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194. Hiroshi Enomoto and Takeshi Yamaguchi*1 : Aerobic Reduction of Cytochrome *c* Preparation by Xanthine Oxidase. II.*2 Prevention of Reoxidation of Reduced Cytochrome *c* by 8-Hydroxyquinoline and *m*-Phenylenediamine.

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1. A study was made of 42 substances, including catalase, metal chelating agents and peroxidase substrates as to their effects on the over-all course of aerobic reduction of cytochrome *c* by xanthine oxidase.

2. It was found that 8-hydroxyquinoline and *m*-phenylenediamine completely prevented the reoxidation of reduced cytochrome *c* in concentrations above 3.3×10^{-5} and 1.7×10^{-5} *M*, respectively. A similar, but less marked, effect was obtained with small amounts of catalase and KCN.

3. This enabled the determination of enzymically reducible cytochrome *c* except polymeric or CO-sensitive cytochrome *c* included in the preparation, by xanthine oxidase, with the use of 8-hydroxyquinoline and *m*-phenylenediamine.

4. 8-Hydroxyquinoline and *m*-phenylenediamine had an inhibitory effect on the peroxidase activity of cytochrome *c*.

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As reported previously,*2 the reduction of cytochrome *c* by xanthine oxidase (xanthine : O_2 oxidoreductase, EC 1. 2. 3. 2) under aerobic conditions includes the reoxidation of reduced cytochrome *c*. It was practically impossible to determine the exact velocity and extent of cytochrome *c* reduction alone without the infiuence of reoxidation. For the elimination of this reoxidation most investigators have used catalase, but it has the disadvantage of markedly inhibiting the reduction of cytochrome *c* in this reaction system.[1,2] Therefore, it is desirable to find another suitable method to inhibit this obstructive side reaction for the exact estimation of enzymically reducible cytochrome *c* with this enzyme.

Present paper describes examinations of the effects of 42 compounds, especially metal chelating agents and substrates of peroxidase, on the reduction and reoxidation of cytochrome *c*. Metal chelating agents were chosen because KCN in a low concentration acted quite similarly to catalase, while peroxidase substrates were selected on the basis of the suggestion[3,4] that free radicals formed from the substrate effect the peroxidase-catalyzed reduction of cytochrome *c*, as discussed in a previous report. It was found that among the substances examined, 8-hydroxyquinoline and *m*-phenylenediamine caused very marked inhibition of reoxidation. It was further proven that these two compounds not only inhibit completely the rapid reoxidation of cytochrome *c* but also under certain experimental conditions accelerate the reduction itself, which does not occur in the presence of catalase.

Experimental

Materials——Xanthine oxidase was prepared from fresh cow's milk as previously described.*2 The cytochrome *c* used routinely was "type III" horse heart muscle preparation obtained from Sigma Chemical

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*2 Part I. S. Muraoka, H. Enomoto, M. Sugiyama, H. Yamasaki : This Bulletin, **15**, 1373 (1967).

1) M.M. Weber, H.M. Lenhoff, N.O. Kaplan : J. Biol. Chem., **220**, 93 (1956).
2) S. Muraoka, H. Enomoto, M. Sugiyama, H. Yamasaki : et Biochim. Biophys. Acta, **143**, 408 (1967)
3) I. Yamazaki : Proc. Int. Symp. Enzyme Chem. (Tokyo and Kyoto, 1957), p. 224 (1958).
4) I. Yamazaki, L.H. Piette : Biochim. et Biophys. Acta, **77**, 47 (1963).

Company, but in certain cases a cruder sample prepared according to the method of Keilin and Hartree[5] was used. The catalase used was a purified beef liver preparation from Sigma Chemical Company. Horseradish peroxidase of B grade was purchased from the California Corporation for Biochemical Research.

Assay Methods——The determination of cytochrome *c* reduction and uric acid formation by xanthine oxidase was done exactly as in the previouse report.*[2]

Results and Discussion

Effect of Catalase——In the presence of relatively large amounts of hypoxanthine and enzyme, in which rapid reoxidation of reduced cytochrome *c* occurs as previously reported,*[2] the effect of catalase was tested on the reduction and reoxidation of cytochrome *c*. As shown in Fig. 1, 20 μg. of catalase completely inhibited the reoxidation. However, with increasing concentrations of catalase there appeared a marked inhibition of the reduction itself. Fridovich and Handler[6] failed to observe such an inhibitory effect of catalase on the reduction of cytochrome *c* by xanthine oxidase, possibly because the concentration of catalase was kept at a very low level. Although it was expected that the amount of catalase required for destroying H_2O_2 generated at low enzyme concentrations would not be large, both the initial velocity and the maximum extent of reduction were in most cases lowered by catalase in an amount sufficient for the complete prevention of rapid reoxidation. Thus, it is practically impossible to select a suitable amount of catalase to inhibit reoxidation without any depressive effect on the reduction.

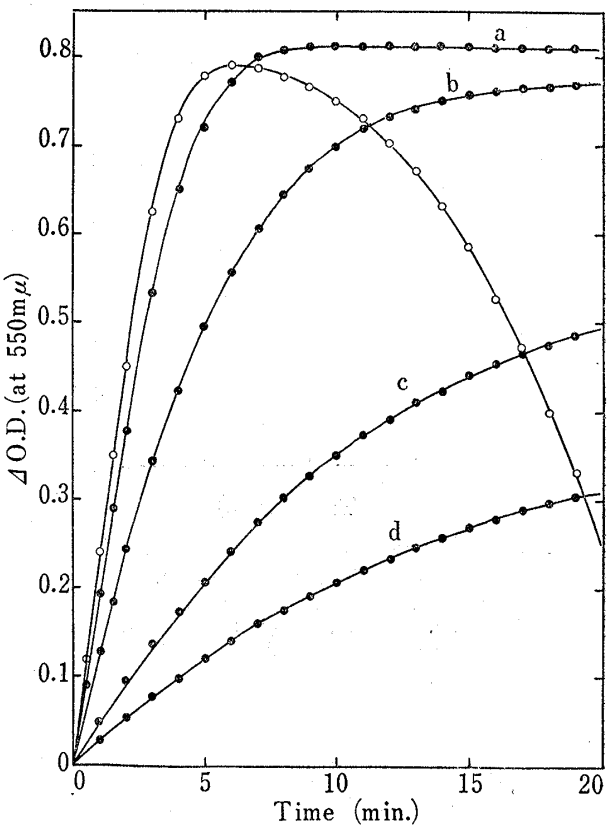

Fig. 1. Effect of Catalase on Reduction of Cytochrome *c* by Xanthine Oxidase

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 20 μg. (a), 100 μg. (b), 500 μg. (c), and 1000 μg. (d) of catalase.

Effect of KCN and Some Other Metal Chelating Agents——In the same reaction system KCN at $3.3\times10^{-4}M$ showed an inhibitory effect on reoxidation similarly to catalase, but with increasing concentrations of KCN the final extent of cytochrome *c* reduction was considerably lower (Fig. 2). The latter effect may be interpreted as being a consequence of its interaction with heme iron of cytochrome *c* in the oxidized form but not with xanthine oxidase, because the decrease in the extent of cytochrome *c* reduction was strengthened when KCN was preincubated with cytochrome *c*. Urate formation and the initial velocity of cytochrome *c* reduction were, rather, accelerated by KCN, but this mechanism remains obscure. The effectiveness of KCN in preventing reoxidation led us to study the effects of several other metal chelating agents in this reaction system. It was found that 8-hydroxyquinoline completely inhibited the reoxidation at concentration above $3.3\times10^{-5}M$

5) D. Keilin, E.F. Hartree : Proc. Roy. Soc. (London), **B 122**, 298 (1937).
6) I. Fridovich, P. Handler : J. Biol. Chem., **237**, 916 (1962).

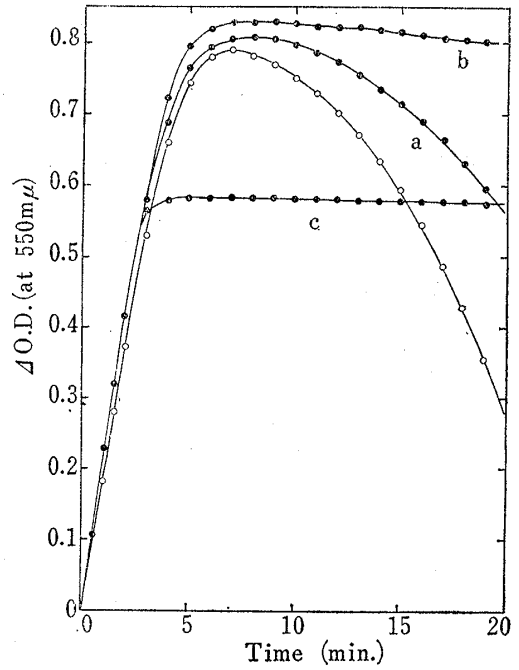

Fig. 2. Effect of KCN on Reduction of Cytochrome *c* by Xanthine Oxidase.
Final content of reaction mixture same as in Fig. 1. ○——○, control; ●——●, with $3.3\times10^{-5}M$(a), $3.3\times10^{-4}M$(b), and $3.3\times10^{-3}M$(c) KCN.

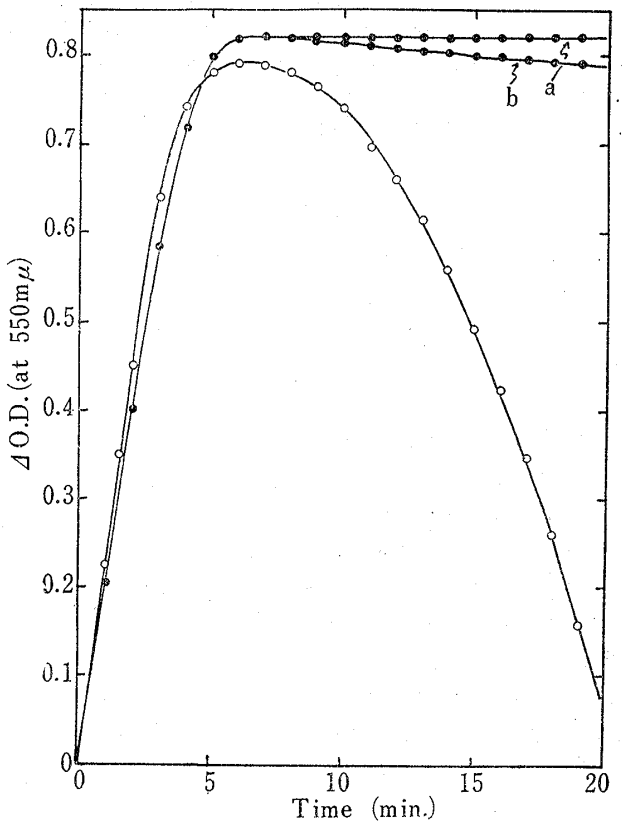

Fig. 3. Effect of 8-Hydroxyquinoline on Reduction of Cytochrome *c* by Xanthine Oxidase.
Final content of reaction mixture same as in Fig. 1. ○——○, control; ●——●, with $3.3\times10^{-5}M$(a) and $3.3\times10^{-6}M$(b) 8-hydroxyquinoline.

(Fig. 3). When a cruder or modified cytochrome *c* sample, such as Keilin-Hartree's preparation was used, the initial velocity as well as the extent of reduction was increased by 8-hydroxyquinoline. At concentrations of hypoxanthine lower than $3.3\times10^{-5}M$, in which reoxidation occurred very slowly, the reoxidation was not suppressed effectively by 8-hydroxyquinoline. Doisy, *et al.*[7] and Westerfeld, *et al.*[8] reported that 8-hydroxyquinoline accelerated the initial velocity of cytochrome *c* reduction by this enzyme, while Fridovich and Handler[6] were of the opposite opinion. The former group of investigators considered that such a stimulating effect revealed the involvement of enzyme iron in the mechanism of the aerobic reduction of cytochrome *c*. In our experiments, the initial velocity of the reaction was slightly inhibited by 8-hydroxyquinoline, as in those of Fridovich and Handler, when highly purified cytochrome *c* was used, but accelerated when less purified cytochrome *c* was used. Therefore, it seems probable that the stimulation of this reaction by 8-hydroxyquinoline is concerned not with the metal cofactor of xanthine oxidase but with cytochrome *c*.

Other chelating agents such as EDTA, histamine, glycine, glycylglycine, citric acid, *o*-phenanthroline, or α,α'-dipyridyl proved to have no influence on the over-all couse of aerobic reduction of cytochrome *c* or on urate formation. However, Tiron, which is a known chelator of iron and molybdenum, inhibited reduction under our experimental conditions, as also reported by Fridovich and Handler.[6]

7) R.J. Doisy, D.A. Richart, W.W. Westerfeld: J. Biol. Chem., **217**, 307 (1955).
8) W.W. Westerfeld, D.A. Richart, E.S. Higgins: *Ibid.*, **234**, 1897 (1959).

Effect of Various Peroxidase Substrates——Various substances which are able to serve as hydrogen donors for plant peroxidase were tested for their effects on urate formation and the reduction of cytochrome *c*.

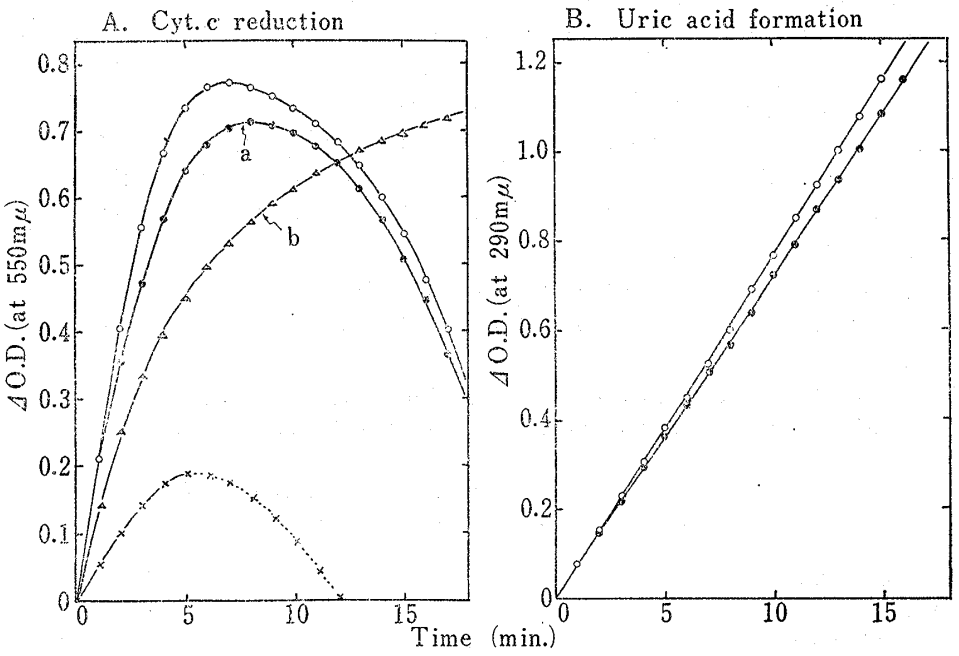

Fig. 4. Effect of Ascorbic Acid on Cytochrome *c* Reduction (A) and Uric Acid Formation (B) by Xanthine Oxidase

Final content of reaction mixture same as in Fig. 1, except that cytochrome *c* was removed in B. ○——○, control; ●——● (a), with $3.3\times10^{-5}M$ ascorbic acid; △——△ (b), with $3.3\times10^{-5}M$ ascorbic acid, without enzyme; ×——×, a—b.

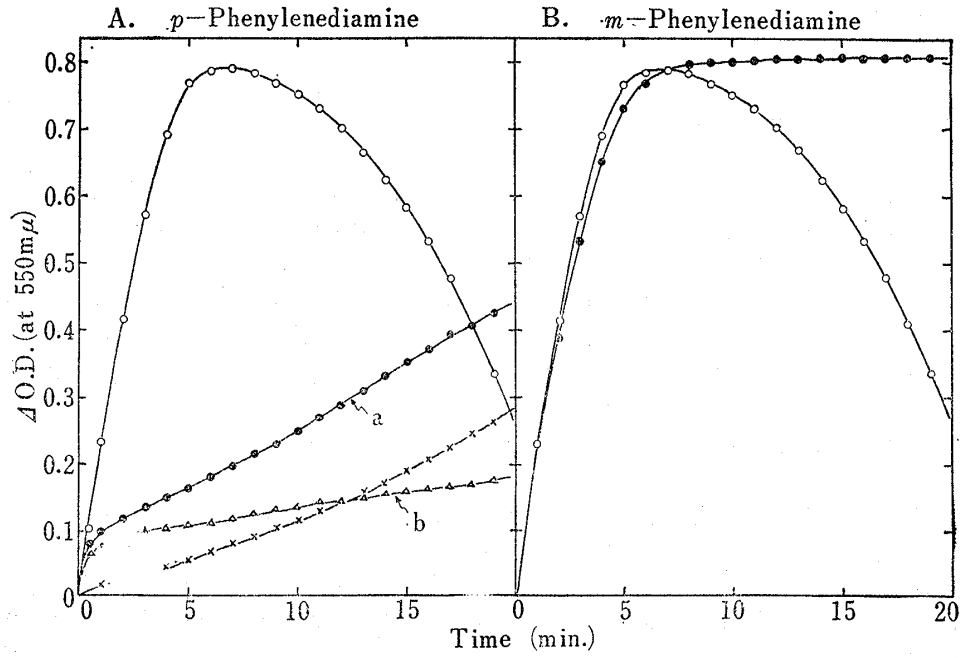

Fig. 5. Effect of *p*-Phenylenediamine (A) and *m*-Phenylenediamine (B) on Reduction of Cytochrome *c* by Xanthine Oxidase

Reaction mixture same as in Fig. 1. A: ○——○, control; ●——● (a), with $3.3\times10^{-5}M$ *p*-phenylenediamine; △——△ (b), with $3.3\times10^{-5}M$ *p*-phenylenediamine, without enzyme; ×——×, a—b. B: ○——○, control; ●——●, with $3.3\times10^{-5}M$ *m*-phenylenediamine.

An example of this study is shown in Fig. 4, which illustrates the effect of ascorbic acid. At neutral pH ascorbate reduces cytochrome *c* non-enzymically, and when this effect is subtracted from the total reaction, its inhibitory effect on the reduction of cytochrome *c* is distinct, although it has no effect on the reoxidation of reduced cytochrome *c* or on urate formation. *p*-Phenylenediamine markedly inhibited the reduction of cytochrome *c*, but what is most interesting is the fact that *m*-phenylenediamine at concentrations above $1.7\times10^{-5}M$ completely inhibited the reoxidation of cytochrome *c* as was also true of 8-hydroxyquinoline (Fig. 5). When Keilin-Hartree's cytochrome *c* preparation was used, *m*-phenylenediamine did accelerate the reduction both in extent and in initial rate, although it had no effect on urate formation. As with 8-hydroxyquinoline, at lower concentrations of hypoxanthine this effect of *m*-phenylenediamine was almost absent.

The effects of various peroxidase substrates subjected to the screening test are summarized in Table I. As is demonstrated in this table, with these compounds

Table I. Effect of Various Peroxidase Substrates on Aerobic Reduction of Cytochrome *c* and Formation of Uric Acid by Xanthine Oxidase

	Reduction of Cytochrome *c*	Reoxidation of Cytochrome *c*	Formation of Uric Acid
Ascorbic Acid	++	−	±
Dopa	+	+	−
Indoleacetic Acid	−	−	−
Hydroquinone	slight st.	slight st.	−
Pyrogallol	−	st.	+++
p-Phenylenediamine	+++	++	−
p-Cresol	+	st.	−
m-Cresol	−	±	−
Guaiacol	+	st.	−
m-Phenylenediamine	+	+++	±
Resorcinol	+	+	−
Phenol	+	−	

+ : inhibition st. : stimulation − : no effect
Reaction mixture same as in Fig. 1.

there is no definite correlation among their effects on the reduction and reoxidation of cytochrome *c* and the urate formation. These results do not also necessarily coincide with those obtained by Yamazaki, *et al.*[3,4] in the reduction of cytochrome *c* by plant peroxidase. They classified peroxidase substrates into two groups, one that reduces cytochrome *c* and the other that oxidizes it, on the basis of the properties of the oxidation intermediates of the substrates. For example, in the plant peroxidase system, such compounds as ascorbic acid, hydroquinone, indoleacetic acid and pyrogallol accelerate the reduction of cytochrome *c*, whereas *m*-phenylenediamine promotes its oxidation.

Effect of 8-Hydroxyquinoline and *m*-Phenylenediamine on the Reduction of a Modified Cytochrome *c* Sample——Fig. 6 represents the assay with the hypoxanthine-xanthine oxidase system conducted on a modified cytochrome *c* sample, prepared by ethanol treatment according to the method of Margoliash and Lustgarten,[9] in the presence or absence of 8-hydroxyquinoline. Exactly the same resuls were obtained as when *m*-phenylenediamine was used. With various cytochrome *c* samples, it was demonstrated that the values obtained by substracting enzymically reducible cytochrome

9) E. Margoliash, J. Lustgarten: J. Biol. Chem., **237**, 3397 (1962).

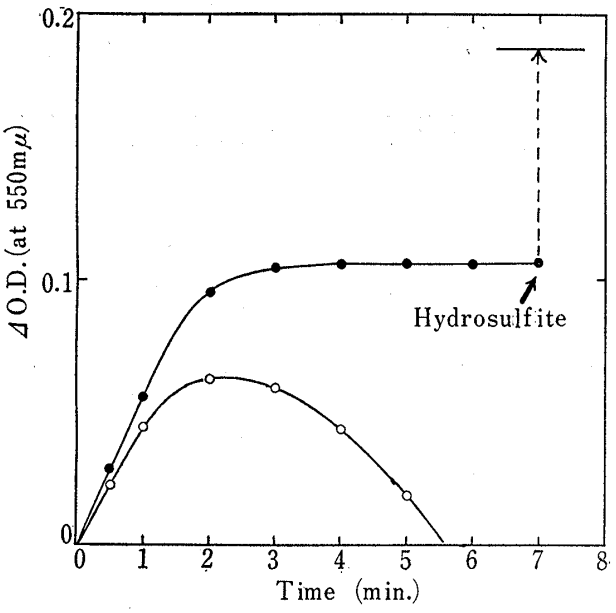

Fig. 6. Effect of 8-Hydroxyquinoline on Reduction of Modified Cytochrome *c* by Xanthine Oxidase

Final contents of hypoxanthine, enzyme and buffer same as in Fig. 1. ○——○, control; ●——●, with $3.3\times10^{-4}M$ 8-hydroxyquinoline.

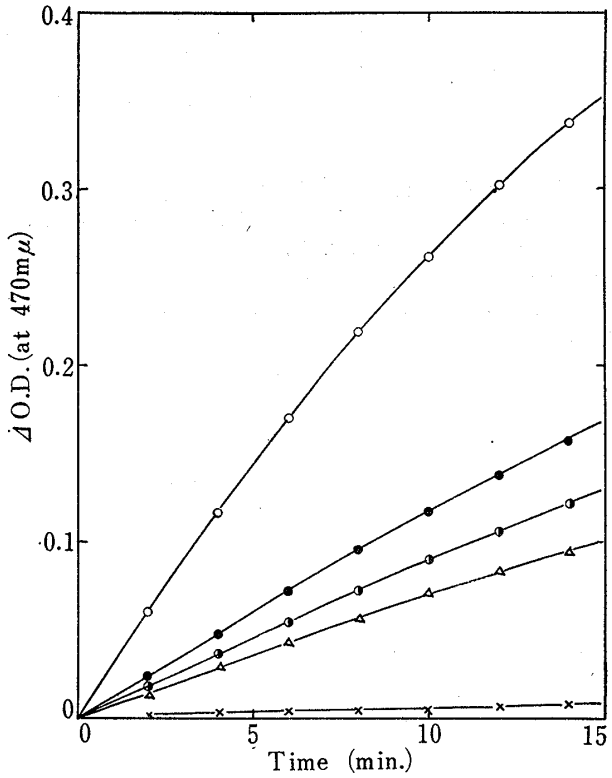

Fig. 7. Inhibitory Effect of 8-Hydroxyquinoline, *m*-Phenylenediamine and KCN on Peroxidase Activity of Cytochrome *c*

Each cuvette contained 0.06 μmoles of cytochrome *c* (Keilin-Hartree's preparation), 0.8 μmoles of guaiacol, 2.4 μmoles of H_2O_2, and 1.0 ml. of $0.1M$ phosphate buffer (pH 7.4) in a final volume of 3.0 ml. ○——○, control; ●——●, with $3.3\times10^{-4}M$ 8-hydroxyquinoline; ◑——◑, with $3.3\times10^{-4}M$ *m*-phenylenediamine; △——△, with $3.3\times10^{-4}M$ KCN; ×——×, with $3.3\times10^{-3}M$ KCN.

c from chemically reducible cytochrome *c* correspond to polymeric or CO-sensitive cytochrome *c*. From the practical standpoint, it is possible, therefore, to determine the content of monomeric cytochrome *c* by xanthine oxidase by using 8-hydroxyquinoline or *m*-phenylenediamine, especially in samples with many impurities.

Effect of 8-Hydroxyquinoline and *m*-Phenylenediamine on the Peroxidase Activity of Cytochrome *c*——As the effect of 8-hydroxyquinoline and *m*-phenylenediamine obtained in the foregoing experiments could not be explained only by their properties as chelating agents or peroxidase substrates, a study was made to see whether the effect of these compounds would primarily be directed to the reoxidation or to the reduction of cytochrome *c*. Since Keilin-Hartree's cytochrome *c* preparation has some peroxidase activity, effects of 8-hydroxyquinoline and *m*-phenylenediamine on the peroxidase activity of cytochrome *c* were tested. Fig. 7 shows that the inhibitory activities of 8-hydroxyquinoline and *m*-phenylenediamine are 52.9 and 62.9%, respectively, at $3.3\times10^{-4}M$. These effects were not potentiated much by increasing the concentration of these compounds. On the other hand, $3.3\times10^{-3}M$ KCN completely inhibited the peroxidase activity. These compounds also inhibited the activity of horseradish peroxidase practically to the same extent. Moreover, it was demonstrated that the velocity of ferrocytochrome *c* oxidation induced by H_2O_2 was likewise suppressed by these compounds.

Since complete inhibition of reoxidation was obtained in the presence of $3.3\times10^{-5}M$ 8-hydroxyquinoline or $1.7\times10^{-5}M$ *m*-phenylenediamine, such an inhibitory action on peroxidase cannot fully explain their striking effect on reoxidation.

The mechanism of the effect of 8-hydroxyquinoline or *m*-phenylenediamine will be dealt with in the following study.

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