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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 a_3, which reacts directly with oxygen, and cytochrome *a*, which reacts with cytochrome a_3 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 a_3 found by Yonetani (1) by static difference spectrophotometry, and led to the conclusions that the initial reaction between oxygen and reduced cytochrome a_3 is very fast indeed, with an apparent second order rate constant of 5×10^7 M^{-1} sec^{-1}, and that the reaction between cytochromes a_3 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 $\text{m}\mu)_{\text{reduced}}$ 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 degassed 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-

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sponding 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 I, 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^{-7} M oxygen) initiates biphasic optical density changes at 445, 550, and 605 mμ. 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 2×10^{-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 mμ is plotted against the change at 605 mμ, a straight line is obtained with a slope, after correction for the contribution at 445 mμ due to the oxidation of cytochrome *c*, of 3.8. This is close to the ratio ΔE_{445} $(a'' - a''')$:ΔE_{605} $(a'' - a''')$ 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 mμ and decreases it at 445 mμ, as compared with the "anaerobic" experiment. In the presence of cyanide, the ratio ΔE_{445}:ΔE_{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_3'', the appearance of photosensitivity is an index of the rate of formation of reduced cyto-

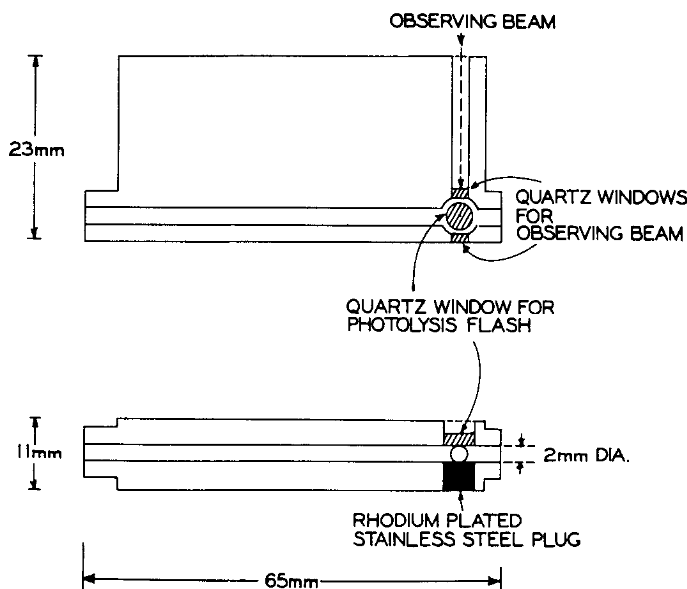

FIG. 1. Sketch of cell for photolysis experiment. For additional information, see "Experimental Procedure."

chrome 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'' 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'' 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 4×10^7 M^{-1} sec^{-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 3×10^6 M^{-1} sec^{-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 mμ 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

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TABLE I

Comparison of some properties of cytochrome oxidase prepared according to Yonetani (7) and Griffiths and Wharton (4)

Reaction studied*	Yonetani preparation†		Griffiths-Wharton preparation	
	Temperature	Rate constant	Temperature	Rate constant
Combination with CO observed at 445 mμ	21°	7.2×10^4 M^{-1} sec^{-1} (Q_{10} = 1.3)	20°	7.5×10^4 M^{-1} sec^{-1}‡ (Q_{10} = 1.4)
Dissociation of CO by oxygen observed at 585 mμ	20	0.023 sec^{-1}	21	0.025 sec^{-1}‡
Reaction of oxygen with reduced cytochrome oxidase observed at 445 mμ				
14 μM O_2	20	6×10^7 M^{-1} sec^{-1}	20	2.7×10^7 M^{-1} sec^{-1}‡
70 μM O_2	20	1×10^7 M^{-1} sec^{-1}	20	5.5×10^6 M^{-1} sec^{-1}‡
Reduction of cytochrome oxidase by cytochrome *c* observed at 445 mμ	21	4×10^6 M^{-1} sec^{-1}	20	4×10^7 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.

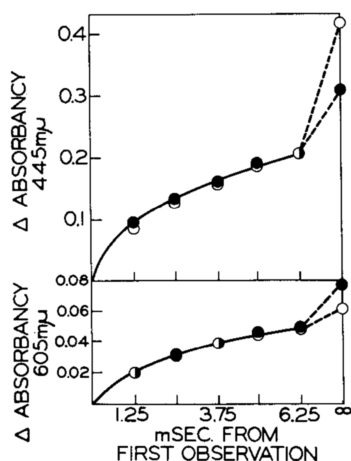

FIG. 2. Reduction of cytochrome oxidase (5.6×10^{-6} M heme *a*) by 11.2×10^{-6} M reduced cytochrome *c* followed in the stopped flow apparatus at 445 and 605 mμ. Temperature, 21°; buffer, 0.1 M phosphate, pH 7.6. The experiment was performed either "anaerobically" (2×10^{-7} M oxygen) (○) or in the presence of 2×10^{-3} M cyanide (●). Concentrations given are those after mixing.

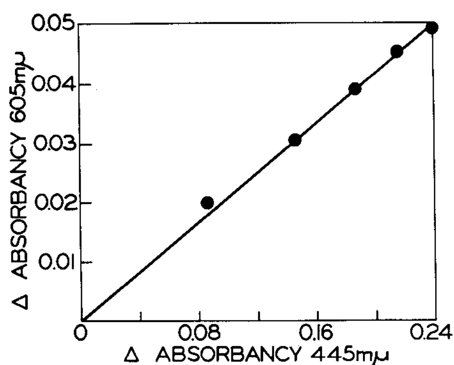

FIG. 3. The relation between the absorbance changes at 445 mμ and those at 605 mμ, obtained from the data of Fig. 2. The slope of the line is 4.8. After correction for the contribution at 445 mμ due to the oxidation of cytochrome *c*, the slope becomes 3.8.

slow rate of electron transfer from a'' to a_3''' 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°, in 0.1 M phosphate, pH 7.4, tetrachlorohydroquinone reduced cytochrome *c* quite efficiently, with a second order rate constant of 2×10^5 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_3 still persists (Fig. 7; note that the scale for the reduction of cytochrome *a* is in milliseconds, and that for cytochrome a_3 in seconds).

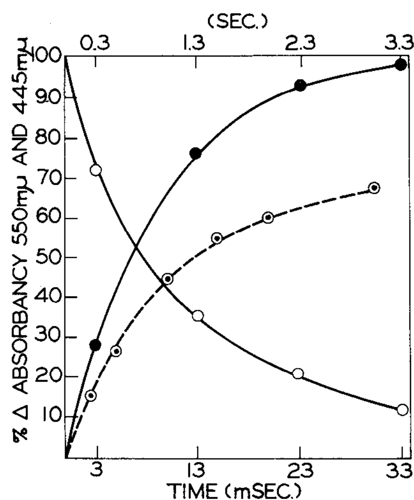

FIG. 4. The rate of appearance of photosensitivity in 2.5×10^{-5} M cytochrome oxidase during reduction by 5×10^{-5} M cytochrome *c* in the presence of 5×10^{-4} M carbon monoxide. The figure shows the absorbance changes at 445 mμ (●) and 550 mμ (○) on the millisecond time scale, and the appearance of photosensitivity (⊙) 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°.

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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 absence of cytochrome c. In one experiment with the Griffiths-Wharton cytochrome oxidase, in which 2×10^{-4} M tetrachlorohydroquinone and 5×10^{-4} 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 a_3 kept closely in step.

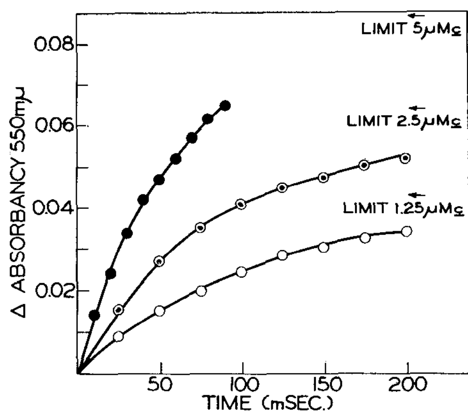

FIG. 5. Oxidation of reduced cytochrome c by 5×10^{-6} M cytochrome oxidase (Yonetani) in the presence of 1×10^{-3} M cyanide. Temperature was 10°. ○, 1.25 μM reduced cytochrome c; ⊙, 2.5 μM reduced cytochrome c; ●, 5 μM reduced cytochrome c. The final changes in optical density are indicated. Path length, 2 cm, 550 mμ; concentrations given are those after mixing.

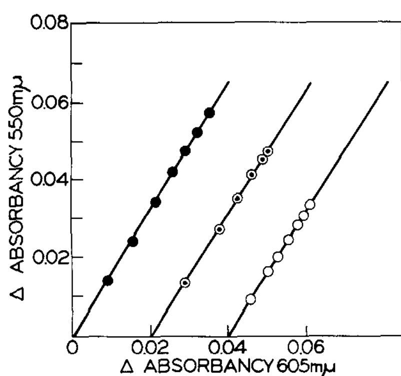

FIG. 6. The relation between the absorbance changes at 550 mμ and the absorbance changes at 605 mμ from the data of Fig. 5. Symbols are the same as in Fig. 5. The scales for ΔE_{605} are displaced for clarity.

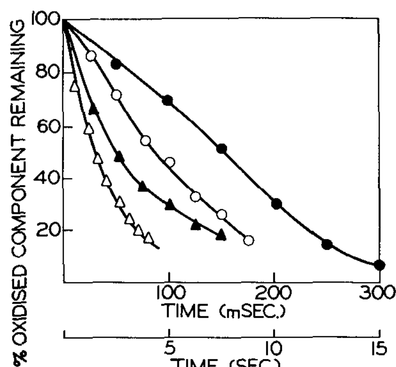

FIG. 7. Time course of reaction of various components on addition of 7×10^{-4} M tetrachlorohydroquinone to 1×10^{-5} M cytochrome c, and 1×10^{-5} M cytochrome c + 5×10^{-6} M cytochrome oxidase (Yonetani). △, cytochrome c alone; ▲, cytochrome c in mixture; ○, cytochrome a; ●, cytochrome a_3. Note that the cytochrome a_3 reaction is on the long time scale. Temperature, 15°; phosphate buffer, 0.1 M, pH 7.4.

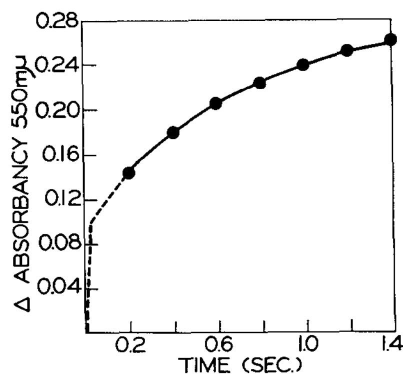

FIG. 8. Extended time course of the absorbance changes at 550 mμ in the stopped flow apparatus from 3 milliseconds after mixing onward. The oxidation of 11.2 μM cytochrome c after mixing with 5.6 μM cytochrome oxidase in the presence of 11 μM oxygen is shown. The buffer was 0.1 M phosphate, pH 7.6. Temperature of the reaction medium was 20°; path, 2 cm.

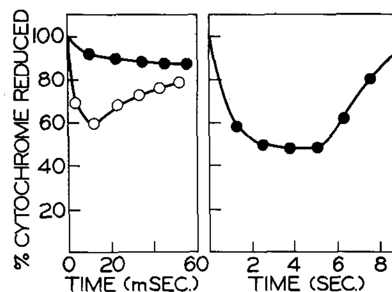

FIG. 9. Reaction of 5×10^{-6} M cytochrome oxidase and 1.5×10^{-5} M cytochrome c with 1×10^{-2} M ascorbate after mixing with 5×10^{-6} M oxygen, followed at 550 and 605 mμ in the stopped flow apparatus at a temperature of 20°. The buffer used was 0.1 M phosphate, pH 7.6. ○, cytochrome a; ●, cytochrome c.

Turnover Experiments—The slow rate of electron transfer from cytochrome a to a_3 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 a_3 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_3 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.

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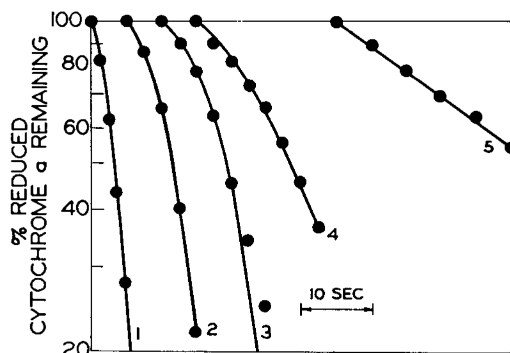

FIG. 10. Effect of enzyme concentration on the rate of reaction of the cytochrome oxidase-CO complex and oxygen. Cytochrome oxidase (Yonetani) (6×10^{-5} M) and cytochrome c (3×10^{-7} M) were reduced with 2×10^{-4} M ascorbate, equilibrated with 50 mm p_{CO}, and subsequently were mixed with an equal volume of 0.1 M phosphate buffer, pH 7.6, equilibrated with 155 mm p_{O_2}. The wave length of observation and the enzyme concentration after mixing are as follows: *Curve 1*, heme $a = 3 \times 10^{-5}$ M, 605 mμ; *Curve 2*, heme $a = 3 \times 10^{-6}$ M, 445 mμ; *Curve, 3* heme $a = 6 \times 10^{-7}$ M, 445 mμ; *Curve 4*, heme $a = 2 \times 10^{-7}$ M, 445 mμ; *Curve 5*, heme $a = 3 \times 10^{-5}$ M, 585 mμ. For *Curve 5*, 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°.

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 8.7 there is an initial fast reduction with a rate of 1.5×10^3 M^{-1} sec^{-1} involving about 70% of the cytochrome c, and a very slow reaction ($k = 4$ M^{-1} 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 ethylenediaminetetraacetic acid (2×10^{-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 O_2, Gibson and Greenwood (8) have presented evidence that electron transport does not follow a strictly linear scheme, $a_3 \leftarrow a \leftarrow c$, 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 *4*) is always faster than the oxidation of cytochrome a_3 (*Curve 5*), 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 4×10^7 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 445 mμ to those at 605 mμ 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

[1] C. Greenwood and G. Palmer, unpublished observations.

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conditions the reduction of cytochrome a_3 is blocked and presumably only cytochrome a is able to react. Finally, the kinetics of appearance of photosensitivity has been used as a means of following the reduction of cytochrome a_3. The slow development of photosensitivity, contrasted with the very rapid optical density changes at 445 mμ, substantiates our presumption, and clearly shows that the transfer of electrons from cytochrome a to a_3 is a slow process under these conditions.

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 experiments 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_3 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 catalytic 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^{3+} and a^{2+}, 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 mμ 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 ΔE_{605} for cytochrome a is 19.5 (12) and ΔE_{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_3 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_3 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_3. Gibson

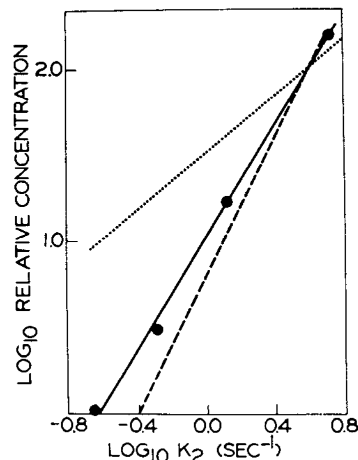

FIG. 11. For description of this figure, see the text. $\cdots\cdot$, k_2 proportional to concentration of reactants; - - -, k_2 proportional to square root of concentration of reactants; ——, best fit to recorded data.

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

$$-\frac{da''}{dt} = k_2a''[1 - \exp(-k_1t)]$$

where k_1 is the rate constant for the dissociation of CO from a_3CO and k_2 is the rate constant for the reaction between a_3''' 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_3CO, multiplied by the proportion of a'' remaining. Then, if the total concentration of heme a is set equal to 1, the full expression becomes

$$-\frac{da''}{dt} = a''(k_2 + (k_1 - k_2)\exp[-k_1t])$$

Examination of this equation, or of its integrated form,

$$a'' = \exp\left[k_2t + \frac{(k_1 - k_2)}{k_1}(1 - \exp[-k_1t])\right]$$

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 $da''/a''dt$ 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_2 contains the concentration of a_3, and if the reaction takes place between independent oxidase molecules, the numerical value obtained for k_2 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_2 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_2 were obtained by using the times for half-oxidation of a'' and substituting these, together with the known value of

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k_1 of 0.025 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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