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INHIBITION OF MITOCHONDRIAL RESPIRATION BY MODEL PHENOLIC COMPOUNDS*

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Abstract—A variety of model phenolic compounds were tested for their ability to inhibit the beef heart mitochondrial NADH-oxidase and succinoxidase enzyme systems *in vitro*. Specifically, we determined the hydroxy and methoxy configurations of the model phenolic compounds that were mandatory for inhibition of mitochondrial respiration. Data are presented that supported the conclusion that the relative potency of inhibition of the beef heart mitochondrial succinoxidase enzyme system was methyl hydroquinone > hydroquinone > 4-methyl catechol > 3-methyl catechol > catechol, whereas *o*-cresol, *p*-cresol, resorcinol, 2-methyl resorcinol and orcinol were non-inhibitory. None of the model phenolic compounds tested were inhibitory toward the beef heart mitochondrial NADH-oxidase enzyme system. These findings indicate that the site of inhibition for the catechol and hydroquinone derivatives is in complex II. Furthermore, it is proposed that a capacity for ortho- or para-quinone formation is mandatory for inhibition of mitochondrial succinoxidase activity by model phenolic compounds.

In a series of investigations on the lignans isolated from *Larrea divaricata* Cav. Fam. Zygophyllaceae (creosote bush), it was shown that nor-dihydroguaiaretic acid (NDGA) [1], nor-isoguaiacin [2] and the partially demethylated dihydroguaiaretic acid [3] were all inhibitory towards the beef heart mitochondrial succinoxidase enzyme system *in vitro*, whereas dihydroguaiaretic acid, dimethyl-dihydroguaiaretic acid and the tetra-acetate derivative of NDGA were not [3]. These findings suggest that the structural configuration of the dihydroxy moieties of the lignans is important for the reported biological activity, since only those lignans possessing a catechol moiety were inhibitory toward the beef heart mitochondrial succinoxidase enzyme system *in vitro* [1–3]. The orthodimethoxy- and ortho-methoxy-phenolic lignans were not inhibitory [3]; therefore, we decided to investigate the structural specificity of the aromatic hydroxy groups required for inhibition of mitochondrial electron transport systems. This report includes our findings on the inhibition of mitochondrial respiration by a series of model phenolic compounds.

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† Asolectin is a soybean phospholipid preparation which was homogenized (40.0 mg) in 20 ml of 20 mM Tris buffer, pH 8.0, that was made 1 mM with respect to EDTA. The homogenate was sonicated for 8 min at maximum output (Biosonic III equiped with a microprobe) with cooling every minute. The sonicate was centrifuged at 100,000 *g* for 45 min and the supernatant fraction was decanted and stored frozen under nitrogen. This micellar preparation was used at the volume of 0.05 ml/assay flask which is equivalent to 0.33 mg/ml in the assay medium.

METHODS

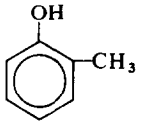
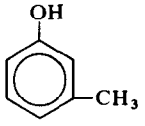
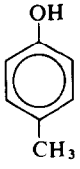
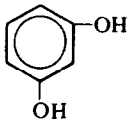
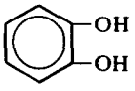
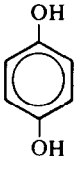
Heavy beef heart mitochondria (HBHM) were isolated by differential centrifugation and stored as described previously [4], and the mitochondria were aged by repeated freezing and thawing so that respiration was uncoupled from phosphorylation. The activities of the HBHM succinoxidase and NADH-oxidase enzyme systems were determined manometrically in the absence and the presence of the various test compounds [5]. The assay medium contained 33 mM Tris buffer, pH 7.5, 166 mM sucrose, 0.33 mg/ml of asolectin,† 33 μM Coenzyme Q_{10}, 66 μg/ml of cytochrome *c*, and 27 μM EDTA for the NADH-oxidase system only. The substrates were 150 μM sodium succinate and 4 μM NADH which were added from the side arm of the reaction vessel. The test compounds were added in ethanol, which was maintained at a constant concentration in all assay flasks (0.1 ml ethanol/3 ml of reaction buffer). In each experiment, a 5-min pre-incubation was employed to insure thermal equilibration and depletion of endogeneous substrates. Mitochondrial protein was assessed by the biuret method [6].

Cytochrome *c*, type III, and β-diphosphopyridine nucleotide, reduced form (NADH), were purchased from the Sigma Chemical Co, St. Louis, MO. Asolectin was obtained from Associated Concentrates, 32–30 61st St., Woodside, Long Island, NY 11377.

RESULTS AND DISCUSSION

The data presented in Table 1 demonstrate that, with three different preparations of HBHM, a concentration of 3.6×10^{-4} M of catechol and hydroquinone depressed HBHM succinoxidase activity to 20 and 13 per

Table 1. Effect of model phenolic compounds on the heavy beef heart mitochondrial succinoxidase system

Compounds added (1 μmole/flask)*	Structures	Enzyme specific activity (μatoms oxygen consumed/min/mg protein)			%
		Batch I†	Batch II	Batch III	
None		0.42 ± 0.02‡	0.41 ± 0.03‡	0.47 ± 0.02‡	100§
o-Cresol		0.40 ± 0.02	0.41 ± 0.02	0.46 ± 0.04	97.7 ± 1.6
m-Cresol		0.49 ± 0.02	0.42 ± 0.04	0.43 ± 0.03	102.8 ± 2.3
p-Cresol		0.41 ± 0.02	0.41 ± 0.03	0.47 ± 0.04	98.6 ± 1.3
Resorcinol		0.41 ± 0.02	0.41 ± 0.02	0.43 ± 0.03	96.3 ± 5.0
Catechol		0.08 ± 0.01	0.08 ± 0.02	0.11 ± 0.01	20.4 ± 1.9
Hydroquinone		0.05 ± 0.01	0.06 ± 0.02	0.07 ± 0.01	12.9 ± 1.5

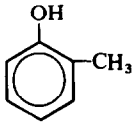
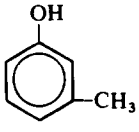
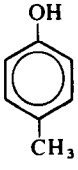
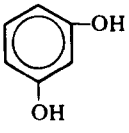
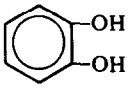
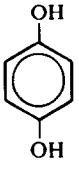
* Final concentration in the reaction flask was 3.6×10^{-4} M.
† Mitochondrial protein batches I, II and III were adjusted to 0.5–0.8 mg/reaction flask.
‡ Average from twelve determinations ± S.E.M.
§ Average per cent of uninhibited controls ± S.E.M.

cent of the controls, respectively, whereas *o*-cresol, *p*-cresol and resorcinol were non-inhibitory. In a similar series of experiments employing the NADH-oxidase enzyme system (Table 2), *o*-cresol, *m*-cresol, *p*-cresol and resorcinol were shown to stimulate the HBHM NADH-oxidase enzyme system, whereas catechol and hydroquinone depressed the enzyme activity to about 80 per cent of the controls at a concentration of 3.6×10^{-4} M.

Similarly, the data in Table 3 indicate that, at a concentration of 3.6×10^{-4} M, 3-methyl catechol, 4-methyl catechol and methyl hydroquinone depressed the HBHM succinoxidase activity to 13, 11 and 9 per cent of the controls, respectively, whereas 2-methyl resorcinol and orcinol monohydrate were essentially non-inhibitory. Conversely, the data in Table 4 indicate that these same dihydroxy analogs were non-inhibitory toward the NADH-oxidase enzyme system at the same concentration (3.6×10^{-4} M).

Any compound that depressed enzyme activity by

Table 2. Effect of model phenolic compounds on the heavy beef heart mitochondrial NADH-oxidase system

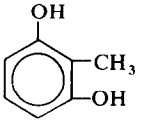
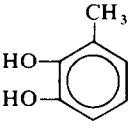
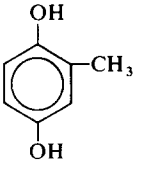
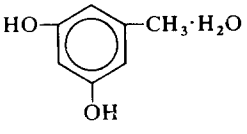
Compounds added (1 μmole/flask)*	Structures	Enzyme specific activity (μatoms oxygen consumed/min/mg protein) Batch I+	Batch II	Batch III	%
None		0.35 ± 0.05‡	0.31 ± 0.04‡	0.28 ± 0.04‡	100§
o-Cresol		0.50 ± 0.06	0.36 ± 0.04	0.32 ± 0.21	124.8 ± 14.8
m-Cresol		0.52 ± 0.02	0.35 ± 0.06	0.32 ± 0.04	123.9 ± 20.0
p-Cresol		0.52 ± 0.04	0.37 ± 0.06	0.32 ± 0.05	128.2 ± 18.2
Resorcinol		0.46 ± 0.04	0.42 ± 0.05	0.31 ± 0.05	126.3 ± 14.0
Catechol		0.26 ± 0.05	0.27 ± 0.03	0.23 ± 0.02	80.8 ± 7.8
Hydroquinone		0.27 ± 0.03	0.26 ± 0.05	0.23 ± 0.03	78.8 ± 4.3

* Final concentration in the reaction flask was 3.6×10^{-4}M.
+ Mitochondrial protein batches I, II and III were adjusted to 0.5–0.8 mg/reaction flask.
‡ Average from twelve determinations ± S.E.M.
§ Average per cent of uninhibited controls ± S.E.M.

70 per cent or greater at a concentration of 3.6×10^{-4} M was considered to be a respiratory chain inhibitor. Any of the test chemicals that did not meet this criterion were considered non-inhibitory and were excluded from additional testing; thus, of the various mono- and dihydroxy model phenolic compounds tested, catechol, hydroquinone, 3-methyl catechol, 4-methyl catechol and methyl hydroquinone were considered to be inhibitory towards the HBHM succinoxidase enzyme system, whereas none of the model phenolic compounds tested were found to be inhibitory towards the HBHM NADH-oxidase enzyme system. These findings indicate that the active model phenolic compounds interact with HBHM respiratory chain at a site in complex II (succinate: Co Q reductase).

Follow-up experiments included titrating the HBHM succinoxidase enzyme system with the various inhibitors to determine the shape of the titration curve

Table 3. Effect of various dihydroxyphenolic compounds on the heavy beef heart mitochondrial succinoxidase system

Compounds added (1 μmole/flask)*	Structures	Enzyme specific activity (μatoms oxygen consumed/min/mg protein) Batch I+	Batch II	Batch III	%
None		0.42 ± 0.02‡	0.41 ± 0.03‡	0.47 ± 0.02‡	100§
2-Methyl resorcinol		0.35 ± 0.04	0.31 ± 0.02	0.35 ± 0.03	76.6 ± 5.7
3-Methyl catechol		0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	13.1 ± 1.9
4-Methyl catechol		0.04 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	11.0 ± 1.0
Methyl hydroquinone		0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	8.6 ± 0.7
Orcinol monohydrate		0.27 ± 0.05	0.29 ± 0.06	0.30 ± 0.07	65.0 ± 3.2

* Final concentration in the reaction flask was 3.6×10^{-4} M.
+ Mitochondrial protein batches I, II and III were adjusted to 0.5–0.8 mg/reaction flask.
‡ Average from eight determinations ± S.E.M.
§ Average per cent of uninhibited controls ± S.E.M.

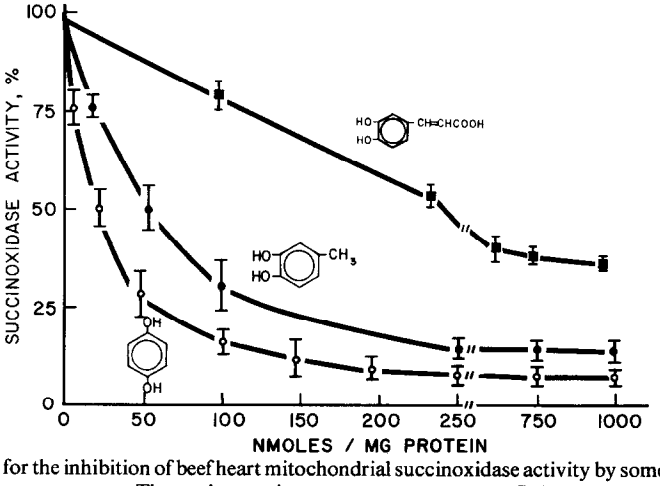
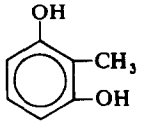
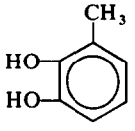
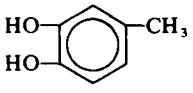
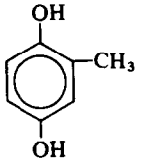
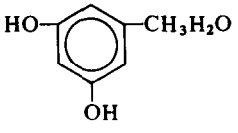


Fig. 1. Titration curves for the inhibition of beef heart mitochondrial succinoxidase activity by some model phenolic compounds. The various points represent averages ± S.E.M.

Table 4. Effect of various dihydroxyphenolic compounds on the heavy beef heart mitochondrial NADH-oxidase system

Compounds added (1 μmole/flask)*	Structures	Enzyme specific activity (μatoms oxygen consumed/min/mg protein) Batch I†	Batch II	Batch III	%
None		0.35 ± 0.05‡	0.31 ± 0.04‡	0.28 ± 0.04‡	100§
2-Methyl resorcinol		0.34 ± 0.04	0.36 ± 0.06	0.30 ± 0.02	106.9 ± 9.1
3-Methyl catechol		0.30 ± 0.05	0.32 ± 0.05	0.22 ± 0.02	89.4 ± 12.0
4-Methyl catechol		0.33 ± 0.04	0.31 ± 0.03	0.27 ± 0.02	101.8 ± 6.1
Methyl hydroquinone		0.36 ± 0.03	0.33 ± 0.02	0.26 ± 0.02	95.6 ± 2.9
Orcinol monohydrate		0.35 ± 0.04	0.37 ± 0.04	0.33 ± 0.04	112.9 ± 11.5

* Final concentration in the reaction flask was 3.6×10^{-4} M.
† Mitochondrial protein batches I, II and III were adjusted to 0.5–0.8 mg/reaction flask.
‡ Average from eight determinations ± S.E.M.
§ Average per cent of uninhibited controls ± S.E.M.

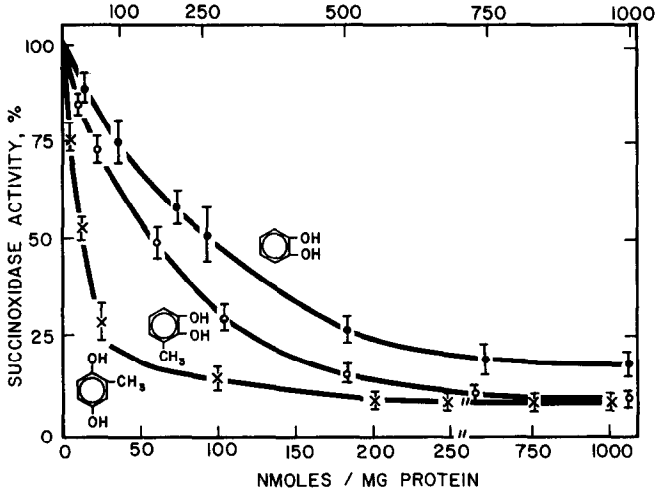
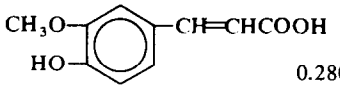
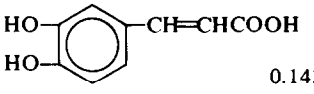
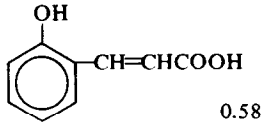
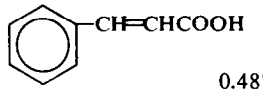
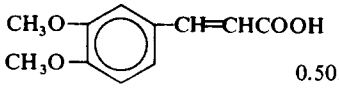


Fig. 2. Titration curves for the inhibition of beef heart mitochondrial succinoxidase activity by some model phenolic compounds. The various points represent averages ± S.E.M.

Table 5. Effect of cinnamic acid derivatives on the heavy beef heart mitochondrial succinoxidase system

Compounds added (1 μmole/flask)*	Structures	Enzyme specific activity (μatoms oxygen consumed/min/mg protein) Batch I+	Batch II	Batch III	%
None		0.551 ± 0.044‡	0.648 ± 0.064‡	0.668 ± 0.027§	100‖
Ferulic acid		0.280 ± 0.028	0.360 ± 0.041	0.374 ± 0.020	54.2 ± 2.8
Caffeic acid		0.143 ± 0.010	0.164 ± 0.008	0.162 ± 0.017	25.2 ± 1.9
Trans -*o*-hydroxy cinnamic acid		0.581 ± 0.021	0.778 ± 0.029	0.668 ± 0.036	108.5 ± 10.4
Trans-cinnamic acid		0.487 ± 0.016	0.632 ± 0.041	0.612 ± 0.025	92.5 ± 4.6
3,4-Dimethoxy cinnamic acid		0.502 ± 0.021	0.728 ± 0.037	0.596 ± 0.016	97.6 ± 12.8

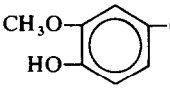
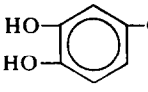
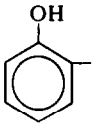
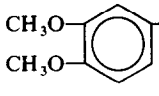
* Final concentration in the reaction flask was 3.6×10^{-4} M.
† Mitochondrial protein batches I, II and III were adjusted to 0.5–0.8 mg/reaction flask.
‡ Average from ten determinations ± S.E.M.
§ Average from six determinations ± S.E.M.
‖ Average per cent of uninhibited controls ± S.E.M.

and the I_{50} value. These data are included in Fig. 1 and 2 and show that all of the mitochondrial complex II inhibitors inhibit the HBHM succinoxidase system in a dose-dependent fashion. The hyperbolic shape of the various titration curves suggests a single site of inhibition for each compound. In addition, these data demonstrate that the relative potency of inhibition of HBHM succinoxidase activity was methyl hydroquinone > hydroquinone > 4-methyl catechol > 3-methyl catechol > catechol, as the I_{50} values were 20, 25, 50, 170 and 250 nmoles/mg of mitochondrial protein respectively. This sequence of relative potency for succinoxidase inhibition does not correlate with the lipophilicity of the various model phenolic compounds, as determined by the water/chloroform partition coefficients [7]. The order of lipophilicity was 4-methyl catechol > 3-methyl catechol > catechol > methyl hydroquinone > hydroquinone, since the water/chloroform partition coefficients were 1.3, 2.2, 5.0, 45.4 and 92.5 respectively.

These findings show that the most potent phenolic inhibitors of the HBHM succinoxidase system contain two free hydroxy groups either in the para- or ortho-configuration. Conversely, the monohydroxy or meta-dihydroxy compounds were non-inhibitory at the highest concentration tested. These findings suggest that the ability to undergo oxidation-reduction may be a prerequisite for succinoxidase inhibition by phenolic compounds since para- and ortho-dihydroxybenzene derivatives are much easier to oxidize than the monohydroxy and meta-dihydroxy analogs. Consistent with this view is the observation that methyl hydroquinone and the methyl catechols were more potent inhibitors of the succinoxidase system than hydroquinone and catechol respectively (Figs. 1 and 2). Branch and Calvin [8] reported that the inductive effect of alkyl group substitution reduces the redox potential of hydroquinones and catechols. On the other hand, the methyl-substituted phenolic compounds are more lipophilic than their respective unsubstituted parent compounds; thus, the increased potency of the methyl-substituted phenolic compounds could relate to their ability to imbed in the lipophilic mitochondrial membrane.

In order to pursue this line of investigation further,

Table 6. Effect of cinnamic acid derivatives on the heavy beef heart mitochondrial NADH-oxidase system

Compound added (1 μmole/flask)*	Structures	Enzyme specific activity (μatoms oxygen consumed/min/mg protein) Batch I†	Batch II	Batch III	%
None		0.288 ± 0.013‡	0.236 ± 0.017‡	0.239 ± 0.022‡	100§
Ferulic acid		0.306 ± 0.020	0.249 ± 0.017	0.282 ± 0.14	116.2 ± 9.2
Caffeic acid		0.258 ± 0.013	0.240 ± 0.018	0.264 ± 0.030	100.4 ± 10.0
Trans-o-hydroxy cinnamic acid		0.324 ± 0.027	0.307 ± 0.028	0.382 ± 0.025	134.1 ± 23.7
Trans-cinnamic acid		0.354 ± 0.022	0.306 ± 0.018	0.406 ± 0.034	140.6 ± 25.2
3,4-Dimethoxy cinnamic acid		0.314 ± 0.017	0.289 ± 0.024	0.305 ± 0.016	120.2 ± 11.5

* Final concentration in the reaction flask was 3.6×10^{-4} M.
† Mitochondrial protein batches I, II and III were adjusted to 0.5–0.8 mg/reaction flask.
‡ Average from six determinations ± S.E.M.
§ Average per cent of uninhibited controls ± S.E.M.

we decided to test the ability of a variety of cinnamic acid derivatives with and without the ability to form a quinone on the HBHM succinoxidase and NADH-oxidase enzyme systems. These data are presented in Tables 5 and 6 respectively. The data in Table 5 indicate that caffeic acid, which was the only catechol tested, and therefore was oxidizable to the *o*-quinone form, depressed succinoxidase activity to below 30 per cent of the uninhibited controls at 3.6×10^{-4} M. Conversely, ferulic, dimethoxy cinnamic, cinnamic, and orthohydroxy cinnamic acids, which represent various analogs of cinnamic acid without the potential for quinone formation, were all non-inhibitory at the same concentration. The data in Table 6 show that all of the cinnamic acid derivatives tested were non-inhibitory toward the NADH-oxidase enzyme system. Thus, caffeic acid interacts somewhere in complex II. These findings support the view that the oxidizability of phenolic compounds is a prerequisite for inhibition of the HBHM succinoxidase enzyme system.

Caffeic acid possessed a water/chloroform partition coefficient of 54.3 and an I_{50} value of 300 nmoles/mg of mitochondrial protein for the succinoxidase system (Fig. 2), which shows that it is less lipophilic and a less potent inhibitor of succinoxidase activity than free catechol; thus, these experiments do not exclude the possibility that lipophilicity is proportional to succinoxidase inhibitory capacity within the catechol series of compounds. We are in the process of obtaining sufficient catechol analogs so that we can subject this preliminary observation to Hansch analysis [9].

The concept that the phenolic mitochondrial complex II inhibitors require the potential for quinone formation is consistent with previous reports from our laboratory that showed that lignans possessing a catechol moiety were inhibitory toward the HBHM succinoxidase enzyme system, whereas those lignans without a catechol moiety were not [1–3].

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