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INFLUENCE OF ALKYLHYDROXYNAPHTHOQUINONES ON THE MITOCHONDRIAL OXIDATION OF TETRAMETHYL-*p*-PHENYLENEDIAMINE

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SUMMARY

1. Those 2-hydroxy-3-alkyl-1,4-naphthoquinones which are saturated at the β bond in the alkyl group inhibit coupled oxidation of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) in the same range of concentration at which they inhibit succinate oxidation.

2. All quinones examined act as uncouplers in that they stimulate mitochondrial ATPase, while those which are saturated at the β bond inhibit succinate and coupled TMPD oxidation at concentrations below those at which uncoupling effects commence.

3. Inhibition of succinate oxidation and of coupled TMPD oxidation appear to reflect a single interaction with the respiratory chain, and this interaction, probably occurring between cytochromes *b* and *c*, should be included in any consideration of phosphorylation sites spanned by the oxidation of TMPD.

INTRODUCTION

Various 2-hydroxy-3-alkyl-1,4-naphthoquinones are inhibitors of mitochondrial electron transport, reacting with the respiratory chain between cytochromes *b* and *c* (ref. 1). The action of these inhibitors may be concerned, at least in part, with energy-transfer reactions in mitochondria, since inhibition is released on addition of uncouplers of oxidative phosphorylation[2,3].

In addition, 2-hydroxy-3-alkyl-1,4-naphthoquinones influence the coupled oxidation of tetramethyl-*p*-phenylenediamine (TMPD) which passes electrons into the respiratory chain in the vicinity of cytochrome *c* (ref. 4). Homologues possessing an alkyl side chain, unsaturated at the β position, uncouple oxidative phosphorylation, stimulating electron transport in the absence of phosphate acceptor. Homologues lacking β unsaturation appear to inhibit oxidative phosphorylation in a manner similar to oligomycin, eliminating the stimulation of respiration produced by phosphate or phosphate acceptor. Both categories of naphtoquinones prevent oxidative ATP synthesis[4].

Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; DNQ, 2-hydroxy-3(3',7'-dimethyloctyl)-1,4-naphthoquinone; CNQ, 2-hydroxy-3-cyclohexyl-1,4-naphthoquinone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

In considering the mechanism of action of these quinones, it is important to know whether their effects on succinate and TMPD oxidation represent a single molecular interaction or several, perhaps unrelated, ones. It is well documented that other respiratory chain inhibitors, such as antimycin, produce a variety of effects, depending upon concentration and the exact character of the system[5–7].

Possible multiplicity of action by quinone inhibitors is of considerable interest as it relates to the location of phosphorylation sites spanned by the oxidation of TMPD. For example, if inhibition by quinones of succinate oxidation (between cytochromes *b* and *c*) and of coupled TMPD oxidation be at the same locus, then the *b–c* region is implicated in energy coupling related to TMPD oxidation. Such a finding would necessarily lead to re-examination of the efficiency and character of phosphorylation coupled to the oxidation of TMPD.

The present communication reports the effect of naphthoquinones on coupled oxidation of TMPD. Results are presented suggesting that all naphthoquinones studied act as uncoupling agents, while those with a saturated β bond in the alkyl side chain inhibit coupled TMPD oxidation as well. These results support the view that inhibition of succinate oxidation and of coupled TMPD oxidation reflect a single interaction between the naphthoquinone and the mitochondrial energy-transfer apparatus.

METHODS

Rat-liver mitochondria were isolated according to the method of MYERS AND SLATER[8] with 0.25 M sucrose as suspension medium. Oxygen consumption was measured polarographically using a Gilson Medical Electronics Oxygraph. The standard Oxygraph reaction mixture contained 15 mM KCl, 2 mM EDTA, 50 mM Tris–HCl, 5 mM $MgCl_2$ and between 2.6 and 5.5 mg mitochondrial protein in a final vol. of 2.0 ml. In experiments with TMPD, a catalytic concentration of TMPD, 60 μM, was employed and it was maintained in a reduced state by the presence of 15 mM ascorbate. Protein was estimated using biuret reagent[9]. Oxidation was measured at 25° and pH 7.4 unless otherwise stated. Inhibitors were added dissolved in ethanol, and in no case did the final ethanol concentration in the reaction mixture exceed 2 %, which was without effect on mitochondrial oxidations.

ATPase activity was estimated, using the method of MYERS AND SLATER[8]. Mitochondrial volume changes were followed at 520 mμ with a Zeiss PMQ II spectrophotometer using the reaction mixture described by AZZONE AND AZZI[10].

Samples of substituted hydroxynaphthoquinones were obtained through the generosity of Prof. L. FIESER. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was the generous gift of Dr. P. G. HEYTLER. Nucleotides were obtained from Nutritional Biochemicals and TMPD from Eastman.

RESULTS

The uncoupling and inhibitory action of naphthoquinones on TMPD-coupled oxidation is illustrated in Table I. The addition of 2-hydroxy-3(3′-methyl-2′-butenyl)-1,4-naphtoquinone (lapachol), which is unsaturated in the β position, leads to stimulation of oxygen consumption in the absence of AMP (Expt. I) or of AMP and phosphate (Expt. II). The fact that a requirement for phosphate has not been demonstrated

TABLE I

EFFECTS OF HYDROXYNAPHTHOQUINONES ON ELECTRON TRANSPORT IN THE PRESENCE OF TMPD AND ASCORBATE

Conditions as described in METHODS section with 4.0 mg protein in the first 2 experiments and 4.3 in the 3rd.

Expt. No.	*Additions*	*Oxygen consumption (μatom/min)*
I	None	0.104
	20 μg lapachol	0.197
	20 μg lapachol + 25 mμmoles rotenone	0.182
	20 μg lapachol + 0.4 μg DNQ	0.139
II	None: omit phosphate	0.115
	20 μg lapachol	0.218
III	None	0.140
	0.7 μmole AMP	0.236
	0.7 μmole AMP + 25 mμmoles rotenone	0.211
	0.7 μmole AMP + 25 mμmoles rotenone + 5 μg DNQ	0.132

for stimulation of respiration by lapachol represents a contrast with the effect of inhibiting quinones on succinate oxidation, where phosphate is required for action at low inhibitor concentrations, probably assisting in permeation of the inhibitor[3].

In addition, the stimulation produced by lapachol is seen in Expt. I to be subject to partial elimination by one of the hydroxynaphthoquinones possessing a saturated β bond, 2-hydroxy-3(3′,7′-dimethyloctyl)-1,4-naphthoquinone (DNQ). Expt. III shows the inhibitory action of DNQ on AMP-stimulated TMPD respiration. Although rotenone, which eliminates endogenous respiration in this system[11], inhibits slightly the rate measured in the presence of AMP, it does not prevent DNQ from producing substantial additional inhibition to a final rate of oxidation about equal to that in the absence of AMP.

Since respiratory chain inhibitors, such as antimycin, are known to exhibit secondary effects at high concentrations[5,7,8], it becomes important to establish concentrations of naphthoquinones necessary to uncouple and inhibit TMPD oxidative phosphorylation compared to concentrations required for effective inhibition of the respiratory chain as spanned by succinate oxidation. Fig. 1 shows the influence of increasing concentrations of 2-hydroxy-3-cyclohexyl-1,4-naphthoquinone (CNQ) on succinate and TMPD oxidation in the presence of AMP. It is clear that one-half maximal inhibition of both activities occurs in the same range of concentration, which suggests that one inhibitory event may be involved. Similar observations were made using other naphthoquinones with saturated side chains.

Fig. 2 illustrates the influence of an increasing concentration of lapachol on TMPD respiration in the absence of AMP, and shows a progressive stimulation of oxygen uptake. It is important that this uncoupling effect is one-half maximal at a concentration of about 0.6 μg/mg protein, while 50 % inhibition of succinate oxidation occurs at a concentration over 10 times as high[3]. Thus, it is clear that the

uncoupling action of the β-unsaturated lapachol is unrelated to its action as a respiratory chain inhibitor.

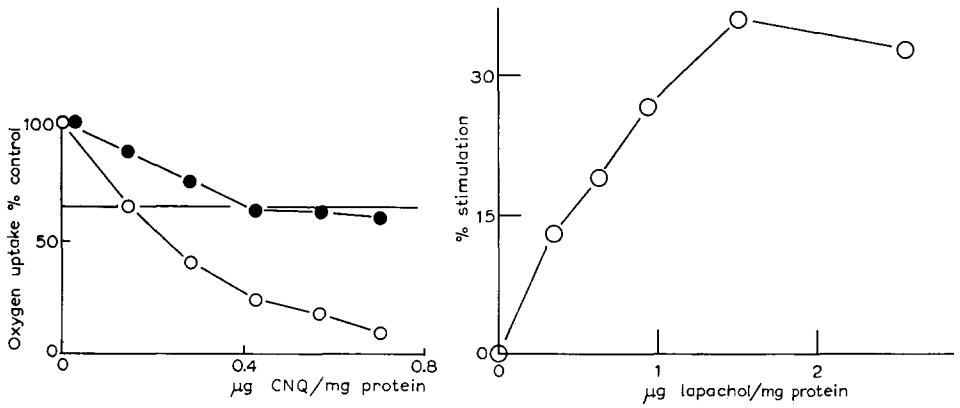

Fig. 1. Effect of increasing concentrations of CNQ on succinate and TMPD oxidation. Conditions as described in METHODS section with 1.1 μmoles AMP, 2.9 mg protein and 60 mM succinate in the case where it was used as substrate. ○, titration curve with succinate as substrate; ●, with TMPD and ascorbate. The horizontal line on the figure represents the rate of TMPD respiration when AMP is omitted.

Fig. 2. Stimulation of TMPD respiration in the absence of AMP induced by increasing concentrations of lapachol. Conditions as described in METHODS section with 3.7 mg of mitochondrial protein.

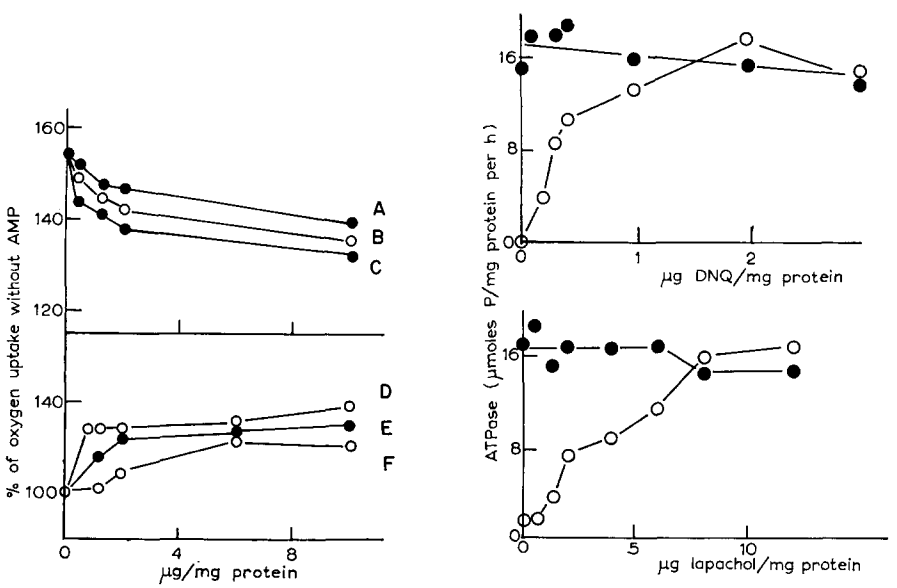

Fig. 3. Influence of pH on the action of naphthoquinones. Conditions as described in METHODS section with 2.6 mg protein. Curves A, B, and C show inhibition by increasing amounts of hydrolapachol of TMPD respiration in the presence of AMP at pH's 7.8, 7.4, and 6.9. Curves D, E, and F show the stimulation of TMPD respiration (absence of AMP) by increasing amounts of lapachol at pH's 7.8, 7.4, and 6.9.

Fig. 4. Stimulation of ATPase by naphthoquinones. Conditions as described in METHODS section with 5.5 mg mitochondrial protein. ●, 250 μM 2.4-dinitrophenol present.

Earlier studies[1,3] have shown that inhibition of succinate oxidation by hydroxynaphthoquinones declines with increasing pH, probably due to decreasing lipid solubility upon ionization of the 2-hydroxyl group. Fig. 3 indicates that inhibition of TMPD respiration in the presence of AMP by hydrolapachol (saturated β bond in the side chain) is likewise most effective at a low pH. On the other hand, lapachol uncouples most effectively at a higher pH, pointing again to a distinction between events resulting in uncoupling and inhibition.

TABLE II

THE EFFECT OF STRUCTURE ON THE ABILITY OF NAPHTHOQUINONES TO INHIBIT OR UNCOUPLE TMPD RESPIRATION

Conditions as in METHODS section with between 3.4 and 5.0 mg protein.

3-Position group of 2-hydroxy-1,4-naphthoquinone	*Unsaturation in 3-position group*	*Concn. of quinone added (µg/mg protein)*	*Observed stimulation (%) (no AMP)*	*Observed inhibition (%) (+AMP)*
3′,7′-Dimethyloctyl (DNQ)	None	1.0	0	45
Cyclohexyl (CNQ)	None	1.0	0	42
3′-Methylbutyl (hydrolapachol)	None	5.3	6	39
3′-Methyl-1′-butenyl (isolapachol)	α	1.7	0	28
CH_3 (phthiocol)	None	17.0	−4	17
3′-Methyl-2′-butenyl (lapachol)	β	2.6	35	1
4′-Hydroxy-3′-methyl-2′-butenyl (lomatiol)	β	5.2	26	8

A number of hydroxynaphthoquinones have been examined with regard to their influence on coupled TMPD oxidation (Table II). The compounds are arranged in order of increasing concentration required to inhibit succinate oxidation 50 % (see ref. 3). Those quinones that inhibit succinate oxidation at a low concentration are the same ones that produce inhibition of coupled TMPD oxidation. β unsaturation in the 3-position side chain renders the quinone inactive as an inhibitor of both succinate and coupled TMPD oxidation, while effective as an uncoupler. Thus, both lapachol and lomatiol stimulate TMPD respiration in the absence of AMP, while hydrolapachol, which differs from lapachol only in the saturation of the β bond, does not, but is a potent inhibitor.

ATPase activity

Inhibitors and uncouplers of oxidative phosphorylation, while alike in eliminating ATP synthesis, differ in their effect on ATPase activity of mitochondria and sub-mitochondrial particles. Uncoupling agents generally stimulate, while inhibitors, such as oligomycin, inhibit mitochondrial ATPase[12]. Fig. 4 indicates that the uncoupling naphthoquinone, lapachol, does, in fact, stimulate mitochondrial ATPase activity in the fashion of uncouplers in general, while remaining without effect on the ATPase stimulated by dinitrophenol. The inhibitory naphthoquinone, hydrolapachol, likewise produces a marked stimulation of ATPase, with no inhibition of dinitrophenol-induced activity. Thus it appears that all naphthoquinones studied act in the manner of uncouplers, in the sense that all stimulate ATPase in the absence of dinitrophenol.

Mitochondrial volume changes

Mitochondria undergo changes in volume which include swelling and shrinking phenomena linked to the availability of intermediates of oxidative phosphorylation. Fig. 5 shows that hydrolapachol does not, by itself, produce significant change in mitochondrial volume, but that inorganic phosphate induces rapid swelling. Such swelling is inhibited (in rate, but not extent) by the addition of ADP which appears to compete effectively for high-energy intermediates of oxidative phosphorylation. The addition of hydrolapachol in the presence of phosphate and ADP restores the rapid swelling obtained with phosphate alone, producing an effect similar to that obtained with the inhibitor atractyloside[10], which appears to interfere with the binding of extramitochondrial ATP and ADP to the mitochondrial membrane[13,14].

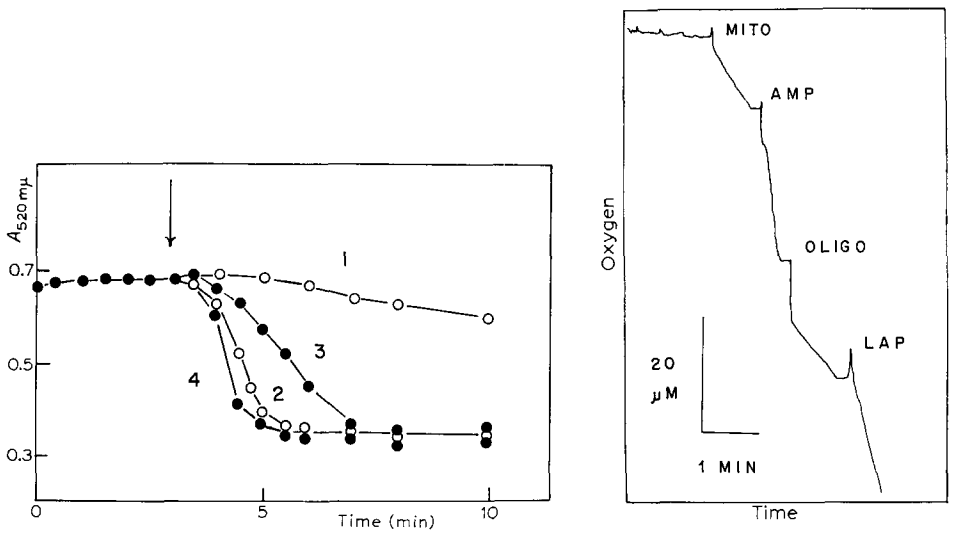

Fig. 5. Influence of hydrolapachol on mitochondrial volume changes. Conditions as described in METHODS section with 2.5 mg mitochondrial protein. Arrow denotes times of addition. Curve 1 shows the influence of 20 μg hydrolapachol. Curve 2 shows that of 6 mM phosphate. Curve 3 shows that of 6 mM phosphate + 0.5 mM ADP. Curve 4 shows that of 6 mM phosphate + 0.5 mM ADP + 20 μg hydrolapachol.

Fig. 6. Inhibition of succinate oxidation by oligomycin and release of inhibition by lapachol. A polarographic trace of oxygen consumption. Conditions as described in METHODS with 60 mM succinate. MITO denotes the addition of mitochondria. AMP denotes that of 0.25 mM AMP, OLIGO that of 20 μg oligomycin, and LAP that of 40 μg lapachol.

Release of inhibition by oligomycin

Fig. 6 is a polarographic tracing and shows that lapachol is able to release inhibition of coupled succinate oxidation by oligomycin, an ability which it shares with other uncouplers of oxidative phosphorylation[12].

DISCUSSION

As well as inhibiting the mitochondrial respiratory chain between cytochromes *b* and *c*, 2-hydroxy-3-alkyl-1,4-naphthoquinones produce important alterations in energy-linked TMPD oxidation. Quinones which are saturated at the β position of

the alkyl side chain are potent inhibitors of succinate oxidation, eliminate ATP synthesis coupled to TMPD oxidation, and inhibit that portion of TMPD respiration which is stimulated by addition of phosphate acceptor. On the other hand, quinones that are unsaturated at the β position, are poor inhibitors of succinate oxidation, produce little effect on phosphate acceptor-stimulated TMPD respiration, but act as classical uncouplers in preventing ATP synthesis and stimulating TMPD respiration in the absence of phosphate or phosphate acceptor. Both inhibitory and uncoupling effects are encountered in the presence of rotenone, which minimizes contribution of endogenous substrates.

Those quinones that inhibit both succinate oxidation and coupled TMPD oxidation do so in the same region of concentration. On the other hand, those that stimulate TMPD respiration do so at concentrations considerably lower than those relatively high concentrations required for their inhibition of succinate oxidation.

These results are taken to suggest that (1) a single molecular event is responsible for inhibition of succinate oxidation and for inhibition of coupled TMPD oxidation and that (2) the inhibitory and uncoupling effects are probably unrelated. The reported stimulation of TMPD oxidation in the absence of AMP is probably a true uncoupling effect, in that the compounds responsible for it also stimulate ATPase, eliminate ATP synthesis[4], and release inhibition induced by oligomycin.

Since all naphthoquinones examined stimulate ATPase, all should be regarded as capable of uncoupling effects. In the case of quinones unsaturated in the β position, uncoupling occurs at a concentration below that required for inhibition, whereas in the case of compounds saturated in that position, the reverse is true. For example, lapachol begins to stimulate ATPase and TMPD respiration at a concentration considerably lower than that required for inhibition of succinate oxidation. On the other hand, hydrolapachol inhibits succinate oxidation and TMPD oxidation at a concentration well below that where uncoupling effects commence.

The inhibiting naphthoquinone, hydrolapachol, while producing little effect on mitochondrial volume, reverses the protective effect of ADP on phosphate-induced swelling, an action reminiscent of that of atractyloside[10] and one that points to additional complexity in the operation of these naphthoquinones.

Lapachol, which is unsaturated in the β position, exerts maximum uncoupling effect at a high pH where lipid solubility might be expected to be lowest. Inhibiting naphthoquinones, on the other hand, are least effective at a higher pH. Furthermore, phosphate is required for action by low concentrations of inhibitory quinones, but not for uncoupling. These observations, together, suggest that uncoupling occurs at a site more accessible to the external aqueous environment of the mitochondrion than does the inhibitory event.

Since naphthoquinones, both saturated and unsaturated at the β position, produce uncoupling effects, it is clear that the character of the side chain does not influence the ability to uncouple, which is more likely related to the 2-position hydroxyl group. On the other hand, β unsaturation with the consequent potential for chromanol ring closure appears inimical to effective inhibition of both succinate and TMPD oxidation. This observation is of considerable interest in view of recent suggestions that chromanols may be intermediates in coupled oxidations[15].

The apparent identity of the inhibitory site in the cases of succinate and TMPD oxidation may shed light on the pathway of energy transfer in the coupled oxidation

of TMPD. Since a portion of TMPD oxidation associated with ATP synthesis is eliminated by a compound that acts between cytochromes *b* and *c*, the cytochrome *b–c*, 'second' phosphorylation site seems implicated in at least a part of TMPD-linked phosphorylation. Since TMPD does not appear to pass electrons directly to cytochrome *b* (ref. 18), the mechanism for such ATP synthesis is unclear. It has been argued that oxidation of endogenous substrates contributes significantly to phosphorylation obtained with TMPD and ascorbate[11,16,17]. Since rotenone, which eliminates such oxidation, does not influence the inhibition of coupled TMPD respiration by naphthoquinones, it is apparent that the proposed involvement of the *b–c* region represents something more than passage of electrons from endogenous substrates through it. Finally, TMPD-linked energy conservation in the cytochrome *b–c* region may be related to the earlier observation of apparent uncoupling of TMPD phosphorylation by antimycin, which was shown to lower P:O ratios by about one-half while stimulating respiration in the absence of phosphate acceptor[18].

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