CHEMICAL
&
PHARMACEUTICAL BULLETIN

Vol. 13 No. 9 September 1965

[Chem. Pharm. Bull. 13(9)1029~1035(1965)]

UDC 547.567.3 : 576.095.18

134. Hikaru Ozawa,*1 Shinsaku Natori,*2 and Kazutaka Momose*1 : Biochemical Studies on Quinone Derivatives. I. Effects of Naturally Occurring Benzoquinone Derivatives on Mitochondrial Preparations.*3

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

Crane, *et al.*[1~3] reported that the lipid factor, ubiquinone named by Morton,[4] is contained in mitochondria and Lester, *et al.*[5,6] confirmed that the ubiquinone is an essential component in the electron transport chain by the experiments of a solvent extraction technique introduced by Nason and Lehman.[7] Water-miscible solvents such as acetone are found to be excellent for ubiquinone extraction without further destruction of the electron transport system. Crane, *et al.*[8] also reported that the activity of the succinate oxidase was inhibited by treating the electron transport system with acetone; that is, the procedure removing 90~100% of ubiquinone; and that for the restoration of the activity it should be necessary to add ubiquinone, cytochrome c, and unidentified lipids. Some investigators described[9~11] on the restoration of the activity by adding ubiquinone homologue and the other derivatives such as methyl and ethyl ubiquinones.

On the other hand, it could be suggested that quinones have a possibility to become an intermediate of oxidative phosphorylation,[12] whereas some quinones act either as an inhibitor or uncoupler on energy coupled electron transport.[13] This paper deals with the experimental effects of naturally occurring benzoquinone derivatives,[14] such

*1 Kitayonbancho, Sendai (小澤 光, 百瀬和享).
*2 Tamagawayoga-machi, Setagaya-ku, Tokyo (名取信策).
*3 A part of this work was presented at the Annual Meeting of the Pharmaceutical Society of Japan at Tokyo, April, 1964 and the other was read at the Annual Meeting of the Japanese Biochemical Society at Nagoya, October, 1964.

1) F. L. Crane, Y. Hatefi, R. L. Lester, C. Widmer : Biochim. et Biophys. Acta, **25**, 220 (1957).
2) Y. Hatefi, R. L. Lester, C. Widmer, F. L. Crane : *Ibid.*, **31**, 490 (1959).
3) F. L. Crane, C. Widmer, R. L. Lester, Y. Hatefi : *Ibid.*, **31**, 476 (1959).
4) R. A. Morton, G. M. Wilson, J. S. Lowe, W. M. F. Leat : Biochem. J., **68**, 16 p. (1958).
5) R. L. Lester, Fleischer : Arch. Biochem. Biophys., **80**, 470 (1959).
6) *Idem* : Biochim. et Biophys. Acta, **47**, 358 (1961).
7) A. Nason, I. R. Lehman : J. Biol. Chem., **222**, 511 (1956).
8) F. L. Crane, W. Fechiner, K. S. Ambe : Arch. Biochem. Biophys., **81**, 277 (1959).
9) K. S. Ambe, F. L. Crane : Biochim. et Biophys. Acta, **43**, 30 (1960).
10) D. Hendlin, T. M. Cook : J. Biol. Chem., **235**, 1187 (1960).
11) F. L. Crane, B. Ehrich : Arch. Biochem. Biophys., **89**, 134 (1960).
12) R. E. Erickson, A. F. Wagner, K. Folkers : J. Am. Chem. Soc., **85**, 1535 (1963).
13) Papers in Ciba Found. Symp., Quinones in Electron Transport, Little, Brown, Boston, 1961.
14) R. H. Thomson : "Naturally Occurring Quinones," Butterworths, London (1957).

as rapanone,[15] maesaquinone,[16] and related compounds, on succinate oxidase of mitochondria under various conditions.

Effects on Intact Mitochondria

As illustrated in Table I, an addition of maesaquinone and rapanone stimulated the succinate oxidase activity of rat liver mitochondria. Since these quinones were slightly soluble in water of pH 7.4 and 6.6, the quinone suspensions were used in the experiments. However, at pH 8.0, these quinones were soluble in water in violet color, and the stimulative action was reduced slightly. Rapanone dimethyl ether and dihydromaesaquinone dimethyl ether, the methylated derivatives at hydroxy groups, did not show any stimulation, but indicated slight inhibition of the succinate oxidase activity of mitochondria. The inhibitory action of the methylated quinones may be considered to be competitive with ubiquinone because methylation of the quinones became more similar to ubiquinone in the chemical structure.

Ubiquinone-7 and ubiquinone-10 also showed a slightly stimulative action on the activity of rat liver and pig heart mitochondria.

Table I. Effects of Quinones on Succinate Oxidase of Rat Liver Intact Mitochondria

pH	No addition	Maesaquinone	Rapanone	Rapanone dimethyl ether	Dihydromaesaquinone dimethyl ether
6.6	1.40	1.93	2.33	—	—
7.4	1.53	1.87	2.13	1.21	1.09
8.0	1.40	1.60	1.80	—	—

The activity represents oxygen uptake (μl./min./2 mg. enzyme protein). All quinones were used at the concentration of 0.5 m*M*. Detailed experimental conditions were described in experimental.

Inhibition of Succinate Oxidase by Acetone Treatment and Reactivation by Quinone Derivatives

When mitochondrial preparations were treated with acetone, more than 95% of ubiquinone in the preparations was removed together with other mitochondrial lipids such as phospholipid, and it resulted in a significant loss of the activity. As shown in Fig. 1, cyclohexane extract of mitochondrial preparations, according to a procedure of Pumphrey and Redfearn,[17] showed the ultraviolet absorption maximum at 275 mμ which disappeared by an addition of sodium borohydride, but it could not be observed in the extract from acetone-treated preparations, suggesting an almost complete removal of ubiquinone by acetone treatment.

In Fig. 2, the representative results which illustrate depletion by acetone extraction and restoration of succinate oxidase activity of mitochondrial preparation were shown by adding ubiquinone-7 and some other benzoquinones in the presence of Torula lipids (TL) which was used as a supplementary lipids.[18] Treatment with acetone reduced the activity and an addition of TL alone to the preparation resulted in a slight reactivation.

Although an aqueous TL itself could not reactivate the succinate oxidase of acetone treated preparations, it had a remarkable potential effect on ubiquinone and other quinones tested for restoration of the activity.

15) M. Asano, K. Yamaguchi: Yakugaku Zasshi, **60**, 585 (1940).
16) M. Hiramoto: *Ibid.*, **59**, 665 (1939); **62**, 460, 464 (1942). H. Ogawa, S. Natori: This Bulletin, **13**, 511 (1965).
17) A. M. Pumphrey, E. R. Redfearn: Biochem. J., **76**, 64 (1960).
18) S. Okui, Y. Suzuki, K. Momose: J. Biochem., **54**, 471 (1963).

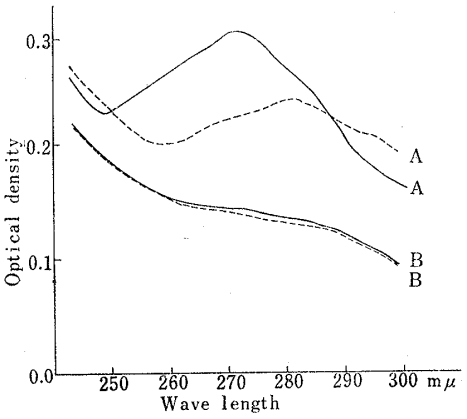

Fig. 1. Ultraviolet Absorption Spectra of Cyclohexane Extracts from Intact and Acetone-treated Pig Heart Mitochondria

A : Extract from intact mitochondria.
B : Extract from acetone-treated mitochondria.
——— oxidized form
– – – – – reduced form

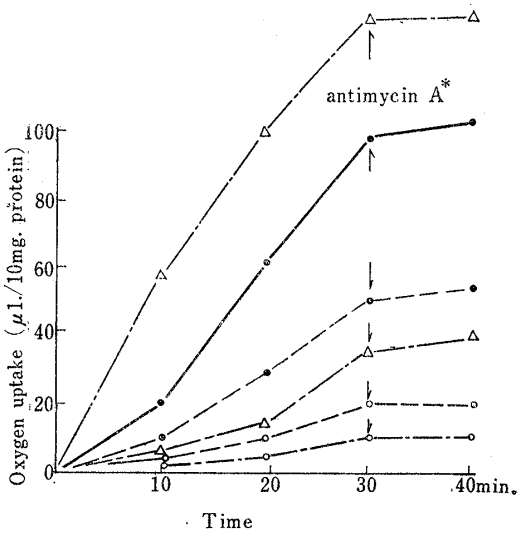

Fig. 2. Reactivation of Succinate Oxidase of Acetone-treated Mitochondria by Various Quinones in the Presence of TL

—▲— ubiquinone —●— rapanone
—●— dihydromaesaquinone
—△— no addition
—○— rapanone dimethyl ether
—○— dihydromaesaquinone dimethyl ether
All quinones were used at the concentration of 0.5 m*M*.
* Antimycin A (2 μg./ml.) was added at the arrow.

In Fig. 3, it shows a dose response curve for the restoration activity to ubiquinone-7 up to the maximum obtained with acetone treated mitochondrial preparations, but rapanone shows an optimal concentration at 0.25～0.5 m*M* which was used in the following experiments. It may be the reason for the inactivation that, at 0.5 m*M* and more higher concentration of rapanone, the deposition of insoluble rapanone to particle protein was observed.

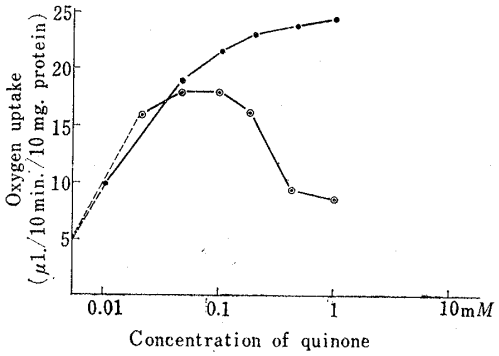

Fig. 3. Dosage response to ubiquinone and rapanone in reactivation of acetone-treated mitochondrial succinate oxidese

—●— ubiquinone-7 —○— rapanone

Table II illustrates the results of reactivation by adding ubiquinone and various benzoquinone derivatives to the succinate oxidase. Restoration activity on the electron transport was observed with rapanone,[15] helicobasidin,[19,20] dihydromaesaquinone,[16] 3-octadecyl-2,5-dihydroxy-*p*-benzoquinone,[21] 2,5-didecyl-3,6-dihydroxy-*p*-benzoquinone.[22]

It was observed that different enzyme preparations afforded the results with some variation. However, less variation was observed between the different experiments with the same preparations and the least variation was found among replicates within an experiment.

From these results, it is suggested that the structure-activity relationship may be as follows : Longer aliphatic group(s) and hydroxy group(s) are necessary and

19) S. Natori, H. Ogawa, K. Yamaguchi, H. Nishikawa : This Bulletin, **11**, 1343 (1963).
20) S. Natori, H. Nishikawa, H. Ogawa : *Ibid.*, **12**, 236 (1964).
21) M. Asano, J. Hase : Yakugaku Zasshi, **60**, 650 (1940).
22) M. Asano, H. Takahashi : *Ibid.*, **61**, 127 (1941).

Table II. Restoration of Succinate Oxidase Activity of Acetone-treated Mitochondria by Various Benzoquinone Derivatives*4

Additives (0.5 m*M*)	Torula lipids		Antimycin A (1 μg./ml.)
	absence	presence (1 mg./ml.)	
No addition	0.07[a]	0.12	0.00
Ubiquinone-0	0.19	0.23	0.00
Ubiquinone-7	0.10	0.87	0.00
Ubiquinone-10	0.09	0.54	0.00
Rapanone[15]	0.07	0.29	0.01
Embelin[23]	0.07	0.25	0.01
Maesaquinone[16]	0.08	0.45	0.00
Maesaquinone dimethyl ether[16]	0.07	0.18	0.00
Dihydromaesaquinone[16]	0.07	0.38	0.00
Dihydromaesaquinone dimethyl ether[16]	0.05	0.31	0.00
Helicobasidin[19,20]	0.07	0.25	0.00
Helicobasidin dimethyl ether[19,20]	0.03	0.03	0.00
3-Octadecyl-2,5-dihydroxy-*p*-benzoquinone (b.q.)[21]	—	0.33	—
2,5-Didecyl-3,6-dihydroxy-b.q.[22]	—	0.35	—
2-Hexadecyl-5-hydroxy-b.q.*5	—	0.27	—
dl-Dihydroperezone[24]	—	0.07	—
2-Dodecyl-6-methoxy-b.q.[23]	—	0.07	—
2-Methyl-3-hydroxy-5-octyl-b.q.[25]	—	0.07	—
Rapanone dimethyl ether[26]	—	0.01	—
Thymoquinone[27]	—	0.07	—
Dihydroxythymoquinone[28]	—	0.07	—

Experimental conditions are as same as described in Table I and Fig. 2.
a) μatom O_2/min./5mg. enzyme.

methylation of the hydroxy group(s) decreases the activity. Imada, *et al.*[29] also reported that the demethylated ubiquinone was effective for reactivation of acetone-treated mitochondrial succinate oxidase activity.

These restored activity was inhibited by the addition of antimycin A at a concentration 1 μg./ml. (0.2 μg./mg. enzyme protein), and an elimination of cytochrome c from incubation medium also reduced the restoration.

Quinol-cytohrome Oxidoreductase

As the quinones described above intermediated electron transport of acetone-treated mitochondria, it was examined whether these quinones might intermediate also in intact mitochondrial electron transport instead of ubiquinone or not.

As shown in Fig. 4, both succinate and ubiquinol could reduce cytochrome c enzymatically with mitochondria, and the both reaction were inhibited by antimycin A addition. However, the antimycin sensitivity of ubiquinol-cytochrome c oxidoreductase varied in different mitochondrial preparations.

*4 A part of these data was shortly communicated. H. Ozawa, *et al.*: Biochim. Biophys. Acta, **86**, 395 (1964).
*5 Synthetic specimen.
23) A. Asano, K. Yamaguchi: Yakugaku Zasshi, **60**, 105 (1940).
24) K. Yamaguchi: *Ibid.*, **62**, 491 (1942).
25) T. Kusaka: *Ibid.*, **62**, 490 (1942).
26) J. Kawamura: Nippon Gakujutsu Kyokai Hokoku, **12**, 377 (1937).
27) Org. Synthesis, Coll. Vol. I, 511.
28) T. Zincke: Ber., **14**, 94 (1881).
29) I. Imada, *et al.*: This Bulletin, **11**, 815 (1963).

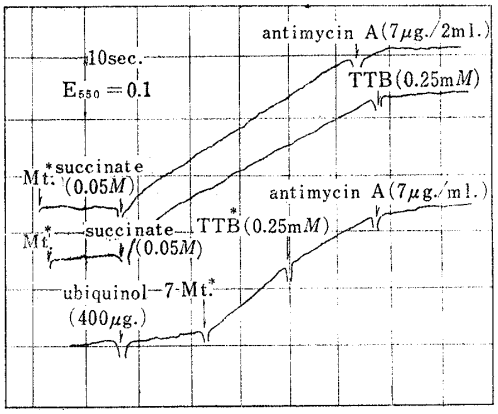

Fig. 4. Succinate- and Ubiquinol-cytochrome Oxidoreductase Activity of Mitochondria

The chart is modified to prevent from an influence by turbidity with additives such as ubiquinol.
* TTB: Tenoyltrifluorobutadien-3-one
Mt.: Mitochondria

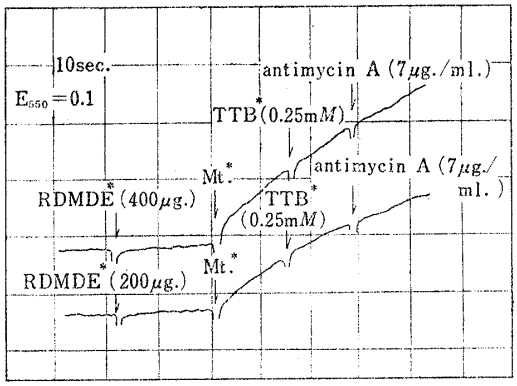

Fig. 5. Reduced Dihydromaesaquinone Dimethyl Ether Cytochrome c Oxidoreductase Activity of Mitochondria

Experimental conditions are as same as described in Fig. 4.
* RDMDE: Reduced dihydromaesaquinone dimethyl ether
Mt.: Mitochondria
TTB: Tenoyltrifluorobutadien-3-one

In the experiments, reduced dihydromaesaquinone dimethyl ether exhibited a similar, but less marked inhibition to ubiquinol by antimycin A, as shown in Fig. 5. Tenoyltrifluorobutadien-3-one, which inhibits succinate-ubiquinol oxidoreductase and is not an inhibitor for ubiquinol-cytochrome oxidoreductase, did not also inhibit the reduction of cytochrome c by this reduced benzoquinone derivative.[30]

The results may suggest that the dihydromaesaquinone dimethyl ether, which is the most similar chemical structure in the quinones described above to ubiquinone, did not take part in the original electron pathway through antimycin A sensitive site

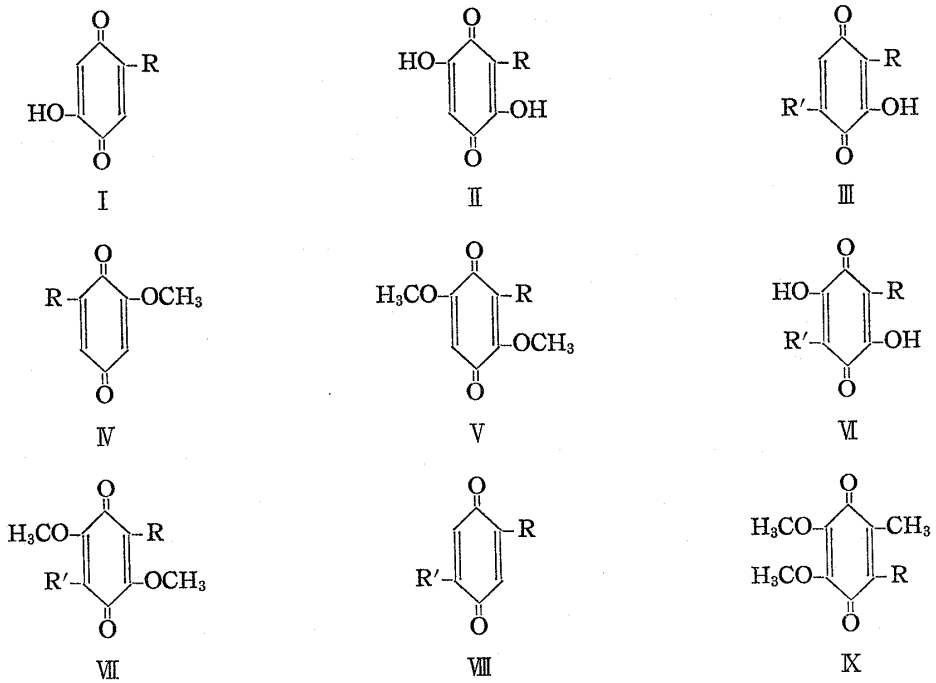

Chart 1.

30) D. R. Sanadi, A. L. Fluharty: Biochemistry, 3, 523 (1963).

under the condition that intact mitochondria was used. The external quinones including ubiquinone are reactive with cytochrome c through other routes, which is approved by the fact that antimycin sensitivity of ubiquinol-cytochrome c oxidoreduction was so affected.

The other quinones except dihydromaesaquinone dimethyl ether could not be used in the experiment because the reduced form of these quinones were reactive nonenzymatically with cytochrome c.

These quinones may be useful for studies of quinone reductase in microsome mithochondria.

Experimental

Materials——Benzoquinone derivatives were synthesized or extracted from plant yeast, fungi and animal, indicated as follows:

2-Hexadecyl-5-hydroxy-*p*-benzoquinone (I : R=$C_{16}H_{33}$), synthetic specimen.*5
Rapanone (II : R=$C_{13}H_{27}$), specimen isolated from *Rapanea Maximowiczii.*[15]
dl-Dihydroperezone (III : R=CH_3, R′=-$CH(CH_3)$-$(CH_2)_3CH(CH_3)_2$), synthetic specimen.[24]
Embelin (II : R=$C_{11}H_{23}$), synthetic specimen.[23]
2-Dodecyl-6-methoxy-*p*-benzoquinone (IV : R=$C_{12}H_{25}$), synthetic specimen.[23]
2-Undecyl-6-methoxy-*p*-benzoquinone (IV : R=$C_{11}H_{23}$), synthetic specimen.[23]
2,5-Didecyl-3,6-dihydroxy-*p*-benzoquinone (VI : R, R′=$C_{10}H_{21}$), synthetic specimen.[22]
Rapanone dimethyl ether (V : R=$C_{13}H_{27}$), prepared from rapanone by the method of Kawamura.[26]
3-Octadecyl-2,5-dihydroxy-*p*-benzoquinone (II : R=$C_{18}H_{37}$), synthetic specimen.[21]
Helicobasidin (VI : R=CH_3, R′=1,2,2-trimethylcyclopentyl), isolated from *Helicobasidium mompa.*[19,20]
Helicobasidin dimethyl ether (VII : R=CH_3, R′=1,2,2-trimethylcyclopentyl), prepared from helicobasidin.[19,20]
2-Methyl-3-hydroxy-5-octyl-*p*-benzoquinone (III : R=CH_3, R′=C_8H_{17}), synthetic specimen.[25]
Maesaquinone (VI : R=CH_3, R′=$(CH_2)_{13}$-CH=CH-$(CH_2)_3$-CH_3), isolated from *Maesa japonica* by the method of Hiramoto.[16]
Dihydromaesaquinone (VI : R′=CH_3, R′=$C_{19}H_{39}$), prepared from maesaquinone.[16]
Maesaquinone dimethyl ether (VII : R=CH_3, R′=$(CH_2)_{13}$-CH=CH-$(CH_2)_3$-CH_3), prepared from maesaquinone.[16]
Dihydromaesaquinone dimethyl ether (VII : R=CH_3, R′=$C_{19}H_{39}$), prepared from dihydromaesaquinone.[16]
Thymoquinone (VIII : R=CH_3, R′=$CH(CH_3)_2$), prepared according to the direction.[27]
Dihydroxythymoquinone (VI : R=CH_3, R′=$CH(CH_3)_2$), prepared by the method of Zincke.[28]
Ubiquinone-10 (IX : R=$(CH_2CH=C(CH_3)CH_2)_{10}H$), prepared from pig heart by direct method.[30]
Ubiquinone-7 (IX : R=$(CH_2CH=C(CH_3)$-$CH_2)_{10}H$), prepared from Torula yeast (Type-A dry powder, Toyo Spinning Co., Ltd.) by direct method[31]
Ubiquinone-0 (IX : R=H), a kind gift of Dr. M. Shimizu[32] of Daiichi Seiyaku Co.

Antimycin A and cytochrome c were purchased from Kyowa Hakko Co., Ltd., and Tokyo Kasei Kogyo Co., Ltd., respectively. Other reagents were of commercial productions.

Mitochondria——Pig heart muscle mitochondria was prepared as follows: Pig heart muscle was minced and blended for 1 min. by Waring blender in 0.25*M* sucrose-0.2 m*M* EDTA solution at 0°. The homogenate was homogenized by Potter teflon homogenizer and the mitochondrial fraction was collected by differential centrifugations. Rat liver mitochondria was prepared in sucrose-EDTA medium according to the method of Schneider.[33]

Preparation of Acetone-treated Mitochondria——Mitochondria was treated with dry cold acetone according to a modified procedure of Okui, *et al.*[18] as follows: Mitochondrial suspension (200 mg. protein in 4.0 ml. of 0.25*M* sucrose) was added dropwise into 96 ml. of dry cold acetone (−20°). The suspension was stirred for 3 min. and collected by centrifugation. The collected preparation was washed with dry cold acetone by the same procedure as before. Finally the mitochondrial precipitate obtained was dried immediately *in vacuo* and stored at −20°.

Succinate Oxidase Activity——The activity was determined manometrically at 30° with the following reaction mixture: 0.25*M* sucrose; 20 m*M* KCl; 2 m*M* $MgCl_2$; 0.2 m*M* EDTA; 5 m*M* phosphate; 10 m*M*

31) R. L. Lester, F. L. Crane: Biochim. et Biophys. Acta, **32**, 492 (1959).
32) M. Shimizu, K. Koshi: This Bulletin, **11**, 404 (1963).
33) W. C. Schneider: J. Biol. Chem., **178**, 259 (1948).

Tris-Cl, pH 7.4. Cytochrome c, TL and enzyme preparation were added to the incubation mixture at the concentrations of 0.5 mg./ml., 1 mg./ml., and 5 mg./ml., respectively. Total volume was 2.0 ml., and 0.2 ml. of 20% KOH was placed in a center well. After preincubation for 10 min., the incubation was started by the addition of 0.2 ml. of 0.5*M* succinate.

Succinate and Quinol Cytochrome c Oxidoreductase Assay——A mixture made up of the following components was introduced into each cuvet : $2\times10^{-2}M$ phosphate buffer, pH 7.4, $3\times10^{-3}M$ KCN, $5\times10^{-4}M$ EDTA, 0.02% bovine plasma albumin. The final volume was made up to 2.0 ml. by addition of water, and 0.5 mg. of enzyme protein in 0.1 ml. of phosphate buffer was added to each cuvet. The reaction was started by the addition of 0.1 ml. of 0.5*M* succinate or 0.2~0.4 mg. of quinols in 0.025 ml. ethanol to one of the two cuvets after 3 min. preincubation, and the optical density at 550 mμ against the control cuvet using Hitachi EPS spectrophotometer was recorded.

Preparation of Reduced Quinones——Quinones were reduced by dithionite according to a procedure of Green, *et al.*[34]

Torula Lipid (TL)——TL was extracted from *Torula utilis* with three volume of EtOH-ether mixture (3:1).[18]

Greatful acknowledgements were made to Dr. K. Yamaguchi, National Institute of Hygienic Science, Osaka, for his encouragement and to Mrs. H. Ogawa, National Institute of Hygienic Science, for her assistance in the experiments.

Summary

Effects of naturally occurring *p*-benzoquinone derivatives on mitochondrial respiration were studied. Plant quinones, such as rapanone and maesaquinone, stimulated succinate oxidase of intact mitochondria. These quinones and other derivatives reactivated succinate oxidase which was inhibited by acetone-treatment of mitochondria. However methylated quinones did not show reactivated efficiently. Reduced dihydroxymaesaquinone dimethyl ether reacted with cytochrome c enzymatically, but the reaction was not inhibited by antimycin A addition. The other reduced quinones were reactive with cytochrome c nonenzymatically.

(Received January 20, 1965)

34) D. E. Green, R. K. Burkhard : Arch. Biochem. Biophys., **92**, 312 (1961).