[Chem. Pharm. Bull. 15(8)1095~1100(1967)] UDC 612.014-06-083 : 547.567.09

135. Hikaru Ozawa,*1 Shinsaku Natori,*2 and Kazutaka Momose*1: Biochemical Studies on Quinone Derivatives. II.*3 Effects of Naturally Occurring Benzoquinone Derivatives on the Respiration of Intact Rat Liver Mitochondria.*4

(*Pharmaceutical Institute, Tohoku University School of Medicine,*1 and National Institute of Hygienic Science*2*)

Effects of naturally occurring benzoquinone derivatives on rat liver mitochondrial respiration were examined manometrically and polarographically comparing with ubiquinone-0. Dihydroxythymoquinone, helicobasidin, and dihydroperezone were found to show significant effect on the respiration.

At the concentration of $1.0\times10^{-5}M$, dihydroxythymoquinone eliminated the State-3 respiration completely and the for 50% inhibition of the State-3 respiration it required the concentration of $3.2\times10^{-6}M$. Helicobasidin was an uncoupler and dihydroperezone showed both an uncoupling action on State-4 respiration and an inhibitory action of State-3 respiration. The other benzoquinone derivatives examined did not show any significant effects on mitochondrial respiration.

(Received February 23, 1966)

Since a discovery of ubiquinone in mitochondria,[1] the significance of quinone derivatives in electron transport system and oxidative phosphorylation has long been discussed. Folkers[2] have recently suggested that ubiquinone is not only an electron carrier but also plays in energy transport reactions of oxidative phosphorylation forming a quinone methine form.

On the other hand, hydro-lapachol[3] and other quinone derivatives including benzoquinone, naphthoquinone, and anthraquinone derivatives are reported as an inhibitor of mitochondrial respiration. A greater part of the quinone derivatives inhibit an electron transport system at a locus between cytochrome b and c_1.

During a course of studies on ubiquinone-like activities of naturally occurring benzoquinone derivatives, it was observed that some quinone derivatives could not restore a respiration of acetone treated mitochondria, but inhibited the respiration which could not be completely eliminated by the acetone treatment.[4,5] Therefore, it was interested to examine the effects of those naturally occurring benzoquinone derivatives on a respiration of intact rat liver mitochondria.

Present communication is concerned with the activities of the twelve benzoquinone derivatives compared with that of ubiquinone-0. Among them, three compounds were found to have significant activities on rat liver mitochondrial respiration determined polarographically and manometrically. Each of them showed depression of State-3

*1 Kitayobancho, Sendai (小澤 光, 百瀬和享).
*2 Tamagawa-yogamachi, Setagaya-ku, Tokyo (名取信策).
*3 Part I: This Bulletin, **13**, 1029 (1965).
*4 A part of this work was presented at the 21st Annual Meeting of Pharmaceutical Society of Japan, October, 1965, Tokushima, and at the 16th Tohoku Branch Meeting of Japanese Biochemical Society, January, 1966, Sendai.

1) T. Moore: Biochem. J., **34**, 335 (1946).
2) K. Folkers: Federation Proc., **24**, 79 (1965).
3) J. L. Howland: Biochim. et Biophys. Acta, **77**, 659 (1963).
4) H. Ozawa, K. Momose, S. Natori, H. Ogawa, K. Yamaguchi: Biochim. et Biophys. Acta, **86**, 395 (1964).
5) H. Ozawa, S. Natori, K. Momose: This Bulletin, **13**, 1029 (1965).

respiration, potent uncoupling action and a mixed type of these both actions, respectively.

Experimental

Preparation of Rat Liver Mitochondria——Fed, male or female Wister rats weighing 150~250 g. were decapitated by a blow on the head and blooded. Livers were immersed in isolating medium containing 0.21*M* mannitol, 0.07*M* sucrose, 0.2 m*M* EDTA, and 10 m*M* tris-HCl (pH 7.4) at 0~2°, and minced to pieces 3~5 mm. in diameter with scissors. Minces were rinsed three times with 50 to 60 volumes of the isolating medium to remove blood. It was then homogenized in 9 volumes of the medium with Teflon homogenizer worked by hand, and the pH was readjusted to 7.4 by the addition of a few drops of 1*M* Tris solution. The homogenate was centrifuged at $1000 \times g$ for 7 minutes. The supernatant was centrifuged again at $10000 \times g$ for 7 minutes. The sediment was saved, washed once in the medium and finally suspended so that mitochondria obtained from 1 g. of liver were contained in 0.5 ml. of 0.3 *M* mannitol solution (pH was adjusted by Tris buffer to 7.2). Mitochondria so prepared were used within two hours after preparation. Protein content was determined with biuret reagent using bovine serum albumin as a standard.

Determination of Oxygen Consumption——The polarographic technique to oxygen consumption measurement was applied using oxygen electrode (Model PO-100, Yanagimoto MFG, Co., Ltd.), according to the procedure of Hagihara.[6]

Warburg manometer was also used for the determination of oxygen consumption.

Benzoquinone derivatives were added as an ethanolic solution, however in no case the final concentration of ethanol in the incubation mixture exceeded two percents in order to prevent the undesirable influence by ethanol.

Materials——Benzoquinone derivatives either chemically synthesized or extracted from plants, yeast, fungi and animal tissues as described below :

Dihydroxythymoquinone (I : R=$CH(CH_3)_2$), prepared by the method of Zincke.[7]

Maesaquinone (I : R=$(CH_2)_{13}CH : CH(CH_2)_4H$), isolated from *Maesa japonica* by the method of Hiramoto.[8,9]

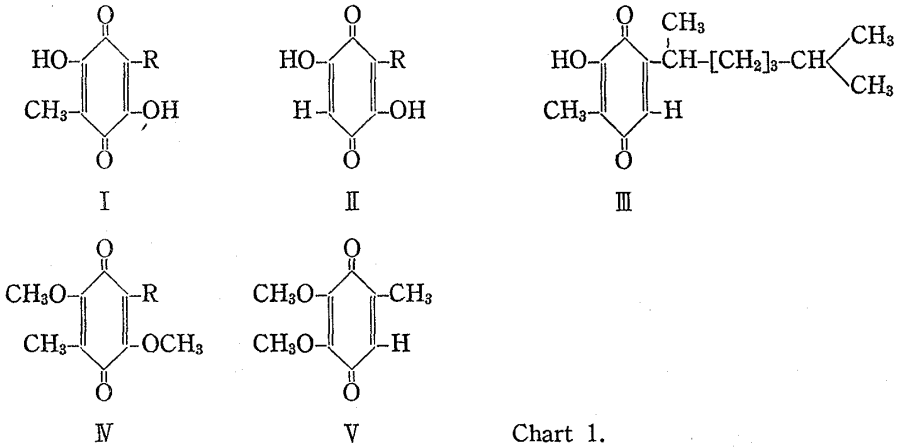
Dihydromaesaquinone (I : R=$C_{19}H_{39}$), prepared from maesaquinone.[8]

Helicobasidin (I : R=1,2,2-trimethylcyclopentyl), isolated from *Helicobasidium mompa.*[10,11]

Emberine (II : R=$C_{11}H_{23}$), synthetic specimen.[12]

Rapanone (II : R=$C_{13}H_{27}$), specimen isolated from *Ramanea Maximowiczii.*[13]

2-Octadecyl-3,6-dihydroxy-*p*-benzoquinone (II : R=$C_{18}H_{37}$), synthetic specimen.[14]



I II III IV V

Chart 1.

6) B. Hagihara : Biochim. et Biophys. Acta, **46**, 134 (1961).
7) T. Zincke : Ber., **14**, 94 (1881).
8) M. Hiramoto : Yakugaku Zasshi, **59**, 665 (1939); **62**, 460, 464 (1942).
9) H. Ogawa, S. Natori : This Bulletin, **13**, 511 (1965).
10) S. Natori, H. Ogawa, K. Yamaguchi, H. Nishikawa : This Bulletin, **11**, 1343 (1963).
11) S. Natori, H. Nishikawa, H. Ogawa : *Ibid.*, **12**, 236 (1964).
12) M. Asano, K. Yamaguchi : Yakugaku Zasshi, **60**, 105 (1940).
13) *Idem* : *Ibid.*, **60**, 585 (1940).
14) M. Asano, J. Hase : *Ibid.*, **60**, 650 (1940).

dl-Dihydroperezone (Ⅲ), synthetic specimen.*5,15)

Rapanone dimethyl ether (Ⅳ : R=$C_{13}H_{27}$), prepared from rapanone by the method of Kawamura.[16]

Dihydromaesaquinone dimethyl ether (Ⅳ : R=$C_{19}H_{39}$), prepared from rapanone by the method of Kawamura.[16]

Helicobasidin dimethyl ether (Ⅳ : R=1,2,2-trimethylcyclopentyl), prepared from helicobasidin.[8~11]

Ubiquinone-0 (Ⅴ), kindly supplied by Dr. M. Shimizu[17] of Daiichi Seiyaku Co., Ltd.

The other chemicals were obtained commercially. Especially, adenosine-5′-diphosphate (ADP), adenosine-5′-*tri*-phosphate (ATP), and hexokinase (Type Ⅲ) were purchased from Sigma Chemical Co., St. Louis, Mo.).

Results

The effects of benzoquinone derivatives listed in Table I on rat liver mitochondrial respiration were determined polarographically. Ubiquinone-0, dihydroxythymoquinone, helicobasidin, and dihydroperezone showed significant effects on the respiration at a concentration of $1.0 \times 10^{-4}M$. The polarographic tracings on oxygen consumption by mitochondria are shown in Fig. 1~3 respiring with succinate in the presence of benzoquinone derivatives. As shown in Fig. 1, the addition of ADP produced a characteristic stimulation of the respiration and the respiratory control index was calculated as 5 (curve A). This stimulation was completely eliminated by the addition of dihydroxythymoquinone at $1.0 \times 10^{-4}M$ (curve B). At a concentration of $3.2 \times 10^{-6}M$ (curve C), about 50% of the inhibition of State-3 respiration was observed. This inhibition by dihydroxythymoquinone ($1.0 \times 10^{-4}M$) was not released by 2,4-dinitrophenol in contrast to the case of oligomycin. Some effects of dihydroxythymoquinone were also observed in oxidation of NAD-linked substrates.

Table I. The Effects of Benzoquinone Derivatives on Rat Liver Mitochondrial Respiration in the Presence or Absence of ADP

Benzoquinone derivatives	Substrate	Condition of mitochondrial respiration		
		State-3 (+ADP)	State-4 (−ADP)	Presence of DNP[a] (0.1 m*M*)
Ubiquinone-0	succinate	↓↓	↑	↓↓
Dihydrothymoquinone	succinate	↓↓↓	—	↓↓↓
	glutamate	↓↓↓	—	↓↓↓
	α-oxoglutarate	↓↓↓	—	↓↓↓
	β-hydroxybutyrate	↓↓↓	—	↓↓↓
Dihydromaesaquinone	succinate	↑	↑	↑
Maesaquinone	succinate	↑	↑	—
Helicobasidin	succinate	—	↑↑↑	—
Emberine	succinate	—	↑	—
Rapanone	succinate	—	↑	—
2-Octadecyl-3,6-dihydroxybenzoquinone	succinate	—	—	—
Dihydroperezone	succinate	↓↓↓	↑↑	↓↓↓
Rapanone dimethyl ether	succinate	↓	—	↓
Dihydromaesaquinone dimethyl ether	succinate	—	—	—
Helicobasidin dimethyl ether	succinate	↓↓	—	↓↓

The oxygen consumption was determined polarographically. Benzoquinone derivatives were added in a final concentration of $10^{-4}M$ in 50 μl. of the ethanolic solution. Detailed conditions are described in Fig. 1.

a) DNP : 2,4-dinitrophenol

Inhibition and stimulation of mitochondrial respiration are indicated as the following scale (% of normal oxygen consumption): ↓↓↓ <30, ↓↓ =30~60, ↓ >60, ↑↑↑ >300, ↑↑ =300~200, ↑ <200, — =negligible.

*5 Recently it was proven that the used dihydroperezone in this experimental was a positional isomer of hydroxy group of natural *dl*-perezone. S. Natori, Y. Inouye, H. Nishikawa : This Bulletin, in press.

15) K. Yamaguchi : Yakugaku Zasshi, **62**, 491 (1942).
16) J. Kawamura : Nippon Gakujutsu Kyokai Hokoku, **12**, 377 (1937).
17) M. Shimizu, K. Koshi : This Bulletin, **11**, 404 (1963).

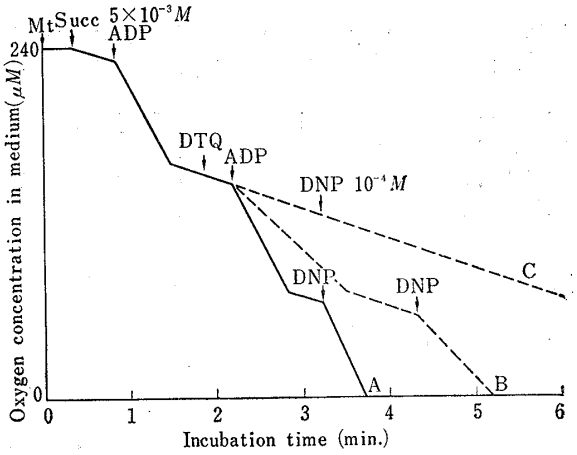


Fig. 1. Polarographic Tracing showing Effects of Dihydroxythymoquinone (DTQ) on Mitochondrial Respiration with Succinate

A, control (without DTQ), B, $3.2\times10^{-6}M$ DTQ; C, $1.0\times10^{-4}M$ DTQ. The reaction mixture was an air-equilibrated solution containing 0.3*M* mannitol, 10 m*M* KCl, 10 m*M* phosphate, 2.5 m*M* $MgCl_2$, and 0.2 m*M* EDTA, in a final volume of 2.3 ml. (pH 7.4). Additions were 0.2 ml. of mitochondrial suspension (3 mg. mitochondrial protein), 25 μl. of 0.5*M* sodium succinate, 10 μl. of 0.05*M* ADP, 50 μl. of DTQ ethanolic solution and 50 μl. of $5\times10^{-6}M$ DNP. The additions are indicated by arrows. Preincubation was carried out and the reaction was initiated by the addition of succinate at 25°.

In Fig. 2 are shown the effects of ubiquinone-0 and dihydroperezone on mitochondrial respiration at a concentration of $10^{-5}M$. Ubiquinone-0 showed a slight uncoupling action. Dihydroperezone was not only a stimulator of State-4 respiration, but also an inhibitor of State-3 respiration. Helicobasidin, as shown in Fig. 3, was a potent uncoupler.

Considering an interaction of quinone derivatives with platinum electrode of polarographic apparatus, it will be necessary to confirm the inhibitory action by the other assay method. Therefore, manometric studies were carried out and the results were shown in Fig. 4A and 4B, indicating similar results to those determined polarographically. In the absence of phosphate acceptor, an inhibitory action by dihydroxythymoquinone was not observed (Fig. 4A), but the respiratory stimulation by the addition of phosphate acceptor (glucose and hexokinase) was inhibited completely at a concentration of $1.0\times10^{-5}M$ (Fig. 4B).

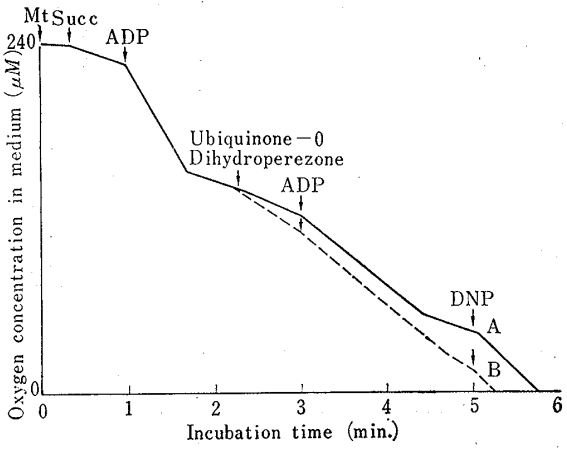


Fig. 2. The Effects Ubiquinone-0 and Dihydroperezone on Mitochondrial Respiration with Succinate. A, Ubiquinone-0; B, Dihydroperezone

The final concentration of the quinone is $1.0\times10^{-5}M$. Detailed conditions are described in Fig. 1.

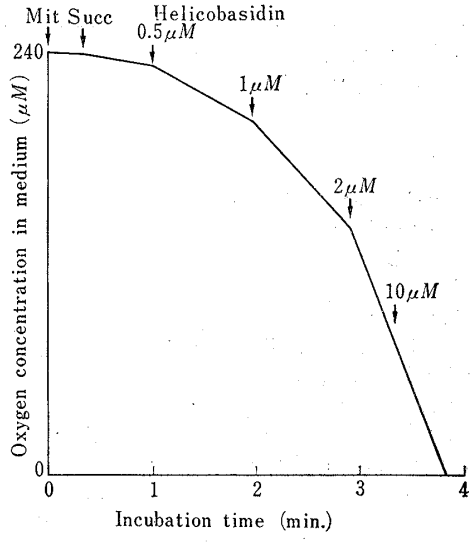


Fig. 3. The Uncoupling Action of Helicobasidin on Mitochondrial Respiraition

The final concentration of added helicobasidin is indicated in the diagram. Detailed conditions are described in Fig. 1.

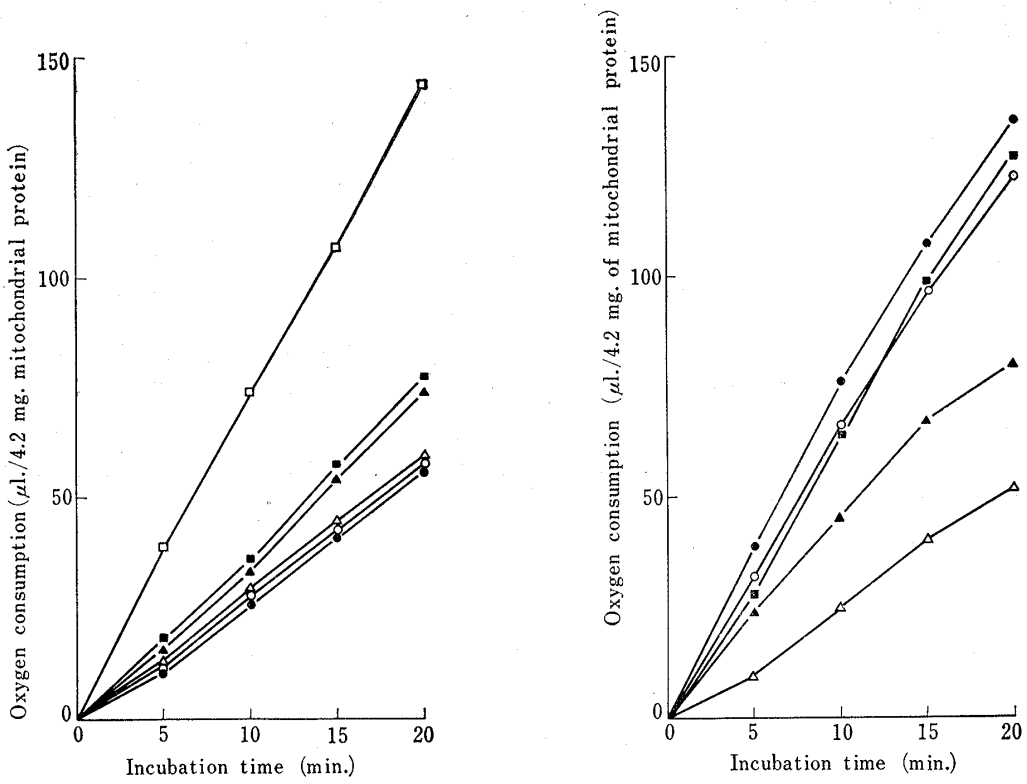


Fig. 4. The Effects of Benzoquinone Derivatives on Mitochondrial Respiration determined Manometrically

A: presence of phosphate acceptor, B: absence of phosphate acceptor. —●— control, —○— ubiquinone-0, —△— dihydroxythymoquinone, —▲— dihydroperezone, —■— helicobasidin —□— DNP. The incubation mixture contained 0.3*M* mannitol, 10 m*M* KCl, 10 m*M* phosphate, 2.5 m*M* $MgCl_2$, 0.2 m*M* EDTA, 1.7 m*M* ATP, 0.03*M* sodium succinate and 4.2 mg. of mitochondrial protein in a final volume of 3.0 ml. at pH 7.4. As a phosphate acceptor, 100 μmloes of glucose and 150 units of hexokinase were added. A 0.2 ml. of 20% KOH was placed in center well. Incubation was carried out at 30° and oxygen consumption was determined manometrically. Benzoquinone derivatives were added at the final concentration of $1.0\times10^{-5}M$ as an ethanolic solution and the final ethanol concentration in the medium was 2%. All controls also contained 2% of ethanol. The concentration of DNP was $1.0\times10^{-4}M$. B was resulted simultaneously with A by the same mitochondrial preparation.

Discussion

It was found that the benzoquinone derivatives which possess a short side chain showed some effects on rat liver mitochondrial respiration in the presence with succinate and NAD-linked substrates. Dihydroxythymoquinone inhibited the respiration which was stimulated by ADP (State-3), whereas State-4 respiration was not inhibited even at the concentration of more than $1.0\times10^{-4}M$. These results indicate that dihydroxythymoquinone may also inhibit oxidative phosphorylation. Helicobasidin was shown to be an uncoupler. The effects of dihydroperezone were a mixed type of uncoupler and inhibitor of State-3 respiration. Ubiquinone-0 showed a slight uncoupling action and the results were identical with that of Smith and Lester.[18] Other benzoquinone derivatives employed here did not show any significant effects on mitochondrial respiration.

It was very interesting to know the facts that dihydroxythymoquinone inhibited only State-3 respiration and that the inhibition was not released by the addition of

18) A. L. Smith, R. L. Lester: Biochim. et Biophys. Acta, 48, 547 (1961).

2,4-dinitrophenol. Recently Howland[19] reported that succinate oxidation in rat liver mitochondria was inhibited by 3-alkyl-1,4-naphthoquinone in 2-hydroxyl group and that inhibition induced by the naphthoquinone could be released to a different extent by various uncoupling agents. However, the action of dihydroxythymoquinone is found to be distinct from that of such naphthoquinone[19] and similar to that of antibiotic DIO-9[20] which inhibits mitochondrial oxidation and is not released by 2,4-dinitrophenol.

The sites of inhibitory action by dihydroxythymoquinone in the electron or energy transfer reactions is under investigation.

Grateful acknowledgments were made to Dr. K. Yamaguchi, Torii Yakuhin Kogyo Co., Ltd., for his encouragement and to Miss. H. Ogawa, National Institute of Hygienic Science, for her assistance in the experiments.

The authors are also indebted to Miss. M. Machida, Pharmaceutical Institute, Tohoku University School of Medicine, for her technical assistance.

19) J. L. Howland: Biochim. et Biophys. Acta, **105**, 205 (1965).
20) R. J. Guillory: *Ibid.*, **89**, 197 (1964).