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INHIBITION OF THE MITOCHONDRIAL RESPIRATORY CHAIN BY ALKYLHYDROXYNAPHTHOQUINES:

REVERSAL ON DISCHARGE OF THE ENERGIZED STATE

J. L. HOWLAND, J. W. LICHTMAN and C. T. SETTLEMIRE

Committee on Biochemistry and Department of Biology Bowdoin College, Brunswick, Me. 04011 (U.S.A.)

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SUMMARY

Inhibition of mitochondrial respiration by alkylhydroxynaphthoquinones may be reversed by addition of a variety of uncouplers including substituted phenols, carbonyl cyanide phenylhydrazones, divalent cations and univalent cations in the presence of ionophoretic antibiotics. A likely explanation for such reversibility is the requirement that the anionic inhibitor be transported to a site of action within the mitochondrion. Support for this view includes (1) failure to obtain reversal of inhibition with submitochondrial particles, (2) release of inhibition by a competing anion, succinate, (3) augmentation of inhibition when a divalent cation is taken up, (4) the chemical diversity of uncouplers that release inhibition and (5) inhibiton by uncoupling compounds of the uptake of labeled alkylhydroxynaphthoquinones. It is suggested that a similar explanation may apply to two other inhibitors of the cytochrome *b–c* region, antimycin and alkylhydroxyquinoline-*N*-oxides.

INTRODUCTION

Inhibition of the mitochondrial respiratory chain by a number of compounds has been shown to be reversible upon addition of uncouplers of oxidative phosphorylation[1]. For example, inhibition produced by amytal, which acts in the region of the first energy-coupling site is prevented by the presence of an uncoupler such as 2,4-dinitrophenol[2]. Likewise, effects of inhibitors with loci in the region of the second site (such as alkylhydroxynaphthoquinones and alkylquinoline-*N*-oxides) and the third site (azide) have all been shown to be reversible on addition of uncoupling agents[3,4]. Moreover, in all cases, reversibility of inhibition is obtained only at relatively low concentrations of inhibitor, and the effect of an uncoupling agent may be overridden by addition of excess inhibitor.

The results mentioned above have been taken to suggest that, at low concentra-

Abbreviations: BNQ, 2-hydroxy-3-α-butenyl-1,4-naphthoquinone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; NQNO, 2-nonyl-4-hydroxyquinoline-*N*-oxide.

tions, the inhibitory agents in question act at the level of energy-conserving reactions, presumably reacting with so-called high-energy intermediates of oxidative phosphorylation[1–4]. According to this point of view, their mechanism of action would be complex since, at higher concentrations, they appear to act directly on the respiratory chain, inhibiting even respiration in such systems as the Keilin–Hartree heart muscle preparation, where energy-linked events are largely lacking[5]. The force of this explanation is, moreover, diminished by the continuing lack of any experimental evidence for the existence of such high-energy intermediate compounds with which the inhibitors should react (see, for example, ref. 6).

Alternately, it has been suggested that certain inhibitors and uncouplers may act at the same locus in the energy-transfer apparatus and that the distinction between them may reside only in their kinetic properties[7]. Thus, reversal of inhibition would result from competition between inhibitor and uncoupler. Such an explanation is attractive in the sense that it predicts the overriding effect of excess inhibitor but becomes somewhat less so upon consideration of the chemical diversity of many compounds in both categories.

Finally, the release of inhibition by uncouplers may reflect a requirement for active translocation of the inhibitor to its site of interaction within the mitochondrion. The uncoupler would, then, prevent concentrative transport and lead to a diminution of the concentration of inhibitor at its locus of action. This paper presents evidence in support of such an explanation as applied to inhibitory hydroxynaphthoquinones which act in the vicinity of cytichrome *b* and, by inference, to other inhibitors acting in that region. Palmieri and Klingenberg[8] have advanced a similar mechanism to explain reversibility of azide inhibition of respiration.

MATERIALS AND METHODS

Rat liver mitochondria were isolated according to the method of Myers and Slater[9] and suspended in 250 mM sucrose. Measurement of oxygen consumption was carried out polarographically using a Gilson medical electronics oxygraph. Oxygen uptake was estimated at 25 °C and at pH 7.5 and the reaction mixture contained 15 mM KCl, 2 mM EDTA, 50 mM Tris–HCl, 5 mM $MgCl_2$, 60 mM succinate, 12 mM phosphate, 25 mM sucrose and from 3.5 to 8.2 mg of mitochondrial protein in a volume of 1.5 ml. Protein concentrations for the individual experiments are given in the legends to tables. Inhibitors were added dissolved in ethanol, and in no case did the final ethanol concentration exceed 2%, a concentration without effect on any of the reactions studied. Protein was measured using Biuret reagent[10].

Submitochondrial particles were obtained from rat liver mitochondria by sonication for 2 min at 0–4 °C at full power employing a Bronwill Biosonik III ultrasonic oscillator. Sonication was carried out in a stainless steel vessel immersed in an ice–acetone mixture and using mitochondria at a protein concentration of about 10 mg/ml suspended in 10 mM Tris–HCl, pH 7.4. The disrupted mitochondria were centrifuged for 20 min at $25000 \times g$ to remove large fragments and the supernatant fluid was then centrifuged at $144000 \times g$ for 30 min and the pellet suspended in 250 mM sucrose to a concentration of about 15 mg/ml. The particles were either used immediately or stored at −60 °C for as long as 60 days before use, there being no differences in results obtained under the two conditions. NADH oxidation was measured

spectrophotometrically at 25 °C using a Perkin–Elmer Model 356 spectrophotometer. The reaction medium and other conditions were identical to those employed in the polarographic experiments except that succinate was replaced with 0.5 mM NADH and the reaction volume was 1.0 ml. Succinate–cytochrome *c* reductase was prepared and assayed as described by Yamashita and Racker[11] employing the Perkin–Elmer Model 356 spectrophotometer and a reaction medium containing 40 mM sodium phosphate, 20 mM sodium succinate, 1 mM EDTA, and 1.5 mg of cytochrome *c* in a volume of 1.0 ml. The reaction was followed by measuring absorbance at 550 nm at pH 7.4 and at 25 °C. The reaction was initiated by addition of enzyme.

The synthesis and purification of ^{14}C-labeled 2-hydroxy-3-α-butenyl-1,4-naphthoquinone (BNQ) was carried out following the method of Hooker[12] using unlabeled 2-hydroxynaphthoquinone and *n*-[1-^{14}C]butylaldehyde which was obtained from Volk Radiochemical Co. Samples of substituted hydroxynaphthoquinones were obtained through the generosity of Professor L. Fieser. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was the generous gift of D. P. G. Heytler and octyldinitrophenol was that of Professor E. C. Slater. Antimycin and 2-nonyl-4-hydroxyquinoline-*N*-oxide (NQNO) were obtained from Sigma Chemical Company; gramicidin and valinomycin were obtained from Calbiochem.

The uptake of BNQ was measured by incubating mitochondria in the presence of BNQ at 25 °C for 30 s in a water bath. The reaction mixture was identical to the standard oxygraph mixture except that it contained 64 nmoles of BNQ corresponding to about 2000 cpm. The reaction was terminated by rapid dilution in 5 volumes of 250 mM sucrose at 0 °C followed by rapid mixing and immediate filtration on millipore filters with a pore diameter of 0.65 μm. The mitochondria on the filter were then washed with 10 ml of 250 mM sucrose at 0 °C. The filters were dried in scintillation vials and radioactivity was measured for a length of time sufficient to give more than 10^3 counts in excess of background. In other experiments, uptake was found to be linear for at least 45s.

RESULTS

Since it has been suggested that release of inhibition could be the result of competition between inhibitors and uncouplers for energized intermediates or for common sites in the respiratory chain[3,7], it was of interest to examine the variety of uncoupling treatments that could release respiratory inhibition. Table I illustrates the range of uncouplers that prevent inhibition by hydrolapachol (2-hydroxy-3-(3-methylbutyl)-1,4-naphthoquinone) and BNQ. It is clear that the list includes, not only classical anionic uncouplers such as 2,4-dinitrophenol, octyldinitrophenol, FCCP and CCCP, but also the permeant cations, Ca^{2+} and Sr^{2+}. In addition, disruption of mitochondrial structure by addition of the detergent, desoxycholate, prevents the inhibitory action of this concentration of hydrolapachol. In other experiments which are not presented, a similar range of uncoupling treatment prevented inhibition by 2-hydroxynaphthoquinones with other 3-position side chains including dimethyloctyl, 3′-methylbutenyl, and cyclohexyl.

Results presented in Table II show that release of hydrolapachol inhibition also occurs on addition of the ionophore, gramicidin, as well as the combined addition of valinomycin and nigericin, there being, in all cases, K^+ in the reaction mixture.

TABLE I

RELEASE OF NAPHTHOQUINONE INHIBITION OF SUCCINATE OXIDATION UNDER UNCOUPLING CONDITIONS

Experimental conditions were as described in Materials and Methods (for polarographic experiments) with 2 mM AMP and 4.5 mg of mitochondrial protein per ml.

Experiment	*Inhibitor*	*Uncoupler*	*Oxygen uptake (μatoms/min)*
I	None	None	0.370
	15 μg hydrolapachol	None	0.026
	15 μg hydrolapachol	100 μM 2,4-dinitrophenol	0.314
	15 μg hydrolapachol	10 μM 6-octyl-2,4-dinitrophenol	0.370
	15 μg hydrolapachol	2.5 μM CCCP	0.370
	15 μg hydrolapachol	2 μM FCCP	0.365
	15 μg hydrolapachol	1 mM $CaCl_2$	0.167
	15 μg hydrolapachol	1 mM $SrCl_2$	0.263
	15 μg hydrolapachol	0.01% deoxycholate	0.270
II	None	None	0.345
	50 μg BNQ	None	0.078
	50 μg BNQ	2.5 μM CCCP	0.144
	50 μg BNQ	500 μM 2,4-dinitrophenol	0.098

TABLE II

RELEASE OF NAPHTHOQUINONE INHIBITION OF SUCCINATE OXIDATION BY IONOPHORETIC ANTIBIOTICS

Conditions are identical to those of Table I but with 3.65 mg of protein per ml.

Additions	*Oxygen uptake (μatoms/min)*
None	0.282
15 μg hydrolapachol	0.020
Hydrolapachol + 20 μg gramicidin	0.133
Hydrolapachol + 0.5 μg nigericin	0.044
Hydrolapachol + 5 μg valinomycin	0.022
Hydrolapachol + valinomycin + nigericin	0.276

These antibiotics, which are oligopeptides, bear no chemical resemblance to the uncouplers listed in Table I and, in fact, do not lead to a release of inhibition in medium lacking added K^+ or Na^+. It is of interest that neither valinomycin nor nigericin added singly is effective in releasing hydrolapachol inhibition since they have been shown to uncouple only in combination, where the action of valinomycin as a K^+ porter, together with that of nigericin as a K^+–H^+ antiporter, results in collapse of the H^+ gradient and membrane potential[13]. Thus, the wide variety of uncoupling treatments which release inhibition renders it unlikely that any simple form of competition will provide a suitable explanation.

Influence of excess succinate

Uncoupling agents have been shown to inhibit respiration when added at relatively high concentrations[14], and the inhibition appears to represent competition between the anionic uncoupler and substrate for entry into the mitochondrion[15]. Data presented in Table III suggests, similary, that competition occurs between succinate and hydrolapachol, with high concentrations of succinate effectively releasing hydrolapachol inhibition. It is clear from Experiment II that the lower concentration of succinate is sufficient to produce a maximum rate of respiration.

TABLE III

REVERSAL OF NAPHTHOQUINONE INHIBITION OF SUCCINATE OXIDATION BY EXCESS SUBSTRATE

Experimental conditions were as described in Materials and Methods with the reaction medium containing, in addition, 10 μM rotenone and 6.3 mg of mitochondrial protein.

Additions	*μatoms oxygen/min*	
	+AMP	*−AMP*
Expt I		
3 mM succinate	0.349	0.10
3 mM succinate + 60 μg hydrolapachol	0.060	0.04
72 mM succinate + 60 μg hydrolapachol	0.099	0.07
144 mM succinate + 60 μg hydrolapachol	0.114	0.09
Expt II		
3 mM succinate	0.34	
72 mM succinate	0.32	

Coupling between hydrolapachol and calcium flux

If respiratory chain inhibitors, such as hydrolapachol, behave as permeant anions, it becomes of interest to consider the relation between their uptake and that of a permeant cation such as Ca^{2+}. In Expt I of Table IV addition of 1 mM Ca^{2+} to mitochondria respiring with succinate produces only slight stimulation of respiration in the absence of phosphate. A sub-inhibitory concentration of hydrolapachol added subsequently produces substantial stimulation. Thus, hydrolapachol appears to replace phosphate in promoting the Ca^{2+}-induced stimulation of respiration.

Since a small addition of hydrolapachol produces a relatively massive increase in oxygen uptake, it is clear that it does not act in a manner strictly analogous to phosphate, whose effect would be stoichiometric. Thus, it appears that the quinone traverses the membrane in a cyclic fashion, possibly accompanying Ca^{2+} in its anionic form and returning in the uncharged state. Such an explanation would necessarily involve a net outward translocation of H^+, the hydrolapachol becoming protonated in the mitochondrial interior. This situation would be in conformity with the well known H^+ efflux associated with Ca^{2+} uptake (see for example, ref. 6).

In this experiment, addition of a similar amount of hydrolapachol produced no change in rate when Ca^{2+} was omitted, Expt II in the same table shows a reversed situation, where addition of a low concentration of $CaCl_2$ promotes hydrolapachol

TABLE IV

COUPLING BETWEEN HYDROLAPACHOL AND CALCIUM FLUX IN THE ABSENCE OF PHOSPHATE

Experimental conditions were as described in Materials and Methods for polarographic measurement of succinate oxidation with 6.9 mg protein in Expt I and 3.5 in Expt II. In both experiments phosphate was omitted from the reaction medium.

Additions	*Succinate oxidation (μatoms/min)*
Expt I	
None	0.090
1 mM $CaCl_2$	0.112
1 mM $CaCl_2$ + hydrolapachol (1.45 μg/mg protein)	0.367
Expt II	
None	0.061
Hydrolapachol (5.7 μg/mg protein)	0.133
Hydrolapachol (5.7 μg/mg protein) + 3 mM $CaCl_2$	0.033
Hydrolapachol (5.7 μg/mg protein) + 6 mM $CaCl_2$	0.128

inhibition of respiration. Addition of a higher concentration of Ca^{2+} leads to release of inhibition as noted above in the case of uncoupling conditions. The apparently paradoxical influence of Ca^{2+} in promoting hydrolapachol inhibition appears due to the hydrolapachol anion being passively drawn into the mitochondria as Ca^{2+} undergoes translocation. The coupling between Ca^{2+} and hydrolapachol transport leads either to stimulation of respiration (as noted in Expt I) or inhibition(Expt II) depending upon concentration of hydrolapachol.

Other inhibitors

Although this communication is mainly concerned witht the reversibility of naphthoquinone inhibition, it is interesting to note that a number of features of these inhibitors are shared with other compounds acting in the same region of the respiratory chain. For instance, inhibition by both antimycin and quinoline-*N*-oxides, which act at a site near, but not identical to, the naphthoquinone locus[16], is reversed under uncoupling conditions (Table V). In both instances, reversal of inhibition has been obtained under a variety of uncoupling treatments including classical uncouplers and induction of energy-linked ion translocation. In neither case, however, has it proved possible to observe reversal of inhibition through the addition of excess succinate, a failure that may be a consequence of the much tighter binding of these inhibitors when compared to naphthoquinones[17]. In addition, the release of antimycin inhibition appears to differ from that of the naphthoquinones in the narrow range of antimycin concentration where it can be observed, as even a small excess of the inhibitor renders it immune to the action of uncouplers (see bottom line of Expt II in the table). Presumably, the difference resides, again, in the extremely tight binding of antimycin to its locus of action, especially in the presence of phosphate. Finally, Zn^{2+} at low concentrations inhibits the respiratory chain between cytochromes *b* and *c*[18] and has also been shown to be, itself, actively translocated[19]. Therefore, it is of interest that Zn^{2+} inhibition is also reversible upon addition of the uncoupler, octyldinitrophenol.

TABLE V

INHIBITION OF SUCCINATE OXIDATION BY ANTIMYCIN, NQNO AND Zn^{2+}: RELEASE BY UNCOUPLING AGENTS

Conditions were as described in Materials and Methods for polarographic experiments with 1.1 mM AMP and 8.2 mg/ml of mitochondria protein. In the experiments with antimycin, phosphate was omitted from the medium which also contained a phosphate trapping system consisting of 30 mM glucose together with 50 units of hexokinase (Sigma Type IV).

Inhibitor	*Uncoupler*	*Oxygen uptake (% control)*
Expt I		
None	None	100
1 μg NQNO	None	12
1 μg NQNO	2.5 μM CCCP	40
1 μg NQNO	0.01% deoxycholate	100
1 μg NQNO	2 mM $CaCl_2$	67
Expt II		
None	None	100
4 ng antimycin	None	39
4 ng antimycin	10 μM CCCP	93
4 ng antimycin	24 μg gramicidin	85
4 ng antimycin	100 μM $CaCl_2$ + 1.5 mM phosphate	92
8 ng antimycin	None	9
8 ng antimycin	10 μM CCCP	9
Expt III		
None	None	100
5 mM $ZnCl_2$	None	3
5 mM $ZnCl_2$	6-Octyl-2,4-dinitrophenol	54

Studies with submitochondrial systems

If the reversibility of naphthoquinone inhibition of the respiratory chain reflects a requirement for active translocation then it would be of obvious interest to study systems where this requirement was lacking—that is, where the site of inhibition was exposed to the reaction medium without a permeability barrier. Such a situation exists in the case of sonically-produced submitochondrial particles which have been clearly shown to be inside out with respect to intact mitochondria[20]. In such particles, inhibition by the naphthoquinone, hydrolapachol, is not reversible by addition of 2,4-dinitrophenol (Table VI) nor by any other uncoupler employed, including octyldinitrophenol, FCCP or valinomycin *plus* nigericin. Indeed, addition of the uncoupler leads to increased inhibition, an observation that would be expected if, in the inside out particles, availability of energy produced an active exclusion of the anionic inhibitor.

It is clear that, on a protein basis, the particles are considerably less sensitive to the inhibitor than are intact mitochondria. To obtain substantial inhibition, it is thus necessary to employ a relatively high hydrolapachol/protein ratio and it is therefore of interest that even small inhibition produced by the lower concentration of hydrolapachol is not reversible on addition of uncoupler.

TABLE VI

NADH OXIDATION BY SONIC PARTICLES: INHIBITION BY HYDROLAPACHOL

Experimental conditions were described in Materials and Methods with 90 μg of protein in a volume of 2.0 ml.

Additions	*NADH oxidation (nmoles/min)*
None	27.60
0.6 μg hydrolapachol	22.60
0.6 μg hydrolapachol + 75 μM 2,4-dinitrophenol	18.21
3 μg hydrolapachol	8.71
3 μg hydrolapachol + 75 μM 2,4-dinitrophenol	3.97

TABLE VII

INHIBITION OF SUCCINATE–CYTOCHROME *c* REDUCTASE BY HYDROLAPACHOL: REVERSAL OF INHIBITION BY UNCOUPLERS

Experimental conditions were described in the Materials and Methods with 40 μg of enzyme protein.

Additions	*Cytochrome c reduction (nmoles/min)*
None	0.724
2 μg hydrolapachol	0.122
2 μg hydrolapachol + 20 μM FCCP	0.714
2 μg hydrolapachol + 20 μM 6-octyl-2,4-dinitrophenol	0.490
2 μg hydrolapachol + 0.15% cholate	0.692

Similar experiments were carried out with a succinate–cytochrome *c* reductase preparation isolated according to the procedure of Yamashita and Racker[11]. Examination of this preparation with the electron microscope reveals it to be vesicular and it is likely that its membrane exhibits the same "sidedness" as intact mitochondria, since it reacts readily with cytochrome *c* which is believed to be on the external surface of the inner mitochondrial membrane[6,21]. Thus, it is of interest that, like intact mitochondria and unlike sonic particles, inhibition of succinate oxidation by hydrolapachol is readily reversed by addition of uncouplers such as FCCP and 6-octyl-2,4-dinitrophenol as well as the detergent, cholate.

Estimation of inhibitor uptake

The uptake of an alkylhydroxynaphthoquinone was measured by incubating mitochondria with ^{14}C-labeled BNQ for 30 s followed by millipore filtration. Such an experiment is illustrated in Table VIII where omission of phosphate produced a small decline in uptake while inclusion of uncoupling agents led to significant inhibition. It should be noted that, of the uncouplers employed, CCCP inhibited uptake most

completely and was also the uncoupler most effective in releasing respiratory inhibition.

TABLE VIII

UPTAKE OF ^{14}C-LABELED 2-HYDROXY-3-α-BUTENYL-1,4-NAPHTHOQUINONE BY RAT LIVER MITOCHONDRIA

Transport was measured as described in Materials and Methods. Duration of the reaction was 30 s and the reaction mixture contained 14.4 mg of mitochondrial protein.

Additions	*Uptake (nmoles)*
None	4.40
None, omit phosphate	3.22
5 μM CCCP	1.01
500 μM 2,4-dinitrophenol	2.53
1 mM 2,4-dinitrophenol	1.48
2 mM $CaCl_2$	2.08

DISCUSSION

Results presented in this paper document the wide diversity of uncoupling conditions that reverse the inhibitory effect of alkylhydroxynaphthoquinones upon mitochondrial respiration, a diversity surely inconsistent with any simple competition between inhibitor and uncoupler. It appears likely that such reversibility of inhibition reflects a requirement that the inhibitory compounds which are anions must be transported to their site of action in an energy-linked fashion. This point of view is supported by the influence of the flux of other ions upon inhibition, where, for example, extensive influx of succinate prevents hydrolapachol inhibition presumably by competing as an anion. On the other hand, low concentrations of Ca^{2+} in the absence of another permeant anion increases the effectiveness of hydrolapachol as an inhibitor, presumably by creating a favorable charge balance for its inward translocation. It is significant that inhibition of respiration in sonic submitochondrial particles which exhibit the opposite membrane polarity from intact mitochondria[6,20] is not able to be released by addition of uncoupling compounds which, indeed, increase the effectiveness of the inhibitor. Thus, it would appear that energy-charged submitochondrial particles actively exclude the anionic naphthoquinones and that the exclusion is eliminated in the absence of energy.

Other indications that active uptake of inhibitors accounts for a number of features of the inhibitory process include the demonstration of apparent uptake of a radioactive inhibitory naphthoquinone, a process which is inhibited by uncouplers and which is stimulated by P_i. In this connection, the requirement for phosphate for effective inhibition at low inhibitor concentrations noted earlier[3] represents a feature in common with the transport of a number of other anions, notably certain organic acids. Also consistent with a requirement for energy-linked translocation is the observation that excess inhibitors, themselves, have been previously shown to give rise to uncoupling effects[3,22].

Although the main body of evidence presented in this communication concerns inhibition by alkylhydroxynaphthoquinones, it also is likely that similar concentrative

translocation is required for inhibition by low concentrations of quinoline-*N*-oxides and antimycin (see Table V and ref. 23). In both cases, phosphate is required for most effective inhibition, inhibition is reversible on addition of a variety of uncoupling compounds and, in neither case, does such reversal occur with sonic particles (unpublished experiments). It is interesting, in this context, that Palmieri and Klingenberg[8] reached similar conclusions with regard to azide inhibition.

Finally, if inhibitors acting in the cytochrome *b–c* region of the respiratory chain must traverse a membrane barrier to be effective, then their site of action cannot reside on the external surface of the inner mitochondrial membrane. Moreover, enhanced inhibition of submitochondrial fragments, when inhibitor *plus* uncoupler is present suggests that the locus can neither be on the surface of the matrix side. Thus, these studies lend support to a locus in the interior of the coupling membrane, a conclusion in conformity with present views of the topology of these membranes which place cytochrome *b* in the hydrophobic interior[24].

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