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THE MECHANISM OF THE REDUCTION OF CYTOCHROME *c* BY XANTHINE OXIDASE

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SUMMARY

1. In the absence of an electron carrier the reduction of cytochrome *c* by milk xanthine oxidase (xanthine: O_2 oxidoreductase, EC 1.2.3.2) requires molecular oxygen. However, the reduction takes place under anaerobic conditions in the presence of various electron carriers such as 2-methyl-1,4-naphthoquinone, 1,4-naphthoquinone, trinitrobenzenesulfonate and methylene blue.

2. Using 2-methyl-1,4-naphthoquinone as an electron carrier, a comparison was made of the direct reduction of cytochrome *c* in the presence of oxygen and the carrier-mediated reduction in the absence of oxygen.

3. The two reactions were found to differ in their pH dependency. The pH optimum for the oxygen-induced reduction was approx. 10.0 while that for the carrier-mediated reduction was 7.5.

4. In the presence of carrier, and in the absence of oxygen, one mole of hypoxanthine reduced 3.4–3.7 moles of cytochrome *c*. The presence of oxygen caused only a slight fall in this value. In the absence of carrier, however, 0.81–0.92 mole of cytochrome *c* was reduced per mole of hypoxanthine under aerobic conditions.

5. Catalase and Tiron markedly inhibited the oxygen-induced reduction but hardly affected the carrier-mediated reduction.

6. In the presence of sufficient 2-methyl-1,4-naphthoquinone, the carrier-mediated reaction was predominant even under aerobic conditions.

INTRODUCTION

The mechanism of reduction of cytochrome *c* by xanthine oxidase is still uncertain. In 1949, HORECKER AND HEPPEL[1] found that molecular oxygen is essential for the reduction, and this was confirmed by WEBER, LENHOFF AND KAPLAN[2] and FRIDOVICH AND HANDLER[3]. In addition, it was found that catalase inhibits the reduction[2]. On the other hand, MORELL[4] and WESTERFELD[5] found that reduction proceeded more rapidly under anaerobic conditions.

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As is to be expected, the reduction of cytochrome *c* by xanthine or hypoxanthine in the presence of both xanthine oxidase and a suitable carrier, such as 2-methyl-1,4-naphthoquinone, does not require oxygen[6]. The present paper is concerned with the mechanism of the direct reduction of cytochrome *c* by xanthine oxidase in the presence of oxygen.

EXPERIMENTAL

Xanthine oxidase was prepared from fresh cow's milk by the method of KUBO *et al.*[7]. Cytochrome *c* was Sigma Type III from horse heart. Beef-liver catalase was also purchased from Sigma Chemical Company.

The reduction of cytochrome *c* was estimated by the increase in absorbance at 550 mμ in a cuvette with 1.0-cm light path at room temperature. 3 ml of reaction mixture usually contained 0.05–1.0 μmole of hypoxanthine, 0.1–0.15 μmole of cytochrome *c*, 1.0 ml of 0.1 M potassium phosphate buffer (pH 7.4) and 20–100 μg of enzyme. Anaerobic reactions were carried out in a photo-cell attached to a Thunberg tube, from which air was completely removed by 4 cycles of evacuation and introduction of purified nitrogen. Purified nitrogen was prepared by passing commercial nitrogen of 99.995 % purity over heated copper and then through alkaline pyrogallol solution. The amount of cytochrome *c* reduced was calculated from the increase in the absorbance at 550 mμ, using $\Delta\varepsilon$ (reduced — oxidized) of 19.5×10^3/M at pH 7.4.

RESULTS

Oxygen requirement for the reduction of cytochrome c

As illustrated in Fig. 1, under aerobic conditions cytochrome *c* is reduced by hypoxanthine in the presence of xanthine oxidase. Under anaerobic conditions cytochrome *c* is not reduced at an appreciable rate. However, vigorous aeration induced rapid reduction of cytochrome *c*. These results show that molecular oxygen is indispensable for the reduction of cytochrome *c* in the absence of an electron carrier, as already reported[1–3].

Reduction of cytochrome c under anaerobic conditions in the presence of an electron carrier

Fig. 2 shows that 2-methyl-1,4-naphthoquinone catalyses the reduction of cytochrome *c* by hypoxanthine in the presence of xanthine oxidase. Oxygen is not necessary for this reaction, which proceeds more rapidly and to a greater extent than the reduction in the presence of oxygen and absence of electron carrier. As well as 2-methyl-1,4-naphthoquinone, phylloquinone, ubiquinone-7, tetramethyl-*p*-phenylenediamine, 1,4-naphthoquinone, trinitrobenzenesulfonate, methylene blue, phenazine methosulfate and 2,6-dichlorophenolindophenol were effective as carriers between the enzyme and cytochrome *c*.

Previously we reported that 8-hydroxyquinoline and *m*-phenylenediamine also act as weak electron carriers[6], since with them a slow reduction was observed under anaerobic conditions. It was further observed that the reduction of cytochrome *c* was markedly accelerated by H_2O_2 in the presence of 8-hydroxyquinoline or *m*-phenylenediamine both under aerobic and anaerobic conditions. The stimulatory

effect of H_2O_2 was recently elucidated as due to the fact that, by the peroxidative action of cytochrome *c*, 8-hydroxyquinoline is hydroxylated to 5,8-dihydroxyquinoline, which acts as a potent electron carrier and promotes the reduction of cytochrome *c* under aerobic and anaerobic conditions[8]. A similar mechanism was suggested in the case of *m*-phenylenediamine[8].

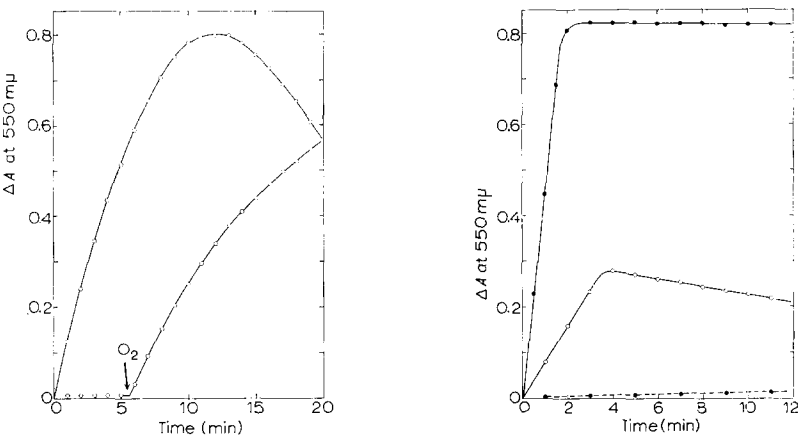

Fig. 1. Oxygen requirement for reduction of cytochrome *c* by xanthine oxidase. The reaction medium contained 1.0 μmole of hypoxanthine, 0.15 μmole of cytochrome *c*, 50 μg of enzyme and 1.0 ml of 0.1 M potassium phosphate buffer (pH 7.4) in a final vol. of 3.0 ml.

Fig. 2. Aerobic and anaerobic reduction of cytochrome *c* by xanthine oxidase in the presence of a limited amount of hypoxanthine. Each cuvette contained 0.05 μmole of hypoxanthine, 0.15 μmole of cytochrome *c*, 50 μg of enzyme and 1.0 ml of 0.1 M potassium phosphate buffer (pH 7.4) in a final vol. of 3.0 ml. ○—○, aerobic (without 2-methyl-1,4-naphthoquinone); ●---●, anaerobic (without 2-methyl-1,4-naphtoquinone); ●—●, anaerobic (with 33 μM 2-methyl-1,4-naphtoquinone)

Stoicheiometry of the reduction of cytochrome c

Table I shows the stoicheiometry of the reaction between xanthine and hypoxanthine with cytochrome *c*, in the presence and absence of carrier, and under aerobic and anaerobic conditions. In the presence of carrier, and in the absence of oxygen, 1 mole xanthine reduced 1.5–1.7 mole of cytochrome *c*, and hypoxanthine 3.4–3.7 moles. The presence of oxygen caused only a slight fall in this value. In the absence of carrier, however, only 0.38–0.46 and 0.81–0.92 mole of cytochrome *c* were reduced per mole of xanthine and hypoxanthine, respectively. From these results it is suggested that the apparent stoicheiometry of the oxygen-induced reduction is quite different from the reduction occurring in the presence of an electron carrier under anaerobic conditions and that in the presence of 2-methyl-1,4-naphthoquinone the reduction of the carrier-mediated type proceeds to a considerable extent under aerobic conditions.

Effect of pH

The carrier-mediated reduction of cytochrome *c* in the absence of oxygen and the direct reduction in the presence of oxygen were found to differ in their pH dependency (Fig. 3, Curves a and d). The addition of carrier in the presence of oxygen

TABLE I

EFFECT OF 2-METHYL-1,4-NAPHTHOQUIONE ON THE AEROBIC AND ANAEROBIC REDUCTIONS OF CYTOCHROME *c* BY XANTHINE OXIDASE

The reaction medium contained 0.15 μmole of cytochrome *c*, 100 μg of enzyme and 1.0 ml of 0.1 M potassium phosphate buffer (pH 7.4) in a final vol. of 3.0 ml.

Substrate (μ*moles*)		*Addition* (μ*moles*)	*Gas phase*	*Cyt. c reduced* (μ*moles*)	*Cyt. c/substrate ratio* (*mole/mole*)
Xanthine	0.01	None	Air	0.0046*	0.46
	0.02	None	Air	0.0077*	0.38
	0.03	None	Air	0.0131*	0.44
Xanthine	0.01	Q, 0.5	Air	0.0154	1.54
	0.02	Q, 0.5	Air	0.0334	1.67
	0.03	Q, 0.5	Air	0.0508	1.69
Xanthine	0.01	Q, 0.5	N_2	0.0169	1.69
	0.02	Q, 0.5	N_2	0.0324	1.62
	0.03	Q, 0.5	N_2	0.0453	1.51
Hypoxanthine	0.01	None	Air	0.0092*	0.92
	0.02	None	Air	0.0162*	0.81
	0.03	None	Air	0.0246*	0.82
Hypoxanthine	0.01	Q, 0.1	Air	0.0284	2.84
	0.02	Q, 0.1	Air	0.0545	2.72
	0.03	Q, 0.1	Air	0.0968	3.23
Hypoxanthine	0.01	Q, 0.1	N_2	0.0362	3.62
	0.02	Q, 0.1	N_2	0.0738	3.69
	0.03	Q, 0.1	N_2	0.1015	3.38

Abbreviations: Q, 2-methyl-1,4-naphthoquinone; cyt., cytochrome *c*.

* This was calculated from the maximum value of the increase in *A* at 550 mμ, since the reduction of cytochrome *c* by xanthine oxidase is invariably accompanied by the reoxidation of reduced cytochrome *c* under aerobic conditions[12].

caused the pH–activity curve to shift towards that obtained with carrier alone (Fig. 3, Curves b and c).

In the absence of 2-methyl-1,4-naphthoquinone the stoicheiometry of the oxygen-induced reaction was considerably influenced by pH as shown in Fig. 4. Maximum reduction of cytochrome *c* was attained at pH 10 and 1 mole of xanthine reduced about 1 mole of cytochrome *c*.

Effect of inhibitors

A and B shown in Figs. 5, 6 and 7 represent the effect of inhibitors on aerobic reduction in the absence of 2-methyl-1,4-naphthoquinone and on anaerobic reduction in the presence of 6.6 μM 2-methyl-1,4-naphthoquinone, respectively. It can be seen from Fig. 5 that 2,4-dinitrophenol inhibits the reduction of both types to the same extent. A similar effect was observed with allopurinol (4-hydroxypyrazolo-[3,4*d*]-pyrimidine), Cu^{2+} and *p*-chloromercuribenzoate. On the other hand, catalase and Tiron markedly inhibited the oxygen-induced reduction, though they hardly affected the carrier-mediated reduction (A and B in Figs. 6 and 7). In the aerobic reduction in

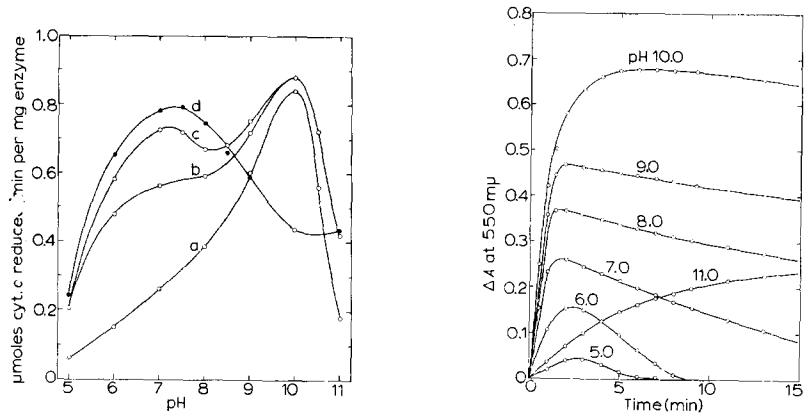

Fig. 3. pH dependency of the aerobic and anaerobic reduction of cytochrome *c* by xanthine oxidase. The reaction medium contained 1.0 μmole of hypoxanthine, 0.15 μmole of cytochrome *c*, 25 μg of enzyme and 1.0 ml of 0.1 M phosphate buffer in a final vol. of 3.0 ml. ○—○, aerobic (a, without 2-methyl-1,4-naphthoquinone; b, with 6.6 μM 2-methyl-1,4-naphthoquinone; c, with 33 μM 2-methyl-1,4-naphthoquinone); ●—●, anaerobic (d, with 6.6 μM 2-methyl-1,4-naphthoquinone).

Fig. 4. Effect of pH on the aerobic reduction of cytochrome *c* by xanthine oxidase in the presence of a limited amount of hypoxanthine. Each cuvette contained 0.05 μmole of hypoxanthine, 0.15 μmole of cytochrome *c*, 100 μg of enzyme and 1.0 ml of 0.1 M phosphate buffer in a final vol. of 3.0 ml.

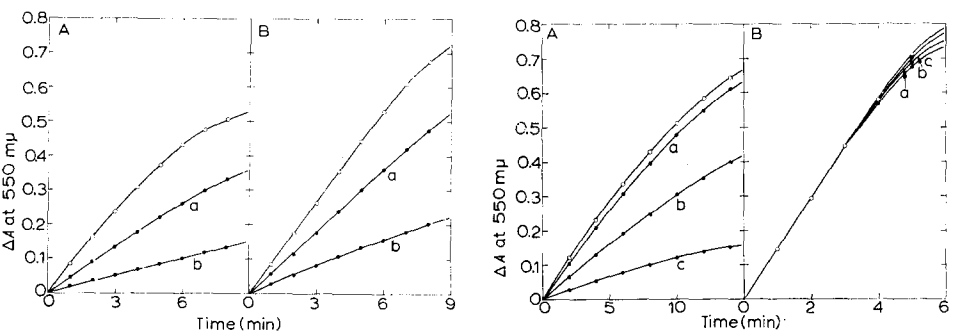

Fig. 5. Effect of 2,4-dinitrophenol on the reduction of cytochrome *c* by xanthine oxidase. Each cuvette contained 1.0 μmole of hypoxanthine, 0.15 μmole of cytochrome *c*, 50 (A) or 20 (B) μg of enzyme and 1.0 ml of 0.1 M potassium phosphate buffer (pH 7.4) in a final vol. of 3.0 ml. 6.6 μM 2-methyl-1,4-naphthoquinone was used in (B). ○—○, control; ●—●, with 0.33 mM (a) and 3.3 mM (b) 2,4-dinitrophenol.

Fig. 6. Effect of catalase on the reduction of cytochrome *c* by xanthine oxidase. The reaction medium was the same as in Fig. 5, except that 30 μg of enzyme was used. ○—○, control; ●—●, with 20 μg (a), 100 μg (b) and 500 μg (c) of catalase.

the presence of 2-methyl-1,4-naphthoquinone it was shown that the inhibitory effect of catalase and Tiron is intermediate between those shown in A and B.

Competition between the oxygen-induced and carrier-mediated reactions

The foregoing experiments on the stoicheiometry, the pH dependency and the inhibitory effects of catalase and Tiron suggest that, in the presence of 2-methyl-

1,4-naphthoquinone, reduction of the carrier-mediated type occurs to a considerable extent even under aerobic conditions. In this case it seems that the two types of cytochrome *c* reduction proceed simultaneously. The rate and stoicheiometry of these mixed reactions under aerobic conditions were investigated at varying concentrations of 2-methyl-1,4-naphthoquinone. It was shown that the maximum extent of cytochrome *c* reduction increased with the carrier concentration, and it reached virtually that of the carrier-mediated reduction in the presence of 17 μM 2-methyl-1,4-naphthoquinone. 2-Methyl-1,4-naphthoquinone also showed a similar effect on the initial velocity of reduction both under aerobic and anaerobic conditions as shown in Fig. 8.

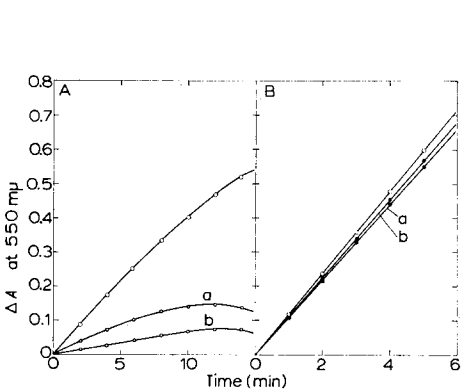

Fig. 7. Effect of Tiron on the reduction of cytochrome *c* by xanthine oxidase. The reaction medium was the same as in Fig. 6. O—O, control; ●—●, with 0.33 mM (a) and 3.3 mM (b) Titron.

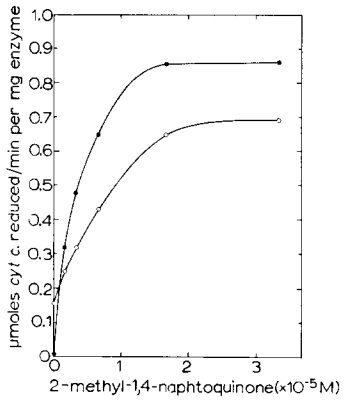

Fig. 8. Effect of 2-methyl-1,4-naphtoquinone on the rate of cytochrome *c* reduction by xanthine oxidase. Each cuvette contained 1.0 μmole of hypoxanthine, 0.15 μmole of cytochrome *c*, 10 μg of enzyme and 1.0 ml of 0.1 M potassium phosphate buffer (pH 7.4) in a final vol. of 3.0 ml. O—O, aerobic; ●—●, anaerobic.

In addition, it was previously reported[6] that in the oxygen-induced reduction of cytochrome *c* by this enzyme oxygen consumption was nearly completely arrested at the stage of cytochrome *c* reduction in the presence of sufficient 2-methyl-1,4-naphthoquinone (33 μM). These findings indicate that the oxygen-dependent reaction is in competition with the carrier-dependent reaction.

DISCUSSION

In the present study with highly purified enzyme, it was found that oxygen is indispensable for the reduction of cytochrome *c* by xanthine oxidase in the absence of an electron carrier. On addition of a suitable electron carrier, such as 2-methyl-1,4-naphthoquinone, the reduction takes place under anaerobic conditions. It is clear that these two reactions occur by entirely different mechanisms, since it was found that (i) the optimum pH of the oxygen-induced reaction is 10, while that of the carrier-mediated one is 7.5, (ii) 0.81–0.92 mole of cytochrome *c* is reduced per mole of hypoxanthine in the oxygen-induced reaction and 3.4–3.7 moles in the carrier-mediated one, and (iii) catalase and Tiron markedly inhibit the cytochrome *c* reduction

in the presence of oxygen and in the absence of carrier, but scarcely affect the carrier-mediated one.

The cytochrome *c* reduction mediated by the electron carrier may be properly represented by the following reactions, as a reaction of the dehydrogenase type,

$$AH_2 + H_2O + \text{Enzyme} \longrightarrow AH_2O + \text{Enzyme-}H_2 \quad (1)$$

$$\text{Enzyme-}H_2 + Q \longrightarrow \text{Enzyme} + QH_2 \quad (2)$$

$$QH_2 + 2 \text{ cyt. } c^{3+} \longrightarrow Q + 2 \text{ cyt. } c^{2+} + 2H^+ \quad (3)$$

where AH_2 is xanthine, AH_2O is uric acid and Q is 2-methyl-1,4-naphthoquinone or another electron carrier. Since stoicheiometric data obtained coincide well with the theoretical values, there is little doubt about these reactions.

To explain the direct reduction of cytochrome *c* in the presence of oxygen, WEBER, LENHOFF AND KAPLAN[2] proposed a mechanism of the peroxidase type, in which free or enzyme-bound H_2O_2 acts as a reductant for cytochrome *c*. This mechanism is based on the facts that this reaction depends strictly on molecular oxygen and is inhibited by catalase. On the other hand, FRIDOVICH AND HANDLER[3] recognized the necessity of molecular oxygen in this reaction but found no inhibitory effect of catalase, and they proposed the formation of a free radical of oxygen (O_2^-) by the action of the non-heme iron of the enzyme in the reduction of cytochrome *c*.

Studies have been made on the reduction of cytochrome *c* by plant peroxidase with the use of ascorbate or dihydroxyfumarate as hydrogen donor. YAMAZAKI *et al.*[9,10] clearly demonstrated by a magnetic method that the reduction of cytochrome *c* by peroxidase is brought about by a free radical of substrate. Some observations tempted us to support a peroxidase mechanism for the reduction of cytochrome *c* by xanthine oxidase; namely, the inhibition caused by catalase, as mentioned in this paper, and the probable formation of a free radical of xanthine reported by ACKERMAN AND BRILL[11]. Another fact that may support this idea is that uric acid induces the reoxidation of reduced cytochrome *c* in the presence of H_2O_2 (ref. 12), since uric acid is classified as an oxidogenic substrate in the plant peroxidase system[9]. When YAMAZAKI's theory is applied to the reduction of cytochrome *c* by xanthine oxidase, the reaction may be represented by the formulae (4)–(7) below,

$$AH_2 + O_2 + H_2O \longrightarrow AH_2O + H_2O_2 \quad (4)$$

$$2AH_2 + H_2O_2 \longrightarrow 2AH^{\bullet} + 2H_2O \quad (5)$$

$$AH^{\bullet} + \text{cyt. } c^{3+} + H_2O \longrightarrow AH_2O + \text{cyt. } c^{2+} + H^+ \quad (6)$$

Net:

$$3AH_2 + O_2 + 2 \text{ cyt. } c^{3+} + H_2O \longrightarrow 3AH_2O + 2 \text{ cyt. } c^{2+} + 2H^+ \quad (7)$$

where $AH^{\bullet}$ represents xanthine with a free radical. If the reaction proceeds exclusively according to these formulae, 1 mole of xanthine would reduce 2/3 mole of cytochrome *c*. However, it seems quite likely that the observed value would be smaller than the theoretical value, since there are possibilities that (i) Reaction 4 alone may proceed independently of Reactions 5 and 6, and (ii) reduced cytochrome *c* is concomitantly reoxidized by uric acid and H_2O_2 (Reactions 8 and 9, where $AHO^{\bullet}$ represents urate with a free radical),

$$2AH_2O + H_2O_2 \longrightarrow 2AHO^{\bullet} + 2H_2O \quad (8)$$

$$AHO^{\bullet} + \text{cyt. } c^{2+} + H^{+} \longrightarrow AH_2O + \text{cyt. } c^{3+} \quad (9)$$

$$2AH^{\bullet} \longrightarrow AH_2 + A \quad (10)$$

and by modified cytochrome *c* which would be present in the cytochrome *c* sample. Reaction 10 would also decrease the amount of cytochrome *c* reduced. The latter possibilities have already been reported[12].

Further consideration must be given to the effect of pH. At neutral pH 1 mole of xanthine reduced 0.38–0.46 mole of cytochrome *c*, while this value increased as the solution became alkaline and at pH 10 about 1 mole of cytochrome *c* was reduced per mole of xanthine. One explanation for this effect would be provided by the possibility that some endogenous substance contaminating the enzyme preparation (perhaps a group on the enzyme) acts as an electron carrier at alkaline pH. This possibility is under investigation.

Finally, returning to the requirement for molecular oxygen in the reduction of cytochrome *c*, it is concluded that the carrier-mediated reaction is far more economical or effective as an electron pathway than the oxygen-induced reaction, both stoicheiometrically and considering the reaction velocity. The oxygen-induced reduction of cytochrome *c* is possibly an artificial reaction due to loss of an electon carrier during purification of the enzyme. In our experiments, the requirement for oxygen seems to become more strict with the purification of enzyme. It is possible that the enzyme preparations used by investigators who presented contrary results concerning the requirement of oxygen contained some electron carrier, although it is also possible that the aged 'inactive enzyme' functions as the carrier. In this connection it is of interest to note that in the enzyme preparations from avian tissues the oxidase activity is far less than the dehydrogenase activity[13–16].

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