Activities were measured on a Unicam spectrophotometer with an external recorder at 550 nm.

However, since C-OH-NQ inhibits reverse electron transport from even coenzyme Q, C-OH-NQ probably interferes with energy transfer. The possibility of inhibition at a cytochrome *b* is indicated by the inhibition of reverse electron transport from DQH_2 to NAD^+ by C-OH-BQ to the same extent as by antimycin A and HOQNO.

Effects on Cytochrome Reduction. Figures 1 and 2 show that like HOQNO and antimycin A, C-OH-BQ and C-OH-NQ also inhibit between cytochromes *b* and c_1. In all cases, when the inhibitor is added the α peaks of reduced cytochromes aa_3 and c_1 but not of cytochromes *b* disappear. Spectrum C-3 (Figure 2) shows that even in the presence of C-OH-NQ, succinate can reduce cytochromes *b*.

TABLE III: NADH to DQ and NADH to $Fe(CN)_6^{3}$ Reductase Activities.[a]

Inhibitor	(μmol of NADH per min per mg of Protein) DQ reductase	$Fe(CN)_6^{3}$ Reductase
None	0.14	8.1
0.26 μg of antimycin A	0.11	7.9
3.9 μg of C-OH-NQ	0.11	7.7
35.0 μg of C-OH-BQ	0.09	7.2

[a] DQ reductase assays contained 4.8 μmol of NaCN, 0.66 mg of protein, and 0.36 μmol of NADH in a total volume of 3 ml. The reactions were started with the addition of 0.30 μmol of DQ. Any rate not requiring DQ was subtracted. $Fe(CN)_6^{3-}$ reductase assays contained 4.8 μmol of NaCN, 0.026 mg of protein, and 2.25 μmol of $Fe(CN)_6^{3-}$ in a total volume of 3.0 ml. The reactions were started with 0.36 μmol of NADH. Any enzyme independent blank rates were subtracted. All rates were measured on a Unicam with external recorder at 340 nm.

TABLE IV: Reverse Electron Transport Activities.[a]

Inhibitor	Electron Source: Succinate	DQH_2 + Dithiothreitol	TMPD + Dithiothreitol	Coenzyme Q + Dithiothreitol
	(μmol of NADH per min per mg of Protein)			
None	0.13	0.09	0.08	0.030
20 μg of HOQNO	0.08	0.05	0.02	
0.26 μg of antimycin A	0.11	0.02	0	0.023
3.9 μg of C-OH-NQ	0.02	0.01	0	0.004
3.5 μg of C-OH-BQ	0.10	0.03	0	

[a] All assays contained 1 mM NAD^+, 1.6 mM NaCN, and 0.66 mg of protein in a total volume of 3.0 ml. The reactions were started with the addition of 10 μmol of ATP and were followed on a Unicam spectrophotometer with external recorder at 340 mμ. The succinate assays also contained 20 μmol of succinate; the DQH_2 assays, 25 μl of methanol saturated at 0° with DQH_2 and 2.4 μmol of dithiothreitol; the TMPD assays, 0.6 μmol of TMPD and 6.0 μmol of dithiothreitol; the coenzyme Q assays, 50 μg of heptyl coenzyme Q and 3.8 μmol of dithiothreitol. The rate with dithiothreitol only was zero.

Hence a strong C-OH-NQ inhibition site in complex II is unlikely. Only with DQH_2 as the reducing agent is there much difficulty in reducing the *b* cytochromes in the presence of C-OH-NQ.

Benzoquinone Bypass Activity. Increasing the C-OH-BQ concentration from 3.5 to 35 μg failed to cause complete inhibition of either NADH or succinate oxidase activities and in both cases at least 25% of each of the activities remained. Figure 3 shows the titrations of NADH and DQH_2 oxidase activities and DQH_2 reverse electron transport activity with C-OH-BQ. Oxidase activities show saturation of inhibition at about 2 μg/ml and reverse electron transport shows maximum inhibition at about 0.6–1 μg/ml. Little additional inhibition by C-OH-BQ on any of these activities in SGM8 medium is experienced at C-OH-BQ concentrations above 5 μg.

Table V shows further studies indicating a complex mode of inhibition by C-OH-BQ. As expected, complex I inhibitors inhibit NADH–cytochrome *c* reductase but not succinate–cytochrome *c* reductase independent of the presence or absence of high levels of C-OH-BQ. However, antimycin A inhibition of both activities is reduced by the presence of high levels of C-OH-BQ. Not shown are similar (although smaller) effects on NADH and succinate oxidase activities. Antimycin A inhibition of DQH oxidase activity is unaffected by C-OH-BQ. High levels of C-OH-NQ and low levels of C-OH-BQ have little effect on antimycin A inhibition of cytochrome *c* reductase activities. Figure 4 shows that C-OH-NQ reverses the antimycin A inhibition rather than preventing the establishment of the inhibition. The above phenomenon can be explained if C-OH-BQ could be enzymatically reduced before the antimycin A site and the hydroquinone form could then reduce cytochrome *c*. Since NADH and succinate oxidase activities in MOP are stimulated 1.6–2.0-fold by cytochrome *c*, the reduced cytochrome

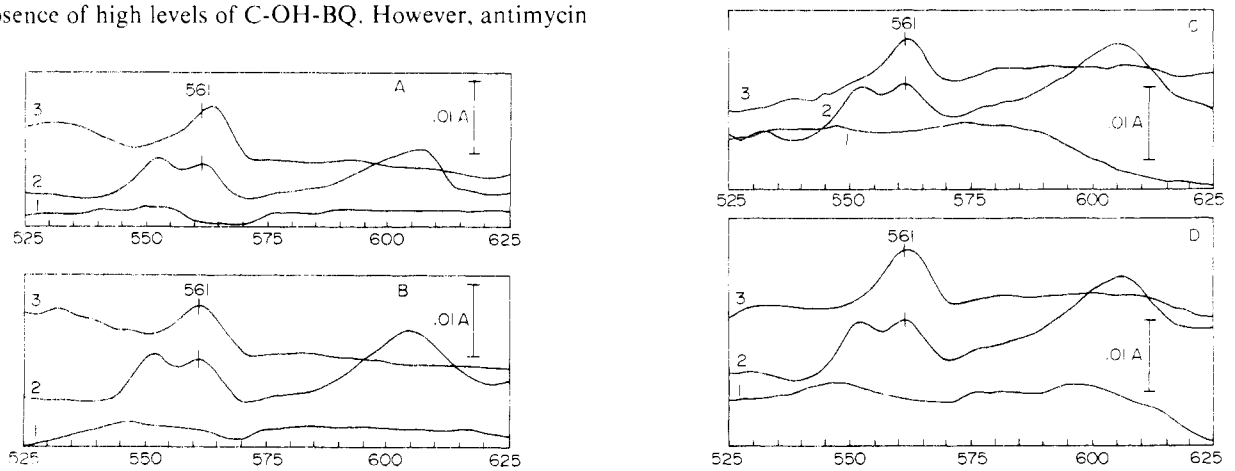

FIGURE 1: For all spectra reference and sample cuvets each contained 1.1 mg of protein in assay medium total volume 1.0 ml. For all spectra sets spectrum 1 was the base line and the sample cuvet in spectrum 2 was reduced with 1.8 μmol of NADH. For spectrum A-3, 2.5 nmol of antimycin A was then added to the sample cuvet; for spectrum B-3, 3.9 μg of C-OH-NQ was added; for spectrum C-3, 3.5 μg of C-OH-BQ was added; and for spectrum D-3, 178 nmol of HOQNO was added. Spectra were recorded on a Cary 15 recording spectrophotometer at room temperature.

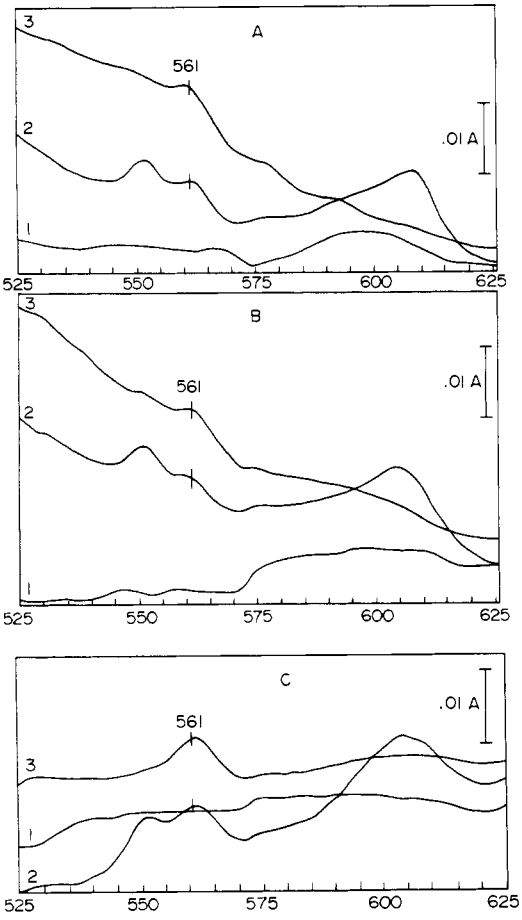

FIGURE 2: For all spectra, reference and sample cuvets each contained 1.1 mg of protein in a total volume of 1.0 ml. For all spectra of spectra sets A and B, all cuvets also contained 6 mM dithiothreitol. Spectra A-1, B-1, and C-1 were base lines. For spectra A-2 and B-2 the sample cuvets were reduced with 25 μl of methanol saturated at 0° with DQH_2. The sample cuvet for spectrum C-2 was reduced with 20 μmol of succinate; 3.9 μg of C-OH-NQ was then added to the reduced sample cuvets to produce spectra A-3 and C-3; 3.5 μg of C-OH-BQ was used instead to produce spectrum B-3.

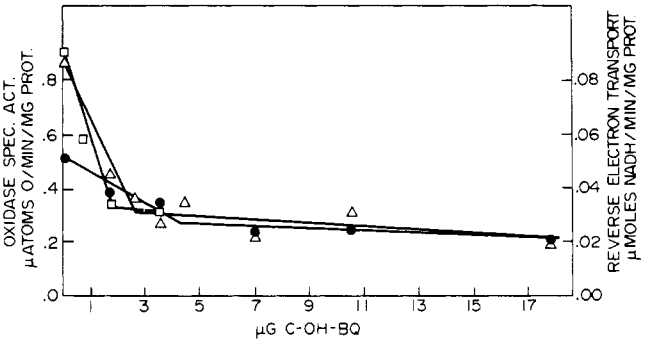

FIGURE 3: DQH_2 and NADH oxidase assays were run as for Table I except that the amounts of C-OH-BQ which were added before the initiation of the 3-min incubation period were varied. Protein was 0.66 mg for the NADH oxidase assays and 0.40 mg for the DQH_2 oxidase assays in 1.8 ml. DQH_2 + dithiothreitol reverse electron transport was run as for Table IV. Protein was 0.66 mg in 3.00 ml. (●) NADH oxidase; (△) DQH_2 oxidase; (□) DQH_2 + dithiothreitol reverse electron transport.

c could be oxidized by the cytochrome oxidase of MOP. A partial bypass around the antimycin A site would then be created by the addition of an electron transport inhibitor, C-OH-BQ. That C-OH-BQ can interact with the electron transport chain in a redox type reaction is indicated in Table VI. While dithiothreitol by itself will not serve as an electron source for reverse electron transport, dithiothreitol can reduce certain quinones such as menadione (Taggart and Sanadi, 1972) or DQ (unpublished work), and the reduced quinones can then donate electrons for ATP dependent reverse electron transport. (Table VI shows that C-OH-BQ can also mediate electron transport from dithiothreitol to MOP for an ATP-dependent reverse electron transport which is completely rotenone sensitive and partially chloroquine sensitive.)

Effect of Coenzyme Q_6 on Inhibition. Folkers *et al.* (Catlin *et al.* 1968; Castelli *et al.*, 1971) have found that inhibition of NADH and succinate oxidase activities by certain other dimethoxy-6-hydroxy-5-alkyl-1,4-benzoquinones can be reversed by various coenzyme Q's. Tables VII and VIII, however, show that for NADH and succinate cytochrome *c* reductase and NADH and succinate oxidase activities such a reversal can occur only if the C-OH-BQ concentration is low. Q_6 also has little effect on the C-OH-BQ bypass of antimycin A inhibition.

Effect of Coupling on Inhibition. Howland *et al.* (Howland, 1965; Howland *et al.*, 1973) have shown that uncouplers will reverse the inhibition by either HOQHO or cer-

TABLE V: Effects of Complex I Inhibitors and Antimycin A on Cytochrome *c* Reductase Activities in the Presence or Absence of High Levels of C-OH-BQ.[a]

	(μmol of Cytochrome *c* per min per mg of Protein)			
	NADH to Cytochrome *c* Reductase		Succinate to Cytochrome *c* Reductase	
Additions	No C-OH-BQ	+35.0 μg of C-OH-BQ	No C-OH-BQ	+35.0 μg of C-OH-BQ
None	0.71	0.30	0.49	0.14
1.5 μmol of chloroquine	0.08	0.08	0.57	0.13
1.2 μg of rotenone + 0 nmol of Q_6		0.06		0.15
1.2 μg of rotenone	0.05		0.56	
60 nmol of Q_6	0.69	0.24	0.60	0.15
0.5 nmol of antimycin A	0.07	0.22	0.02	0.09

[a] All assays were run according to procedures used for Table II. All inhibitors were added before the initiation of the 3-min incubation period. Protein was 0.13 mg.

TABLE VI: Reverse Electron Transport from Dithiothreitol Reduced Benzoquinone.[a]

Benzoquinone	Inhibitor	μmol of NAD per min per mg of Protein
Experiment 1		
DQH_2	None	0.078
None	None	0
35 μg of C-OH-BQ	None	0.013, 0.008
35 μg of C-OH-BQ	0.5 mM chloroquine	0.005
Experiment 2		
DQH_2	None	0.024
17.5 μg of C-OH-BQ	None	0.014
None	None	0
17.5 μg of C-OH-BQ	Rotenone	0

[a] Assays for experiment 2 were performed according to the procedure for Table IV. The above values represent rate increases upon addition of ATP. A significant ATP and enzyme independent rate was observed in the presence of C-OH-BQ. Assays for experiment 1 were performed according to the procedure for experiment 2 except that an Amino SPF 125 fluorometer with external recorder was used. No significant C-OH-BQ dependent blank rate was observed using this instrument. Protein was 0.57 mg. For all assays, 6 μmol of dithiothreitol was present.

tain naphthoquinones of electron transport in whole coupled mitochondria. Table IX shows that MOP are coupled, but CCCP only slightly stimulates the C-OH-BQ and C-OH-NQ inhibited oxidase activities. For an uncoupler to truly reverse the effect of an inhibitor, not only must the uncoupler stimulate the inhibited activity, but the uncoupler must stimulate the inhibited activity more than it stimulates the uninhibited activity. However, the uncoupled rate is inhibited to a greater extent than is the uncoupled rate especially at low inhibitor levels.

Discussion

Assays of electron transport activities and spectra of MOP using the conventional electron sources, NADH, succinate, and reduced TMPD, show that the major sites of inhibition by both C-OH-BQ and C-OH-NQ are in the cytochromes b–c_1 region. Activities, such as NADH to DQ and NADH to ferricyanide reductases, succinate reverse electron transport, and cytochrome oxidase, which do not require cytochromes b–c_1 are not inhibited by C-OH-BQ. Of the C-OH-BQ insensitive activities C-OH-NQ only inhibits the ATP requiring reverse electron transport activities. Since C-OH-NQ inhibits reverse electron transfer from coenzyme Q, a reaction which is chloroquine stimulated (unpublished data), C-OH-NQ probably inhibits this activity by interferring with energy transfer. All activities which involve the cytocrome b–c_1 region (NADH and succinate oxidases, NADH and succinate to cytochrome c reductases, and reduced TMPD reverse electron transport) are inhibited by both C-OH-BQ and C-OH-NQ. Both C-OH-NQ and C-OH-BQ cause the disappearance of the cytochrome c_1 and aa_3 peaks in spectra of NADH, succinate, or DQH_2 reduced MOP. These inhibition patterns are similar to those of the known cytochrome b–c_1 inhibitors, HOQNO and antimycin A. Antimycin A has been shown to specifically bind in the cytochrome b–c_1 region (Pumphrey, 1962; Gupta and Rieske, 1973). Other alkylhydroxy-1,4-naphthoquinones besides C-OH-NQ have previously been shown to be cytochrome b–c_1 region inhibitors (Ball *et al.*, 1949; Estabrook, 1958; Tappel, 1960; Howland, 1965; Howland *et al.*, 1973).

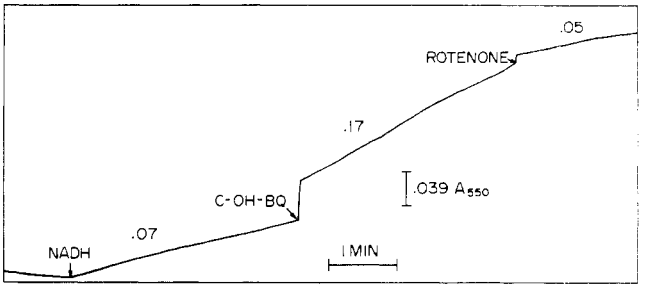

FIGURE 4: The cytochrome c reductase assay was performed according to the procedure for Table II except that 60 nmol of Q_6 was added prior to incubation. The protein was 0.13 mg. The amount of rotenone used was 1.2 μg. The amount of antimycin A used was 0.5 nmol. The amount of C-OH-BQ used was 35.0 μg.

TABLE VII: Effect of Q_6 on Cytochrome c Reductases.[a]

	(μmol of Cytochrome c per min per mg of Protein)			
	NADH to Cytochrome c Reductase		Succinate to Cytochrome c Reductase	
Inhibitor	No Q_6	60 nmol of Q_6	No Q_6	60 nmol of Q_6
Experiment 1				
None	0.86	0.99		
3.5 μg of C-OH-BQ	0.40	0.47		
Experiment 2				
None	0.71 (av 3 runs)	0.69	0.50	0.60
0.25 μg of antimycin A	0.07	0.07	0.02	0.03
35 μg of C-OH-BQ	0.30	0.24	0.14	0.15
35 μg of C-OH-BQ + 0.26 μg of antimycin A	0.22	0.23	0.09	0.08

[a] All assays were performed according to the procedure for Table II. For both experiments protein was 0.13 mg. Also, Q_6 and any inhibitors were added prior to the initiation of the 3-min incubation period.

TABLE VIII: Effect of Q_6 on NADH and Succinate Oxidase Activities in EDTA–Phosphate Buffer.[a]

	(μmol of $2e^-$ per min per mg of protein)			
	NADH Oxidase		Succinate Oxidase	
Inhibitor	No Q_6	60 nmol of Q_6	No Q_6	60 nmol of Q_6
None	1.55	1.50	0.33	0.31
0.52 μg of Antimycin A	0.04	0.03	0.02	0.03
4 μg of C-OH-BQ	0.07	0.10	0.03	0.03
4 μg of C-OH-NQ	0	0	0.01	0.01

[a] Assays were run as for Table I except that the assay medium was 83 μM EDTA–83 mM phosphate (pH 7.4). Protein was 0.15 mg.

Other dimethoxy-6-hydroxy-5-alkyl-1,4-benzoquinones have been previously shown to inhibit succinate and NADH oxidase activities (Catlin *et al.*, 1968; Castelli, 1971). Based on the differential reversal by various coenzyme Q's of these inhibitions of these two activities the site of inhibition was though to be at coenzyme Q. C-OH-BQ does not appear to inhibit at coenzyme Q as NADH to DQ reductase activity which requires coenzyme Q (Ruzicka and Crane, 1971) is not inhibited significantly more by C-OH-BQ than by antimycin A. However, the antimycin A bypass created by C-OH-BQ may begin at or just below coenzyme Q, as this bypass is inhibited by chloroquine, a coenzyme Q inhibitor (Skelton *et al.*, 1968).

The incomplete inhibition by C-OH-BQ of activites using either succinate or NADH as electron sources may be due to any of a number of possibilities. The bypass phenomenon can account for much of the C-OH-BQ insensitive NADH and succinate oxidase and NADH and succinate to cytochrome *c* reductase activities. Related to the above phenomenon is the possibility that reduced C-OH-BQ may be a poor inhibitor. Because of insufficient quantities of C-OH-BQ, we were unable to demonstrate enzymatic reduction of C-OH-BQ by first exposing C-OH-BQ to substrate reduced enzyme, then extracting the reduced C-OH-BQ. Also, the ionized C-OH-BQ may be a poor inhibitor compared to the un-ionized form. In acidic assay media C-OH-BQ becomes a better inhibitor than is HOQNO in basic assay media. This pH effect has been previously demonstrated for certain alkyl-hydroxy-1,4-naphthoquinones. However, the pH effect for C-OH-BQ may be a pH effect only on the ability of ETPH to reduce C-OH-BQ.

The partial inhibitions by C-OH-BQ and C-OH-NQ on activities using DQ and DQH_2 become consistent with the above inhibition and bypass sites if one considers the possibility that there may be at least two sites for DQH_2 dehydrogenation, one at a non-heme iron discovered by J. Hare (Hare and Crane, 1971, 1973) just before cytochromes *b* and a second site in the cytochromes *b* region as indicated by Boveris *et al.* (Boveris *et al.*, 1971, 1972). That the antimycin-insensitive, NADH to DQ reductase activity, which was first discovered by F. Ruzicka (Ruzicka and Crane, 1970; 1971) is four to five times greater than the antimycin A sensitive reductase activity in MOP is one indication that Hare's and Ruzicka's site is active in MOP. In the presence of dithiothreitol at least 20% of the DQH_2 reverse electron transport activity becomes insensitive to C-OH-BQ, antimycin A, and HOQNO at inhibitor concentrations sufficient to completely inhibit reverse electron transport from reduced TMPD (this paper as well as unpublished data). That both DQH_2 dehydrogenation sites are at the same and/or higher potential than the proposed C-OH-BQ reduction site is indicated by the fact that C-OH-BQ does not reverse the antimycin A inhibition of DQH_2 oxidase and also by the facts that the extent of C-OH-BQ inhibition of DQH_2 oxidase is greater than the extent of C-OH-BQ inhibition of NADH oxidase (Tables I and IX) and also is equal to the extent of antimycin A inhibition of DQH_2 oxidase activity.

The use of C-OH-BQ and C-OH-NQ should shed some light on the functioning of the cytochromes b–c_1 region. For instance, in a Wikström (Wikstrom and Berden, 1972) type experiment where the combination of both anaerobicity and an electron mediator such as PMS or TMPD prevent the antimycin A induced, increased reduction of cytochrome b_{556}, C-OH-BQ could conceivably function in the place of both antimycin A and the electron mediator.

Acknowledgments

We thank Dr. T. H. Porter and Dr. K. Folkers of the In-

TABLE IX: Effect of Uncoupling on C-OH-BQ and C-OH-BQ Inhibition of Oxidase Activities.[a]

	(μatoms of O per min per mg of Protein)			
	NADH Oxidase		DQH_2 Oxidase	
	No CCCP	1.02 μg of CCCP	No CCCP	1.02 μg of CCCP
Experiment 1				
None	0.47	0.84	0.66	1.01
4.0 μg of C-OH-NQ	0 (100%)	0.01 (99%)	0.13 (80%)	0.15 (85%)
35 μg of C-OH-BQ	0.12 (74%)	0.13 (84%)	0.14 (79%)	0.16 (84%)
Experiment 2				
None	0.49	0.79	0.85	1.38
1.8 μg of C-OH-BQ	0.38 (22%)	0.54 (32%)	0.45 (47%)	0.55 (58%)
7.0 μg of C-OH-BQ	0.22 (54%)	0.34 (56%)	0.21 (75%)	0.23 (82%)

[a] All assays were run according to the procedure for Table I. All assay contained 180 μg of cytochrome *c*. Protein for NADH and oxidase activities were respectively 0.66 and 0.40 mg. Values in parentheses represent percent inhibition.

stitute for Biomedical Research, University of Texas, for kindly supplying us with heptyl coenzyme Q (2,3-dimethoxy-5-methyl-6-heptylbenzoquinone), C-OH-NQ, and C-OH-BQ.

References

Ball, E. G., Afinsen, C. B., and Cooper, O. (1949), *J. Biol. Chem. 168,* 257.
Bogentoft, C., von Klaudz, A., and Folkers, K. (1972), *J. Med. Chem. 15,* 1135.
Boveris, A., Oshino, R., Erecinska, M., and Chance, B. (1971), *Biochim. Biophys. Acta 245,* 1.
Boveris, A., Erecinska, M., and Wagner, M. (1972), *Biochim. Biophys. Acta 256,* 223.
Castelli, A., Bertoli, E., Littarru, G. P., Lenaz, G., and Folkers, K. (1971), *Biochem. Biophys. Res. Commun. 42,* 806.
Catlin, J. C., Pardini, R. S., Daves, G. D., Jr., Heidker, J. C., and Folkers, K. (1968), *J. Amer. Chem. Soc. 90,* 3572.
Estabrook, R. W. (1958), *J. Biol. Chem. 230,* 735.
Fieser, L. F., Berliner, E., Bondhus, F. T., Chang, F. C., Dauben, W. C., Ettlinger, M. G., Fawaz, G., Fields, M., Heidelberger, C., Heyman, H., Vaugham, W. R., Wilson, A. G., Wilson, E., Wu, M. I., Leffler, M. T., Hamlin, K. E., Matson, E. J., Moore, E. E., Moore, M. B., and Zaugg, H. E. (1948), *J. Amer. Chem. Soc. 70,* 3174.
Gupta, U. D., and Rieske, J. S. (1973), *Biochem. Biophys. Res. Commun. 54,* 1247–1254.
Hansen, M., and Smith, A. L. (1964), *Biochim. Biophys. Acta, 81,* 214.
Hare, J. F., and Crane, F. L. (1971), *Bioenergetics 2,* 317.
Hare, J. F., and Crane, F. L. (1973), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31,* 595.
Howland, J. L. (1965), *Biochim. Biophys. Acta 105,* 205.
Howland, J. L., Lichtman, J. W., and Settlemire, C. T. (1973), *Biochim. Biophys. Acta 314,* 154.
King, T. E. (1967), *Methods Enzymol. 10,* 203.
King, T. E., and Howard, R. L. (1967), *Methods Enzymol. 10,* 275.
Lightbrown, J. W., and Jackson, F. L. (1956), *Biochem. J. 63,* 130.
Low, H., and Vallin, I. (1963), *Biochim. Biophys. Acta 69,* 361.
Pumphrey, A. M. (1962), *J. Biol. Chem. 237,* 2384.
Ruzicka, F. J., and Crane, F. L. (1970), *Biochim. Biophys. Acta 223,* 71.
Ruzicka, F. J., and Crane, F. L. (1971), *Biochim. Biophys. Acta 226,* 221.
Skelton, F. S., Pardini, R. S., Heidker, J. C., and Folkers, K. (1968), *J. Amer. Chem. Soc. 90,* 5334.
Taggart, W. V., and Sanadi, D. R. (1972), *Biochim. Biophys. Acta 267,* 439.
Tappel, A. L. (1960), *Biochem. Pharmacol. 3,* 289.
Thorn, M. B. (1956), *Biochem. J. 63,* 420.
Thorn, M. B., and Jackson, F. L. (1959), *Biochim. Biophys. Acta 35,* 65.
Tisdale, H. E. (1967), *Methods Enzymol. 10,* 213.
Wikstrom, M. K. F., and Berden, J. A. (1972), *Biochim. Biophys. Acta 283,* 403.
Yonetani, T. (1961), *J. Biol. Chem. 236,* 1680.

Modification of Histone Binding in Calf Thymus Chromatin by Protamine†

Thomas K. Wong and Keiji Marushige*

ABSTRACT: When calf thymus chromatin is incubated with protamine, the protein binds to DNA, forming a chromatin–protamine complex. The binding reaches a saturating level at the weight ratio of protamine to DNA of approximately 0.5. Although the saturated binding of protamine to DNA does not cause major displacement of histones from calf thymus chromatin, examination of the dissociation profiles by salt in combination with urea of protamine-treated chromatin shows that the histone–DNA interactions are markedly altered by such binding. The dissociation of histones from the chromatin–protamine complex requires less NaCl but the same concentration of urea as that for untreated chromatin, suggesting that the electrostatic interactions between the histones and DNA are decreased as a result of protamine binding. When protamine concentration is increased beyond that required for saturated binding to DNA during *in vitro* exposure of calf thymus chromatin to protamine, lysine-rich histone is completely displaced.

Chromosomal DNA becomes tightly packaged during transformation of spermatids into spermatozoa at the terminal stages of spermatogenesis. In the salmonid and related fish, the packaging of DNA occurs as a result of replacement of the entire complement of somatic-type histones by protamine (Alfert, 1956; Ingles *et al.*, 1966; Marushige and Dixon, 1969). The mechanism by which somatic-type histones are totally displaced has not been fully understood. Histones isolated from nucleohistone portions of trout spermatid chromatin show chromatographic profiles and amino acid compositions indistinguishable from those of calf thymus chromatin (Marushige and Dixon, 1971). Further-

† From the Laboratories for Reproductive Biology and the Department of Biochemistry, Division of Health Affairs, University of North Carolina, Chapel Hill, North Carolina 27514. *Received July 9, 1974.* This work was supported in part by a grant from the Rockefeller Foundation to the Laboratories for Reproductive Biology, University of North Carolina, and in part by grants from the North Carolina United Community Services and from the University of North Carolina Research Council.