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176. Saburo Muraoka,*1 Hiroshi Enomoto,*2 Mie Sugiyama,*1 and Hidemasa Yamasaki*1 : Aerobic Reduction of Cytochrome *c* Preparation by Xanthine Oxidase. I. Reduction and Reoxidation of Cytochrome *c*.

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1. The over-all course of the aerobic reduction of cytochrome *c* by xanthine oxidase was investigated under various conditions, especially to clarify the mechanism of reoxidation of reduced cytochrome *c*. The higher the concentration of enzyme or hypoxanthine and the lower the pH, the more marked was the reoxidation of reduced cytochrome *c*.

2. The reoxidation was also dependent upon the properties of the cytochrome *c* sample. The more marked reoxidation was observed with samples having greater reactivity with CO.

3. With Amberlite CG-50 column chromatography, Keilin-Hartree's cytochrome *c* preparation gave two enzymically reducible and less reoxidizable monomeric fractions, and a monomeric and a polymeric fraction. The latter two fractions were less active as electron acceptors and are thought to be mainly responsible for the reoxidation.

4. Reoxidation of reduced cytochrome *c* has been found to be caused in the following two ways : the peroxidation due to modified cytochrome *c* originally contaminating and also probably formed during the reaction, and the acceleration of peroxidation by uric acid which is the oxidation product of the substrate.

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Xanthine oxidase (xanthine: O_2 oxidoreductase, EC 1.2.3.2) can reduce cytochrome *c* under aerobic conditions. Although there have been some conflicting reports on the requirement for molecular oxygen in this reaction, Muraoka, *et al.*[1] have recently demonstrated that cytochrome *c* can be reduced even under anaerobic conditions on the addition of a suitable electron carrier and therefore have suggested that the reduction of cytochrome *c* might be performed by two distinct electron pathways, an O_2-dependent "aerobic type" and an electron carrier-dependent "anaerobic type".

An attempt has been made to apply the aerobic reaction with the xanthine oxidase system to one of the routine methods of enzymic assay of ferricytochrome *c* in biological materials and drug preparations. It is known, however, that the aerobic reduction of cytochrome *c* is invariably accompanied by the reoxidation of reduced cytochrome *c*.[2,3] The fact that this reoxidation directly affects the initial rate and extent of the reduction has presented one of the difficulties in the enzymic assay or in the study of the mechanism of aerobic reduction of cytochrome *c*. As catalase inhibits this side reaction, Horecker and Heppel[2] and Weber, *et al.*[3] have attributed its cause to the action of lactoperoxidase contaminating the milk enzyme. However, we have observed that this reoxidation cannot be avoided even with the use of highly purified enzyme.

This paper describes experiments designed especially to clarify the factors inducing the reoxidation of reduced cytochrome *c* in the xanthine oxidase system.

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1) S. Muraoka, M. Sugiyama, H. Yamasaki : Biochem. Biophys. Res. Commun., **19**, 346 (1965).
2) B. L. Horecker, L. A. Heppel : J. Biol. Chem., **178**, 683 (1949).
3) M. M. Weber, H. M. Lenhoff, N. O. Kaplan : *Ibid.*, **220**, 93 (1956).

Experimental

Xanthine Oxidase——The enzyme was prepared from fresh cow's milk by the method by Kubo, *et al.*[4] The A_{280}/A_{450} of the preparation was 5.7~5.9, and its specific activity was essentially the same as that of a crystalline preparation. This enzyme did not exhibit peroxidase activity in the system with guaiacol as its substrate, nor did it stimulate peroxidation of ferrocytochrome *c*.

Cytochrome *c*——The cytochrome *c*'s used were the type III preparation of Sigma Chemical Company and Keilin-Hartree's preparation[5] both from horse heart muscle. Further purification was performed using column chromatography on Amberlite CG-50 (type 2), which was fully oxidized by sodium hypochlorite and equilibrated with 0.25*N* ammonium phosphate buffer (pH 7.0), according to the method of Hagihara, *et al.*[6] Ferrocytochrome *c* was obtained by passage through a column of Sephadex G-25 gel after chemical reduction with sodium hydrosulfite.

Manometric assay of cytochrome *c* was carried out in the succinoxidase system, which was prepared from pig heart muscle by thorough washing of the minced muscle to remove endogenous cytochrome *c*.

Oxidase Activity of Xanthine Oxidase——The formation of uric acid from hypoxanthine was determined by the increase in the absorbancy at 290 mμ in a cuvette with 1.0 cm. light path at room temperature. The reaction mixture contained 1.0 μ mole of hypoxanthine, 1~50 μg. of enzyme and 1.0 ml. of 0.1*M* phosphate buffer (pH 7.4) in a final volume of 3.0 ml.

Reduction of Cytochrome *c* by Xanthine Oxidase——The increase in the absorbancy at 550 mμ was determined in a cuvette with 1.0 cm. light path at room temperature. The reaction mixture of 3.0 ml. usually contained 0.05~1.0 μ moles of hypoxanthine, 0.1~0.15 μ moles of cytochrome *c* and 1.0 ml. of 0.1*M* phosphate buffer (pH 7.4).

Carbon Monoxide Binding Capacity of Cytochrome *c*——The reactivity of cytochrome *c* with CO was determined by the method of Tsou[7] and was expressed as the percentage of cytochrome *c* which combines with CO (CO%).

Peroxidase Activity of Cytochrome *c*——The formation of tetraguaiacol was determined by the increase in the absorbancy at 470 mμ in the system containing guaiacol and H_2O_2 by a modification of the method of Kondo and Morita.[8] 3.0 ml. of the reaction mixture contained 0.8 μ moles of guaiacol, 2.4 μ moles of cytochrome *c* and 1.0 ml. of 0.1*M* phosphate buffer.

Results

Effect of Enzyme Concentration——With the system containing $3.3\times10^{-4}M$ hypoxanthine and $5\times10^{-5}M$ cytochrome *c*, the absorbancy at 550 mμ was traced in the presence of various amounts of the enzyme to demonstrate the over-all course of the reaction. As shown in Fig. 1, a decrease in the absorbancy appeared in a later stage of the incubation period. It was also found that the larger the amount of enzyme added, the more marked was the reoxidation of reduced cytochrome *c*.

Effect of Substrate Concentration——In the presence of a sufficient amount of enzyme, both the rate of reoxidation and the maximum extent of reduction of cytochrome *c* were accelerated with increasing amounts of hypoxanthine in the reaction mixture (Fig. 2). Stimulation of the reoxidation of reduced cytochrome *c* by excess amounts of enzyme and substrate suggests that the increase in formation of the reaction products, uric acid and/or H_2O_2, is responsible for such a rapid reoxidation.

4) H. Kubo, K. Shiga, A. Isomoto, M. Uozumi, K. Kadota, Y. Kondo : Proceedings of Symposia on Enzyme Chemistry (Japan), p. 37 (1962).
5) D. Keilin, E. F. Hartree : Proc. Roy. Soc. (London), **B 122**, 298 (1937).
6) B. Hagihara, I. Morikawa, K. Tagawa, K. Okunuki : "Biochemical Preparations," ed. by C. S. Vestling, Vol. 6, p. 1 (1958). John Wiley & Sons, Inc., New York.
7) C. L. Tsou : Biochem. J., **49**, 362 (1951).
8) K. Kondo, Y. Morita : Bull. Res. Inst. Food. Sci., Kyoto Univ. (in Japanese), **4**, 12 (1951).

In the reduction of cytochrome *c* no excess substrate inhibition was seen, which is observable in the oxidase reaction with oxygen as an electron acceptor in the absence of cytochrome *c*.[9~12)]

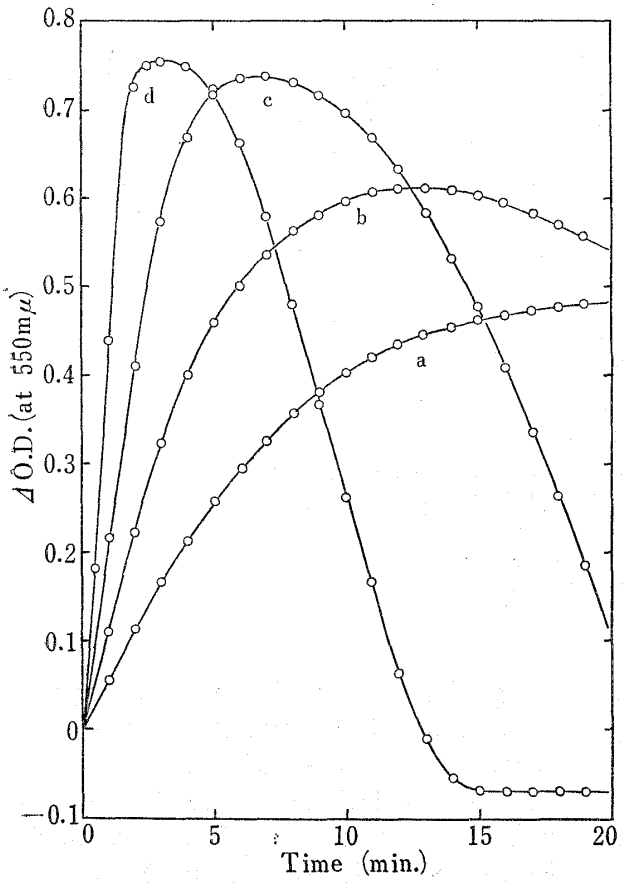

Fig. 1. Reduction of Cytochrome *c* by Xanthine Oxidase in Various Concentrations

Each cuvette contained 1.0 μmole of hypoxanthine, 0.15 μmoles of cytochrome *c*, 1.0 ml. of 0.1*M* phosphate buffer (pH 7.4), and enzyme (a, 25 μg.; b, 50 μg.; c, 100 μg.; d, 200 μg.) in a final volume of 3.0 ml.

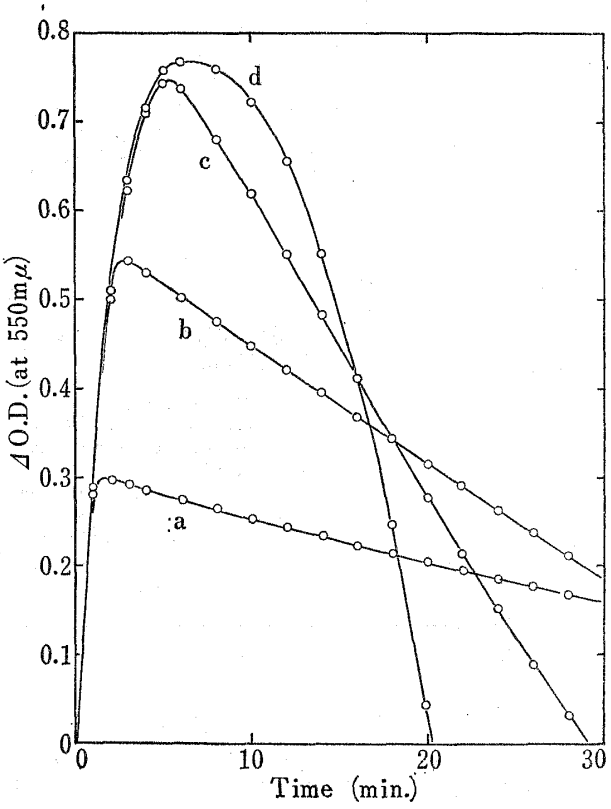

Fig. 2. Effect of Substrate Concentration on Reduction of Cytochrome *c* by Xanthine Oxidase

Each cuvette contained 0.15 μmoles of cytochrome *c*, 100 μg. of enzyme, 1.0 ml. of 0.1*M* phosphate buffer (pH 7.4), and hypoxanthine (a, 0.05 μmoles; b, 0.1 μmoles; c, 0.2 μmoles; d, 0.5 μmoles) in a final volume of 3.0 ml.

Effect of H_2O_2 and Uric Acid——Further experiments were conducted to determine the effects of H_2O_2 and uric acid on the reduction and reoxidation of cytochrome *c*. It was found that H_2O_2 decreased both the initial velocity and the extent of reduction of cytochrome *c*. When H_2O_2 was added during the course of the reaction, it accelerated the reoxidation nearly in proportion to the amount of H_2O_2 added.

In Fig. 3 is shown the effect of uric acid when the reaction was allowed to proceed in the presence of a low concentration of hypoxanthine, followed by the addition of excess amounts of hypoxanthine after slow reoxidation. This figure shows that uric acid not only accelerates the reoxidation but also suppresses the extent of reduction. This effect of uric acid was also demonstrated when it was added during the course of the reaction. Fig. 4 illustrates the effect of uric acid and H_2O_2 on the peroxidation of fer-

9) I. Fridovich, P. Handler : J. Biol. Chem., **237**, 916 (1962).
10) M. Dixon, S. Thurlow : Biochem. J., **18**, 976 (1924).
11) S. Muraoka : Biochim. Biophys. Acta, **60**, 445 (1962).
12) *Idem* : *Ibid.*, **73**, 17 (1963).

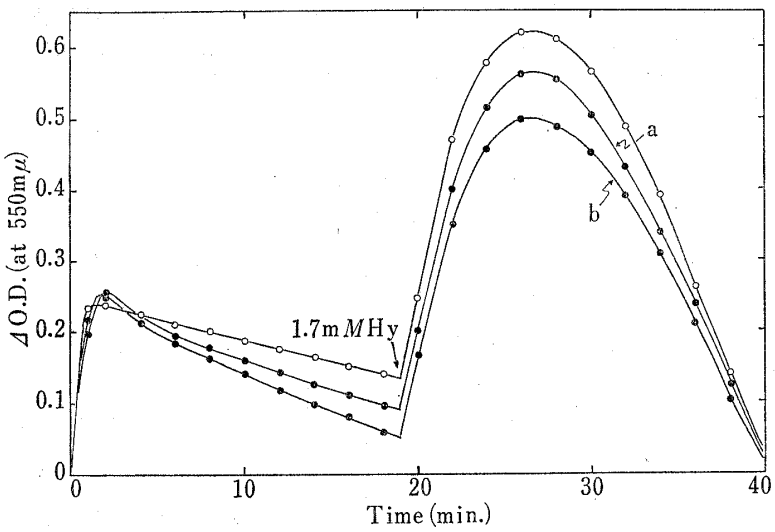

Fig. 3. Effect of Uric acid on Reduction and Reoxidation of Cytochrome *c* by Xanthine Oxidase

Each cuvette contained 0.05 μmoles of hypoxanthine, 0.1 μmoles of cytochrome *c*, 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 0.5 μmoles (a) and 1.0 μmole (b) of uric acid. 5.0 μmoles of hypoxanthine was admitted at the indicated time.

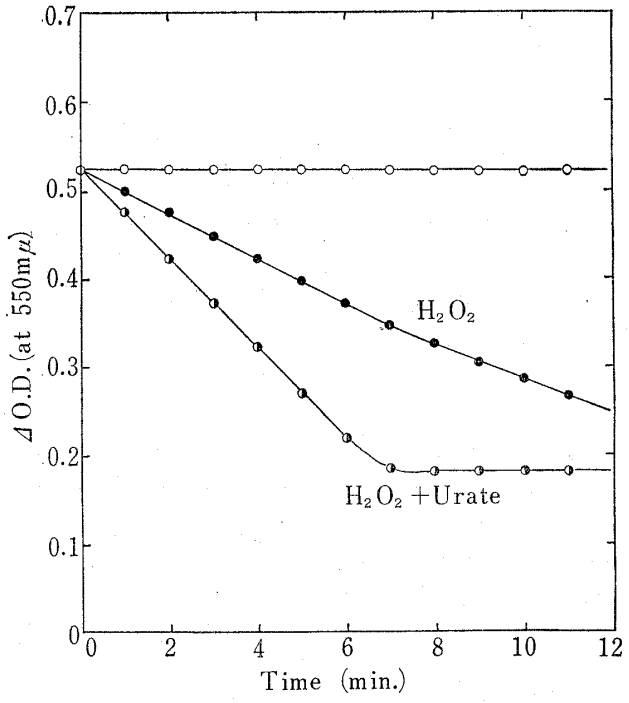

Fig. 4. Effect of Uric Acid on Peroxidation of Ferrocytochrome *c*

Each cuvette contained 1.0 ml. of 0.1*M* phosphate buffer (pH 7.4) in a final volume of 3.0 ml. ○——○, control; ●——●, with $1.7\times10^{-4}M$ H_2O_2; ◑——◑, with $1.7\times10^{-4}M$ H_2O_2 and $3.3\times10^{-4}M$ uric acid.

rocytochrome *c*, which was confirmed to be stable to autoxidation, in the absence of xanthine oxidase, indicating that the oxidation of cytochrome *c* is markedly stimulated by uric acid. However, in the absence of H_2O_2, ferrocytochrome *c* was not oxidized by uric acid alone.

Effect of the Degree of Purity of Cytochrome *c* Samples——Subsequently, it was found that reoxidation had almost no relation to the degree of enzyme purification but was closely related to the purity, especially to the CO%, of the cytochrome *c* sample used. Fig. 5 shows the column chromatography of Keilin-Hartree's preparation on Amberlite CG-50, together with the fluctuation of CO% values. The fraction of the first peak (F-1) was rich in reduced cytochrome *c*. Both F-1 and the fraction of the second peak (F-3) were equally active in the succinoxidase system and were considered to be of monomeric form.[13] The last frac-

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

tion (F-4), which was not eluted with 0.25 *N* ammonium ion, was thought to be of dimeric or polymeric form.[13] In Fig. 6 is illustrated the reaction patterns of the cytochrome *c* samples in tubes no. 35 (F-1, CO 8.5%, curve b), no. 46 (F-2, CO 50.7%, curve c), no. 64 (F-3, CO 3.2%, curve d), and no. 144 (F-4, CO 36.2%, curve e), as well as in the original sample (CO 11.6%, curve a). With sample F-3 reoxidation was very mild, but there were two fractions (F-2 and 4) whose reoxidation was prominent as shown by curves c and e. The fact that the latter two fractions showed high reactivity with CO suggests that the ligand field of the iron atom of the cytochrome *c* molecule is involved in the reoxidation.

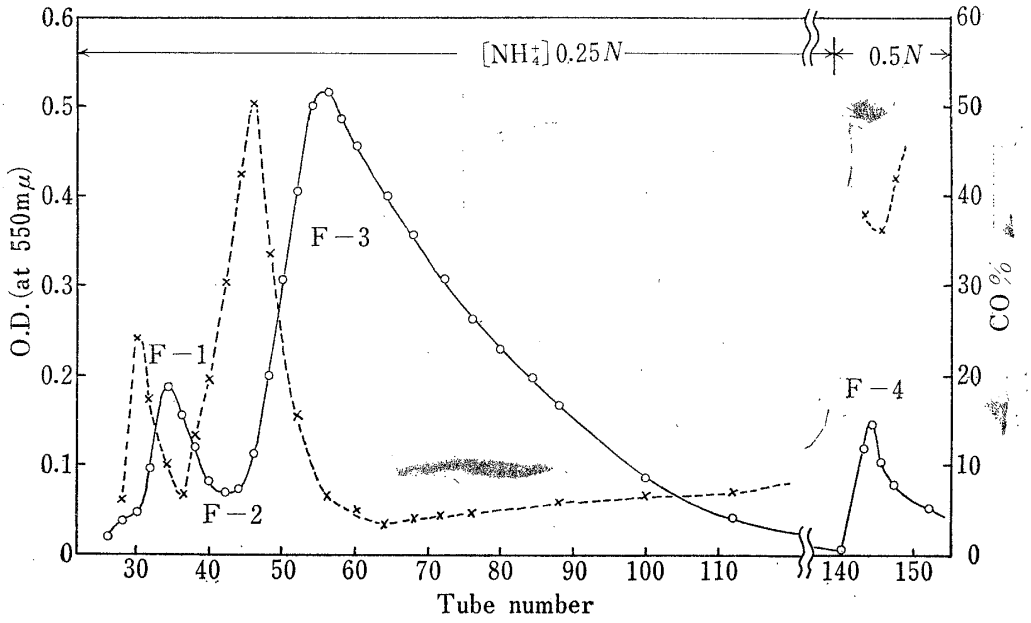

Fig. 5. Column Chromatography of Keilin-Hartree's Preparation of Cytochrome *c* by Amberlite CG-50

1.0 g. of the sample was applied on a column of the resin (mesh 300~400, 2.5×50 cm.), equilibrated with 0.25*N* ammonium phosphate buffer (pH 7.0). ○—○, Absorbancy at 550 mμ (reduced form); ×---×, CO%.

In regard to cytochrome *c* prepared by trichloroacetic acid treatment according to Keilin and Hartree's procedure,[5] there are some reports[14,15] that a somewhat irreversible structural alteration of the protein moiety may occur at acidic pH's which would destroy the natural contribution of the ligands to the binding with the heme iron. Furthermore, since reoxidation invariably occurs, no matter how purified the cytochrome *c* is, especially at higher concentrations of the enzyme, it seems possible that such a denaturation of the protein structure might also occur to a considerable degree during the course of the reaction.

Effect of pH on the Reduction of Cytochrome *c* by Xanthine Oxidase and the Peroxidase Activity of Cytochrome *c*——As the cytochrome *c*'s used in the present experiment were found to show peroxidase activity to a considerable extent, it was presumed that the reoxidation of reduced cytochrome *c* might be caused by the peroxidatic action of denatured cytochrome *c*. This hypothesis was supported by a comparison of the pH dependency of the peroxidase activity of cytochrome *c* with that of its reduction and reoxidation pattern in the xanthine oxidase system. Thus, as is shown in Fig. 7, the more acidic the pH, the less is the extent of reduction and the marked the reoxidation,

14) K. G. Paul: Acta Chem. Scand., **2**, 430 (1948).
15) H. Theorell, A. Åkesson: J. Am. Chem. Soc., **63**, 1818 (1941).

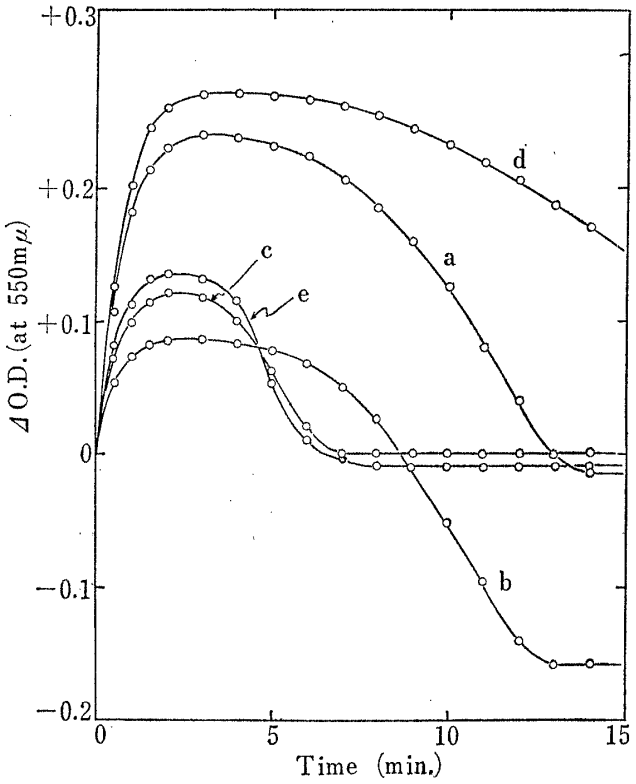

Fig. 6. Reduction and Reoxidation of the Various Cytochrome *c* Samples separated in Fig. 5

Final content of reaction mixture same as in Fig. 3, except that of cytochrome *c* was *ca.* 0.05 μmoles by colorimetric estimation. a, Keilin-Hartree's preparation; b, tube no. 35; c, tube no. 46; d, tube no. 64; e, tube no. 144.

while at pH's above 8.0 the initial rate and extent of reduction increases with the pH up to 10.0 and there is almost no reoxidation. On the other hand, the peroxidatic action of cytochrome *c* was marked at acidic pH's but paractically disappeared at pH's above 8.0 (Fig. 8, curve a), suggesting an intimate correlation between the initial rate and extent of cytochrome *c* reduction and the peroxidase activity of the cytochrome *c* used. However, for the explanation of the apparent pattern of the overall reaction, consideration must be given not only to the pH dependency of the peroxidase activity of cytochrome *c* but also to that of the xanthine oxidase activity itself (Fig. 8, curve b). The initial velocity of the reduction of cytochrome *c* seems to depend chiefly on the latter factor, especially at alkaline pH's.

Discussion

In the reduction of cytochrome *c* by hypoxanthine *via* xanthine oxidase under aerobic conditions, incubation was carried out for a relatively long time to observe two phases of the reaction, namely the reduction and reoxidation of cytochrome *c*. It was observed that the larger the amount of enzyme or substrate, the more marked was the reoxidation,and it seemed that the reaction products, most probably H_2O_2 and uric acid, are involved in the reoxidation. It was further demonstrated that the degree of reoxidation was dependent on the purity of the cytochrome *c* used; the sample of cytochrome *c* with a greater CO binding capacity had a higher peroxidase activity, especially at acidic pH's. It is known that intact or native cytochrome *c* is fairly resistant to autoxidation or to peroxidation by H_2O_2. In addition, the fact that the purified xanthine oxidase used in our experiments did not exhibit any peroxidase activity precluded the possibility of lactoperoxidase contamination in the enzyme preparation as suggested by Horecker and Heppel,[2] and Weber, *et al.*[3] These results indicate that the reoxidation of reduced cytochrome *c* occurring in the hypoxanthine-xanthine oxidase system is probably elicited by the action of denatured or modified cytochrome *c* in the presence of generated H_2O_2 and that this reoxidation of reduced cytochrome *c* catalyzed by modified cytochrome *c* is stimulated by uric acid, an oxidation product of hypoxanthine. This is certainly consistent with the intimate correlation noted between the pH dependency of the cytochrome *c* reduction pattern in the xanthine oxidase system and the peroxidase activity of cytochrome *c*.

Reoxidation could not be avoided even when cytochrome *c* was purified by repeated Amberlite CG-50 chromatography. Thus it is reasonable to assume that the modified or denatured cytochrome *c*, which is the principal causative factor in reoxidation, is not only an original contaminant in the sample but is possibly formed during the reaction.

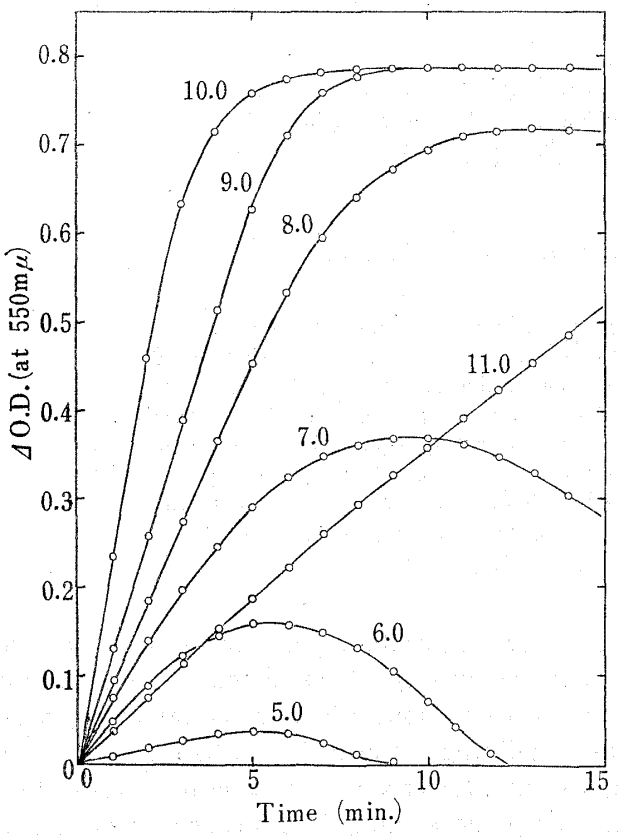

Fig. 7. Effect of pH on Reduction of Cytochrome *c* by Xanthine Oxidase

Each cuvette contained 0.6 μmoles of hypoxanthine, 0.15 μmoles of cytochrome *c*, 30 μg. of enzyme, and 1.0 ml. of 0.1*M* phosphate buffer of the indicated pH value in a final volume of 3.0 ml.

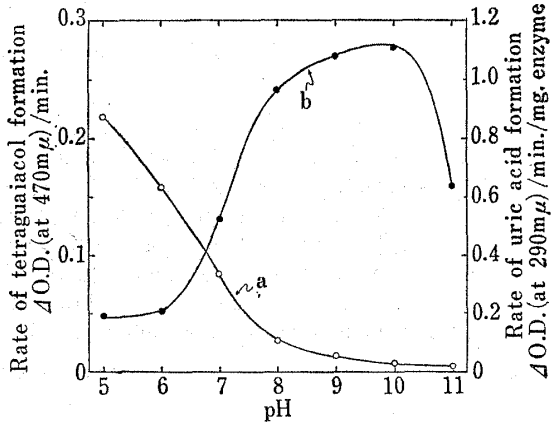

Fig. 8. Effect of pH on Peroxidase Activity of Cytochrome *c*. (○—○) and Hypoxanthine-O_2 Oxidoreductase Activity of Xanthine Oxidase (●—●)

Concerning the accelerating effect of uric acid on the peroxidation of reduced cytochrome *c*, interesting research has been done by Yamazaki, *et al.*,[16,17] who studied the mechanism of reduction of cytochrome *c* by plant peroxidase with system containing H_2O_2 and various hydrogen donors such as ascorbate, triose reductone and dihydroxyfumarate, to demonstrate that the reduction of cytochrome *c* is induced by a free radical derived from the substrate. These investigators have divided a series of compounds, which are capable of serving as hydrogen donors of plant peroxidase, into two groups : a group of substrates, free radicals from which are formed in the peroxidase reaction to effect the reduction of ferricytochrome *c*; and another group that effects the oxidation of ferrocytochrome *c* in the plant peroxidase system. Uric acid is an "oxidogenic" substrate according to this theory, which is very helpful in explaining the effect of uric acid against reoxidation of cytochrome *c* in the xanthine oxidase system. If this is the case, uric acid should remain intact throughout the reaction in the reaction medium. However, it was demonstrated that uric acid disappeared gradually in systems containing H_2O_2 and cytochrome *c* or horseradish peroxidase. On the other hand, there is some evidence that uric acid is oxidized by various peroxidases to allantoin and further degradation products.[18] Therefore, it is not possible, at present, to explain conclusively the effect of uric acid.

16) I. Yamazaki : Proc. Int. Symp. Enzyme Chem. (Tokyo and Kyoto, 1957), p. 224 (1958).
17) I. Yamazaki, L. H. Piette : Biochim. Biophys. Acta, **77**, 47 (1963).
18) E. S. Canellakis, A. L. Tuttle, P. P. Cohen : J. Biol. Chem., **213**, 397 (1955).