Inhibition of Mitochondrial Respiratory Chain by Arylthiolated 2,3-Ethylenedioxy-1,4-benzoquinones[1)]

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A series of arylthiolated 2,3-ethylenedioxy-1,4-benzoquinones as a coenzyme Q (CoQ) antagonist was tested for inhibition of succinate oxidase and reduced nicotinamide adenine dinucleotide (NADH) oxidase systems in the mitochondrial respiratory chain. The following characteristics were revealed: (1) 2,3-ethylenedioxy, 5-arylthio and 5,6-diarylthio groups were confirmed to be favorable for inhibition of both systems; (2) these analogs were more effective in the succinate oxidase system than in the NADH oxidase system; (3) 4′ substituents on the benzene side ring had little effect on inhibitory activity; (4) the acting sites of these analogs had no strict stereospecificity. The reduced minus oxidized difference spectra revealed that these analogs inhibited the succinate oxidase system at the site between succinate and CoQ, and the NADH oxidase system at the site after cytochrome a+a_3, suggesting these analogs might act as antagonists of CoQ in the succinate oxidase system. However, 5-(4′-nitrophenylthio)-2,3-ethylenedioxy-1,4-benzoquinone (If) strongly inhibited only the succinate oxidase system at the site after cytochrome a+a_3.

Keywords coenzyme Q analog; arylthiolated 2,3-ethylenedioxy-1,4-benzoquinone; succinate oxidase; NADH oxidase; cytochrome; beef heart mitochondria; difference spectra; respiratory chain; structure–activity

Many kinds of coenzyme Q (CoQ) analogs have been synthesized and several analogs were found to have a variety of biological activities. In the previous paper,[1)] alkylthio- (alkyl: *n*-C_8H_{17}, *n*-$C_{12}H_{25}$, *n*-$C_{18}H_{37}$) and arylthio-2,3-ethylenedioxy-1,4-benzoquinones (aryl: phenyl, β-naphthyl) were tested for the inhibition of succinate oxidase and reduced nicotinamide adenine dinucleotide (NADH) oxidase systems in the mitochondrial respiratory chain. The structural difference between these analogs and CoQ is the presence of 2,3-ethylenedioxy and 5- or 5,6-di alkylthio or arylthio groups on the quinone ring instead of 2,3-dimethoxy, 5-decaprenyl and 6-methyl groups of CoQ_{10}. It was found that among these compounds, two arylthiolated homologs mentioned above showed potent inhibitory activities toward both enzyme systems by inhibiting some sites between succinate and CoQ, and after cytochrome a+a_3. However, the diverse results are reported for the inhibitory sites by CoQ analogs. For example, several halogenated benzoquinones[2)] inhibit the site between succinate and CoQ, although several hydroxylated benzoquinones and naphthoquinones[3)] inhibit the site between cytochromes b and c_1. The mechanisms of inhibitions are also not yet understood. On the basis of the above situation, we have subsequently investigated a series of arylthiolated 2,3-ethylenedioxy-1,4-benzoquinones with the various substituents on the benzene side ring at 4′ position as a CoQ antagonist. The present report extends our investigations of these points where we have mentioned them in our previous paper.

Results and Discussion

Inhibition of Mitochondrial Respiratory Chain 5-(4′-X-Phenylthio- (X: CH_3, Cl, OCH_3, NH_2, OH, NO_2), 5-(2′-X-phenylthio)- (X: COOH) and 5,6-di-(4′-X-phenylthio)-2,3-ethylenedioxy-1,4-benzoquinone (X: CH_3, Cl) derivatives were evaluated for the inhibition of succinate oxidase and NADH oxidase systems in the mitochondrial respiratory chain. The inhibitory activities were monitored by measuring the rates of oxygen consumption, and Fig. 1 shows the effect of 5-2′-carboxyphenylthio-2,3-ethylenedioxy-1,4-benzoquinone (Ig) toward both enzyme systems. Ig exhibited 50% inhibition of the succinate oxidase and NADH oxidase systems at concentrations of 8.7 and 106.4 nmol/mg mitochondrial protein, respectively. The inhibitory activities were expressed as both a nmol of inhibitor per mg protein which causes 50% inhibition of enzyme activity and the antimetabolite CoQ index (A.I.).[4)] The results of the analogs are summarized in Table I. The following charac-

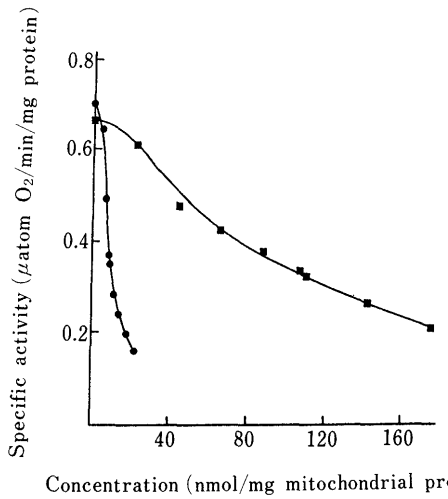

Fig. 1. Relationship between the Concentration of Ig and the Oxygen Consumptions

●, succinate oxidase; ■, NADH oxidase.

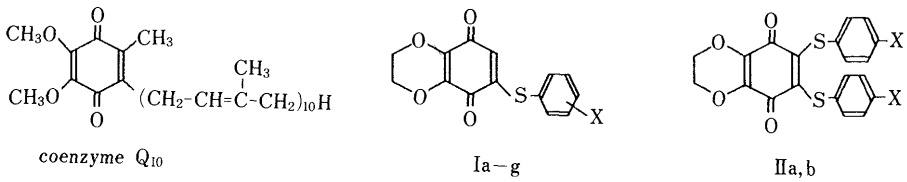

Chart 1

TABLE I. Effects of Arylthiolated 2,3-Ethylenedioxy-1,4-benzoquinones on Succinate Oxidase and NADH Oxidase Systems in Beef Heart Mitochondria

Compd. No.	X	Succinate oxidase		NADH oxidase	
		ID_{50}[a]	A.I.[b]	ID_{50}[a]	A.I.[b]
5-(4′-X-Phenylthio)-2,3-ethylenedioxy-1,4-benzoquinones					
Ia	CH_3	17.4	4.0	37.1	8.5
Ib	Cl	28.3	6.5	32.7	7.5
Ic	OCH_3	15.3	3.5	21.8	5.0
Id	NH_2	24.0	5.5	34.9	8.0
Ie	OH	24.0	5.5	48.0	11.0
If	NO_2	30.5	7.0	>654[c]	>150[c]
5-(2′-X-Phenylthio)-2,3-ethylenedioxy-1,4-benzoquinone					
Ig	COOH	8.7	2.0	106.4	24.4
5,6-Di-(4′-X-phenylthio)-2,3-ethylenedioxy-1,4-benzoquinones					
IIa	CH_3	34.9	8.0	48.0	11.0
IIb	Cl	43.6	10.0	41.4	9.5

a) nmol of inhibitor per mg of mitochondrial protein which causes 50% inhibition of enzyme activity. *b*) A.I. (antimetabolite coenzyme Q index) is defined as the nmol of inhibitor per nmol of mitochondrial CoQ which causes 50% inhibition of enzyme activity. *c*) The mark (>) means that ID_{50} or A.I. is greater than the number shown.

teristics were revealed: (1) arylthiolated 2,3-ethylenedioxy-1,4-benzoquinones except 5-(4′-nitrophenylthio-2,3-ethylenedioxy-1,4-benzoquinone (If) exhibited potent inhibitory activities toward both enzyme systems. The introduction of various substituents into the benzene side ring at 4′ position had little effect on inhibitory activity; (2) contrary to 2,3-dimethyl-1,4-benzoquinone analog,[5] these quinones were more effective in the succinate oxidase system than in the NADH oxidase system; (3) the introduction of 2,3-ethylenedioxy, 5-arylthio and 5,6-diarylthio groups into the quinone ring were again confirmed to be more favorable for inhibitory activity than 2,3-dimethoxy, 2,3-dimethyl, 5-alkyl, 5-alkylthio and 5,6-dialkylthio groups[1]; (4) there were no apparent differences in the inhibitory activities between 5-mono and 5,6-disubstituted quinones, suggesting that the acting sites of these quinones have no strict stereospecificity.

Difference Spectra of Submitochondrial Particles The difference spectra of reduced minus oxidized forms of cytochromes were investigated to identify the site of action of inhibitors in the respiratory chain. The freeze-thawed mitochondria are capable of electron transfer in the respiratory chain but are incapable of respiratory control. However, the mitochondria suspensions are so turbid that they are unsuitable for measuring the absorption spectra with ordinary apparatus. Therefore, in order to obtain sharp spectra, we used submitochondria particles which are not so turbid and maintain the mitochondrial respiratory chain, although they have the sides opposite that of mitochondria. In our previous paper,[1] 5-phenylthio- and 5-β-naphthylthio-2,3-ethylenedioxy-1,4-benzoquinones were found to inhibit some sites between succinate and CoQ, and after cytochrome $a+a_3$. We have subsequently investigated acting sites of a series of arylthiolated 2,3-ethylenedioxy-1,4-benzoquinones. Figure 2 shows the effects of Ig on the difference spectra of submitochondrial particles with succinate and NADH as a substrate. The peaks of reduced

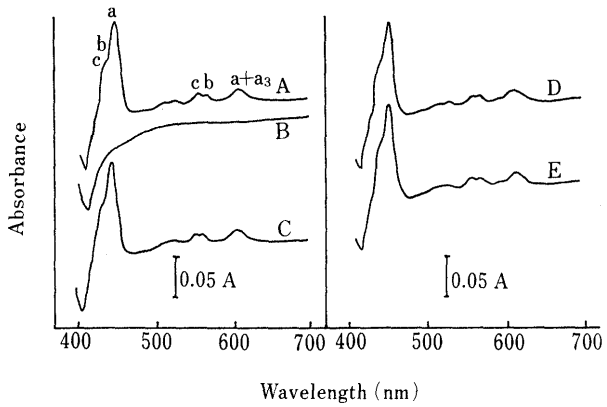

Fig. 2. Effects of Ig on the Reduced Minus Oxidized Spectra of Submitochondrial Particles with Succinate and NADH as the Substrate

Difference spectra after the addition of succinate (5 μmol) (A), subsequent addition of Ig (0.16 μmol) (B), and further addition of NADH (1.3 μmol) (C); difference spectra after addition of NADH (1.3 μmol) (D) and subsequent addition of Ig (0.2 μmol) (E).

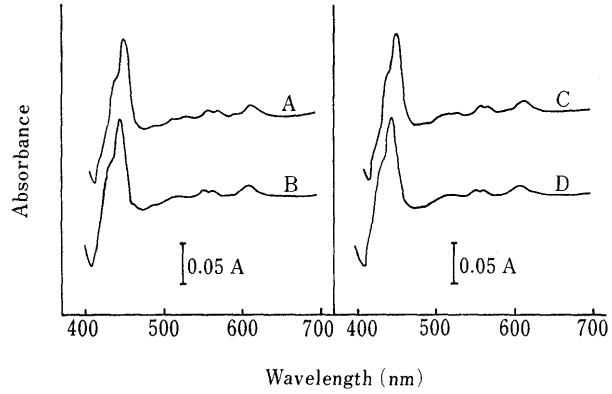

Fig. 3. Effects of If on the Reduced Minus Oxidized Spectra of Submitochondrial Particles with Succinate and NADH as the Substrate

Difference spectra after the addition of succinate (5 μmol) (A) and subsequent addition of If (0.15 μmol) (B); difference spectra after addition of NADH (1.3 μmol) (C) and subsequent addition of If (0.15 μmol) (D).

cytochromes a, a_3, b and c after the addition of succinate (spectrum A) almost disappeared after a subsequent addition of Ig (spectrum B) which inhibited the succinate oxidase system, and were restored by a further addition of another substrate, NADH (spectrum C). Such spectral changes mean that the respiratory chain was first inhibited by Ig between succinate and CoQ, and then restored by replacing the section with a route between NADH and CoQ. However, in the NADH oxidase system, the peaks of the reduced cytochromes did not change before (spectrum D) and after (spectrum E) the addition of Ig at a concentration which inhibited the NADH oxidase system, suggesting that the inhibitory site is located after cytochrome $a+a_3$. Figure 3 shows the effects of If on the difference spectra and that the peaks of reduced cytochromes did not change before (spectrum A) and after (spectrum B) the addition of If at higher concentrations than those which inhibited the succinate oxidase system. This means that If inhibits the succinate oxidase system at the site after cytochrome $a+a_3$. In the NADH oxidase system, the inhibitory activity of If was so weak that no spectral changes (spectrum D) were observed even at the highest concentration which normally dissolves in a reaction mixture. Therefore, the inhibitory site could not be determined. The difference spectra of

TABLE II. Physicochemical Data for Arylthiolated Ethylenedioxybenzoquinones

Compd. No.	X	Yield (%)	mp (°C)	Formula	Analysis (%) Calcd (Found) C	H	MS (M^+)	IR (KBr, cm^{-1}) $\nu_{C=O}$
5-(4′-X-Phenylthio)-2,3-ethylenedioxy-1,4-benzoquinones								
Ia	CH_3	13	191—193	$C_{15}H_{12}O_4S$	62.49 (62.37)	4.20 (4.05)	288	1650
Ib	Cl	34	187—189	$C_{14}H_9ClO_4S$	54.47 (54.54)	2.94 (3.03)	308	1645
Ic	OCH_3	44	186—188	$C_{15}H_{12}O_5S$	59.20 (59.17)	3.97 (3.93)	304	1650
Id	NH_2	25	228—230	$C_{14}H_{11}NO_4S$	58.12 (57.70)	3.83 (3.74)	289	1635
Ie	OH	38	202—204	$C_{14}H_{10}O_5S$	57.93 (57.82)	3.47 (3.55)	290	1635
If	NO_2	60	248—250	$C_{14}H_9NO_6S$	52.67 (52.97)	2.84 (2.74)	319	1640
5-(2′-X-Phenylthio)-2,3-ethylenedioxy-1,4-benzoquinone								
Ig	COOH	26	200—202	$C_{15}H_{10}O_6S$	56.60 (56.65)	3.17 (3.11)	318	1635
5,6-Di-(4′-X-phenylthio)-2,3-ethylenedioxy-1,4-benzoquinones								
IIa	CH_3	20	176—178	$C_{22}H_{19}O_4S_2$	64.21 (64.30)	4.65 (4.30)	410	1670
IIb	Cl	22	175—177	$C_{20}H_{12}Cl_2O_4S_2$	53.22 (52.36)	2.68 (2.72)	451	1650

other quinones were also measured in the same manner as above.

From the results described above, we conclude as follows that: Arylthiolated 2,3-ethylenedioxy-1,4-benzoquinones, except If, inhibit the sites between succinate and CoQ in the succinate oxidase system, and after cytochrome $a+a_3$ in the NADH oxidase system. The results and their structural similarity with CoQ suggested the possibility that these quinones act as antagonists of CoQ in the succinate oxidase system. If the respiratory chain from CoQ to oxygen is common for two oxidase systems, If ought to inhibit both enzyme systems to the same extent. In such a case there may be a separate respiratory chain for succinate oxidase and NADH oxidase systems, that is, CoQ compartmentalizes in the mitochondrial membrane as has been reported by Lenaz *et al.*[6] and Castelli *et al.*[7] Nevertheless, this subject should be investigated from different angles such as hydrophobicity, lipid solubility and impermeability of mitochondrial membrane of CoQ analogs.

Experimental

Synthesis of 5-Arylthio- and 5,6-Diarylthio-2,3-ethylenedioxy-1,4-benzoquinone Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Infrared (IR) spectra were taken in KBr with a Hitachi 260-30 spectrophotometer. Mass spectra (MS) were measured with a Hitachi M-60 spectrometer. New 5-arylthio- (Ia—g) and 5,6-diarylthio-2,3-ethylenedioxy-1,4-benzoquinones (IIa, b) were synthesized by the methods which were previously described.[1] The reaction gave 13—60% and about 20% yields of Ia—g and IIa, b, respectively. The following method for the synthesis of Ib and IIb is a typical procedure.

A solution of 1 eq of 4-chlorophenylthiol (261 mg) in EtOH (15 ml) was added to a solution of 2 eq of 2,3-ethylenedioxy-1,4-benzoquinone (300 mg) in EtOH (15 ml) at room temperature. After being stirred for 1 h, the reaction mixture was evaporated to dryness under reduced pressure. The residue was dissolved in acetone (15 ml), and a solution of 2 eq of Fremy's salt (1 g), 1 N sodium acetate (2 ml) and H_2O (60 ml) was added to the above solution. After being stirred for 10 min, the reaction mixture was extracted with $CHCl_3$. The extract was evaporated to dryness under reduced pressure and was purified by column chromatography on silica gel with benzene–$CHCl_3$ (4 : 1) as the eluent. The eluate (violet in color) was evaporated to dryness and the residue was recrystallized from dilute EtOH to afford Ib. The filtrate was evaporated to dryness and the residue was purified again by column chromatography with the same conditions as above. The eluate (deep purple in color) was evaporated to dryness and the residue was recrystallized from EtOH to afford IIb. The physicochemical data are summarized in Table II.

Inhibition of Mitochondrial Respiratory Chain[8] Mitochondria were isolated from fresh beef hearts by the usual procedures[9] and kept frozen in 0.25 M sucrose until used. Phospholipid micelles were prepared by sonication of commerical soybean phospholipids (Asolectin)[10] and used instead of mitochondrial phospholipids. The amount of protein and CoQ_{10} in the mitochondrial preparation was determined by the methods of Lowry[11] and Okamoto *et al.*,[12] respectively. The mitochondria contained 4.36 nmol of CoQ_{10}/mg of mitochondrial protein. The inhibition of the respiratory chain by quinones was determined manometrically in a Gilson respirometer[9] by a procedure described previously.[4] A total of 2.6 ml of the reaction mixture in the main compartment of each 15 ml flask contained: 1.0 ml of 0.1 M Tris–HCl buffer (pH 7.6); 0.5 ml of 1 M sucrose; 0.1 ml of 0.8 mM ethylenediaminetetraacetic acid (EDTA); 0.05 ml of Asolectin (20 mg/ml); 0.1 ml of inhibitor dissolved in ethanol; 0.1 ml of 0.2% cytochrome c; 0.08 ml of mitochondria (0.460 mg of protein); 0.67 ml of H_2O. Then, 0.2 ml of 0.75 M succinate or 0.075 M NADH was put in the side arm and 0.2 ml of 6 N KOH was put in the center well. The reaction was initiated by the addition of the substrate from the side arm into the reaction mixture at 30 °C. The rates of oxygen consumption were measured at several concentrations, and the concentrations of quinones required for 50% inhibition of succinate oxidase and NADH oxidase systems were determined.

Difference Spectra of Submitochondrial Particles The reduced minus oxidized difference spectra of the submitochondrial particles were measured by the method of Chance[13] with a Hitachi 340 double-beam spectrophotometer. A pair of the reference and the sample cuvettes contained 1 mg of submitochondrial particles as a protein content and 50 μmol of potassium phosphate buffer (pH 7.4) in a total volume of 0.95 ml, and were maintained at 37°C. Difference spectra at 400—700 nm of the control were recorded 3 min after the addition of succinate (5 μmol) or NADH (1.3 μmol) as a substrate into the sample cuvette. Then, the inhibitor was added to both cuvettes, and the spectral changes were measured again 3 min after incubation.

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