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EFFECT OF BUTYLATED HYDROXYANISOLE ON ELECTRON TRANSPORT IN RAT LIVER MITOCHONDRIA

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Abstract—The effects of butylated hydroxyanisole (BHA), a commonly used food antioxidant, on oxygen consumption, ATPase activity, and the redox state of some electron carriers of rat liver mitochondria have been studied. It was observed that BHA slightly stimulated state 4 respiration but strongly inhibited ADP- and uncoupler-stimulated respiration on NAD^+- and FAD-linked substrates. ATPase activity and vectorial H^+ ejection were affected only slightly by BHA, suggesting that BHA predominantly inhibits mitochondrial electron flow. Experiments to determine its site of action showed that BHA did not noticeably affect electron flow through cytochrome oxidase; in contrast, NADH:duroquinone reductase activity and electron flow through ubiquinone-cytochrome *b*–cytochrome *c* complex were inhibited strongly because the oxidation of duroquinol was affected markedly. The BHA block of electron transport was bypassed by both *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine and 2,6-dichlorophenolindophenol. Also, the presence of BHA changed the redox state of cytochrome *b* and c_1 to a more oxidized level. These observations suggest that electron transport is inhibited by BHA at the NADH–ubiquinone and at the ubiquinone–cytochrome *b* levels. From Hill plots, it is clear that more than one binding site is involved in complete inhibition; in addition, available evidence suggests that there may be two sites at the substrate side of ubiquinone and another two sites at the oxygen side of ubiquinone. Consequently, mitochondrial ATP synthesis would be interrupted. This event could be related to the toxicity of BHA.

Because of its antioxidant properties, butylated hydroxyanisole (BHA) is used frequently as a preservative in fat- and oil-containing foods. Despite its presumed low toxicity [1–4], BHA exerts a variety of effects on cellular functions. It enhances the hepatic and peripheral activities of several detoxification enzymes [5–9] and protects against certain types of toxins and carcinogens in various organs of rats and mice [4, 10–12]. Moreover, BHA and other phenolic compounds inhibit the growth of several human tumor cell lines [13] and the growth and oxygen consumption of rodent tumor cells and trypanosome parasites [14–16].

Much attention has been paid to the antitumorigenic and antimutagenic actions of phenolic antioxidants but almost none to the possible adverse biological effects. Only recently have cytotoxic effects been observed in isolated rat hepatocytes by BHA. Thompson and Moldéus [17] reported that BHA is able to both inhibit state 3 and stimulate slightly state 4 mitochondrial respiration, when succinate behaves as an electron donor. It also dissipates membrane potential across the mitchondrial membrane and causes the release of calcium ions, mitochondrial swelling, and the decrease of ATP levels in intact hepatocytes, which precede cell death. In the present study, the effects of BHA on the rate of ADP- and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)-stimulated respiration, the flow of electrons through specific segments of the respiratory chain, ATPase activity, and the redox state of cytochromes *b* and c_1 in isolated liver mitochondria are shown. The experiments were designed to localize the sites of respiratory inhibition by BHA. The observations show that BHA primarily inhibited the action of NAD^+- and FAD-linked dehydrogenase systems. Extensive inhibition of both dehydrogenases led, of course, to the cessation of oxidative phosphorylation, the rapid depletion of the ATP supply, and cytotoxic effects.

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MATERIALS AND METHODS

Chemicals. ADP, ATP, antimycin, ascorbate, BHA, CCCP, diadenosine pentaphosphate, 2,6-dichlorophenolindophenol (DCIP), duroquinone, ethylene glycol-bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA), glutamate, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes), malate, rotenone, succinate, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) and valinomycin were purchased from the Sigma Chemical Co. (St. Louis, MO). Duroquinol was prepared from duroquinone as described by Boveris *et al.* [18]. BHA was dissolved in dimethyl sulfoxide. The solvent had no effect at any of the concentrations employed. All the other reagents were of the highest purity commercially available.

Preparation of mitochondria. Male Wistar rats of 180–230 g were used throughout. Mitochondrial suspensions of 50 mg protein/mL were prepared from liver following the procedure of Pedersen *et al.* [19]. Protein concentration was determined by a modified

biuret reaction standardized with serum albumin [20].

Assay of oxygen consumption. The rates of oxygen consumption were determined with a Clark oxygen electrode (Yellow Springs Instrument Co.). The 2.0-mL reaction medium contained final concentrations of 200 mM sucrose, 50 mM KCl, 3 mM Hepes (pH 7.1), 0.5 mM EGTA, 3 mM potassium phosphate, 2 mM magnesium chloride, and 1.0 to 1.3 mg/mL of mitochondrial protein [21]. Substrate concentrations were: 2.5 mM glutamate + 2.5 mM malate or 5 mM succinate or 1 mM duroquinol or 5 mM ascorbate + 0.5 mM TMPD or 25 μM DCIP or 0.1 mM TMPD; when added, the concentrations of ADP and CCCP were 0.18–0.24 mM and 1 μM, respectively, as indicated in the figures. The system was equilibrated with mitochondria at 25° for 2 min; then BHA was added and all measurements were made after a 6-min preincubation with the antioxidant. Each point is the mean ± SD of at least three separate experiments. Respiratory control indices and ADP/O ratios were determined as described by Lessler and Brierley [22].

Assay of ATPase activity. This activity was evaluated by measuring the rate of H^+ ejection accompanying ATP hydrolysis, as described by Floridi and Lehninger [23]. The 2.0-mL reaction medium contained final concentrations of 200 mM sucrose, 50 mM KCl, 3 mM Hepes (pH 7.1), 0.5 mM EGTA, 2 mM magnesium chloride, 4 μM rotenone, 0.2 nmol antimycin/mg protein, 0.01 μM diadenosine pentaphosphate to inhibit adenylate kinase, and 4 mg protein. After 30 sec at 25° 1 μg valinomycin (to provide charge compensation for the ejected H^+) and BHA were added while stirring the reaction. The reaction was started 6 min later by the addition of 400 nmol ATP, and the rate of the subsequent H^+ ejection was recorded. Known amounts of HCl were added as internal standard to calibrate the pH electrode response in all experiments.

Assay of NADH–duroquinone reductase. This activity was evaluated by measuring the rate of NADH oxidation coupled to the duroquinone reduction at 340–370 nm [23, 24]. The 3.0-mL reaction medium contained final concentrations of 200 mM sucrose, 50 mM KCl, 3 mM Hepes (pH 7.1), 3 mM potassium phosphate (pH 7.1), 2 mM magnesium chloride, 0.5 mM EGTA, and 2.0 mg of mitochondrial protein. After 2 min at 25°, BHA was added with stirring; 6 min later, 2.5 mM glutamate + 2.5 mM malate, 0.4 nmol antimycin and 0.1 mM potassium cyanide were added to reduce the endogenous NADH. Then, 0.1 μM CCCP was added, the reaction was started by the addition of 0.3 μmol duroquinone and the rate of the subsequent NADH oxidation was recorded.

Determination of the redox level of cytochromes b *and* c_1. The oxidation of cytochrome *b* was followed at 430–410 nm, whereas that of cytochrome c_1 was followed at 553–541 nm [25] by dual-wavelength spectrophotometry (Aminco DW-2). The cuvette (10 nm light path) was provided with magnetic stirring. The 3.0-mL reaction medium was the same as the one used for the oxygen consumption assay, but the protein concentrations were 0.65 and 0.83 mg/mL for cytochrome *b* and cytochrome c_1 determinations, respectively, and 2.5 mM succinate as substrate. The additions of succinate, cyanide and BHA, as indicated in the figure legends, were made by rapid injection from microsyringes in such a way as to achieve the shortest possible mixing time.

RESULTS

Effects of BHA concentration on the rate of mitochondrial respiration. Figure 1 shows the effects of BHA concentration on the oxidation of glutamate + malate, succinate, and ascorbate + TMPD by rat liver mitochondria in state 4, state 3, and the respective respiratory control indices (state 3 respiration/state 4 respiration). The effects of BHA concentration on mitochondrial state 4 are shown in Fig. 1A. The glutamate + malate oxidation was 7.8 natoms/min/mg protein. BHA began to stimulate the state 4 rate of oxygen utilization at 70 μM, and the maximal stimulation of state 4 respiration was approximately 295% at about 210–280 μM BHA, indicating an uncoupling effect, after which further increases of BHA concentration resulted in a concentration-dependent inhibition of the stimulated oxygen consumption. The addition of CCCP only stimulated slightly, or not at all, further BHA-enhanced state 4 respiration. An essentially similar effect was observed with succinate. The control state 4 respiration rate was 20.7 natoms 0/min/mg, and the maximal stimulation of state 4 respiration was approximately 250% at about 300 μM BHA, results which are in accord with the findings reported by Thompson and Moldéus [17]. With ascorbate + TMPD, the state 4 respiration rate was 126.6 natoms 0/min/mg and BHA stimulated only slightly the state 4 rate of oxygen consumption (at about 500 μM BHA, only 37% stimulation was observed).

However, ADP-stimulated glutamate + malate and succinate oxidations were inhibited strongly by BHA. Hyperbolic inhibition curves were observed, which are shown in Fig. 1B. The addition of BHA decreased the rate of oxygen consumption of mitochondria oxidizing glutamate + malate. The half-maximal rate was obtained at 200 μM and almost complete inhibition by 550 μM. The effect of BHA on the rate of succinate oxidation was essentially the same, as the half-maximal inhibition was attained by 300 μM and maximal inhibition by 700 μM. The effect of BHA on cytochrome oxidase activity coupled to the ADP phosphorylation was studied using ascorbate + TMPD as electron donors. Figure 1B indicates that the phenolic antioxidant was less able to inhibit the rate of oxygen consumption. A biphasic inhibition curve was observed when the BHA concentration was increased, reaching a plateau at 40–50 μM where only 25% inhibition was obtained. Further increases in BHA concentration resulted in a concentration-dependent inhibition; at 900 μM only 45% inhibition was observed. Since BHA was able to both stimulate state 4 and inhibit state 3 respiration, respiratory control indices were also inhibited, as shown in Fig. 1C. The inhibition curves were much steeper in the case of

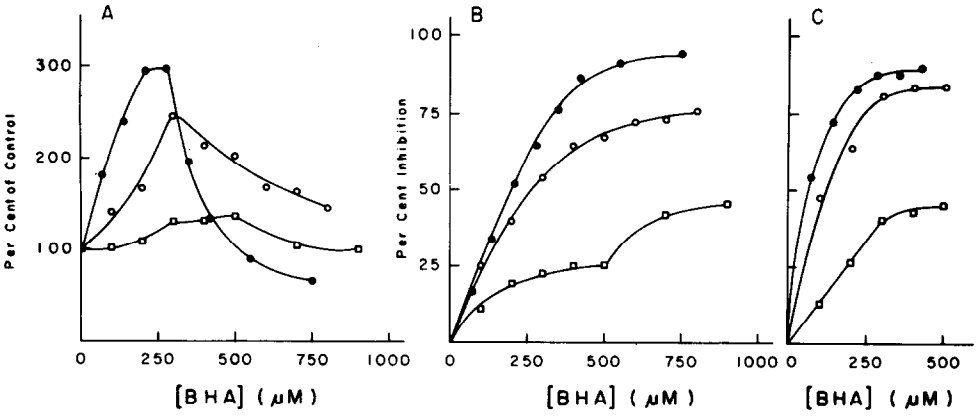

Fig. 1. Effects of BHA concentration on mitochondrial respiration. Control rates for ADP-stimulated glutamate + malate (●), succinate (○), and ascorbate + TMPD (□) oxidations were: 80.6 ± 3.2, 126.6 ± 5.4, and 331.6 ± 8.3 natoms oxygen/min/mg protein respectively. Initial respiratory control indices were: 9.6 ± 0.8, 5.8 ± 0.5, and 1.9 ± 0.2. (A) Stimulation of state 4 respiration by BHA. (B) Inhibition of state 3 respiration by BHA. (C) Inhibition of respiratory control indices. For other details, see Materials and Methods.

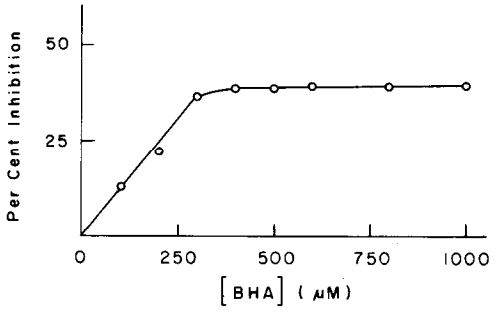

Fig. 2. Effect of BHA concentration on ATPase activity. Details are described under Materials and Methods. Control rate for H^+ ejection was 110 nmol H^+/min/mg. Each point was averaged from three different mitochondrial preparations.

glutamate + malate and succinate oxidations. Respiratory control indices were lost completely at about 350 μM BHA. Only a slight inhibitory effect was observed when ascorbate + TMPD were the electron donors. Consequently, when the electron donors were glutamate + malate and succinate, ADP/O ratios were decreased dramatically from 2.95 to 1.58 and 1.97 to 1.34, respectively, by 350 μM BHA. However, the ADP/O ratio was not affected by the oxidation of ascorbate + TMPD up to 400 μM BHA.

Effect of BHA on H^+ translocation coupled to ATPase activity. To exclude the possibility that BHA inhibits ADP-stimulated oxidation of different electron donors by inhibiting the ATP synthetase or the adenine nucleotide translocase, the effect of BHA on the vectorial ejection of H^+ coupled to the hydrolysis of ATP was investigated. Figure 2 shows the effect of increasing concentrations of BHA on H^+ ejection coupled to the hydrolysis of added ATP. In the absence of BHA, the rate of H^+ ejection of 110 nmol H^+/min/mg was obtained. At 300 μM BHA, only 37% inhibition was observed, which remained practically constant up to 1000 μM BHA. This degree of inhibition was much less than that observed for ADP-stimulated respiration in the presence of substrates which donate electrons to energy-conserving site 1 and site 2. This inhibitory effect was probably caused by a slight increase in the H^+ permeability of the mitochondrial membrane, corresponding to an incomplete uncoupling effect observed in state 4 experiments, since BHA had no direct effect on uncoupler-stimulated mitochondrial ATPase activity (results not shown). Also, there may be a small detergent effect of BHA, since it interacts with inner mitochondrial membrane producing solubilization of protein [26]. It has been suggested that this effect is due to the high lipid solubility of BHA [17, 26]. Consequently, the effective concentration of BHA in the mitochondrial lipid bilayer would be much higher than the concentration added to the reaction medium. This finding could explain the first phase of the inhibitory curve of the ADP-stimulated ascorbate + TMPD oxidation. The strong inhibitory effect of BHA on ADP-stimulated respiration was evidently not due to inhibition of either the ATP synthetase or the adenine nucleotide translocase, an activity required for ATP entry prior to ATPase action.

Effect of BHA on mitochondrial electron flow. The findings presented above demonstrate that BHA predominantly inhibits the ADP-stimulated oxidation of those substrates donating electrons to the energy-conserving sites 1 and 2 of the respiratory chain. Moreover, the fact that BHA inhibited only partially H^+ ejection coupled to the ATP hydrolysis indicates that the primary effect of BHA is on the electron flow *per se*, and is not a secondary consequence of the inhibition of ATP synthesis. It also indicates that the predominant site of action of BHA, at least at the concentration used, could be located somewhere in the respiratory chain prior to cytochrome *c*.

To determine the effect of BHA on electron flow

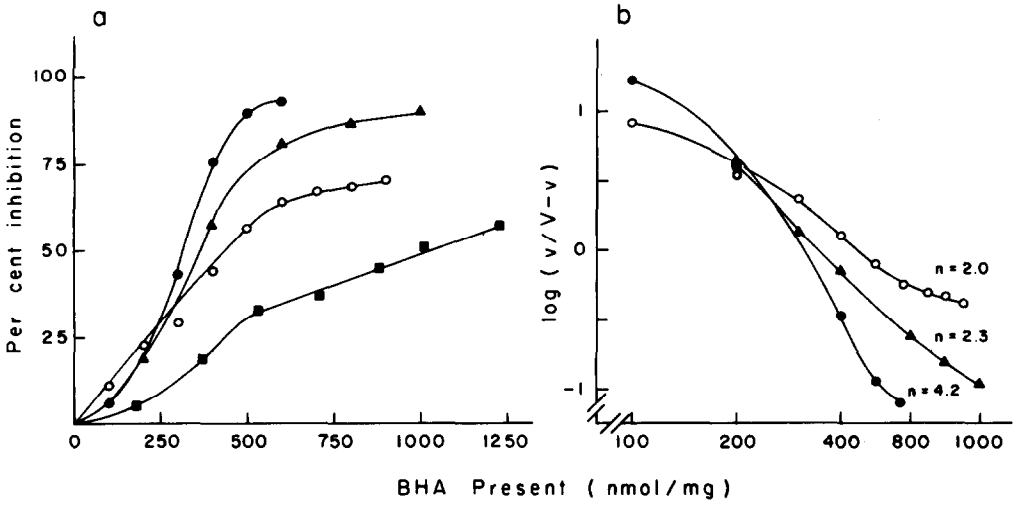

Fig. 3. Effect of BHA concentration on CCCP-stimulated mitochondrial respiration. (a) Control rates for CCCP-stimulated glutamate + malate (●), succinate (○), duroquinol (▲), and ascorbate + TMPD (■) oxidations were: 91.5 ± 3.4, 168.8 ± 8.7, 235.7 ± 12.4 and 400.8 ± 13.4 natoms oxygen/min/mg protein respectively. (b) Data plotted in accord with the Hill equation. For other experimental conditions, see Materials and Methods.

through the respiratory chain, CCCP-stimulated oxidation of different substrates was studied. Figure 3a summarizes the concentration dependence of the effect of BHA. When glutamate + malate were the electron donors, CCCP-stimulated oxygen consumption was strongly sensitive to BHA, and a sigmoidal inhibitory curve was observed. Half-maximal inhibition was obtained by 320 nmol/mg and almost complete inhibition (93%) by 600 nmol/mg BHA. The CCCP-stimulated oxidation of succinate was also sensitive to BHA. The inhibition was intensified progressively with increasing BHA concentrations; half-maximal inhibition was attained by 450 nmol/mg, and at 800 nmol/mg BHA it was inhibited by 70%. The most direct test of the action of BHA on site 2 was the measurement of duroquinol oxidation, which donates electrons directly to ubiquinone [23]. A sigmoidal inhibitory curve of the CCCP-stimulated duroquinol oxidation was observed (Fig. 3a); half-maximal inhibition was obtained by 370 nmol/mg and almost complete inhibition (90%) by 1000 nmol/mg BHA. On the other hand, when ascorbate + TMPD were the electron donors, the inhibition of the oxygen consumption practically did not appear at relatively low BHA concentrations; for instance, at 500 nmol/mg only 32% inhibition was observed. This degree of inhibition is less than that observed for the corresponding ADP-stimulated respiration, which was probably caused by slight inhibition of the ATP synthesis activity (Fig. 2). Above this BHA concentration, inhibition was increased slightly. Half-maximal inhibition was attained by 1000 nmol/mg. These results indicate that BHA does not predominantly inhibit electron flow from cytochrome *c* to oxygen.

Inhibition curves of CCCP-stimulated oxygen consumption by BHA are sigmoidal in nature, thus indicating a cooperative inhibitory effect on the electron flow, which would suggest the existence of more than one site of inhibition. One of the alternative methods that may be used to determine the minimum number of inhibitor molecules required for full inhibition of electron flow is the Hill plot [27], as shown in Fig. 3b. The sigmoidicity observed here is not uncommon; completely linear Hill plots are expected only in cases where maximal cooperativity exists either in binding the modulator or in the effect of binding on the activity [28, 29]. When glutamate + malate were the electron donors, the slope at the midpoint in Fig. 3b was about 4.2. This fact indicates that BHA binding at more than one site is required for complete inhibition, and it could be suggested that four binding sites are involved in the inhibition from NADH to cytochrome *c*. In return, the slopes at midpoint of the inhibitory curves of the CCCP-stimulated succinate and duroquinol oxidations were 2.0 and 2.3, respectively, suggesting that only two binding sites are involved in the inhibition on energy-conserving site 2, that BHA does not inhibit electron flow through the complex II, i.e. succinate : dehydrogenase, and that two binding sites would be involved in the inhibition by BHA prior to ubiquinone, i.e. NADH : dehydrogenase.

Effect of BHA on electron flow through site 1. To study the possibility that BHA inhibits the NADH–ubiquinone span, the effect of BHA concentration on the rate of oxidation of mitochondrial NAD(P)H at 340–370 nm [23] coupled to reduction of duroquinone [24] was investigated. Antimycin and cyanide were added to inhibit CCCP-stimulated electron flow through energy-conserving sites 2 and 3. The Hill plot for the inhibition of NADH : duroquinone reductase activity by BHA is shown in Fig. 4. The slope at midpoint is about 1.9; in other experiments, values from 1.8 to 2.0 were found. Thus, it indicates that two binding sites are involved in the inhibition on the NADH : dehydrogenase segment of the mitochondrial electron transport system.

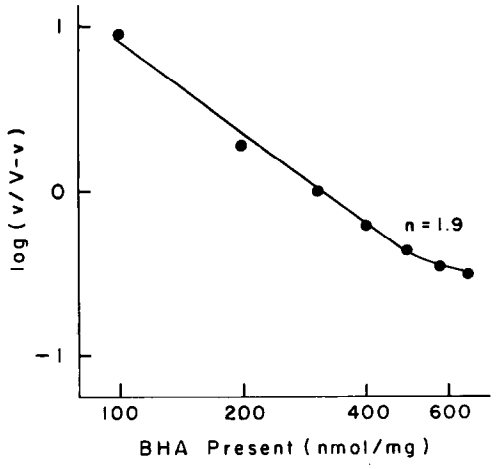

Fig. 4. Hill plot for the inhibition of NADH:duroquinone reductase activity by BHA. Details are described under Materials and Methods.

Effect of BHA on electron flow through site 2. Experiments were carried out to establish more precisely the inhibitory site within the Q—c span. TMPD can carry electrons around the antimycin block of the respiratory chain from either NAD^+-linked substrates or succinate [23]. The oxidized form of TMPD, Wurster's blue (WB^+), accepts electrons from ubisemiquinone, transferring them to cytochrome *c* [30]. Figure 5 shows that BHA strongly inhibited electron flow both from glutamate + malate and from succinate to oxygen in the absence of TMPD, as was observed above. The inhibition of CCCP-stimulated glutamate + malate oxidations by BHA was partially relieved by the addition of TMPD, presumably because BHA also inhibits the energy-conserving site 1, preventing ubiquinol regeneration. However, the inhibition of succinate oxidation was relieved almost completely by the addition of TMPD. Similar results were obtained when the respiration was inhibited by antimycin (AA) in the presence of CCCP and then reactivated by TMPD. DCIP can transfer electrons around the antimycin block of the respiratory chain from its non-enzymatic reductant ascorbate-succinate couple, giving them to the Rieske FeS center, and thence to cytochrome c_1 [30]. Figure 5 also shows that the BHA block of electron flow can be bypassed by DCIP. Since BHA does not inhibit electron flow through site 3, the observations strongly indicate that BHA inhibits electron flow at some point of the *b–c* complex.

The following experiments also excluded cytochrome *b* and cytochrome c_1 as the site of BHA action. Figure 6 shows two typical spectrophotometric traces obtained by monitoring the redox state of both cytochromes *b* (430–410 nm) and cytochrome c_1 (553–541 nm). The addition of BHA caused an immediate large decrease in the absorbance of both cytochromes *b* and c_1; these results thus show that these cytochromes become more oxidized when BHA is added. No noticeable increases of cytochrome oxidation levels were observed when new amounts of BHA were added.

These experiments not only support the conclusion that BHA inhibits electron flow at some point before cytochrome *b* and that it does not inhibit from cytochrome *b* to oxygen, but they also indicate that BHA inhibits between ubisemiquinone and cytochrome b_T.

DISCUSSION

The observations recorded in the present study show that BHA is mainly an inhibitor of the oxidation of NAD^+-linked substrates and succinate, but much less so an inhibitor of the oxidation of ascorbate-TMPD by rat liver mitochondria. The inhibition was exerted only on ADP- or uncoupler-stimulated respiration, and not on state 4 respiration. BHA inhibited only slightly the H^+ ejection associated with ATPase activity on added ATP; therefore, it does not interfere with phosphorylation mechanisms *per se* or with the adenine nucleotide translocase. Since BHA exerts a perturbing action on membranes which causes structural modifications [26], this partial inhibition was probably caused by a slight increase in the H^+ permeability of the mitochondrial membrane, as judged by the small uncoupling observed in state 4 experiments. If ATPase activity coupled to H^+ ejection is partially inhibited, it could be assumed that the phosphorylation of ADP also could be partly inhibited. This would explain the hyperbolic shapes of the inhibition curves of ADP-stimulated oxygen consumption by BHA. On the other hand, those curves corresponding to uncoupler-stimulated oxygen consumption have sigmoidal shapes, indicating a cooperative inhibitory effect on the electron flow. This would suggest the existence of more than one site of inhibition. From the Hill plots, it could be assumed that there may be two sites on the substrate side of ubiquinone, i.e. NADH–ubiquinone span, and another two sites on the oxygen side of ubiquinone, i.e. ubiquinone–cytochrome *c* span, although there is no direct evidence of cooperativity in binding. In fact, the structure of BHA closely resembles that of ubiquinol [13, 14], and a competitive relationship between the ubiquinone/ubiquinol couple and BHA would be expected. Consequently, the inhibitory sites within the NADH–ubiquinone span of BHA presumably would be situated between the NADH:dehydrogenase molecule and ubiquinone. The binding of BHA to the sites would involve both the NADH:dehydrogenase molecule and lipids, as it is for rotenone and piericidin A [31].

Analysis of the effect of BHA on different segments of the respiratory chain showed that energy-conserving site 3, the cytochrome oxidase reaction, was unaffected. On the other hand, electron flow through the NADH–ubiquinone complex was inhibited by BHA as indicated by the ability of BHA to inhibit the NADH:duroquinone reductase activity. Also, BHA inhibited the electron flow through the energy-conserving site 2, as indicated by the inhibitory effect on the oxidation of duroquinol. The inhibitory sites of BHA within the ubiquinone–cytochrome c_1 span are presumably situated between ubisemiquinone and cytochrome b_T indicated by: (i) the failure of BHA to inhibit electron transfer via

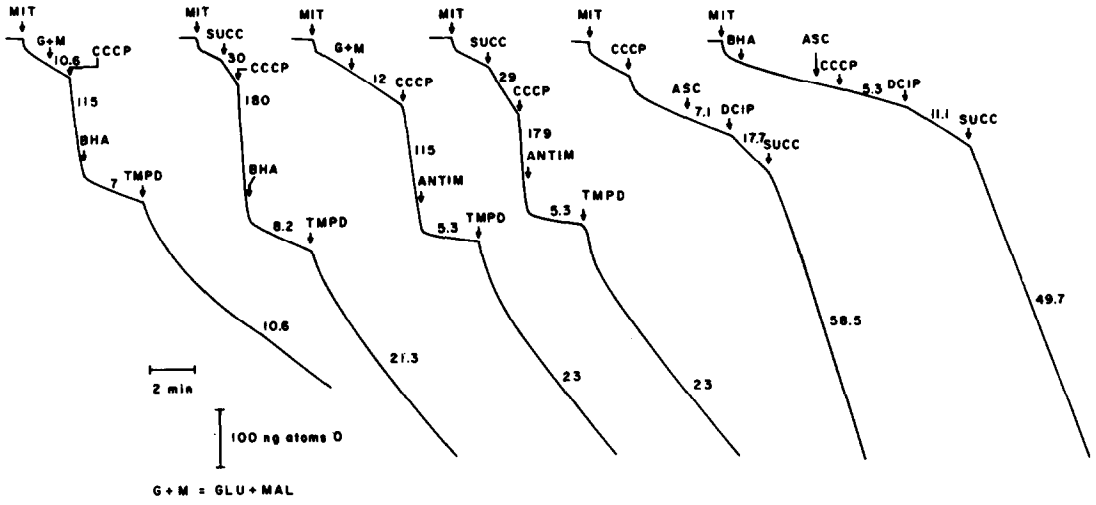

Fig. 5. Bypasses of the BHA block by reduced TMPD or DCIP within the ubiquinone–cytochrome *c* span. Electron transfer from glutamate + malate or succinate to oxygen. When indicated, 2.2 mg of mitochondrial protein, 2.5 mM glutamate + 2.5 mM malate (G + M), 5.0 mM succinate (SUCC), 1.0 mM BHA, 0.1 μM CCCP, 0.1 mM TMPD, 0.5 μg antimycin, and 25 μM DCIP were added. For details, see Materials and Methods.

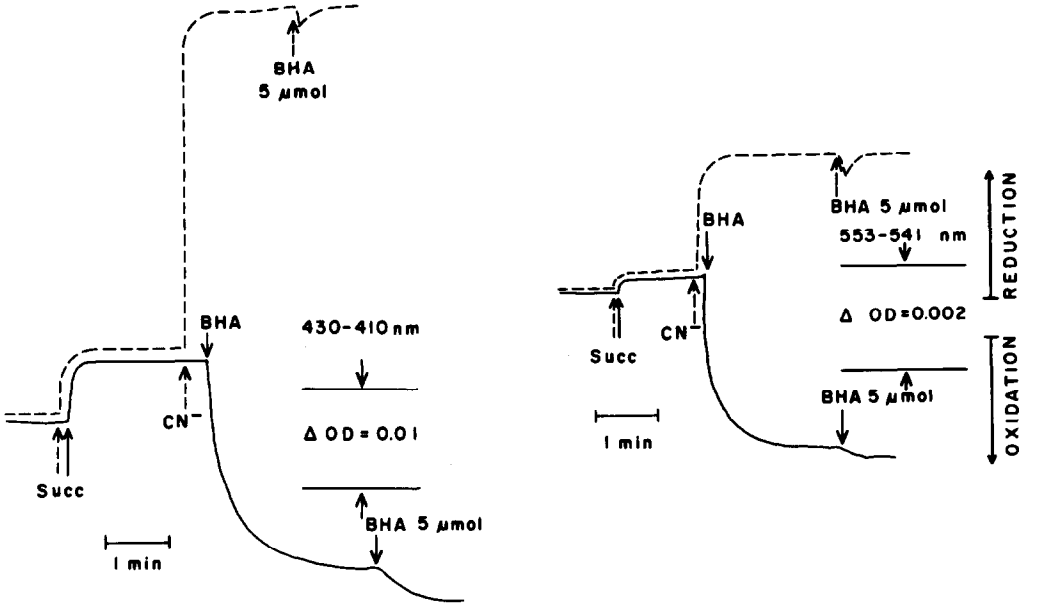

Fig. 6. Effect of BHA on the redox states of cytochromes *b* and c_1. At the points indicated by the arrows, 10 μmol BHA, 2.5 mM succinate and 10 mM cyanide (dashed traces) were added.

both the TMPD and DCIP bypasses, and (ii) the fact that the cytochromes *b* and c_1 became more oxidized when BHA was added. These observations are consistent with current models of the Q cycle [32]. Figure 7 shows an abbreviated respresentation of the electron carrier systems in mitochondria, including the apparent sites of action of BHA.

The redox reactions of components in the respiratory chain generate hydrogen ions which are ejected to the outside of the coupling membrane. The electrochemical potential difference resulting from the asymmetrical distribution of the hydrogen ions is used (a) for chemical work of the ATP and PP_i syntheses and reverse electron transport against

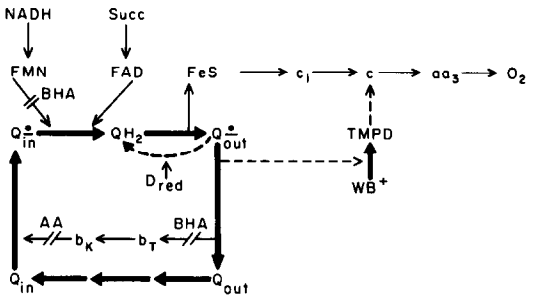

Fig. 7. Pathways of electron flow of DCIP and TMPD bypasses of the BHA block. In the DCIP bypass, ubisemiquinone ($Q^{\cdot}_{out}$) is constantly rereduced to ubiquinol (QH_2) by reduced DCIP (D_{red}), as shown by the dashed arrows. Electron flow from QH_2 to cytochrome *c* takes place via the Rieske FeS center and cytochrome c_1. D_{red} is constantly regenerated by ascorbate or succinate oxidation. In the TMPD bypass, one electron from QH_2 passes to the Rieske FeS center and the other one passes from $Q^{\cdot}_{out}$ to WB^+, which is reduced to TMPD. As a result, both electrons reduce cytochrome *c*. Scheme is based on material from Alexandre and Lehninger (Fig. 8) [30].

a redox gradient; (b) osmotic work of uphill transport of a great variety of compounds; (c) mechanical work of mitochondrial motility, and (d) heat production [33]. Chemical work of the ATP synthesis is responsible for most of the cellular oxygen consumption, and it is the main source of cellular ATP in higher organisms [34]. BHA inhibited primarily the action of NAD^+- and FAD-linked dehydrogenase systems, leading to the cessation of oxidative phosphorylation and a rapid depletion of the ATP supply, which, in turn, would lead to a decrease in the activity of cellular energy-dependent processes. As a consequence, the complex chain of events which results in cell death could be triggered by this compound.

In conclusion, it has been demonstrated that BHA predominantly affects mitochondrial electron flow around ubiquinone, but the BHA concentrations used to observe these effects were extremely high compared with those that would be ingested as food preservatives and antioxidants [2, 4].

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