THE CYTOCHROME C-CYTOCHROME OXIDASE COMPLEX

By ELMER STOTZ, AARON M. ALTSCHUL, AND T. R. HOGNESS

(From the George Herbert Jones Chemical Laboratory of the University of Chicago, Chicago)

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In the preceding paper the oxidation of substrates by "indophenol oxidase" was demonstrated to be a joint action of cytochrome and cytochrome oxidase. It was further shown that with a given amount of oxidase the velocity of hydroquinone oxidation reached a maximum as the amount of added cytochrome was increased. The latter fact immediately suggested the probability that the rapid aerobic oxidation of reduced cytochrome C in the presence of the oxidase involved the formation of an enzyme-substrate complex (cytochrome oxidase-cytochrome C).

In this paper we are presenting a study of the oxidation of hydroquinone by this complex and the influence of such factors as KCN, CO, and O₂ upon the reaction.

EXPERIMENTAL

The experimental arrangement was the same as in the previous paper. The oxidase preparations used had gone through at least two successive acetate precipitations, so that they were very low in cytochrome C content and, without the addition of cytochrome C, produced no appreciable increase in the velocity of oxidation over the autoxidation level of hydroquinone.

Since the reduction of cytochrome C by the hydroquinone was visibly very fast, the aerobic oxidation of cytochrome C in the presence of its oxidase must be the limiting velocity which controls the rate of oxygen consumption in this system. It must be emphasized that all rates recorded pertain to initial velocities which never exceeded 25 per cent oxidation of the hydroquinone. The velocity, for any given set of conditions, was always constant.
Cytochrome C-Cytochrome Oxidase

for at least this part of the reaction. The function of the hydroquinone in such experiments is essentially that of maintaining the cytochrome C in the reduced condition. The same phenomenon of reaching maximum velocity with increasing cytochrome C concentration is also observed if p-phenylenediamine is used as the reductant, but this latter substance cannot be used advantageously, since its use introduces complications due to the cytochrome B component (see the preceding paper). As in the hydroquinone experiments recorded previously, autoxidation blanks were determined and subtracted from the values obtained with the catalytic system.

**Cytochrome C-Cytochrome Oxidase Complex**

The addition of cytochrome C to cytochrome oxidase increased the velocity of oxidation of hydroquinone to a point above which further additions were without effect. The maximum velocity reached was directly proportional to the amount of oxidase preparation used, which in the preceding paper was demonstrated as
a method for quantitatively determining the enzyme. Illustrative curves are shown in Fig. 1.

According to the Lineweaver and Burk (1) analysis of enzyme-substrate relations, a plot of $1/v$ against $1/s$ should, in the simplest case of a complex, yield a straight line since

\[
\frac{1}{v} = \frac{K_m}{V_{\text{max}} \cdot S} + \frac{1}{V_{\text{max}}} \tag{1}
\]

derived from the Michaelis-Menten equation

\[
v = \frac{V_{\text{max}} \cdot S}{K_m + S}
\]

where $v$ is the velocity obtained at a given substrate concentration $S$; $K_m$, the Michaelis constant of the complex; and $V_{\text{max}}$, the maximum velocity obtainable with the given amount of enzyme. Fig. 1 shows the results of this analysis of two representative experiments. Different total enzyme concentrations were used in the two cases.

The character of the curves obtained can be interpreted to be the result of an enzyme-substrate complex formation in the aerobic oxidation of cytochrome C by the oxidase. The Michaelis constant of this complex has been calculated to be approximately $5.8 \times 10^{-5}$ molar. This constant includes the true dissociation constant and may be identical with it (2).

Many such curves have been determined for a variety of different enzyme preparations. Successive acetate precipitations, dialysis, and heat treatment, although producing great differences in the oxidase activity, yielded preparations showing no appreciable change in the Michaelis constant for the reaction. These

* This equation is based upon the assumption that the substrate concentration is large as compared with that of the enzyme; the free and total substrate concentrations can then be taken as practically identical. However, if such is not the case, the equation becomes

\[
\frac{1}{v} = \frac{K_m}{V_{\text{max}} \cdot \left( S_t - e \frac{v}{V_{\text{max}}} \right)} + \frac{1}{V_{\text{max}}}
\]

where $S_t$ is total substrate concentration, and $e$ is total enzyme concentration. The fact that the $1/v$ against $1/S_t$ plots give a straight line is an indication that $e$ is relatively small as compared with $S_t$.
results, while indicating that the oxidase is probably a single
substance, do not preclude the possibility that the oxidase, on
further fractionation, may not be found to consist of more than
one component. These particular experiments constitute a part
of a search to determine a possible function for cytochrome A
in this oxidation or detect the presence of another necessary factor,
such as Warburg's oxygen-carrying ferment (see "Discussion").

**Fig. 2.** The effect of added cytochrome on the system partially in-
hibited by KCN. ○ represents no KCN; ●, 20 × 10⁻⁶ mM of KCN total.
T = 38°, pH 7.15, hydroquinone 0.033 mM total.

**Effect of Cyanide**

The inhibiting effect of KCN could be the result of its combining
with the cytochrome, or with the oxidase component of the com-
plex. To test whether the inhibition was due to a combination
with cytochrome C we reasoned that if a definite amount of KCN
caused a partial inhibition of the reaction with a given small
amount of cytochrome C, then further additions of cytochrome C
should relieve the inhibition. An increase in the cytochrome C concentration would increase the ratio Cyto to Cyto(CN) and the rate should increase as the total cytochrome concentration increased with the result that the same point of maximum velocity should then be reached at a higher level of cytochrome concentration. Since, as shown in Fig. 2, the original velocity is not reached upon further addition of cytochrome C, it is apparent that the point of attack of the cyanide is not the cytochrome.

On the other hand, if cyanide formed a compound with the oxidase constituent of the complex and if cytochrome C does not effectively displace the cyanide, then the rate should approach a constant value with increasing cytochrome C concentration. The lower maximum velocity obtained under these conditions strongly suggests that KCN inactivated a definite fraction of the oxidase, which was then "saturated" by lower concentrations of cytochrome.

**Effect of Carbon Monoxide**

A similar problem arises in the case of carbon monoxide inhibition. Experiments analogous to those with cyanide were carried out in which the gas space was 80 per cent CO + 20 per cent O₂, with an 80 per cent N₂ + 20 per cent O₂ mixture for the controls. The experiments were performed in the dark.

These experiments were of special interest in view of those reported by Altschul and Hogness (3) from this laboratory. In their experiments it was found that CO produced a change in the spectrum of reduced cytochrome C, and that the cytochrome was saturated with CO at relatively low CO pressures. The present experiments indicate that carbon monoxide inhibition is not due to the union with the free cytochrome. In all the cases tested, varying from a "saturation" of the oxidase with cytochrome to conditions in which the oxidase was operating at only a few per cent of its total capacity, as measured in the presence of excess cytochrome, inhibitions varying only from 55 to 65 per cent were obtained. Since the velocity increments produced by successive additions of cytochrome depend entirely upon the oxidase already "saturated," the percentage inhibition produced by CO under these conditions should also vary greatly if the free cytochrome alone were affected. This is not the case.
Further proof of the fact that free cytochrome was not greatly affected lies in the character of the lower curve in Fig. 3. Assuming a constant concentration of carbon monoxide and that a cytochrome-CO complex would not combine with the oxidase, a given percentage inhibition of free cytochrome would result in an increase in the amount of "available" cytochrome as the total cytochrome was increased. This should result in a steadily rising curve, which is not the case.

On the other hand, if the Cyto(CO) compound possessed an affinity for the oxidase, and the resulting complex were not catalytically active, a constant percentage of the oxidase would be inactivated. Since the CO concentration is constant, Cyto to Cyto(CO) must be constant with the result that the ratio of active
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to inactive complex must also be constant. A limiting velocity would be the result of increased concentration of cytochrome. However, the results of Altschul and Hogness showed that the cytochrome is saturated with CO at pressures below those used in this experiment, and we should therefore expect much greater inhibition than we have obtained. We are therefore led to the conclusion that the CO combines with the oxidase constituent, as in the case of cyanide inhibition.

![Graph](https://example.com/graph.png)

**Fig. 4.** The effect of cytochrome oxidase on the velocity of oxidation in the presence of a constant amount of cytochrome C. $T = 38^\circ$, pH 7.15, cytochrome $15 \times 10^{-4}$ mm total, hydroquinone 0.033 mm total.

Such evidence, bearing on the point of attack of cyanide and carbon monoxide, is of value in understanding the nature of the oxidase. Our evidence so far indicates that the oxidase itself is attacked by these reagents, and supports the view that this enzyme is a metal-containing compound.

In the recent paper by Keilin and Hartree (4), in which the effect of CO was discussed, small percentage inhibitions were found for the oxidation of $p$-phenylenediamine by a crude oxidase
preparation. This was attributed to the relative excess of oxidase to cytochrome. In view of the proportionality existing between the oxidase concentration and velocity of oxidation (see Fig. 4), and in view of the contribution of cytochrome B in this oxidation, as demonstrated in the preceding paper, this explanation does not appear satisfactory. The variation in carbon monoxide sensitivity of this oxidation more likely would seem due to the relative part of the oxidation concerned with cytochrome B.

![Graph](image)

**Fig. 5.** The effect of oxygen tension on the catalytic ability of the oxidase-cytochrome C complex. ○ represents 32 units of oxidase + 116 × $10^{-6}$ mm cytochrome C total; ●, 64 units of oxidase + 11.6 × $10^{-6}$ mm cytochrome C total. $T = 38^\circ$, pH 7.15, hydroquinone 0.033 mm total.

**Effect of Oxygen Tension**

Since an "oxygen-carrying ferment" should operate efficiently at the relatively low oxygen tensions of the cell, it was of interest to study the effect of oxygen on the rate involving the oxidase-cytochrome C complex. This effect was studied under conditions in which there were high and low concentrations of oxidase with respect to the cytochrome. The results are illustrated in Fig. 5. In both cases the system reached its maximum catalytic activity
at approximately 70 mm. of O₂ and reached approximately 60 per cent of its maximum activity at 40 mm. of O₂, the oxygen tension estimated to exist in the cell.

This fact is not in itself sufficient evidence to distinguish whether this system is the oxygen-carrying ferment or is secondary to it. Although Warburg and Kubowit (5) have shown that certain cells respire at a maximum rate at much lower tensions, the oxygen requirements in various cases may not demand the maximum action of the oxidase-cytochrome system.

**DISCUSSION**

Our experiments lead to the hypothesis that there is a simultaneous adsorption or complex formation of cytochrome and oxygen with the oxidase, and that carbon monoxide inhibits the catalytic ability of the complex by displacement of the oxygen from the enzyme surface.

The existence of an oxidase-cytochrome complex is of considerable interest from the standpoint of tissue respiration. Keilin (6) found difficulty in attributing the different properties displayed by indophenol oxidase to one substance. If the oxygen-transporting enzyme is actually a complex between the oxidase and cytochrome C, then the difficulty is partly overcome, but the identity of this complex with Warburg's oxygen-carrying ferment is by no means proved. It must be remembered that Warburg's enzyme operates at much lower oxygen tensions (5) than found for the oxidase-cytochrome C complex and that it is defined by a particular CO spectrum (7) and by a band at 589 mμ (8) for its reduced form. Such spectra and properties have never been shown for cytochrome oxidase nor for any combination of it with the cytochromes. However, it is still possible that the oxidase itself is Warburg's oxygen-carrying ferment in the living cell. Unless further analysis of the known factors can yield a combination of properties (in addition to CO and cyanide sensitivity) approaching those of the oxygen-transporting enzyme, it seems inadequately proved that cytochrome C, either free or in complex form, is identical with Warburg's system. It may not even be justified to extend Warburg's conclusions to oxidations produced by heart muscle extract, since his oxygen-transporting enzyme has not been demonstrated in this tissue, and CO sen-
sitivity in white light is in itself insufficient evidence for the presence of this factor.

We are in complete agreement with the recent comments of Keilin and Hartree (4) concerning the views of the Japanese school; we could not confirm the isolation of a CO-insensitive indophenol oxidase reported by Yamagutchi, Tamiya, and Ogura (9).

SUMMARY

Evidence has been given to show that the aerobic oxidation of reduced cytochrome C in the presence of its oxidase operates through a cytochrome oxidase-cytochrome C complex. The point of attack of cyanide and carbon monoxide in the inhibition of hydroquinone oxidation was found not to be the free cytochrome, but the oxidase. The relation of oxygen tension to the rate of oxidation was studied, and the significance of these findings to tissue respiration is discussed.

BIBLIOGRAPHY

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