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234. Hikaru Ozawa, Kazutaka Momose, and Motoko Machida*1 : Biochemical Studies on Benzoquinone Derivatives. III.*2 Effects of Dihydroxythymoquinone on Rat Liver Mitochondria in the Electron Transport System.*3

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Dihydroxythymoquinone (DTQ) showed a marked inhibitory action on State-3 respiration of rat liver mitochondria with succinate, whereas State-4 respiration was not inhibited. This inhibition was neither released by 2,4-dinitrophenol nor other uncoupling agents. Completely DTQ inhibited oxidation of succinate by electron transport particles but the rate of inhibition was less than that of State-3 respiration by intact mitochondria. Reduction of cytochrome c by mitochondria with succinate was also inhibited. Succinate dehydrogenase was stimulated by DTQ.

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In the course of studies of effects of naturally occurring benzoquinone derivatives on the mitochondrial functions[1,2], it was shown that, as previously reported by the present authors, some of the quinone derivatives gave a significant effect on rat liver mitochondrial respiration.[3] One of these compouns, dihydroxythymoquinone (DTQ), showed a marked inhibitory action on State-3 respiration and, in contrast to the case of oligomycin, the inhibition was not released completely by 2,4-dinitrophenol (DNP).

It was reported that a number of quinone derivatives have been proved to be a potent inhibitor in the mitochondrial electron transfer system between cytochrome b and c_1.[4] Considering from these facts, it is possible to consider that reduction of oxygen comsumption by DTQ may also be due to the similar effects.

This paper deals with the effects of DTQ on intact mitochondria of rat liver and its non-phosphorylating preparation.

Experimental

Materials——DTQ (dihydroxythymoquinone, 2-methyl-5-isopropyl-3,6-dihydroxy-*p*-benzoquinone) was synthesized according to Zincke's procedure from thymoquinone.[5] DTQ (mol. wt. 196.20, m.p. 223~224°) is orange needles, and when dissolved in buffer solution of above pH 4.9, it gives a violet solution. The solubility in water at pH 7.2 was more than $4\times10^{-4}M$.

The other chemicals were obtained commercially : in particular, disodium adenosine-5′-diphosphate (ADP) and disodium adenosine-5′-triphosphate (ATP) from Sigma Chemical Co. (St. Louis, Mo.), phenazine methosulfate (PMS) from N. B. C. (Cleveland, Ohio), cytochrome c from Daiichi Pure Chemicals Co., Ltd. (Tokyo), and hexokinase (Type III) from Sigma Chemical Co. (St. Louis).

Methods——Rat liver mitochondria were isolated according to the procedure previously reported by the authors, using an isolation mixture containing 0.21*M* mannitol, 0.07*M* sucrose, 0.2 m*M* EDTA, and Tris-HCl (pH 7.4)[3] An amount of protein was determined by using biuret reagent with a standard of bovine serum albumin (Fraction-V, Armor Laboratories).[6]

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*1 Kitayobancho, Sendai (小澤 光, 百瀬和享, 町田征子).
*2 Part II : This Bulletin, **15**, 1095 (1967).
*3 This work was presented at the Tohoku Branch Meeting of Pharmaceutical Society of Japan, May 21, 1966.

1) H. Ozawa, K. Momose, S. Natori, K. Yamaguchi, H. Ogawa : Biochim. et Biophys. Acta, **86**, 395 (1964).
2) H. Ozawa, S. Natori, K. Momose : This Bulletin, **13**, 1029 (1965).
3) *Idem* : *Ibid.*, **15**, 1095 (1967).
4) J. L. Howland : Biochim. et Biophys. Acta, **73**, 665 (1963).
5) T. Zincke : Ber., **14**, 815 (1881).
6) T. Yonetani : J. Biol. Chem., **236**, 1680 (1961).

Mitochondrial respiration was determined polarographically at 25° using an oxygen electrode (Model PO-100, Yanagimoto MFG Co., Ltd., Tokyo).[7] For calculation of the rates, the oxygen concentration of an air-saturated medium was taken as 240 μM. Degree of the phosphorylation (ADP/O ratio) was estimated by the range of the stimulated respiration (State-3), produced by the addition of a known amount of ADP, the concentration of which was measured by the absorbancy at 260 mμ ($\varepsilon M = 15.4 \times 10^{-3}$ at pH 7).

Electron transfer particles were prepared by the procedure, shown in Fig. 1, according to the method of Crane, *et al.*[8] An oxygen consumption by electron transport partiices was determined manometrically as follows: a 2.7 ml. of incubation mixture contained 0.25M sucrose, 10 mM KCl, 10 mM phosphate, 2.5 mM $MgCl_2$, 0.2 mM EDTA (pH 7.3) and electron transport particles of rat liver mitochondria (25~30 mg. protein) were preincubated for 5 minutes at 30°, and then the reaction was initiated by the addition of 0.3 ml. of 0.5M succinate.

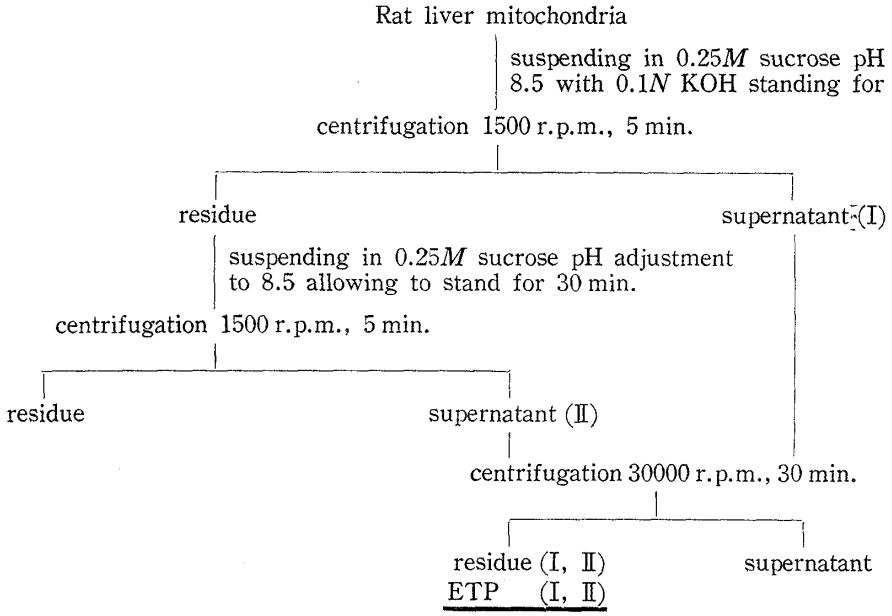

Fig. 1. Preparation Procedure of Electron Transpot Particles (ETP) from Rat Liver Mitochondria

ETP (I) was mainly used in the Experiments.

Succinate dehydrogenase activity was determined manometrically at 30° using PMS.[9,10]

Succinate-cytochrome c oxidoreductase activity was determined photometrically measuring reducing rate of cytochrome c externally added at 550 mμ with a recording spectrophotometers (Model MPS-50, Shimadzu, Kyoto, and Model EPS-3, Hitachi) at room temperature.

The basic reaction mixture used in most of experiments were composed of 0.3M mannitol, 10 mM KCl, 0.2 mM EDTA, 2.5 mM $MgCl_2$, and 10 mM phosphate (pH 7.2). DTQ was added as an ethanolic solution and, in any cases the final concentration of ethanol did not exceed more than 2%, otherwise the higher concentration gives effects on mitochondrial oxidations or phosphorylation.

Results

In Fig. 2 polarographic tracings of oxygen consumption by rat liver mitochondria respiring with succinate are shown. An addition of ADP produced a characteristic stimulation of the respiration (State-3[11]), and when the ADP added was phosphorylated completely to ATP, the stimulated respiration was reduced to the original level (State-4[11]). A respiratory control index (R.C.) was obtained as 5.5. When 3 μM DTQ was added to State-4 respiration, the State-3 respiration could not be induced even by the successive

7) B. Hagihara: Biochim. et Biophys. Acta, **46**, 134 (1961).
8) F.L. Crane, J.L. Glenn, D.E. Green: Biochim. et Biophys. Acta, **22**, 475 (1956).
9) H. Ozawa, K. Asami: Seikagaku, **34**, 243 (1962).
10) W.D. Bonner: Methods in Enzymol., **1**, 722 (1955).
11) B. Chance, G.R. Williams: Adv. in Enzymol., **17**, 65 (1956).

addition of ADP (curve A). When DTQ was added to State-3 respiration condition, a similar inhibition was also observed (curve B). On the other hand, little effect of DTQ on State-4 respiration was observed at any concentration used in the experiments.

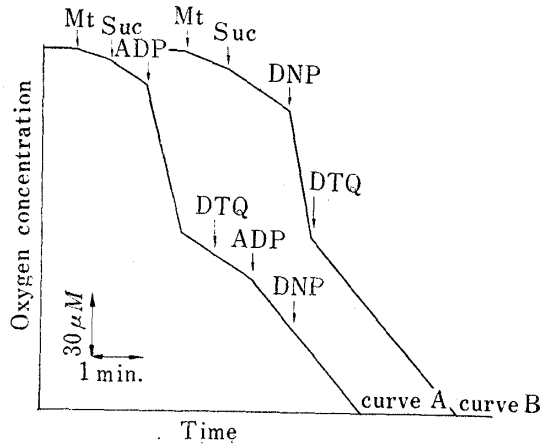

Fig. 2. Polarographic Tracing Showing Effects of Dihydroxythymoquinone on Mitochondrial Respiration with Succinate.

The basic mixture is described in experimental. Additions were 0.2 ml. of mitochondrial suspension (Mt., 36.4 mg. of protein per ml.), 0.02 ml. of 0.5*M* succinate (Suc), 10 μl. of 0.05*M* adenosine-5′-diphosphate (ADP). The final concentrations of dihydroxythymoquinone (DTQ) and 2,4-dinitrophenol (DNP) are $3\times10^{-6}M$ and $10^{-4}M$ respectively. The final volume was 2.5 ml. Temperature, 25°; pH 7.2; ADP : O ratio, 1.9; Respiratory control index, 5.5~5.7.

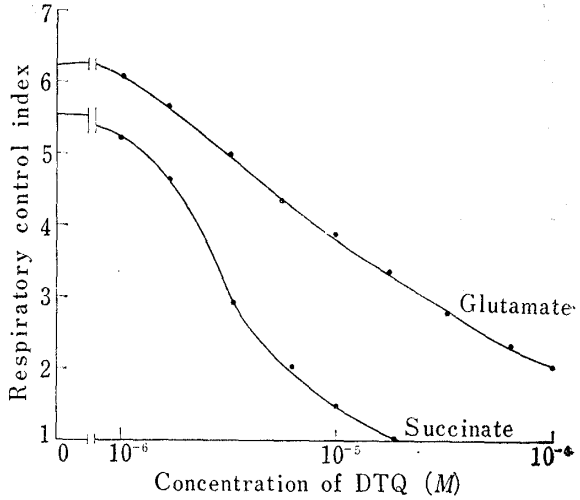

Fig. 3. Effect of DTQ Concentration on the State-3 Respiration with Succinate and Glutamate.

Oxygen consumption was determined polarographically. The concentration of both substrate was 5.0 m*M*. The other detailed conditions are indicated in the experimental. The State-4 respiration was not affected by DTQ at the concentration as indicated in the diagram. Mitochondrial content : 3.6 mg. protein.

An influence of concentration of DTQ on respiratory control index is shown in Fig. 3, in which inhibition of succinate oxidation is seen to be almost complete at a low concentration of inhibitor, the titration curve being of a sigmoid character, whereas inhibition of glutamate oxidation is not completely accomplished. The other NAD-linked substrates, *i.e.* β-hydroxybutyrate and α-oxoglutarate, gave the same results as glutamate. A stronger inhibition of succinate oxidation was also confirmed manometrically as shown in Fig. 4, and the inhibitory action was coincident with that of the polarographic results.

One-half maximal inhibition of succinate oxidation calculated by the both manometric and polarographic methods was found to be at a concentration of about 3μ*M*.

In contrast to the case of oligomycin, this inhibition could not be completely released by the addition of any uncoupling agents such as DNP (0.1 m*M*), carboxycyanide phenylhydrazone (CCP, 1 μ*M*), pentachlorophenol (20 μ*M*) and oleate (10 μ*M*). Moreover, a released respiration by an preceding addition of DNP ($10^{-4}M$) was also inhibited by DTQ and the rates of inhibition were quitely as same as that of the case when DTQ was added to State-3 respiring condition. It was also observed that the stimulated respiration by calcium ions was reduced by DTQ to the same extent observed in the case of State-3 respiration.

Detailed values for oxygen consumption and ADP/O ratios calculated by polarographic tracing in the presence of 3.2 and 6.4 μ*M* DTQ are shown in Table I, which also demonstrated that DTQ inhibition of succinate oxidation is not released completely even by the addition of DNP, but ADP/O ratios were found not to be variable when DTQ was added at these concentrations in which State-3 respiration was not completely eliminated.

Effects of DTQ on non-phosphorylating preparation, *i.e.* electron transport particles (ETP), were examined both manometrically and polarographically. Manometric results

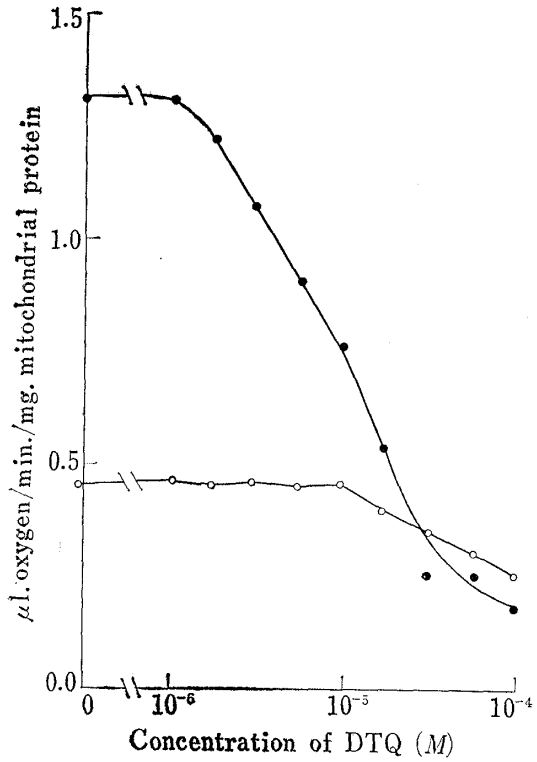

Fig. 4. Effect of DTQ on Succinate Oxidation by Rat Liver Intact Mitochondria.

The basic mixture contained 750 μmoles of sucrose, 40 μmoles of phosphate, 30 μmoles of KCl, 7.5 μmoles of $MgCl_2$, 0.6 μmoles of EDTA, 100 μmoles of sodium succinate, 50 μmoles of ATP, and mitochondrial preparation (4.5 mg. protein), in a final volume of 3.0 ml. pH 7.4. In the center well, 0.2 ml. of 20% KOH was placed. Incubation was inhibited by the addition of succinate at 30° after 4 minutes of preincubation. —●— presence of glucose (100 μmoles) and hexokinase (150 units); —○— absence of the glucose and hexokinase. DTQ was added with 50 μl. of ethanol.

are shown in Fig. 5, which indicating the titration curve of DTQ inhibition on ETP and also less inhibitory action than the case of State-3 inhibition of intact mitochondria. The inhibition did not depend upon the amounts of protein presents.

Succinate dehydrogenase was stimulated by the presence of more than 1 μ*M* DTQ as shown in Fig. 6, whereas the mitochondrial respiration was inhibited. As illustrated in the diagram, however, DTQ alone could not catalyzed electron transfering path way from succinate dehydrogenase to oxygen as PMS does.

A reduction of external cytochrome c by mitochondria with succinate was also stimulated by the addition of ADP or released by DNP, and both the stimulated and the released reducing activity were eliminated by DTQ. But even in the absence of ADP, DTQ inhibited a reduction of cytochrome c. In Fig. 7, titration curve of DTQ inhibition on cytochrome c reduction in the absence of ADP are shown. This pattern of inhibition was similar to that in the case of the electron transport particles as described above.

Effect of DTQ on oxidation of ferrocytochrome c by rat liver mitochondria was also investigated employing a reduction system, composed of ascorbate and minimum catalyzable amount of N,N,N′,N′-tetramethyl-*p*-phenylenediamine, and DTQ was observed to

Table I. Effect of DTQ on Mitochondrial Respiration with Succinate

Exptl.	without DTQ			
	State-3	State-4	R.C.	ADP:O
1	190	34	5.7	1.9
2	156	30	5.5	1.9

Exptl.	with DTQ					
	Conc. of DTQ (μ*M*)	State-3	State-4	R.C.	Add. of DNP (0.1 m*M*)	ADP:O
1	3.2	72	32	2.2	73	1.9
2	6.4	48	34	1.4	51	1.9

Oxygen consumption was measured polarographically. Detailed conditions are described in Fig. 1. Rate of oxygen consumption is represented as mμ atom oxygen uptake per minute per mg. of mitochondrial protein. R.C. represents a respiratory control index.

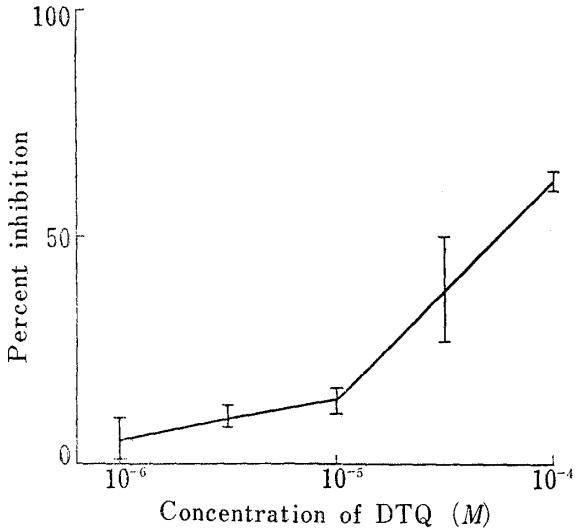

Fig. 5. Effect of DTQ on ETP Preparation.

Detailed conditions are described in experimental.

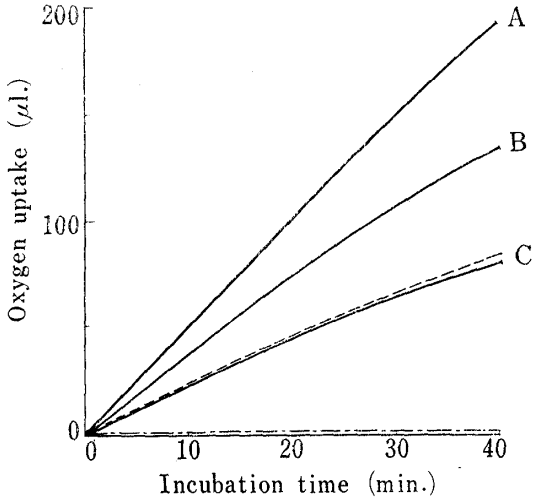

Fig. 6. Effect of DTQ on Succinate Dehydrogenase.

The reaction mixture consisted of 0.25*M* sucrose, 10 m*M* KCl, 10 m*M* phosphate, 2.5 m*M* $MgCl_2$, 0.2 m*M* EDTA, 50 m*M* succinate, 1 m*M* KCN, and 0.1% PMS. The reaction was started by the addition of mitochondrial suspension. Final volume, 3.0 ml. Temperature, 30°. – – – – – control; ——— presence of DTQ at the concentration of $10^{-4}M$ (A), $10^{-5}M$ (B), and $10^{-6}M$ (C); —•—•— without PMS and with DTQ at $10^{-5}M$.

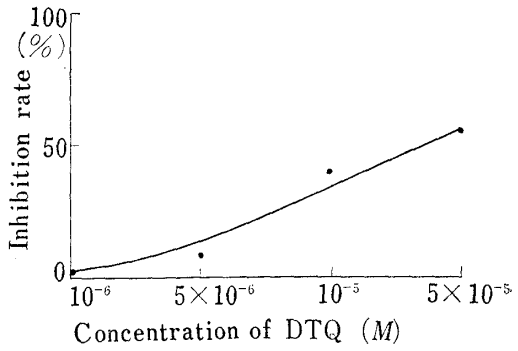

Fig. 7. Effect of DTQ on Reduction of External Cytochrome c by Rat Liver Mitochondria.

The system contains basic mixture, mitochondria (2 mg. protein), 50 m*M* succinate, 1 m*M* KCN, and 0.9 mg. cytochrome c. pH 7.4; total volume 3.0 ml.; at room temperature; DTQ was added with 50 μl. of ethanol.

show a tendency to inhibit ADP-stimulated oxygen consumption. However, since the respiratory control index of the system was lower than 2, it was difficult to decide the effect of DTQ on the ADP-induced respiration quantitatively.

Effects of DTQ on different spectra of liver mitochondria was recorded with and without DTQ according to method by Chance, *et al.*[12] with a recording spectro photometer (Model MPS-50, Shimadzu, Kyoto), but any remarkable effect on the spectra was not observed even by the addition of DTQ.

Discussion

It is very extraordinal that DTQ inhibits only State-3 respiration but not State-4. Howland[4,13] reported a resembling inhibitor, hydrolapachol (alkylhydroxynaphthoquinone) which inhibits mitochondrial respiration in the presence of succinate, but the inhibition by hydrolapachol is released by various uncoupling agents to a various extent. It has been suggested that hydrolapachol is an inhibitor in energy converting reactions. In contrast to the case of either hydrolapachol or oligomycin,[14] the DTQ induced inhibition could not be completely released by any uncoupling agents employed in the experiments, and the results may suggest that DTQ is an inhibitor in electron transport system. However,

12) B. Chance, G. R. Williams: Adv. Enzymol., **17**, 74 (1956).
13) J. L. Howland: Biochim. et Biophys. Acta, **105**, 205 (1963).
14) H. A. Lardy, D. Johnson, W. C. Mcmurry: Arch. Biochem. Biophys., **78**, 587 (1958).

the fact that DTQ give effect only to State-3 respiration seems to be unsuitable for DTQ to be an inhibitor in respiratory system. But supposing that it is difficult to distinguish between normal and inhibited State-4 respiring rate because of its low rate of respiration, it may be agreeable with the above argument. Indeed, mitochondrial preparations, which showed a loose coupling, showed larger State-4 respiration and inhibition was observed by a concentration of $10^{-4}M$ DTQ. But concentration below $10^{-5}M$ showed little inhibition of the State-4 respiration.

Since it is difficult to prove a direct action inhibitors in electron transport system by application of intact mitochondria, ETP was attempted to the experiments, and an inhibitory action was observed in the system. In addition to the irreversible action by uncoupling agents, the above results may suggest that the DTQ would be an electron transport inhibitor. It is also one of the significant evidences for being an electron transport inhibitor that the succinate cytochrome c oxidoreduction was inhibited by DTQ. But it is inconsistent that the inhibition of State-3 respiration by intact mitochondria is very stronger than the inhibition of oxygen consumption by ETP and succinate-cytochrome oxidoreductase activity.

On the other hand, a failure to obtain reverse action of inhibition by uncoupling agents should not be cited so enough evidences that an inhibitor does not react with a part of the energy-transfer enzymes. It is one of the represented examples that HQNO (2-heptyl-4-hydroxyquinoline N-oxide) appears to give some effects on both energy preserving reactions and electron transport itself although inhibition of electron transport has been shown in non-phosphorylating preparations.[15]

Considering from these results, it may be suggested that high concentrations of DTQ could inhibit the respiratory system (at a place between cytochrome b and c_1). However such a specific character of DTQ that act upon the State-3 respiration only at low concentrations might indicate that the some inhibitory effects other than the inhibition of respiratory chain may be involved, therefore it is possible to consider that DTQ may show inhibitory action in energy transfering reactions.

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15) J. W. Lightbown, F. L. Jackson : Biochem. J., **63**, 130 (1956).