THE RÔLE OF THE CYTOCHROMES IN THE ACTION OF "INDOPHENOL OXIDASE"

BY ELMER STOTZ, A. E. SIDWELL, JR., AND T. R. HOGNESS

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

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The enzyme "indophenol oxidase" is the name given the indefinite factor or factors of the Warburg-Keilin respiratory system which lie between the cytochromes and oxygen. Keilin (1, 2) implied that he believes it to be a single substance and postulated that it is affected in the cyanide and carbon monoxide inhibition of respiring cells and heart muscle extract. Although he demonstrated a cooperation of indophenol oxidase and cytochrome C in the aerobic oxidation of cysteine, and Stotz et al. (3) reported similar results in the oxidation of ascorbic acid, the absolute dependence of oxidase action upon the presence of cytochrome C, up to the present time, has not been adequately proved. Indophenol oxidase is characterized by its ability to catalyze the aerobic oxidation of Nadi reagent to form indophenol blue, and of p-phenylenediamine (4, 5). These reagents have been commonly used to determine its activity, and because of lack of any other criterion have been implicitly assumed to be specific for this one substance. Mori et al. (6), in spite of the large number of substrates reported, have even gone so far as to ascribe a p-phenylenediamine-like structure to cytochrome C, on the basis of the similarity of the reactions between either of these two substances and indophenol oxidase.

In view of the high potential of cytochrome C (+0.262 volt at pH 7.4) found in this laboratory (7), it became apparent that the non-specificity of indophenol oxidase may be attributed to reduction of traces of cytochrome C by the various substrates of lower potential with subsequent enzymic oxidation of the reduced cytochrome C. This is more tenable when the state of purity of oxidase preparations used in the past (crude tissue
Cytochromes extracts) is considered. This assumption was substantiated by experiments in our laboratory on the rate of oxidation of leuco indophenols by crude oxidase preparations. The rate of oxidation changed with pH in such a way that for those pH values for which the potential of the dye was greater than that for cytochrome C, the reaction was very slow, while it was rapid at those pH values for which the potential of the dye was lower than that for cytochrome C. The most rapid change in velocity with changing pH occurred when the potential of the dye and that for cytochrome C were equal. It appeared, therefore, that the oxidation of these dyes was directly dependent upon cytochrome C and in a secondary way upon an oxidase.

Furthermore, these results convinced us that by using a substrate with a potential immediately below that of cytochrome C we might eliminate other factors of lower potential, such as cytochrome B, and thereby obtain a more unambiguous method of estimating the activity of cytochrome C oxidase. This procedure proved to be successful.

This paper, then, deals with the enzymic oxidation of hydroquinone and p-phenylenediamine, and attempts to demonstrate the multiplicity of the factors actually engaged in these systems, with a new concept of the function of the oxidase. Oxidase preparations have been made which alone were unable to cause appreciable oxidation of hydroquinone or p-phenylenediamine after the removal of cytochromes B and C. It was found that cytochrome C (and oxidase) was involved in the oxidation of both hydroquinone and p-phenylenediamine, and that cytochrome B which does not oxidize hydroquinone could function quite independently of this system in the oxidation of p-phenylenediamine.

During the drafting of this paper, Keilin and Hartree (8) published results which led them to the same conclusion with respect to this system. In a study of the oxidation of several substrates they concluded that “the oxidation of all these compounds, therefore, is not catalyzed directly by indophenol oxidase but through co-operation with cytochrome.” Their main evidence for this consisted of the facts that some of the substrates were oxidized relatively slowly by the crude indophenol oxidase preparations, although p-phenylenediamine was oxidized rapidly, and
that all the substrates were oxidized more rapidly upon addition of cytochrome C. Adequate proof for the theory would involve the preparation of oxidase which showed no activity with these substrates without addition of cytochrome C, together with a satisfactory account of other factors present and involved in the oxidation of the various substrates.

EXPERIMENTAL

The reactions were carried out in Warburg-Erlenmeyer type vessels with Barcroft manometers at 38° in an atmosphere of air. The substrates, placed in the side arm of the vessels, were un-buffered solutions of hydroquinone or p-phenylenediamine (0.033 mM total). After the usual equilibration and addition of the side arm contents, the total volume of reactants was always 3.3 cc. The reacting mixture was strongly buffered with 0.1 M phosphate solution of pH 7.15. The rate of shaking was 120 to 130 complete oscillations per minute, which permitted sufficient oxygenation of the solution for the highest velocities recorded in this paper.

The rate of oxygen uptake of these substrates in the presence of oxidase-cytochrome preparations was constant for at least the first 100 c.mm. The velocities, reported as c.mm. of O₂ per hour, are calculated from the initial rates of oxygen uptake.

Pure cytochrome C was prepared from beef heart according to the method of Keilin and Hartree (9) and conformed with the criteria of purity described in our earlier paper (7).

The crude indophenol oxidase preparation was prepared by an alkaline phosphate extraction of washed heart muscle, as described by Stotz and Hastings (5). This was the starting material for all the enzyme preparations used in these experiments and will be referred to as crude oxidase extract.

Oxidation of Hydroquinone

Hydroquinone itself showed a considerable rate of autoxidation in phosphate buffer of pH 7.15 and therefore this rate was necessarily determined as a blank in each of the hydroquinone experiments. The extent of this autoxidation is shown in Fig. 1, which also illustrates many of the arguments concerning the oxidation of hydroquinone by the oxidase-cytochrome system.
The addition of cytochrome C alone produced no change in the rate of autoxidation of hydroquinone. Upon addition of the hydroquinone to the cytochrome-containing solution, the solution changed color at a very fast rate, indicating that the reaction between hydroquinone and cytochrome C was a very fast one. Therefore, the slow rate of autoxidation of cytochrome C alone must account for its inability to catalyze the oxidation of hydroquinone.

Heat-inactivated (85° for 5 minutes) crude oxidase extract was also without effect on the autoxidation of hydroquinone, even upon the subsequent addition of cytochrome C.

Addition of crude oxidase extract alone to hydroquinone, however, caused a definite increase in the oxidation rate (Point 1, Fig. 1).
In different experiments this increase varied from 25 to 75 c.mm. of O$_2$ per hour. This variation was largely dependent on the thoroughness of washing of the tissue before phosphate extraction. A similar fact was noticed previously (3) in testing the activity of such a preparation towards succinate oxidation. Very probably exhaustive washing of the tissue removes some of the cytochrome C present, although inefficiently.

The addition of both cytochrome C and crude oxidase extract to the hydroquinone solution produced marked increases in the oxidation (curved line, Fig. 1). There is therefore a heat-labile cytochrome oxidase present in the preparation, which by itself very probably causes no oxidation of hydroquinone but which requires the cooperation of cytochrome C. The curve can be logically extrapolated (dotted line) to the autoxidation level to indicate the presence of small amounts of cytochrome in the oxidase preparation.

By a 2-fold precipitation of the oxidase with an equal volume of 0.2 M acetate buffer (pH 4.5) and resuspension in 0.1 M phosphate buffer (pH 7.4), the oxidase preparation produced by itself only 4 to 7 c.mm. of O$_2$ per hour increase in hydroquinone oxidation—practically no increase, since the individual determinations of the autoxidation of hydroquinone showed this much variation. Addition of cytochrome C to this preparation was still able to cause large increases in the oxidation rate of hydroquinone.

Observations with a Steinheil 3-prism short arm spectrograph showed the presence of cytochrome C in the supernatant phase (treated with hydrosulfite) from the first acetic acid precipitation, while a similar examination of crude oxidase extract showed cytochromes A, B, and C, with an especially strong C band (550 m$\mu$). After two acetic acid precipitations, the bands of cytochromes A and B were still visible in the resuspended precipitate, but the cytochrome C band was very faint.

The experiments illustrated in Fig. 1 offer a possibility of estimating the amount (in arbitrary units) of the oxidase factor present, which in itself cannot oxidize hydroquinone, but in conjunction with cytochrome C can produce a rapid oxidation. Since with a given amount of oxidase preparation a limiting velocity was reached upon the addition of cytochrome C, the velocity of oxidation should therefore be proportional to the...
oxidase concentration when cytochrome C is in excess. In making such a test a total amount of cytochrome C equal to $1 \times 10^{-4}$ mM was chosen with a maximum enzyme concentration represented by a velocity (excluding the autoxidation of hydroquinone) not exceeding 600 c.mm. of O$_2$ per hour. A blank autoxidation of hydroquinone was in each case determined in the presence of the same amount of enzyme preparation treated at 85° and subtracted from the velocity obtained with the untreated enzyme and cytochrome C. Under the conditions specified the velocity of oxidation was not affected by additional amounts of cytochrome C which might occur in crude oxidase preparations. The proportionalities obtained are recorded in Table I.

**Table I**

*Estimation of Oxidase Activity*

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Velocity*</th>
<th>Ratio of velocities</th>
</tr>
</thead>
<tbody>
<tr>
<td>cc.</td>
<td>c.mm. O$_2$ per hr.</td>
<td></td>
</tr>
<tr>
<td>0.4</td>
<td>568</td>
<td>4.05</td>
</tr>
<tr>
<td>0.2</td>
<td>276</td>
<td>1.97</td>
</tr>
<tr>
<td>0.1</td>
<td>140</td>
<td>(1.00)</td>
</tr>
<tr>
<td>0.3</td>
<td>592</td>
<td>2.98</td>
</tr>
<tr>
<td>0.15</td>
<td>298</td>
<td>1.51</td>
</tr>
<tr>
<td>0.1</td>
<td>198</td>
<td>(1.00)</td>
</tr>
</tbody>
</table>

*After the autoxidation rate of hydroquinone is subtracted.

For convenience we shall designate a unit of oxidase as that amount which, under the conditions specified, produces a 10 c.mm. of O$_2$ per hour increase in excess of the autoxidation rate of hydroquinone.

*Oxidation of p-Phenylenediamine*

p-Phenylenediamine, although possessing a much lower potential than hydroquinone (10), shows in itself no autoxidation under the conditions of our experiments. However, the addition of crude oxidase extract produces a rapid oxidation, and it is this reaction which has been so widely used in the past for the estimation of indophenol oxidase activity. This oxidation was found
to be due, at least in part, to cytochrome C, since addition of
the latter caused marked increases in the velocity of oxidation
(in the presence of oxidase). p-Phenylenediamine, like hydro-
quinone, reduces cytochrome C rapidly.

A comparison of the cyanide sensitivity of hydroquinone and
p-phenylenediamine oxidations by the same amounts of crude
oxidase extract and cytochrome C revealed significant results.

![Graph showing sensitivity to cyanide](http://example.com/graph.png)

**Fig. 2.** The sensitivity of hydroquinone and p-phenylenediamine
oxidations to cyanide. ● represents hydroquinone; □, p-phenylene-
diamine. \( T = 38^\circ \), pH 7.15, 15 units of oxidase, \( 23.5 \times 10^{-4} \) mm cytochrome C total.

The per cent inhibitions produced by increasing amounts of
cyanide are shown in Fig. 2. In contrast to the rapid approach
to 100 per cent inhibition of hydroquinone oxidation, the p-phenyl-
ylenediamine oxidation, after an initial cyanide-sensitive cataly-
sis, showed a relatively cyanide-insensitive reaction. Further
large increases in the cyanide concentration were able to cause
complete inhibition of this reaction also. These facts indicate
that p-phenylenediamine oxidation involves a relatively non-
cyanide-sensitive catalyst, besides cytochrome C and its oxidase, which is present in the oxidase preparation and is non-operative in hydroquinone oxidation.

It is believed from the following evidence that this factor is cytochrome B.

Ball (11) estimated the potentials of the three cytochromes in a preparation comparable with our crude oxidase extract. He gave the figure $-0.04$ volt for cytochrome B, which would exclude it as an efficient catalyst for the hydroquinone oxidation. As indicated by spectrographic observation, $p$-phenylenediamine reduces cytochrome B.

Keilin (1) demonstrated, and we have confirmed, that when the reduced cytochromes present in a crude oxidase preparation are treated with small amounts of cyanide and subsequently exposed to air, only the cytochrome B is oxidized, as indicated by the disappearance of its characteristic band. This indicates that cytochrome B is relatively insensitive to cyanide, is autoxidizable, and as a catalytic agent can act independently of the cytochrome C oxidase.

![Diagram](http://www.jbc.org/)
A study was made of the effect of successive acetate precipitations on the ability of the resulting preparation to oxidize \( p \)-phenylenediamine. Each preparation was tested with hydroquinone for its activity in units of oxidase and an equal number of units were used in the \( p \)-phenylenediamine test. At the same time, the effect of small amounts of cyanide was tested. The results are shown in Fig. 3. The relative amounts of cyanide chosen for these experiments were based upon the previous determinations illustrated by Fig. 2. The first rapid drop very probably represents inhibition of the oxidase-cytochrome C system, and the remaining oxidation, which is relatively insensitive to cyanide, represents cytochrome B catalysis. Keeping in mind that all these preparations contained equal amounts of oxidase (hydroquinone + cytochrome C test), we find the acetate precipitations must have eliminated the cytochrome C for the most part (the points on the ordinate with no cyanide added) and much of the cytochrome B (right portions of curves). These conclusions were confirmed by spectrographic observations on thick suspensions of the enzyme.

By a combination of three acetate precipitations (100 cc. of oxidase + 100 cc. of 0.2 M acetate buffer of pH 4.5 + 200 cc. of \( \text{H}_2\text{O} \)) and a 65 to 70 hour dialysis against water, with a final resuspension in phosphate buffer (pH 7.4), a preparation was obtained which was nearly completely unable to oxidize \( p \)-phenylenediamine (6 to 10 c.mm. of \( \text{O}_2 \) per hour). Addition of cytochrome C to such a preparation established rapid oxidation of either hydroquinone or \( p \)-phenylenediamine. Such a preparation showed very weak bands of cytochromes B and C even in thick suspension, but a strong band at 603 m\( \mu \) (cytochrome A). Thus \( p \)-phenylenediamine oxidation can occur both through the oxidase-cytochrome C system and independently through that involving cytochrome B.

The effect of temperature on the oxidase was studied. Employing the rate of oxidation of \( p \)-phenylenediamine as a measure of indophenol oxidase, Keilin (1) concluded earlier that this enzyme was destroyed by heat at 65°, but this test is a combined measure of the oxidase, as we now define it, and of cytochromes C and B. We have now found that heating an oxidase preparation at 52° for 20 minutes completely destroys its ability to cooperate with...
Cytochromes

cytochrome C in the oxidation of hydroquinone. Such a preparation nevertheless still oxidizes p-phenylenediamine, though at a considerably lower rate. Spectrographically, all three cytochromes are intact in such a preparation. Since only cytochrome B is autoxidizable, the remaining catalysis must be due to this component.

Cytochrome A

We have considered the possible rôle of cytochrome A in these oxidation systems, but have not been able to attribute to it any special function. The following points were considered to substantiate such a viewpoint.

Neither the true oxidase content (cytochrome C addition on hydroquinone oxidation) nor the ability of the preparations alone to oxidize p-phenylenediamine or hydroquinone showed any obvious relationship to the concentration of cytochrome A as determined by the intensity of the cytochrome A band. (We are still limited to the spectrograph for the detection and even definition of cytochrome A.)

Active oxidase preparations containing little or no cytochrome C, but nevertheless a strong cytochrome A band, were unable to catalyze the oxidation of hydroquinone. This was true in spite of the favorable potential (+0.29 volt) of cytochrome A reported by Ball (11).

It would appear then that although cytochrome A undoubtedly undergoes oxidation and reduction during the oxidase-cytochrome C catalysis of hydroquinone oxidation, a catalytic function of cytochrome A would not only be dependent on the oxidase, but upon cytochrome C as well. It would appear from the work thus far that cytochrome A is not essential to the catalytic function of the systems studied.

SUMMARY

It has been shown that the oxidation of hydroquinone by heart muscle extract, which has been attributed to an enzyme, indophenol oxidase, is actually due to two factors: cytochrome C and a cytochrome oxidase. The oxidation of p-phenylenediamine by such an enzyme, which has also been attributed to the entity indophenol oxidase, in this case is due not only to cytochrome C and its oxidase,
but also to cytochrome B. The oxidase appears to be specific only for the oxidation of cytochrome C. In conjunction with cytochrome C, this system, from thermodynamic considerations, can oxidize a variety of substances (due to the high potential of the "electromotively active" cytochrome C) followed by rapid aerobic oxidation of cytochrome C in the presence of its oxidase. The enzymic portion of the catalytic system should be referred to as cytochrome oxidase and the term indophenol oxidase abandoned. This term has been suggested before and is used by some, but without adequate experimental basis.

Active preparations of the oxidase have been made which alone lack the ability to oxidize either hydroquinone or p-phenylenediamine, but produce rapid oxidations upon the addition of cytochrome C. A method has been devised for the estimation of cytochrome oxidase activity which is independent of the cytochrome B and C content of the preparations and which is based on a more fundamental character of the oxidase. The use of former tests for indophenol oxidase would hardly be expected to be used to advantage in the concentration of the oxidase itself, since they are dependent on both the oxidase and cytochromes present. The use of this more unambiguous test for the oxidase is expected to aid in the concentration and isolation of this important respiratory enzyme.

Whatever the dependence of the cytochromes in intact cellular respiration, we have shown that the oxidase-cytochrome C system can function independently of cytochrome B in the oxidation of hydroquinone or p-phenylenediamine. Conversely, cytochrome B can act catalytically without the intervention of the oxidase system. The rôle of cytochrome A has not been evident from these experiments but has been discussed.

BIBLIOGRAPHY

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