The Reaction of Cytochrome Oxidase with Cytochrome c*

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In recent work on the reaction between oxygen and reduced cytochrome oxidase made by the method of Yonetani (1), Gibson and Greenwood (2) found that the reaction is complex and that at least two spectroscopically distinguishable reactions must take place. The kinetic changes were interpreted in terms of a scheme proposed by Keilin and Hartree (3), according to which cytochrome oxidase preparations contain two components: cytochrome a₃, which reacts directly with oxygen, and cytochrome a, which reacts with cytochrome a₃ and cytochrome c but not with oxygen. The experiments afforded a semiquantitative confirmation of the difference spectra between the oxidized and reduced forms of cytochromes a and a₃ found by Yonetani (1), and led to the conclusions that the initial reaction between oxygen and reduced cytochrome a₃ is very fast indeed, with an apparent second order rate constant of 5 x 10⁷ M⁻¹ sec⁻¹, and that the reaction between cytochromes a₃ and a (presumed first order) has a rate constant of 750 sec⁻¹ at 20°.

Yonetani's preparation is soluble only in the presence of the nonionic detergent Emasol 4130 (Kao Soap Company, Tokyo), and the results obtained with it, like those obtained with other soluble preparations, are subject to criticism as artifacts. The active, deoxycholate-stabilized preparation of Griffiths and Wharton (4) has now been compared with Yonetani's preparation, and has been found to agree closely with it in the properties examined. This agreement between enzymes prepared by different procedures from different starting materials goes some way to meet the criticism of artificiality, since there is no a priori reason why such close parallelism between the preparations should have been found unless their behavior is a reflection of the underlying properties of the enzyme. In addition to the experiments on the oxidation of the enzyme, we also report on its reaction with cytochrome c, and on some properties of a more complex system with oxygen, oxidase, cytochrome c, and ascorbic acid.

EXPERIMENTAL PROCEDURE

Cytochrome oxidase was prepared by the method of Griffiths and Wharton (4) or Yonetani (1). Concentrations are expressed in terms of heme a based on a value of E (605 mμ minus 630 mμ) reduced of 16.5 mM⁻¹ cm⁻¹. In the experiments with the Griffiths-Wharton enzyme, 0.1% recrystallized deoxycholate was present in all solutions, and 0.1% Emasol was present in experiments with Yonetani's preparation. The buffers used were 0.1 M sodium phosphate, pH 7.6, and 0.1 M Tris, pH 8.1. The monomeric form of horse heart cytochrome c was obtained either as a gift from Dr. E. Margoliash or by isolation from the Sigma type III product by chromatography on Amberlite CG-50 (5). Reduced cytochrome c was prepared by the addition of a slight excess of solid potassium ascorbate to a concentrated (approximately 10 mg per ml) solution of cytochrome c. This was dialyzed overnight against degased phosphate buffer in a closed vessel. The product was better than 99% reduced. Tetrachlorohydroquinone was an Eastman Kodak product. Ascorbate was routinely used as the potassium salt (6).

The stopped flow and flash flow spectrophotometers and associated equipment have been described (2, 7). In order to study the time course of appearance of photosensitivity on reduction of the oxidase in the presence of CO, a special cell was constructed (Fig. 1). This had two windows, which allowed passage of the observation beam from the monochromator to the photomultiplier, while a third window, placed at right angles to the other two, admitted the photolysis light. The latter came from a high voltage discharge tube which was filled with argon at a pressure of 5 cm of Hg and had a flat window at one end. The discharge energy of 100 joules was stored in a 4 microfarad condenser, and was released with a spark gap and trigger as described previously (2). The photolysis path length was 2 mm, and a rhodium-plated mirror opposite the entry window increased the efficiency of energy transfer. The volume between the mixer and the point of observation was 50 μl, and with a flow rate of 30 ml per second, photochemical excitation of the reaction mixtures was possible about 2 milliseconds after the reactants were mixed.

RESULTS

Comparison of Preparations—One object of this work was to compare the Griffiths-Wharton preparation with that of Yonetani, and experiments were carried out to examine its reactions with oxygen and carbon monoxide under conditions similar to those used previously by Gibson and Greenwood (2). The results are summarized in Table I, and show that there is a close similarity of general behavior in all the reactions examined, with the exception of the reduction of Yonetani's preparation by cytochrome c, which is about 10 times slower than the corre-
spending reaction of the Griffiths-Wharton preparation, under the same conditions.

In particular, it should be noted that the measured rate of the oxygen reaction, expressed as a second order rate constant, is lower at high oxygen concentrations for both preparations. The rate of reaction with carbon monoxide, on the other hand, was accurately proportional to the concentration of ligand, and showed a similar temperature dependence in both cases. The Griffiths-Wharton preparation, however, showed some evidence of heterogeneity, about 10 to 20% of the oxidase reacting less rapidly than the remainder.

Gibson and Greenwood (8) have examined the reaction of the oxidase-CO compound with O$_2$ in detail. Their somewhat complex results cannot readily be summed up in tabular form, except for the numerical value of the dissociation velocity constant for CO. As shown in Table 1, this value was similar for the two preparations. In addition, the other features of the reaction, in particular the dependence of the spectrophotometric changes on wave length of observation, agreed well.

**Reaction of Cytochrome Oxidase with Cytochrome c**—Addition of reduced cytochrome c to cytochrome oxidase under "anaerobic" conditions (approximately $10^{-2}$ M oxygen) initiates biphasic optical density changes at 445, 550, and 605 m$\mu$. The fast stage of these changes is substantially complete within 10 milliseconds (Fig. 2), while the slower stage requires several seconds for completion. Analysis of the changes suggests that the rapid phase represents the reduction of cytochrome a. Thus, repetition of the reaction in the presence of 2 x $10^{-3}$ M cyanide gives a similar rapid phase under conditions in which the reduction of cytochrome a$_3$ may be excluded (cf. Yonetani (1)). This interpretation is further borne out by detailed analysis of the spectrophotometric changes at the other wave lengths studied. Thus, Fig. 3 shows that when the absorbance change at 445 m$\mu$ is plotted against the change at 605 m$\mu$, a straight line is obtained with a slope, after correction for the contribution at 445 m$\mu$ due to the oxidation of cytochrome c, of 3.8. This is close to the ratio $\Delta E_{445}/E_{605}$ of 4.4 found by Yonetani (1), Horie and Morrison (9), and Lemberg et al. (10), who give values of 4.5, 3.7, and 3.1, respectively.

A similar analysis of the slow phase of the reaction, i.e., from 6 milliseconds after initiation to completion some 10 seconds later (point marked ~ in Fig. 2), suggests that in the absence of cyanide the slow phase represents the reduction of cytochrome a$_3$. Thus, it is strongly influenced by cyanide, which increases its extent at 605 m$\mu$ and decreases it at 445 m$\mu$, as compared with the "anaerobic" experiment. In the presence of cyanide, the ratio $\Delta E_{445}/E_{605}$ is again about 4, which is consistent with changes involving cytochrome a only, while in the absence of cyanide the ratio is about 16, which is within the range of ratios found for changes involving cytochrome a$_3$ given by Lemberg et al. (10), who found an average value of 19 and a range of 15 to 27, and is close to values of 13 (1) and 18.4 (9) reported by others.

**Appearance of Photosensitivity** The correctness of the allocation of the slow and fast phases of the reaction between cytochrome oxidase and cytochrome c to changes involving cytochromes a$_3$ and a, respectively, has been further confirmed by following this development of photosensitivity when the reaction is carried out anaerobically in the presence of CO.

Since CO can combine only with a$, the appearance of photosensitivity is an index of the rate of formation of reduced cytochrome a$_3$, provided that the rate of combination of a$_3''''$ with CO is rapid compared with the rate of formation of a$_3''''$ from a$_3'''''$. When cytochrome oxidase equilibrated with CO was allowed to react with reduced cytochrome c, the half-time for formation of a$_3''''$ was about 5 milliseconds. However, the half-time for the formation of a$_3''''$/CO was 1.2 seconds (Fig. 4). As the half-time for combination of a$_3''$ and CO under these conditions is about 20 milliseconds, it would appear that the transfer of electrons from a$_3''$ to a$_3''''$ is a comparatively slow process and is rate-limiting under the conditions of the experiment.

**Reaction between Cytochromes a and c**—The rate constant for the reaction is approximately $4 \times 10^7$ M$^{-1}$ set$^{-1}$ for the Griffiths-Wharton preparation, with the concentration of oxidase expressed in terms of heme a. This rate is probably within an order of magnitude of that expected for a diffusion-controlled reaction between molecules of this size (cf. Alberty and Hammes (11)). Cytochrome oxidase prepared according to Yonetani (1) also reacts rapidly with cytochrome c, and the results at three levels of cytochrome a are shown in Fig. 5. The reaction does not go to completion even at the lowest concentration of reducing agent when the ratio of oxidase heme to cytochrome c is 4:1.

To measure the rate of the reaction, tangents were drawn to the initial part of the curve. The reaction is second order with a rate of $3 \times 10^9$ M$^{-1}$ set$^{-1}$, with the enzyme concentration expressed in terms of heme a, and on the assumption that cyanide blocks one-half of the heme a and one-half of the enzyme copper (12). In Fig. 6 the changes in absorbance at 550 and 605 m$\mu$ are plotted against one another, and lie closely on parallel straight lines for the three experiments. This result will be considered further (see "Discussion").

The effect of temperature on the reaction between cytochrome c and Yonetani's preparation has been determined by carrying out experiments similar to those of Figs. 5 and 6 at temperatures between 0° and 30°. The results indicate an apparent heat of activation of 15 kcal per mole. As the rate constant is somewhat larger than would be expected to correspond to this value, the reaction may perhaps proceed through an intermediate complex.

**Reduction of Cytochrome a$_3$**—One possible interpretation of the
TABLE I
Comparison of some properties of cytochrome oxidase prepared according to Yonetani (7) and Griffiths and Wharton (4)

<table>
<thead>
<tr>
<th>Reaction studied*</th>
<th>Yonetani preparation†</th>
<th>Griffiths-Wharton preparation</th>
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<td>Temperature</td>
<td>Rate constant</td>
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| Combination with CO observed at 445 m
| 21°C            | 7.2 × 10³ M⁻¹ sec⁻¹ (Q₁₀ = 1.3) | 20°C          | 7.5 × 10³ M⁻¹ sec⁻¹† (Q₁₀ = 1.4) |
| Dissociation of CO by oxygen observed at 585 m
| 20               | 0.023 sec⁻¹ | 21             | 0.025 sec⁻¹‡ |
| Reaction of oxygen with reduced cytochrome oxidase observed at 445 m
| 14 μM O₂       | 6 × 10⁷ M⁻¹ sec⁻¹ | 20          | 2.7 × 10⁷ M⁻¹ sec⁻¹‡ |
| 70 μM O₂       | 1 × 10⁶ M⁻¹ sec⁻¹ | 20          | 5.5 × 10⁶ M⁻¹ sec⁻¹‡ |
| Reduction of cytochrome oxidase by cytochrome c observed at 445 m
| 21              | 4 × 10⁶ M⁻¹ sec⁻¹ | 20          | 4 × 10⁷ M⁻¹ sec⁻¹† |

* For details of the techniques employed, see References 2 and 8.
† Buffer was 0.1 M phosphate, pH 7.6.
‡ Buffer was 0.1 M Tris, pH 8.1.

Fig. 2. Reduction of cytochrome oxidase (9.6 × 10⁻⁸ M heme a) by 11.2 × 10⁻⁶ M reduced cytochrome c followed in the stopped flow apparatus at 445 and 605 m. Temperature, 21°C; buffer, 0.1 M phosphate, pH 7.6. The experiment was performed either "anaerobically" (2 × 10⁻⁷ M oxygen) (O) or in the presence of 2 × 10⁻⁵ M cyanide (●). Concentrations given are those after mixing.

Fig. 3. The relation between the absorbance changes at 445 m and those at 605 m, obtained from the data of Fig. 2. The slope of the line is 4.8. After correction for the contribution at 445 m due to the oxidation of cytochrome c, the slope becomes 3.8.

Fig. 4. The rate of appearance of photosensitivity in 2.5 × 10⁻⁵ M cytochrome oxidase during reduction by 5 × 10⁻⁴ M cytochrome c in the presence of 5 × 10⁻⁴ M carbon monoxide. The figure shows the absorbance changes at 445 m (●) and 550 m (O) on the millisecond time scale, and the appearance of photosensitivity (O) on the time scale in seconds. The buffer was 0.1 M phosphate, pH 7.6, containing 0.1% deoxycholate; path, 4 mm; temperature, 20°C.

slow rate of electron transfer from a'' to a''' is the establishment of an unfavorable oxidation-reduction equilibrium because of the presence of both oxidized and reduced cytochrome c. This supposition is not supported by the results of experiments in which tetrachlorohydroquinone was mixed with oxidized cytochrome c and oxidized cytochrome oxidase.

At 15°C, in 0.1 M phosphate, pH 7.4, tetrachlorohydroquinone reduced cytochrome c quite efficiently, with a second order rate constant of 2 × 10⁵ M⁻¹ sec⁻¹ (Fig. 7). This rapid reaction indicates that free oxidized cytochrome c must have a short half-life in the complete system, which tends to minimize possible effects due to equilibria. Even so, the slow reduction of cytochrome a still persists (Fig. 7; note that the scale for the reduction of cytochrome a is in milliseconds, and that for cytochrome a₂ in seconds).
As tetrachlorohydroquinone is able to react directly with cytochrome oxidase, albeit relatively slowly, it was of interest to determine the rates of appearance of photosensitivity in the

![Figure 5](http://www.jbc.org/)

**Fig. 5.** Oxidation of reduced cytochrome c by $5 \times 10^{-4} \text{M}$ cytochrome oxidase (Yonetani) in the presence of $1 \times 10^{-4} \text{M}$ cyanide. Temperature was $10^\circ$. ○, $1.25 \mu \text{M}$ reduced cytochrome c; ○, $2.5 \mu \text{M}$ reduced cytochrome c; ●, $5 \mu \text{M}$ reduced cytochrome c. The final changes in optical density are indicated. Path length, 2 cm; concentrations given are those after mixing.

![Figure 6](http://www.jbc.org/)

**Fig. 6.** The relation between the absorbance changes at 550 mp and the absorbance changes at 605 mp from the data of Fig. 5. Symbols are the same as in Fig. 5. The scales for $\Delta E_{655}$ are displaced for clarity.

![Figure 7](http://www.jbc.org/)

**Fig. 7.** Time course of reaction of various components on addition of $7 \times 10^{-4} \text{M}$ tetrachlorohydroquinone to $1 \times 10^{-3} \text{M}$ cytochrome c, and $1 \times 10^{-3} \text{M}$ cytochrome c + $5 \times 10^{-4} \text{M}$ cytochrome oxidase (Yonetani). ○, cytochrome c alone; △, cytochrome c in mixture; ○, cytochrome a; ●, cytochrome a3. Note that the cytochrome a3 reaction is on the long time scale. Temperature, $15^\circ$; phosphate buffer, $0.1 \text{M}$, pH 7.4.

![Figure 8](http://www.jbc.org/)

**Fig. 8.** Extended time course of the absorbance changes at 550 mp in the stopped flow apparatus from 3 milliseconds after mixing onward. The oxidation of $11.2 \mu \text{M}$ cytochrome c after mixing with $5.6 \mu \text{M}$ cytochrome oxidase in the presence of $11 \mu \text{M}$ oxygen is shown. The buffer was $0.1 \text{M}$ phosphate, pH 7.6. Temperature of the reaction medium was $20^\circ$; path, 2 cm.

![Figure 9](http://www.jbc.org/)

**Fig. 9.** Reaction of $5 \times 10^{-6} \text{M}$ cytochrome oxidase and $1.5 \times 10^{-5} \text{M}$ cytochrome c with $1 \times 10^{-2} \text{M}$ ascorbate after mixing with $3 \times 10^{-3} \text{M}$ oxygen, followed at 550 and 605 mp in the stopped flow apparatus at a temperature of $20^\circ$. The buffer used was $0.1 \text{M}$ phosphate, pH 7.6. ○, cytochrome a; ●, cytochrome c.

In one experiment with the Griffiths-Wharton cytochrome oxidase, in which $2 \times 10^{-4} \text{M}$ tetrachlorohydroquinone and $5 \times 10^{-4} \text{M}$ CO were used, the half-time both of the reduction of cytochrome a and of the onset of photosensitivity was approximately 15 seconds. Thus, in this case the reduction of a and a3 kept closely in step.

**Turnover Experiments**—The slow rate of electron transfer from cytochrome a to a3 has a pronounced effect on turnover experiments. Thus, when reduced cytochrome c is mixed with cytochrome oxidase in the presence of excess oxygen, there is an initial rapid oxidation of cytochrome c by a, followed by a slow prolonged oxidation of c as electrons are slowly transferred to a3 and thus to oxygen (Fig. 8).

Yonetani (13) has reported studies of the system containing his preparation of cytochrome oxidase, ascorbic acid, cytochrome c, and oxygen. Using the Griffiths-Wharton enzyme, we have performed parallel experiments, one of which is illustrated in Fig. 9. These data show that when oxygen is introduced into the mixture containing ascorbate, cytochrome c, and cytochrome oxidase, there is an initial rapid oxidation of cytochromes c and a during the first 10 milliseconds or so. These changes may be accounted for by the oxidation of cytochrome a by molecular oxygen at an estimated initial rate of 150 sec$^{-1}$, followed by a transfer of electrons from cytochrome a at a rate of 750 sec$^{-1}$ and from c to a at the rate measured in the earlier experiments.
After this rapid oxidation (about 10 milliseconds), there follows a phase of slow reduction of cytochrome $a$. During this second reaction, cytochrome $c$ continues to be oxidized, although much less rapidly than in the initial rapid phase. These observations can be explained by supposing that the rate-limiting step has been shifted from the reduction of cytochrome $c$ by ascorbate (during the initial rapid phase) to the reduction of cytochrome $a_3$ by cytochrome $a$ (during the second phase). The transfer of the rate-limiting step is attributed to the formation of a slowly reacting complex of cytochromes $a$ and $c$ (cf. References 14 and 15) and is dealt with under “Discussion.”

**Reaction of Cytochrome $c$ with Ascorbate**—The conclusion that an inhibitory complex is present is further supported by the turnover number, which is of the order of 1 electron equivalent per second per mole of heme iron. These low turnover numbers are due, in part, to the rather slow reaction between ascorbate and cytochrome $c$, which has been studied in some detail by Greenwood and Palmer.1 The most striking feature of their results is the pronounced pH dependence, the rate increasing by over two orders of magnitude when the pH was raised from 7.2 to 9.4. In addition, it was noticed that the reaction changed from a simple monotonic function at pH 7.2 to a markedly biphasic reaction under the more alkaline conditions. Thus, at pH 9.4, there is an initial slow reduction with a rate of 1.5 X 10^-7 M^-1 sec^-1 involving about 70% of the cytochrome $c$, and a very slow reaction ($k = 4$ X 10^-7 sec^-1) accounting for the remainder. The extent of the slow reaction was independent of cytochrome $c$ concentration but increased with increasing pH. The fast reaction is not affected by either ethylene-diaminetetraacetic acid (2 X 10^-4 M) or deoxycholate (0.1%), but is reduced in the presence of phosphate. In one experiment, 0.05 M phosphate decreased the rate to about 50% of that found in Tris alone. This is in qualitative agreement with previous results (16).

**Evidence of Branching between Cytochromes $a$ and $a_3$**—In studies of the reaction between the CO compound of cytochrome oxidase and oxygen. Cytochrome oxidase (Yonetani) (6 X 10^-9 M) and cytochrome $c$ (3 X 10^-7 M) were reduced with 2 X 10^-4 M ascorbate, equilibrated with 50 mm phosphate buffer, pH 6.5, and subsequently were mixed with an equal volume of 0.1 M phosphate buffer, pH 7.2, equilibrated with 150 mm phosphate. The wavelength of observation and the extinction coefficients after mixing are as follows: Curve 1, heme $a = 2$ X 10^-5 M, 605 mυ; Curve 2, heme $a = 5$ X 10^-5 M, 644 mυ; Curve 8, heme $a = 6$ X 10^-7 M, 445 mυ; Curve 7, heme $a = 2$ X 10^-7 M, 445 mυ; Curve 8, heme $a = 3$ X 10^-7 M, 685 mυ. For Curve 4, the ordinate is percentage of $a_3$ CO remaining. The lines were calculated from the equation given in the text. The light path was 2 cm, and the temperature 22.5°C.

And O$_2$, Gibson and Greenwood (8) have presented evidence that electron transport does not follow a strictly linear scheme, $a_3 \rightarrow a \rightarrow c$, and that branching between $a_3$ and $a$ must occur. Their kinetic experiments did not show whether the cross-linking reaction of $a_3$ and $a$ took place between separate oxidase units or within stable groups of oxidase units having a group life-time that was long compared with the time of oxidation of cytochrome $a$, though they favored the former interpretation because of the large size of the group (about 8 oxidase units) required for the second. We have now extended the analysis of Gibson and Greenwood (who were chiefly concerned to determine the number of spectrophotometrically distinguishable changes going on in the reaction) and have examined the effect of concentration of the reagents on the oxidation of heme $a$ when $O_2$ is mixed with the CO compound of cytochrome oxidase (Fig. 10). The figure shows the general form of the results. The oxidation of cytochrome $a$ (Curves 1 through 5) is always faster than the oxidation of cytochrome $a_3$ (Curve 6), and is concentration-dependent, though rather mildly so. Further, the oxidation of cytochrome $a$ accelerates markedly at first, and then settles to an apparently constant rate. These results are considered further below.

**DISCUSSION**

During the last few years the availability of purified preparations of cytochrome oxidase has stimulated a considerable amount of experimentation (cf. References 1, 2, 8, 13, 17, 19-21). While the elementary properties of these preparations have tended to a common norm, the rather unconventional techniques employed in their isolation have justified reservations about the significance of such data. The finding that two of these preparations, despite important differences in source and method of purification, exhibit very similar responses in reactions with oxygen and carbon monoxide is of considerable encouragement to those trying to elucidate the mechanism of this system. Thus, in Table I, we find that the two preparations have a similar rate of combination with CO. In addition, the complicated spectral changes occurring during the reaction of CO oxidase with $O_2$ (8) are observed with both preparations, and the displacement of CO by $O_2$ is equal in rate with enzyme prepared by either method. Furthermore, both enzymes are very rapidly oxidized by molecular oxygen, the 2-fold faster reaction of the Yonetani preparation, although probably significant, is not certainly so, since variations of the order of 20% were found in earlier work (2). There may be an appreciable difference in the ability of these two proteins to react with cytochrome $c$, the Griffiths-Wharton preparation reacting as much as 10 times faster than the Yonetani preparation, although it must be emphasized that even the slower rate is still rapid for a reaction between two proteins.

Addition of reduced cytochrome $c$ to the Griffiths-Wharton oxidase produces a very rapid reduction of cytochrome $a$ with a rate of about 4 X 10^-8 M^-1 sec^-1. (when the enzyme concentration is expressed in electron equivalents). The conclusion that this fast reaction involves cytochrome $a$ alone, and not cytochrome $a_3$, is based on three considerations. First, the ratio of absorbance changes at 645 mυ to those at 605 mυ agrees closely with the ratio predicted from the extinction coefficients of cytochrome $a$. Thus we would have expected ratios of about 4 for cytochrome $a$ alone, of about 15 for cytochrome $a_3$ alone, and of about 7 for cytochrome $a$ and $a_3$ together (1, 9, 10). Second, the rapid changes are duplicated in the presence of cyanide; under these
other complexes do not exist. The only complex discussed here is that between $c^+$ and $a^+$, for which would be expected to be a first order process. Although slow dissociation of the cytochrome $c$-cytochrome $a$ complex, lytic oxidation by cytochrome oxidase is a consequence of the first order disappearance of ferrocytochrome $c$ during cata-
quent reaction is very slow. It is a reasonable supposition that an initial oxidation of cytochrome $c$ by cytochrome $a^+$, the subse-
experiments in the absence of ascorbate (Fig. 8), in which, after,further consequence of complex formation is observed in turnover of cytochrome oxidase is then controlled by the very slow rate
and a are rapidly oxidized. The reduced cytochrome $c$ then binds to cytochrome $a^+$, transfers its electron, and forms the inactive cytochrome $c^{'''}$-cytochrome $a^{''}$. Subsequent turnover of cytochrome oxidase is then controlled by the very slow rate of dissociation of cytochrome $c^{'''}$ from the complex $a^{'''}-c^{''}$. A further consequence of complex formation is observed in turnover experiments in the absence of ascorbate (Fig. 8), in which, after an initial oxidation of cytochrome $c$ by cytochrome $a$, the subse-
ion that $A_{E200}$ for cytochrome $a$ is 19.5 (12) and $A_{E550}$ for
ments employed, with a slope of 1.6 in each case. On the assumption that $A_{E200}$ for cytochrome $a$ is 19.5 (12) and $A_{E550}$ for cytochrome $c$ is 21 (15), a slope of 1.1 would have been antici-
data.

The discovery that, in the presence of cytochrome $c$, electron transfer from $a$ to $a_3$ is slow is somewhat unexpected in view of the observation (2) that during reoxidation experiment the rate of this reaction is at least 750 sec$^{-1}$. Perhaps the simplest explanation of this discrepancy and one in accordance with other work (14, 15) is that a complex is formed between $c^{'''}$ and $a^+$, and that this complex does not readily transfer electrons to $a_3^{'''}$. Such an explanation is consonant not only with the results of the experiments in which oxidized oxidase was allowed to react with $c''$ anaerobically, but also with the experiments in the presence of excess $O_2$ (Fig. 8).

The experiments with the complex reaction system (ascorbate, cytochrome $c$, cytochrome oxidase, oxygen) also fit in with the suggestion of complex formation. Initially, when oxygen is added to reduced oxidase and reduced cytochrome $c$, both $a_2$ and $a$ are rapidly oxidized. The reduced cytochrome $c$ then binds to cytochrome $a^+$, transfers its electron, and forms the inactive cytochrome $c^{'''}$-cytochrome $a^{''}$. Subsequent turnover of cytochrome oxidase is then controlled by the very slow rate of dissociation of cytochrome $c^{'''}$ from the complex $a^{'''}-c^{''}$. A further consequence of complex formation is observed in turnover experiments in the absence of ascorbate (Fig. 8), in which, after an initial oxidation of cytochrome $c$ by cytochrome $a$, the subsequent reaction is very slow. It is a reasonable supposition that the first order disappearance of ferrocytochrome $c$ during cata-
lytic oxidation by cytochrome oxidase is a consequence of the slow dissociation of the cytochrome $c$-cytochrome $a$ complex, which would be expected to be a first order process. Although the only complex discussed here is that between $c^{''}$ and $a^{''}$, for which evidence is presented, no implication is intended that other complexes do not exist.

As the reaction between reduced cytochrome $c$ and Yonetani's preparation is significantly slower than that with the Griffiths-Wharton enzyme, it is possible to correlate the absorbance changes at 550 and 605 m$\mu$ with some precision (Fig. 6). The points lie closely on a straight line at each of the three concentrations employed, with a slope of 1.6 in each case. On the assumption that $\Delta E_{200}$ for cytochrome $a$ is 19.5 (12) and $\Delta E_{550}$ for cytochrome $c$ is 21 (15), a slope of 1.1 would have been anticipated. The observed slope indicates that groups in the protein other than the heme are also accepting electrons. There is a substantial body of evidence to implicate copper as this additional electron acceptor (18–21). The observation that the slope is a straight line also indicates that the reduction of the copper must be linked dynamically with that of the heme.

The experiments designed to examine the possibilities of inter-
and intramolecular reaction between cytochromes $a_2$ and $a$ may be treated by supposing that two reactions are going on: (a) a linear reaction in which, as each CO molecule dissociates, $a_2$ is oxidized and reacts rapidly ($k = 750$ sec$^{-1}$) with its associated $a$ (if this is in the reduced state), and (b) the branching reaction, in which oxidized oxidase molecules accept electrons from heme $a$ in oxidase molecules in which CO is still bound to $a$. Gibson and Greenwood (8) have discussed the branching reaction, and have shown that the rate of oxidation of $a''$, on the assumption that the reaction in solution is between separate oxidase molecules, is

$$-\frac{da''}{dt} = k_3a''[1 - \exp (-k_1t)]$$

where $k_1$ is the rate constant for the dissociation of CO from $a_CO$ and $k_2$ is the rate constant for the reaction between $a''''$ and $a''$ (in a different oxidase molecule). Although not exact, a sufficiently good approximation to the rate of oxidation of $a''$ in the linear reaction is obtained by supposing that it is equal to the rate with which CO dissociates from $a_CO$, multiplied by the proportion of $a''$ remaining. Then, if the total concentration of heme $a$ is set equal to 1, the full expression becomes

$$-\frac{da''}{dt} = a''(k_1 + (k_1 - k_3) \exp [-k_1t])$$

Examination of this equation, or of its integrated form,

$$a'' = \exp \left[ k_3t + \frac{(k_1 - k_3)}{k_1} (1 - \exp [-k_1t]) \right]$$

shows that the points in the first four lines in Fig. 10 should lie on a curve and that the asymptotic value of the rate constant $da''/dt$ should not be approached until the end of the reaction. Although no exact analysis can be attempted, some conclusions may, nevertheless, be drawn from Fig. 10. First, in the form in which it has been derived, $k_3$ contains the concentration of $a_2$ and if the reaction takes place between independent oxidase molecules, the numerical value obtained for $k_3$ should be directly proportional to the oxidase concentration. On the other hand, if the reaction takes place entirely within preformed stable groups of oxidase molecules, the size of which is independent of oxidase concentration, a single value of $k_3$ independent of concentration should be observed. It is at once clear from Fig. 10 that this is not the case. To permit a further analysis, approximate values of $k_3$ were obtained by using the times for half-oxidation of $a''$ and substituting these, together with the known value of

![Fig. 11. For description of this figure, see the text. ---, $k_3$ proportional to square root of concentration of reactants; ---, $k_2$ proportional to square root of concentration of reactants; ---, best fit to recorded data.](image-url)
$k_1$ of $0.025 \text{ sec}^{-1}$, in the integrated form of the rate equation. The logarithms of these values of $k_2$ are shown plotted against the logarithms of the relative concentrations in Fig. 11. In this figure, lines are also drawn showing the effect which would have been expected if $k_2$ had been proportional to the concentration or to the square root of the concentration of the enzyme. It is clear that $k_2$ varies much more nearly with the square root of the concentration than with the concentration itself. The result is thus intermediate between that expected of oxidase molecules free in solution and that expected for fixed groups of oxidase molecules without intergroup reactions, and is compatible with the reversible formation of complexes of cytochrome oxidase molecules with a half life shorter than the half-time for the reaction.

**SUMMARY**

1. A comparison of some kinetic properties of cytochrome oxidase made both by the method of Yonetani (1) and according to Griffiths and Wharton (4) indicates that the preparations are very similar.

2. The spectroscopic changes observed when reduced cytochrome $c$ is added to cytochrome oxidase indicate a very rapid reduction of cytochrome $a$ and a much slower reduction of cytochrome $a_3$.

3. Evidence has been obtained which indicates that the slow reduction of cytochrome $a_3$ is due to a complex between reduced cytochrome $a$ and oxidized cytochrome $c$.

4. From an analysis of the kinetics of oxidation in the presence of CO, it is inferred that there is a dynamic equilibrium among the cytochrome oxidase aggregates in solution.

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The Reaction of Cytochrome Oxidase with Cytochrome c
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