[Chem. Pharm. Bull. 15(10)1521～1528(1967)] UDC 577.158.1 : 577.15.04

195. Hiroshi Enomoto*1 : Aerobic Reduction of Cytochrome *c* Preparation by Xanthine Oxidase. III.*2 Mechanism of 8-Hydroxyquinoline in Activating the Aerobic Reduction of Cytochrome *c*.

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1. In the presence of 8-hydroxyquinoline the reduction of cytochrome *c* by xanthine oxidase was markedly accelerated by H_2O_2 under both aerobic and anaerobic conditions.

2. The accelerating effect of H_2O_2 was induced only when H_2O_2 was preincubated with 8-hydroxyquinoline and cytochrome *c*, and this effect was dependent on the concentration of each component and on the period of preincubation time.

3. Thin-layer chromatography showed that 5,8-dihydroxyquinoline and 5,8-quinolinedione were present both in aerobically incubated medium containing hypoxanthine, xanthine oxidase, cytochrome *c* and 8-hydroxyquinoline and in aerobically or anaerobically incubated medium containing cytochrome *c*, 8-hydroxyquinoline and H_2O_2. These two quinoline derivatives were proved to be potent electron carriers between xanthine oxidase and cytochrome *c*.

4. As a plausible explanation for the mechanism of action of 8-hydroxyquinoline in inhibiting the reoxidation and in accelerating the reduction of cytochrome *c*, it was proposed that 8-hydroxyquinoline is hydroxylated to 5,8-dihydroxyquinoline by the peroxidative action of cytochrome *c*, and this reaction product acts as an electron carrier which promotes the anaerobic type of reduction of cytochrome *c*.

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It was reported in a previous paper*2 that in the aerobic reduction of cytochrome *c* by xanthine oxidase (xanthine : O_2 oxidoreductase, EC 1.2.3.2) the concomitant occurrence of the rapid reoxidation of reduced cytochrome *c* was effectively inhibited by 8-hydroxyquinoline and *m*-phenylenediamine, especially in the presence of large amounts of hypoxanthine or enzyme. As one of the explanations for this effect, it was assumed that 8-hydroxyquinoline and *m*-phenylenediamine inhibit the peroxidase activity of modified cytochrome *c*. However, this does not completely explain their striking effect.

During the course of studies on the reduction of cytochrome *c* by xanthine oxidase, it was demonstrated that the reduction of cytochrome *c* was markedly accelerated by H_2O_2 in the presence of 8-hydroxyquinoline or *m*-phenylenediamine under both aerobic and anaerobic conditions. It was thought that this phenomenon indicated the direct involvement of H_2O_2 in the mechanism of the aerobic reduction of cytochrome *c*[1,2] and also served as an important clue for the clarification of the reoxidation inhibiting mechanism of these compounds.

The present paper is concerned with the elucidation of this stimulating effect of H_2O_2 in the presence of 8-hydroxyquinoline.

Experimental

Materials——Milk xanthine oxidase was prepared as described previously.[3] Cytochrome *c* was from "type III" horse heart muscle preparation of Sigma Chemical Company, unless otherwise stated.

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*2 Part II. H. Enomoto, T. Yamaguchi : This Bulletin, **15**, 1514 (1967).

1) S. Muraoka, M. Sugiyama, H. Yamasaki : Biochem. Biophys. Res. Commun., **19**, 346 (1965).
2) S. Muraoka, H. Enomoto, M. Sugiyama, H. Yamasaki : Biochim. et Biophys. Acta, **143**, 408 (1967).
3) Part I. S. Muraoka, H. Enomoto, M. Sugiyama, H. Yamasaki : This Bulletin, **15**, 1373 (1967).

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Horseradish peroxidase was purchased from the California Corporation for Biochemical Research. 5,8-Dihydroxyquinoline and 5,8-quinolinedione were prepared from 8-hydroxyquinoline according to the methods of Kostanecki[4] and Fischer and Renouf.[5] 2,8-Dihydroxyquinoline was also synthesized from 8-hydroxyquinoline by the method of Phillips, *et al.*[6]

Assay Methods——Reduction and reoxidation of cytochrome *c* were traced by the change in the absorbancy at 550 mμ as reported previously.[3] The reaction mixture routinely contained 0.05 μmoles of hypoxanthine, 0.15 μmoles of cytochrome *c*, 50~200 μg. of enzyme, and 1.0 ml. of 0.1 *M* phosphate buffer (pH 7.4) in a final volume of 3.0 ml. The number of moles of cytochrome *c* reduced was calculated from the change in the absorbancy at 550 mμ using ε (reduced-oxidized) $= 1.95 \times 10^{-4}/M$.

Anaerobic reaction was performed in a photo-cell attached to a Thunberg tube, in which the air was completely replaced with deoxygenated N_2 gas by repeated evacuation and filling. The reaction was started by adding xanthine oxidase from the side chamber, while all the other components were placed in the photo-cell. N_2 gas was purified by passing commercial N_2 gas of 99.995% purity through an alkaline pyrogallol solution and then over heated copper.

Detection of 5,8-Dihydroxyquinoline and 5,8-Quinolinedione by Thin-layer Chromatography——The incubated reaction mixture was treated by rapid deep-freezing and drying. The dried residue was extracted with ethanol and subjected to thin-layer chromatography on plates covered with a layer of 0.3 mm. silica gel G-Stall (E. Merck, Darmstadt) in solvent systems of ethyl acetate-formic acid (5:1) and ethyl acetate (100%). Chromatoplates were activated by heating at 120° for 30 min. immediately before use. Spots were visualized by spraying $FeCl_3$ solution or diazotized sulfanilic acid solution for phenolic compounds and by Craven's test for quinones.

Results

Stimulation of Cytochrome *c* Reduction by H_2O_2 in the Presence of 8-Hydroxyquinoline——In a previous paper*[2] it was demonstrated that in the aerobic reduction of cytochrome *c* by xanthine oxidase 8-hydroxyquinoline and *m*-phenylenediamine not only prevent the concomitant reoxidation of reduced cytochrome *c* but also accelerate the reduction itself, especially when highly CO-sensitive cytochrome *c* is used. In the present experiment it was found that H_2O_2, which is the oxidant for the reoxidation of reduced cytochrome *c*,[3] has a marked stimulating effect on cytochrome *c* reduction under both aerobic and anaerobic conditions in the presence of 8-hydroxyquinoline or *m*-phenylenediamine. Fig. 1 shows the effect of H_2O_2 under aerobic conditions, indicating that the maximum extent of the reduction of cytochrome *c* is enhanced by 1.7~6.6*M* H_2O_2. However, this reaction is extremely sensitive to the quantity of H_2O_2, so in the presence of over $1.7 \times 10^{-4} M$ H_2O_2, reoxidation was too strong and reduction was even depressed. This activating effect of H_2O_2 was not observed in the absence of 8-hydroxyquinoline. Fig. 2 illustrates the effect of H_2O_2 under anaerobic conditions, revealing that the initial rate of reduction was increased about 15-fold by $1.7 \times 10^{-4} M$ H_2O_2. It should be noted here that 8-hydroxyquinoline acts as a weak electron carrier from the enzyme to cytochrome *c*, since with 8-hydroxyquinoline the slow reduction of cytochrome *c* was observed (Fig. 2). A similar effect could also be obtained even with *m*-phenylenediamine, but not with iron chelating agents such as EDTA, *o*-phenanthroline, and α,α'-dipyridyl.

This accelerating effect was most marked when 8-hydroxyquinoline, cytochreme *c* and H_2O_2 were preincubated together, and when anyone of them was added slightly later, the effect was diminished. No such effect could be achieved when xanthine oxidase was preincubated together with H_2O_2 and/or 8-hydroxyquinoline.

Analyses of the Effects of H_2O_2 and 8-Hydroxyquinoline under Anaerobic Conditions——In order to clarify the role of H_2O_2 in the above phenomenon, some kinetic and stoichiometric studies were carried out with the addition of H_2O_2 under anaerobic

4) St. v. Kostanecki: Ber., **24**, 150 (1891).
5) O. Fischer, E. Renouf: Ber., **17**, 1644 (1884).
6) J.P. Phillips, E.M. Barrall, R. Brease: Trans. Kentucky Acad. Sci., **17**, 135 (1956).; C.A., **51**, 11349c.

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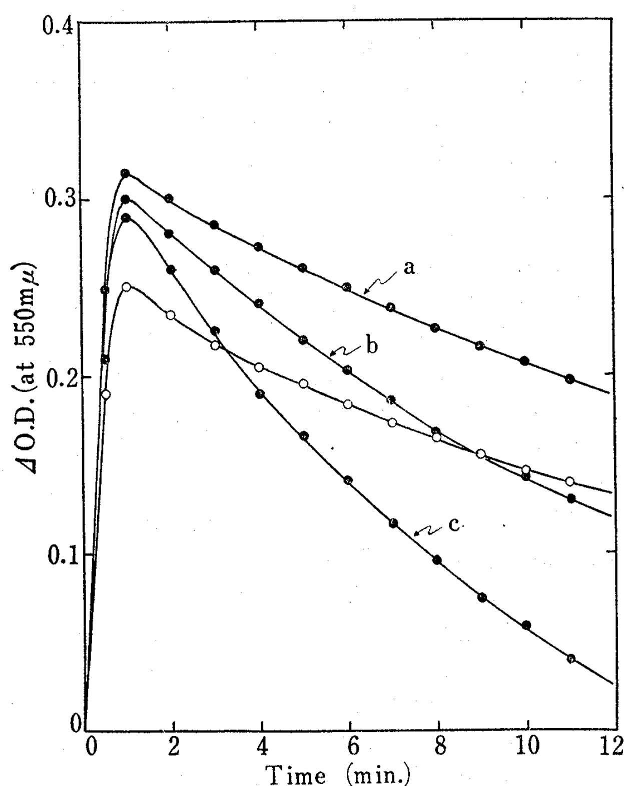

Fig. 1. Effect of H_2O_2 on Aerobic Reduction of Cytochrome *c* by Xanthine Oxidase in the Presence of 8-Hydroxyquinoline

Each cuvette contained 0.05 μmoles of hypoxanthine, 0.15 μmoles of cytochrome *c*, 1.0 μmole of 8-hydroxyquinoline, 200 μg. of enzyme, and 1.0 ml. of 0.1*M* phosphate buffer (pH 7.4) in a final volume of 3.0 ml. ○——○, control; ●——●, with $1.7\times10^{-5}M$ (a), $3.3\times10^{-5}M$ (b), and $6.6\times10^{-5}M$ (c) H_2O_2.

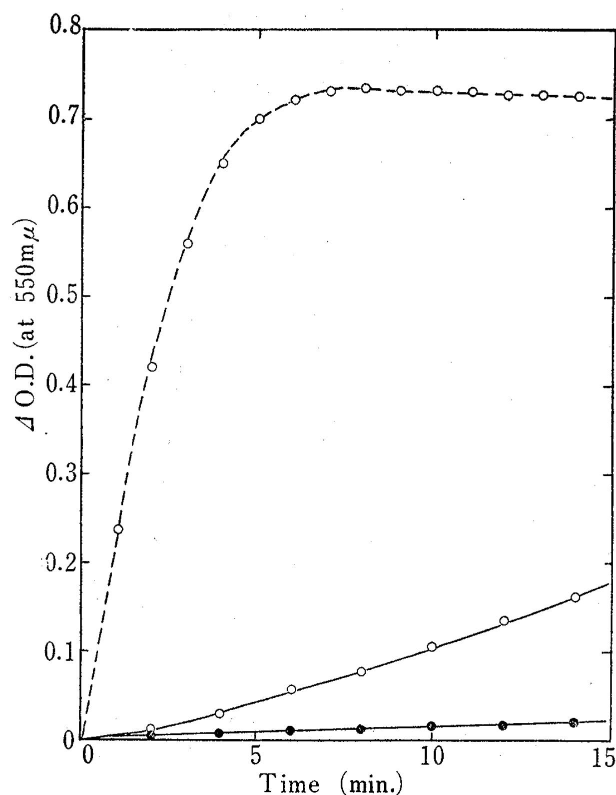

Fig. 2. Effect of H_2O_2 on Anaerobic Reduction of Cytochrome *c* by Xanthine Oxidase in the Presence of 8-Hydroxyquinoline

Reaction mixture same as in Fig. 1, except that 50 μg. of enzyme was used. ○——○, control; ○⋯⋯○, with $3.3\times10^{-5}M$ H_2O_2; ●——●, without 8-hydroxyquinoline and H_2O_2.

conditions, because H_2O_2 is inevitably formed during the reaction under aerobic conditions.

i) Stoichiometry: The number of moles of cytochrome *c* reduced per mole of substrate was, as shown in Table I, irrelevant to the amount of H_2O_2 added, 1 mole of xanthine and hypoxanthine reducing about 2 and 4 moles of cytochrome *c*, respectively. It was reported previously[2] that the reduction of cytochrome *c* is performed *via* two different mechanisms, the aerobic type of reduction in the absence of an electron carrier

Table I. Stoichiometry of Anaerobic Reduction of Cytochrome *c* by Xanthine Oxidase in the Presence of 8-Hydroxyquinoline and H_2O_2

Substrate	(μmoles)	Cytochrome *c* reduced (μmoles)	Cytochrome *c*/Substrate (mole/mole)
Xanthine	0.02	0.0396	1.97
	0.04	0.0774	1.96
	0.06	0.1146	1.91
Hypoxanthine	0.01	0.0406	4.06
	0.02	0.0806	4.03
	0.03	0.1187	3.96

3.0 ml. of reaction mixture contained 0.15 μmoles of cytochrome *c*, 1.0 μmole of 8-hydroxyquinoline, 0.1 μmoles of H_2O_2, 150 μg. of enzyme and 1.0 ml. of 0.1*M* phosphate buffer (pH 7.4).

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and the anaerobic type in the presence of a suitable electron carrier, and that these two reactions can be easily distinguished from one another by their stoichiometries and pH optima. It had been demonstrated that 1 mole of xanthine and hypoxanthine can reduce about 2 and 4 moles of cytochrome *c* anaerobically, and 0.5 and 0.9 moles of cytochrome *c*, respectively, aerobically.[2] It is evident, therefore, that the reaction is stoichiometrically of the anaerobic type even in the presence of H_2O_2, and that H_2O_2 only greatly accelerates the initial rate of the reduction.

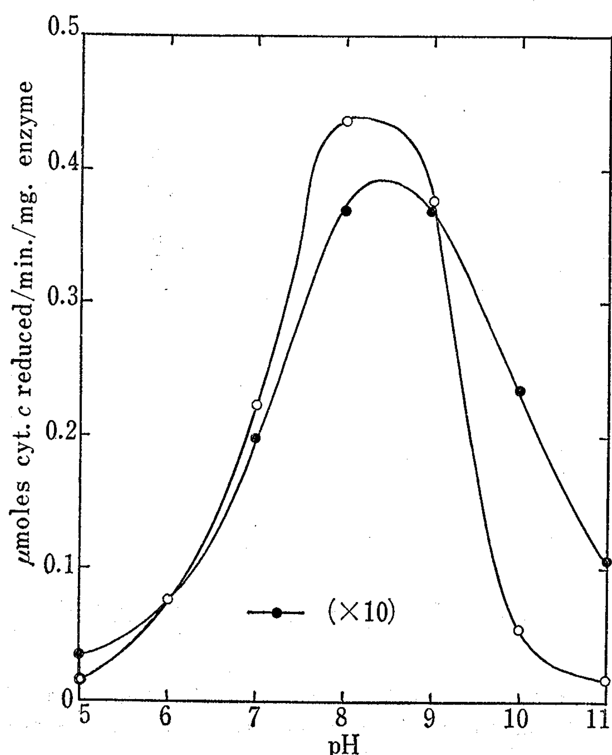

Fig. 3. Effect of pH on Initial Rate of Anaerobic Reduction of Cytochrome *c* by Xanthine Oxidase in the Presence of 8-Hydroxyquinoline

Final content of reaction mixture same as in Fig. 2. ○——○, with $1.7\times10^{-5}M$ H_2O_2; ●——●, without H_2O_2.

ii) **pH Dependency**: The initial rate of the anaerobic reduction was elevated 10-fold or more at various pH's by $3.3\times10^{-4}M$ H_2O_2, but the pH optimum was scarcely influenced (Fig. 3). The optimum pH of the aerobic reduction has been proved to be 10, while that of the anaerobic reduction is 7.5. These results indicate that in the presence of 8-hydroxyquinoline, H_2O_2 only effects the initial velocity but not the other aspects of the nature of the anaerobic reduction of cytochrome *c* by xanthine oxidase, and that the added H_2O_2 may not be, at least directly, involved in the mechanism of the aerobic reduction of cytochrome *c*.

iii) **Effects of H_2O_2 and 8-Hydroxyquinoline Concentrations**: In the presence of $3.3\times10^{-4}M$ 8-hydroxyquinoline, the initial rate of anaerobic reduction was in direct proportion to the concentration of H_2O_2 in a range of $0\sim6.6\times10^{-5}M$ (Fig. 4). In addition, when the concentration of H_2O_2 was fixed, the rate also varied with the concentration of 8-hydroxyquinoline. Lineweaver and Burk's plot gave $3.2\times10^{-6}M$ as the *Km* for 8-hydroxyquinoline (Fig. 5).

Effects of Preincubation Time and Cytochrome *c* Concentration on the Aerobic Reduction of Cytochrome *c*——It was already noted that in the presence of 8-hydroxyquinoline the extent of the aerobic reduction of cytochrome *c* is increased by H_2O_2 (Fig. 1). Such stoichiometric changes are thought to indicate that the "anaerobic type" of reduction occurs concomitantly even under aerobic conditions as in the presence of menadione.[2] In this experiment, before the initiation of the reaction by the addition of enzyme, the mixture containing hypoxanthine, cytochrome *c*, 8-hydroxyquinoline and H_2O_2 was preincubated at room temperature (23°) for varying lengths of time to elucidate the relationship between the incubation time and the maximum extent of reduction. It was found that for up to 60 minutes of incubation the maximum extent of reduction was approximately proportional to the incubation time (Fig. 6), and that this effect was roughly doubled when the incubation temperature was raised to 37°. When the reduction was made to proceed, after incubating the mixture of hypoxanthine, 8-hydroxyquinoline, H_2O_2 and varying amounts of cytochrome *c* for 30 minutes, and then more cytochrome *c* was added to make the final concentration 0.2 μmoles immediately before the addition of xanthine oxidase, it was demonstrated that the maximum extent of the

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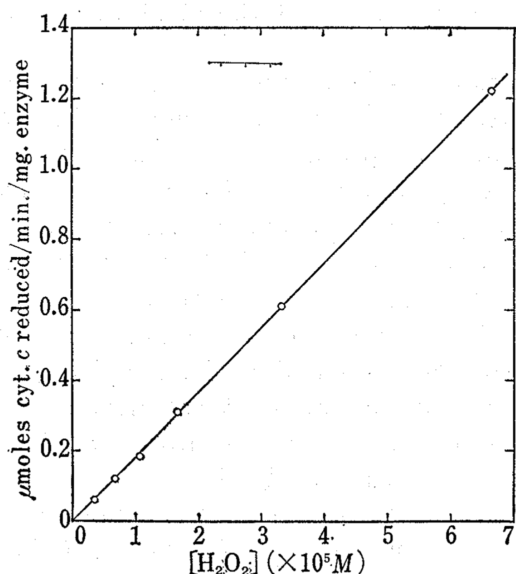

Fig. 4. Effect of H_2O_2 Concentration on Initial Rate of Anaerobic Reduction of Cytochrome *c* by Xanthine Oxidase in the Presence of 8-Hydroxyquinoline

Final content of reaction mixture same as in Fig. 2.

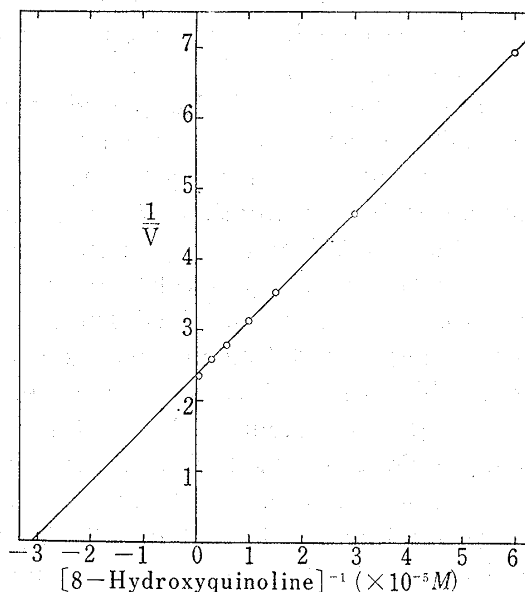

Fig. 5. Effect of 8-Hydroxyquinoline Concentration on Initial Rate of Anaerobic Reduction of Cytochrome *c* by Xanthine Oxidase in the Presence of H_2O_2

Each cuvette contained 0.05 μmoles of hypoxanthine, 0.15 μmoles of cytochrome *c*, 0.1 μmoles of H_2O_2, 50 μg. of enzyme, and 1.0 ml. of 0.1*M* phosphate buffer (pH 7.4) in a final volume of 3.0 ml.

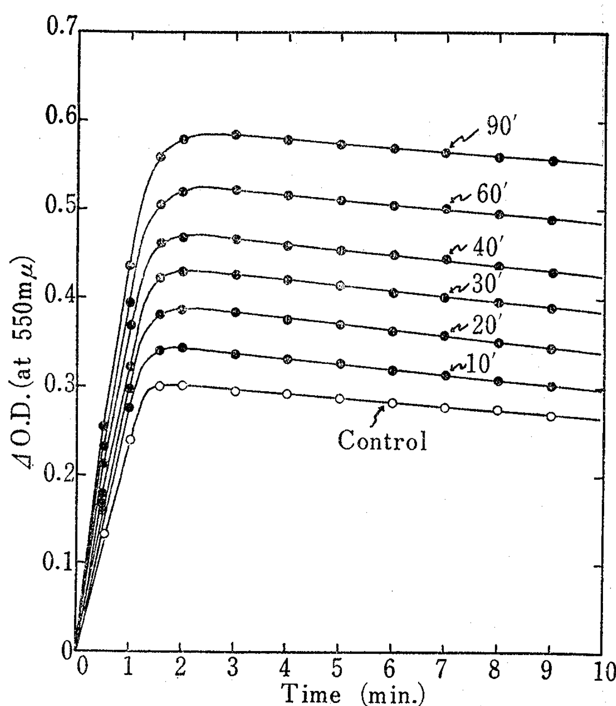

Fig. 6. Effect of Preincubation of Reaction Mixture in Aerobic Reduction of Cytochrome *c* by Xanthine Oxidase

Each cuvette contained 0.05 μmoles of hypoxanthine, 0.15 μmoles of cytochrome *c*, 1.0 μmole of 8-hydroxyquinoline, 0.1 μmoles of H_2O_2, 100 μg. of enzyme, and 1.0 ml. of 0.1*M* phosphate buffer (pH 7.4) in a final volume of 3.0 ml. ○——○, control; ●——●, before start of reaction by addition of enzyme reaction mixture was preincubated for indicated period.

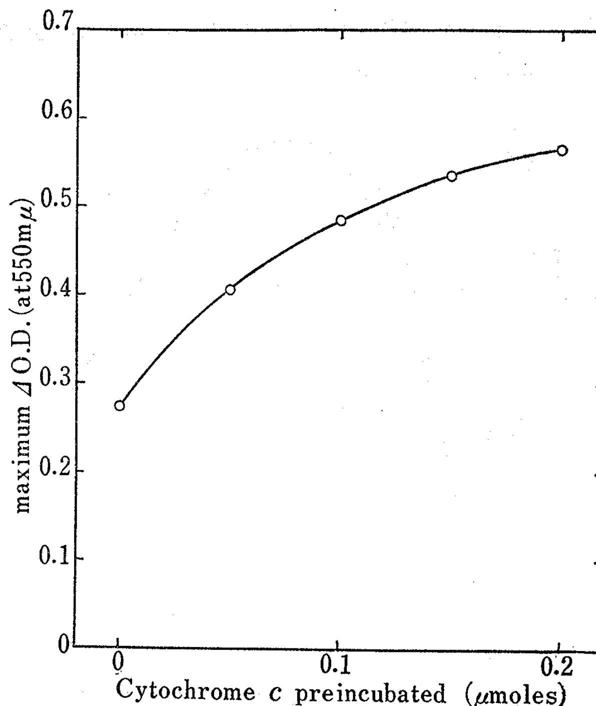

Fig. 7. Effect of Cytochrome *c* Concentration in Preincubation Mixture on Extent of Aerobic Reduction of Cytochrome *c* by Xanthine Oxidase in the Presence of H_2O_2 and 8-Hydroxyquinoline

Final content of reaction mixture, except that of cytochrome *c*, same as in Fig. 6. The medium containing the indicated dose of cytochrome *c* was preincubated for 30 min., and then more cytochrome *c* was added to make a final content of 0.2 μmoles immediately before start of reaction.

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reduction increased with the amount of cytochrome *c* present at the start of preincubation, as illustrated in Fig. 7.

Effects of Synthetic Hydroxylated Derivatives of 8-Hydroxyquinoline——The above findings indicate that during the preincubation period a reaction, independent of xanthine oxidase, must have taken place among cytochrome *c*, H_2O_2 and 8-hydroxyquinoline to produce the active stimulation of the reduction of cytochrome *c*. It was most reasonable to deduce that 8-hydroxyquinoline has been hydroxylated and converted to a potent electron carrier by the peroxidative action of cytochrome *c*. A few derivatives of 8-hydroxyquinoline were provided synthetically, and observations were made on their behavior during reoxidation and on their activity as electron carriers under anaerobic conditions. The derivatives examined were 2,8-dihydroxyquinoline, 5,8-dihydroxyquinoline and 5,8-quinolinedione. Fig. 8 illustrates inhibitory effects of these derivatives on the rapid reoxidation observable in the presence of excess hypoxanthine during the aerobic reaction. The effect of 2,8-dihydroxyquinoline was nearly the same as that of 8-hydroxyquinoline, while that of 5,8-dihydroxyquinoline and 5,8-quinolinedione was far greater than that of 8-hydroxyquinoline. Furthermore as is obvious from Fig. 9, 5,8-dihydroxyquinoline and 5,8-quinolinedione showed high activity as electron carriers from enzyme to cytochrome *c* under anaerobic conditions.

Identification of 5,8-Dihydroxyquinoline and 5,8-Quinolinedione in the Reaction Mixture——The supposition described above has been verified, since 5,8-dihydroxyquinoline and 5,8-quinolinedione were detected in the reaction mixture. Fig. 10 shows the

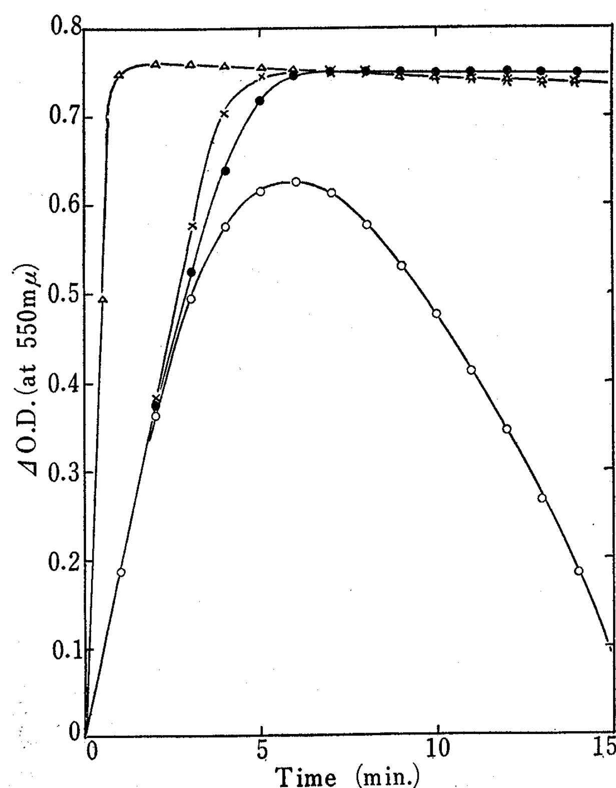

Fig. 8. Effect of 8-Hydroxyquinoline Derivatives on Aerobic Reduction of Cytochrome *c* by Xanthine Oxidase

Each cuvette contained 1.0 μmole of hypoxanthine, 0.15 μmoles of cytochrome *c*, 100 μg. of enzyme, and 1.0 ml. of 0.1*M* phosphate buffer (pH 7.4) in a final volume of 3.0 ml. ○——○, control; ●——●, with $3.3\times10^{-4}M$ 8-hydroxyquinoline; △——△, with $3.3\times10^{-4}M$ 5,8-dihydroxyquinoline or 5,8-quinolinedione; ×——×, with $3.3\times10^{-4}M$ 2,8-dihydroxyquinoline.

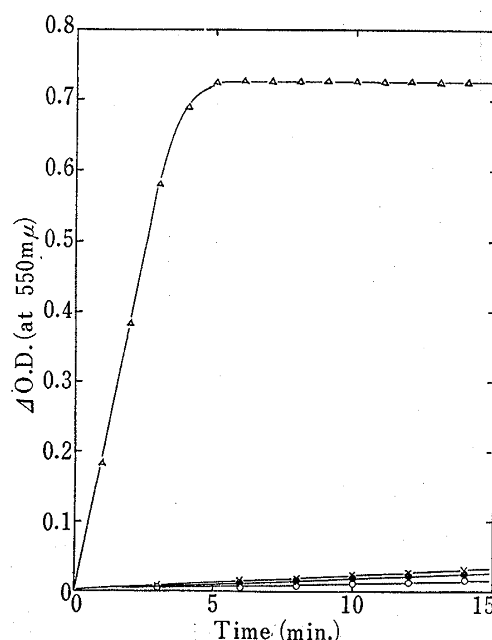

Fig. 9. Effect of 8-Hydroxyquinoline Derivatives on Anaerobic Reduction of Cytochrome *c* by Xanthine Oxidase

Reaction mixture same as in Fig. 8, except that 50 μg. of enzyme was used. ○——○, control; ●——●, with $3.3\times10^{-4}M$ 8-hydroxyquinoline; △——△, with $3.3\times10^{-4}M$ 5,8-dihydroxyquinoline or 5,8-quinolinedione; ×——×, with $3.3\times10^{-4}M$ 2,8-dihydroxyquinoline.

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thin-layer chromatograms of the reaction products extracted from the mixture containing cytochrome *c*, 8-hydroxyquinoline and H_2O_2 after 30 minutes of incubation at room temperature, and developed with two solvent systems, ethyl acetate-formic acid (5:1) and ethyl acetate (100%). In (I) of this figure sample B shows 2 spots, the one with the smaller Rf value representing true 5,8-dihydroxyquinoline and the other with the larger Rf value being 5,8-quinolinedione formed by autoxidation. When developed with ethyl acetate alone, true 5,8-dihydroxyquinoline is demonstrated as a pale band with long tailing (II). Both 5,8-dihydroxyquinoline and 5,8-quinolinedione were found to be partially converted into two unidentified products by the action of H_2O_2 (samples D and E). In the chromatogram of sample G spots of 5,8-dihydroxyquinoline, 5,8-quinolinedione and their unidentified products induced by H_2O_2 are clearly demonstrated. It was also found that increasing the amount of H_2O_2 or prolonging the incubation caused a gradual diminution of the spots of 5,8-dihydroxyquinoline and an increase in 5,8-quinolinedione and its unidentified products (sample F). The same results were obtained with the aerobically incubated medium containing xanthine oxidase, hypoxanthine, cytochrome *c* and 8-hydroxyquinoline.

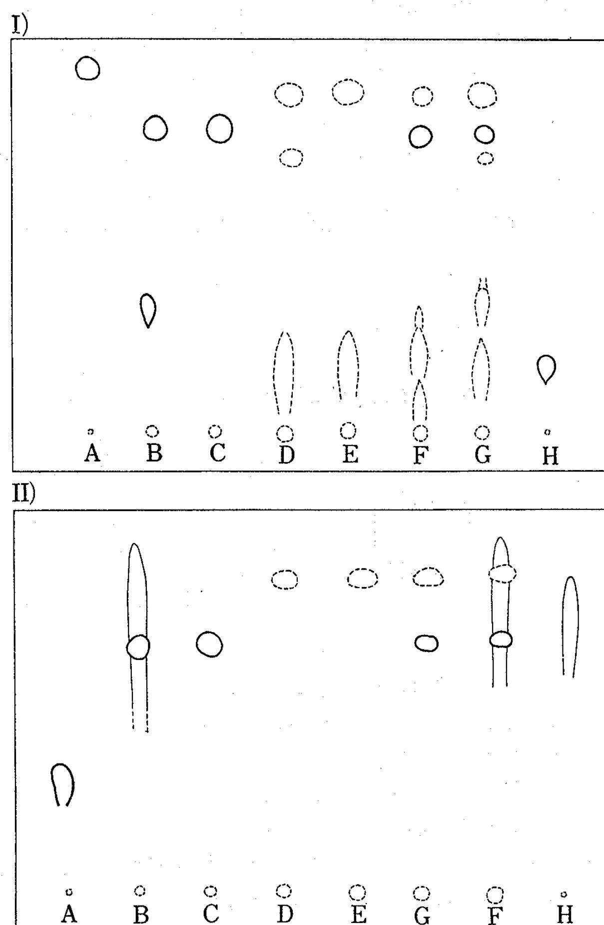

Fig. 10. Thin-layer Chromatogram of Incubated Reaction Mixture

Solvent systems: I) ethyl acetate-formic acid (5:1)
II) ethyl acetate (100%)

A: 2,8-dihydroxyquinoline;
B: 5,8-dihydroxyquinoline;
C: 5,8-quinolinedione;
D: 5,8-dihydroxyquinoline +4 m*M* H_2O_2;
E: 5,8-quinolinedione +4 m*M* H_2O_2;
F: 0.1 m*M* cytochrome *c* +2 m*M* 8-hydroxyquinoline +2 m*M* H_2O_2 +33 m*M* phosphate buffer (pH 7.4);
G: 0.1 m*M* cytochrome *c* +2 m*M* 8-hydroxyquinoline +4 m*M* H_2O_2 +33 m*M* phosphate buffer (pH 7.4);
H: 8-hydroxyquinoline;
D, E, F, G, incubated for 30 min. at room temperature.

Discussion

As it was found that the reduction of cytochrome *c* by xanthine oxidase was accelerated by H_2O_2 in the presence of 8-hydroxyquinoline or *m*-phenylenediamine, this phenomenon was supposed to show the direct participation of H_2O_2 in the mechanism of aerobic reduction of cytochrome *c* by this enzyme. Another basis of support for this hypothesis was our confirmation, in a previous paper,*2 of the findings of Weber, *et al.*[7] that catalase inhibits the reduction of cytochrome *c* under aerobic conditions. Such a possibility, however, has become questionable, since it was demonstrated in this paper that H_2O_2 had no effect on the stoichiometry or pH dependency of the anaerobic reduction of cytochrome *c*; 1 mole of hypoxanthine reduced about 4 moles of cytochrome *c*, and its optimum pH was about 8 in the presence of H_2O_2

7) M.M. Weber, H.M. Lenhoff, N.O.Kaplan: J. Biol. Chem., **220**, 93 (1956).

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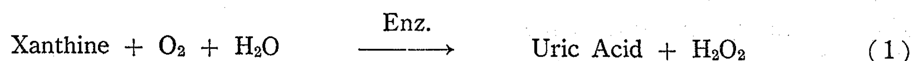
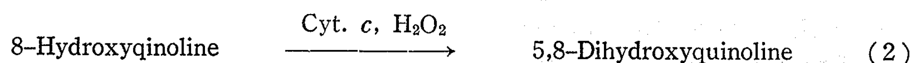
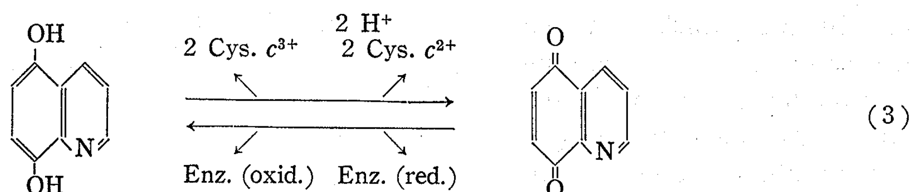
It was demonstrated that the effect of 8-hydroxyquinoline in inhibiting the reoxidation of reduced cytochrome *c* under aerobic conditions is not due to its inhibitory action on the peroxidase activity of modified cytochrome *c* but to the accelerating effect on the reduction itself. It was proved, as the mechanism of the latter effect, that 8-hydroxyquinoline is hydroxylated to 5,8-dihydroxyquinoline, and that this compound acts as a potent electron carrier to promote the anaerobic type of reduction of cytochrome *c*, as illustrated in the following reactions (1~3).







In our reaction systems containing 8-hydroxyquinoline, horseradish peroxidase,[8] horseradish peroxidase+H_2O_2, Fe^{2+}+EDTA, and Fe^{2+}+EDTA+H_2O_2[9] did not show any such accelerating effect. Therefore, the above hydroxylation of 8-hydroxyquinoline is most probably catalyzed chiefly by the peroxidative action of cytochrome *c*. However, there is some possibility that the mechanism of oxidation by Fenton's reagent is partially involved, since Fe would be liberated from cytochrome *c* by H_2O_2-induced degradation of its heme-structure. In any case, it is very probable that the 5-position, the most electronegative site, of 8-hydroxyquinoline is attacked by either ferryl ion or an OH radical.

In this connection it is of interest to note the hydroxylation of various quinoline derivatives, including 8-hydroxyquinoline, by crude aldehyde oxidase of rat liver, reported by Knox.[10] In this case only 2-hydroxylated derivatives are produced as reaction products in almost every case.

Since 8-hydroxyquinoline and *m*-phenylenediamine showed a very similar effect under varying conditions, the latter compound was assumed also to be hydroxylated by the cytochrome *c*-H_2O_2 system. However, as substances believed to be hydroxylation products of *m*-phenylenediamine were apt to polymerize, more precise studies were impossible.

It still remains obscure by what mechanism 8-hydroxyquinoline and *m*-phenylenediamine, in their intact forms, can accelerate, even though only slightly, the reduction of cytochrome *c* under anaerobic conditions.

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8) H.S. Mason, J. Onopryenko, D.H. Buhler: Biochim. et Biophys. Acta, **24**, 225 (1957).
9) H.S. Mason: "Advances in Enzymology" ed. by F.F. Nord, Vol. 19, p. 79 (1957). Interscience Publ., Inc., New York.
10) W.E. Knox: J. Biol. Chem., **163**, 699 (1946).

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