Reactions of Cytochrome Oxidase with Oxygen and Carbon Monoxide

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Although spectrophotometric determinations of steady states together with turnover rates have allowed Chance (1952) to calculate that the rate of reaction of reduced cytochrome oxidase with oxygen must be about 10^7 M^{-1} sec.$^{-1}$, no direct measurements of this rate appear to have been reported. This is due to the difficulty of obtaining the enzyme in a form which can be studied spectrophotometrically without the special double-beam methods of Chance (1951) for turbid preparations. In addition to the intrinsic interest of the rate measurements it was hoped that experiments with stoicheiometric quantities of enzyme and oxygen might help to resolve the vexed questions of the existence and function of cytochromes *a* and a_3, and of the role of copper. Some of the experiments have been done with the venom-digest preparation of Greenwood (1963), but most have been carried out with the preparation of Yonetani (1960*a*, 1961), which is stabilized by a non-ionic detergent. Preliminary accounts of part of this work have been given by Gibson, Greenwood & Massey (1960) and by Gibson & Greenwood (1962).

EXPERIMENTAL

Cytochrome-oxidase preparations. Two types were used: the venom preparation described by Greenwood (1963) (referred to below as the 'Greenwood preparation'), and the preparation of Yonetani (1960*a*, 1961) (referred to below as the 'Yonetani preparation'). As Emasol 4130, a product of the Kao Soap Co., Tokyo, could not be obtained, the preparation was stabilized with Tween 80 (L. Light and Co. Ltd., Colnbrook, Bucks.), a poly(oxyethylene sorbitan mono-oleate).

Buffers. Experiments with the Yonetani preparation were performed in the presence of sodium phosphate buffer, pH 7·4, a stock solution of which was prepared by mixing 0·3 M solutions of Na_2HPO_4 and NaH_2PO_4 (81:19, v/v). The buffer used in the preparation of and in experiments with the Greenwood preparation was tris–HCl, pH 8·5 at 23°, a stock solution of which was prepared by mixing 0·2 M-tris with 0·1 N-HCl (5:3, v/v). All solutions were prepared and subsequently diluted with glass-distilled water.

Reduction of cytochrome-oxidase preparations. The Greenwood preparation was reduced by adding 2,3-dimercaptopropanol (BAL) to give a final concentration of 1 mM. It was not readily reduced by cytochrome *c*–ascorbic acid. The Yonetani preparation was reduced by adding ascorbic acid (final concn. 0·1 mM) and cytochrome *c* (final concn. 0·2 μM). For experiments with CO and cyanide both preparations were reduced by adding 1 mg. of sodium dithionite/ml.

Preparation of solutions of gases. Solutions containing small known amounts of O_2 and CO were prepared by degassing a large volume (usually 500 ml.) of buffer in a 1 l. tonometer. Small measured volumes of water in equilibrium with room air or with 1 atm. of CO were then added to the tonometer. Concentrations of O_2 and CO in the stock water solutions were obtained by reference to the Tables in the *Handbook of Chemistry and Physics* (Chemical Rubber Co.).

Stopped-flow kinetic apparatus. Stopped-flow measurements were carried out in an apparatus similar to that of Gibson & Antonini (1960), by using a water-jacketed observation tube with a 2 cm. light-path. A 2 mm. tube was used in some experiments.

Apparatus for photochemical determinations. The flash-photolysis apparatus used was as described by Gibson (1959) but with modifications for the study of cytochrome oxidase. To measure the rate of reaction of O_2 with reduced cytochrome oxidase by the photochemical method, the compound of the enzyme with CO, in equilibrium with a p_{CO} of about 50 mm. Hg, was placed in a large syringe, *A* (Fig. 1). A solution of O_2 of 10 times the concentration required for reaction was placed in a small syringe, *B*. The two syringes were then driven in by a pushing block, *P*, when their contents flowed into a 5 cm. observation tube, *O*, which lay within the magnesium oxide-coated box of the flash-photolysis apparatus. The outflow from the observa-

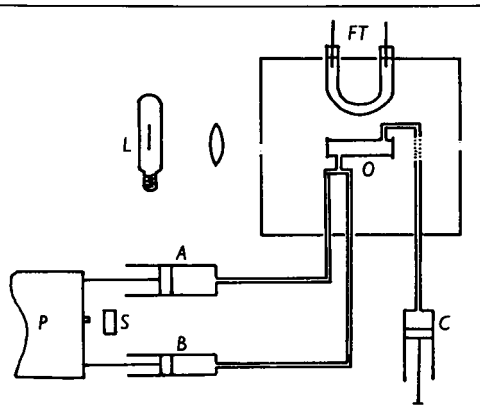

Fig. 1. Block diagram of flow–flash apparatus. *A*, Large syringe to contain the CO compound of the reduced enzyme; *B*, small syringe for O_2 solution; *C*, receiving syringe; *P*, pushing block; *S*, switch of flash-photolysis apparatus; *FT*, flash tube; *L*, light source for recording the reaction; *O*, 5 cm. observation tube.

tion chamber led into a large receiving syringe, *C*. The pushing block had a contact at its end which closed the firing switch, *S*, of the flash-photolysis apparatus when the last of the O_2 and enzyme had been driven into the observation tube. When the photolysis flash was fired, energy up to 200 *J* was discharged in about 20 μsec. This removed CO from the cytochrome oxidase–CO compound. The free reduced cytochrome oxidase was then able to react with O_2 and a record of the extinction changes associated with this reaction was made with a photomultiplier and cathode-ray oscillograph. The rate of reaction of cytochrome oxidase with O_2 is so much greater than that with CO that the latter could be neglected at all the O_2 concentrations used. After the record of the reaction with O_2 had been obtained, the mixture was kept in the observation tube until the O_2 added had been consumed by the enzyme. The compound with CO was then re-formed and a line corresponding to its extinction was added to the oscillograph record. A second photolysis flash was fired and a reference line giving the extinction of the reduced compound recorded. Finally, the light-beam from the monochromator was interrupted and a line corresponding to zero light added to the record. The mixture of enzyme plus one-tenth of its volume of water, which had collected in the receiving syringe, *C*, was next returned to the syringe, *A*, the small syringe, *B*, was detached and refilled with a fresh portion of oxygenated water, and the whole procedure repeated. The method may be regarded as a combination of the regenerative stopped-flow method of Chance (1955) with the flash-photolysis procedure.

Spectrophotometric measurements. Spectrophotometry was carried out at fixed wavelengths in the visible region with a Unicam SP. 600 spectrophotometer, and spectra over more extended ranges were recorded with an Optica Recording Spectrophotometer [Optica (U.K.) Ltd., Team Valley, Gateshead on Tyne]. The extinction coefficients used in determining the concentrations of cytochrome oxidase were those of Yonetani (1961), which were assumed to apply to our Yonetani preparation, and of Greenwood (1963) for the venom preparation. All concentrations are expressed in terms of haem content (Greenwood, 1963).

Determination of the carbon monoxide-binding capacity of the cytochrome oxidase. The principle used was to equilibrate the reduced form of the enzyme with CO in the chamber of the Van Slyke & Neill (1924) manometric apparatus, to release bound CO by oxidation with ferricyanide, and to measure the total volume of gas at the 0·5 ml. mark. The extracted gases were transferred to the capillary of a Scholander & Roughton (1943) syringe and analysed for CO by the procedure of Roughton & Root (1945). The detailed procedure was to raise the mercury reservoir and transfer 2 ml. of mercury to the cup. A tonometer containing CO, fitted with a two-way tap and a long capillary with a rubber tip at its end, was settled in position in the cup. The capillary stem was cleared of air by raising the mercury reservoir, and 0·5–2 ml. of CO was drawn from the tonometer into the chamber of the Van Slyke apparatus by lowering the mercury reservoir. The tonometer tap was closed, and the tonometer removed. The sample of cytochrome oxidase (3 ml.) was delivered into the cup and drawn into the chamber of the apparatus. The bottom of the cup was touched with a glass rod smeared with a trace of Dow–Corning antifoam AF (Dow–Corning Chemical Co.). The enzyme was reduced by drawing in 0·2 ml. of a 0·5% solution of $Na_2S_2O_4$ in 0·1 M-phosphate buffer, pH 7·4. The top tap of the Van Slyke apparatus was sealed with mercury, and the pressure of gas in the chamber measured at the 2 ml. mark. or at the 10 ml. mark when 2 ml. of CO had been used. The mercury was then lowered to the 50 ml. mark, and the chamber was covered with a black cloth and shaken for 3 min. to equilibrate the enzyme with the gas phase. The excess of CO, together with the extracted gases, was then expelled from the chamber through a mercury seal, and 3 ml. of a de-aerated 4% (w/v) solution of $K_3Fe(CN)_6$ in 1 N-NaOH was run into the cup. The ferricyanide reagent (1 ml.) was drawn into the chamber and the top tap sealed with mercury. The mercury was again lowered to the 50 ml. mark and the chamber shaken for 3 min. to extract CO. The pressure due to the extracted gases was read at 0·5 ml. and the gas bubble transferred to the syringe capillary. The O_2 was removed with a solution of 1% $Na_2S_2O_4$ plus 0·1% sodium β-anthraquinone sulphonate in 1 N-NaOH, and the excess of reagent was rinsed from the syringe with water. The percentage of CO in the extracted gases was then determined by absorbing it in Winkler's reagent (20 g. of CuCl, 25 g. of NH_4Cl and 75 g. of water). Replicate determinations on amounts of CO up to 10 μl. agreed within 0·5 μl., which is not quite so good as the precision obtained by Roughton & Root (1945). The results require correction for CO in physical solution. This correction was calculated from the measured p_{CO} and the solubility coefficients taken from the *Handbook of Chemistry and Physics*. The correction was 10–30% of the total CO measured.

RESULTS

Reaction of reduced cytochrome oxidase with carbon monoxide

The rate of combination of reduced cytochrome oxidase with carbon monoxide was measured for the Greenwood and Yonetani preparations. Both were examined by the flash-photochemical method and by the stopped-flow method; similar rates were found (Table 1). The time-course of the com-

Table 1. *Combination of reduced cytochrome oxidase with carbon monoxide*

In these experiments the concentration of enzyme (expressed as haem) was in the range 2–4 μM for the stopped-flow measurements (2 cm. light-path) and in the range 1–2 μM for the flow–flash determinations (5 cm. light-path). All determinations were made with light of mean wavelength 445 mμ.

Preparation	Buffer	Method	Temperature	Rate constant (M^{-1} sec.$^{-1}$)
Greenwood	Tris, pH 8·5	Flow–flash	21°	$5·6 \times 10^4$
Greenwood	Tris, pH 8·5	Stopped flow	22	$8·0 \times 10^4$
Yonetani	Phosphate, pH 7·4	Flow–flash	22	$7·8 \times 10^4$
Yonetani	Phosphate, pH 7·4	Stopped flow	21	$7·2 \times 10^4$

bination of carbon monoxide is accurately second order. The temperature-dependence has been investigated for the Yonetani preparation only and the results in Fig. 2 yield an activation energy of 6·4 kcal. This is rather lower than would be expected from the rate of the reaction and is only about two-thirds of that measured for the corresponding reaction of sheep haemoglobin by Gibson & Roughton (1957). The combination rates for both preparations are rather higher than those obtained for the Keilin–Hartree heart-muscle preparation by Chance (1953) by a photochemical method. The agreement between the flash-photochemical and stopped-flow methods indicates that photochemical after-effects of the kind observed in sheep haemoglobin by Gibson (1959) do not occur with the cytochrome oxidase.

Dissociation of carbon monoxide from cytochrome oxidase. A knowledge of the rate of this reaction is necessary to justify the use of the flow–flash method for determining the rate of reaction of oxygen with reduced cytochrome oxidase and is also needed for evaluating experiments in which the reduced enzyme is titrated with carbon monoxide. The rate of dissociation was measured by replacing carbon monoxide with nitric oxide: the course of the replacement reaction was accurately first order (Fig. 3) and was unaffected by a tenfold variation in the concentration of nitric oxide. The reaction was followed in experiments other than those of Fig. 3 to 80% completion. It appeared that the affinity of the cytochrome oxidase for carbon monoxide was much less than its affinity for nitric oxide, and that the observed rate of replacement of carbon monoxide by nitric oxide could be taken as equal to the rate of dissociation of carbon monoxide from reduced cytochrome oxidase without any need to apply the procedures developed by Gibson & Roughton (1955) for dealing with the situation where two competing ligands have comparable affinities for a haemoprotein. The high affinity of nitric oxide can in part be accounted for by its very high rate of combination with reduced cytochrome oxidase; the rate constant was approx. 4×10^7 M^{-1} sec.$^{-1}$ at 21° and pH 7·4.

It should be possible to determine the rate of dissociation of carbon monoxide from its com-

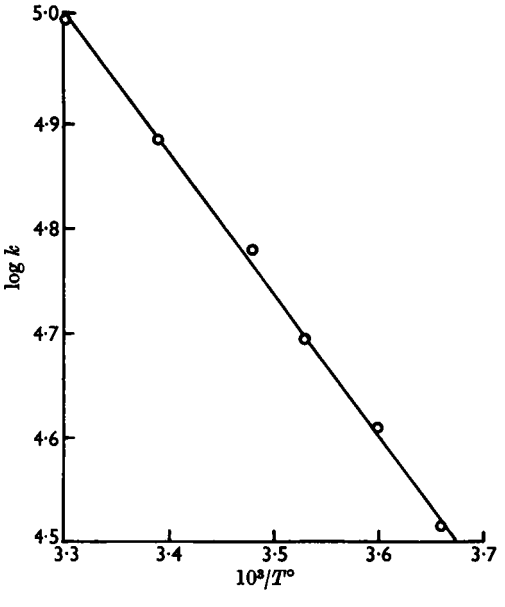

Fig. 2. Effect of temperature on the rate of combination of CO with reduced cytochrome oxidase (Yonetani preparation). The conditions were as for Fig. 1, except that the temperature was varied. The results give an activation energy of 6·4 kcal.

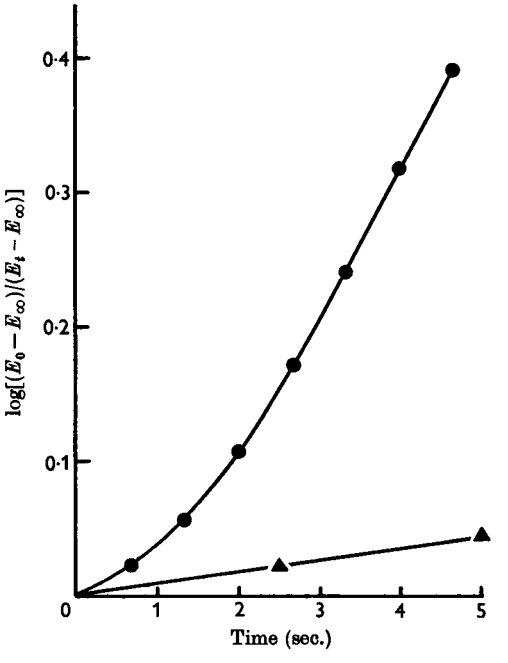

Fig. 3. Displacement of CO from reduced cytochrome oxidase (Yonetani preparation) by NO (▲), and the oxidation of the CO compound of reduced cytochrome oxidase by O_2 (●). The CO compound of reduced cytochrome oxidase [4 μM (expressed as haem) after mixing] was mixed in the stopped-flow apparatus with 300 μM-NO and the reaction followed with light of mean wavelength 430 mμ at 21° in 0·1 M-phosphate buffer, pH 7·4; a 2 cm. light-path was used. In the oxidation experiment the same enzyme solution was mixed with air-equilibrated phosphate buffer to give a concentration of O_2 after mixing of 140 μM. The reaction was followed with light of mean wavelength 445 mμ. The Figure shows $\log[(E_0 - E_\infty)/(E_t - E_\infty)]$ plotted against time in each case. The first-order rate constant for the replacement of CO by NO was 0·023 sec.$^{-1}$.

pound with reduced oxidase by oxidizing the iron of the free reduced oxidase with oxygen instead of replacing carbon monoxide by nitric oxide, and, provided that any changes consequent on the combination of oxygen with the enzyme in place of carbon monoxide take place rapidly as compared with the dissociation of carbon monoxide from the enzyme, the same rate constant should be obtained with oxygen as with nitric oxide. In practice, although the displacement reaction began with much the same speed when oxygen was used, the extinction change observed accelerated rapidly to a rate about ten times that with nitric oxide. It may be that the oxidation of one group within the enzyme is able to influence the dissociation of carbon monoxide from other groups. The increase in the rate of dissociation was spread over several seconds (Fig. 3) but no detailed study of this effect has yet been made.

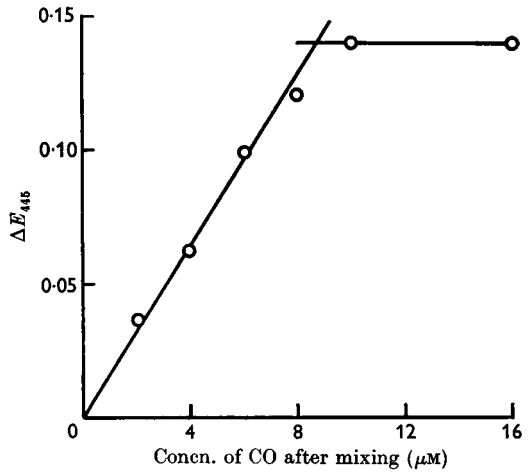

Fig. 4. Titration of reduced cytochrome oxidase (Yonetani preparation) with CO. The enzyme [20·5 μM (expressed as haem) after mixing] was allowed to react at 24° in the stopped-flow apparatus with various concentrations of CO dissolved in 0·1 M-phosphate buffer, pH 7·4. The reaction was followed at 445 mμ in a 2 mm. light-path and the total change in extinction is plotted against the concentration of CO.

Table 2. *Carbon monoxide-binding capacity of the Yonetani preparation of cytochrome oxidase*

The enzyme solution was equilibrated with CO in the Van Slyke–Neill apparatus as described in the Methods section, and the CO bound was calculated after allowance for CO held in physical solution. The enzyme iron content was obtained by using the extinction coefficients of Yonetani (1961), averaging the values for 445 mμ (reduced enzyme), 422 mμ (oxidized enzyme) and 605 mμ (reduced enzyme).

Preparation	p_{CO} (mm. Hg)	Fe (μM)	CO bound (μM)	Fe:CO ratio
I	36	215	71	3:0·99
I	39	215	77	3:1·08
II	9·4	405	149	3:1·10
II	9·4	405	132	3:0·98
II	8·5	405	138	3:1·02

Titration of the enzyme with carbon monoxide. The reduced enzyme was mixed with low concentrations of carbon monoxide in the stopped-flow apparatus and the change in extinction at 445 mμ recorded. When such a titration experiment was carried out with enzyme of the concentration used in most of the kinetic experiments with the stopped-flow apparatus in which the 2 cm. light-path was employed, considerable corrections were required for the amount of carbon monoxide which remained in physical solution, especially at the higher percentage saturations with carbon monoxide. The amount of correction required can be much reduced by increasing the concentration of cytochrome oxidase, and Fig. 4 shows the result of an experiment carried out with a 2 mm. light-path with correspondingly increased amounts of carbon monoxide and enzyme. These results, when combined with Yonetani's (1961) correlation between the spectrum of his preparation and its iron content, allow the calculation of the carbon monoxide:iron ratio in the cytochrome oxidase–carbon monoxide compound. The experiment of Fig. 4 shows that less than half of the iron is able to combine with carbon monoxide.

Carbon monoxide:iron ratio in cytochrome-oxidase preparations. Because of the importance of the carbon monoxide:iron ratio in establishing the proportion of iron available for reaction with ligands, this quantity was determined directly by measuring the amount of gas which can combine with a given quantity of enzyme. The results of five determinations of carbon monoxide-binding capacity on two preparations (Table 2) show that about one-third of the total iron is able to combine with carbon monoxide.

Reaction of cytochrome oxidase with oxygen

Most of the experiments were done with the Yonetani preparation and the rather complex results can be summarized by considering the results with low and high oxygen concentrations separately.

(*a*) Experiments with low oxygen concentrations (less than 5 μM) have mostly been made with the stopped-flow apparatus. The results depend, not only on the concentration of oxygen, but also on the wavelength of the light used for following the reaction. Fig. 5 shows the reaction of 2·7 μM-oxygen with an approximately equivalent amount

of enzyme. The reaction appeared to take place faster when followed at 605 mμ than when followed at 445 mμ. The second-order rate constant, when calculated over successive intervals of time, rises rapidly for the observations at 445 mμ but shows less change at 605 mμ.

(*b*) At higher oxygen concentrations (20 μM or more) in observations by the flow–flash method, the results are almost exactly the converse of those shown in Fig. 5. The time-course of the reaction with 25 μM-oxygen (Fig. 6) shows that the changes at 445 mμ take place much more rapidly than those at 605 mμ. If the second-order rate constant is worked out for successive intervals, the values for the 445 mμ results show a continuing downward trend as the reaction proceeds.

(*c*) The effect of oxygen concentration on the apparent second-order rate constant, calculated over the first time-interval for which observations are available, is shown in Fig. 7. These results are based on observations at 445 mμ. In the low oxygen concentration range (covered by the stopped-flow method) the rate constant rises with oxygen concentration, whereas in the higher range

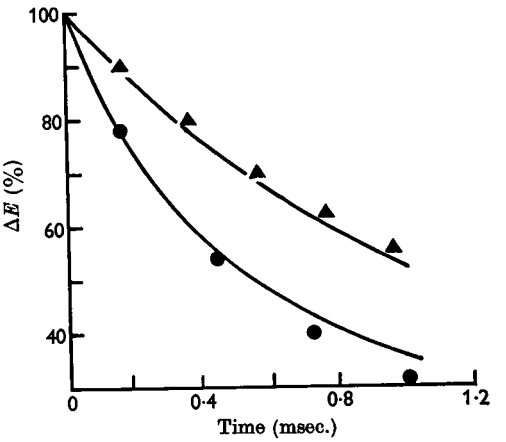

Fig. 6. Reaction of reduced cytochrome oxidase (Yonetani preparation) with O_2 followed by the flow–flash method at 605 mμ (▲) and at 445 mμ (●). The concentration of enzyme was 2·6 μM (expressed as haem) for observations at 605 mμ and 0·6 μM (expressed as haem) for observations at 445 mμ. The concentration of O_2 was 25 μM in each case. Other conditions were: temperature, 21°, 0·1 M-phosphate buffer, pH 7·4; light-path, 5 cm.; p_{CO}, 40 mm. Hg. The lines were calculated with the same rate constants and scheme as used for Fig. 5.

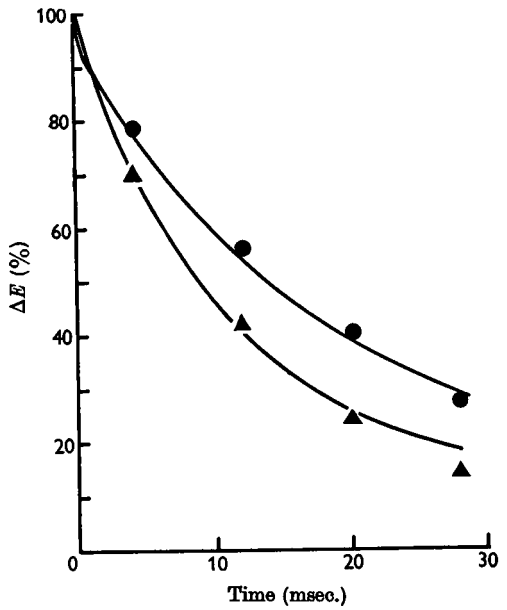

Fig. 5. Reaction between reduced cytochrome oxidase (Yonetani preparation) and O_2 followed in the stopped-flow apparatus at 605 mμ (▲) and at 445 mμ (●). The concentration of enzyme was 4·5 μM (expressed as haem) and that of O_2 2·7 μM, after mixing. The other conditions were: temperature, 21°; 0·1 M-phosphate buffer, pH 7·4; 2 cm. light-path. The lines were calculated from a scheme given in the text with $k_1 = k_3 = k_5 = 1{\cdot}5 \times 10^8\ \text{M}^{-1}\ \text{sec.}^{-1}$, and $k_2 = k_4 = 1{\cdot}5 \times 10^3\ \text{sec.}^{-1}$.

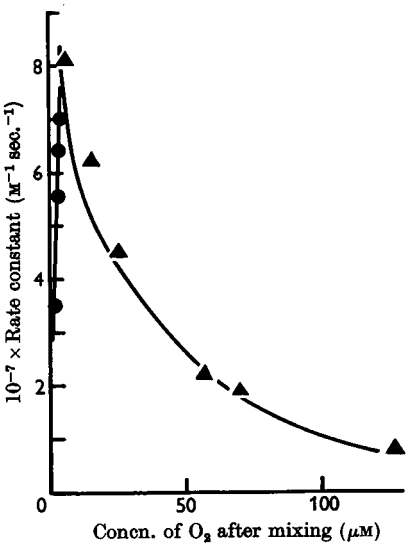

Fig. 7. Effect of O_2 concentration on the apparent second-order rate constant for the reaction of O_2 with reduced enzyme (Yonetani preparation). ●, Experiments with the stopped-flow apparatus; ▲, experiments by the flow–flash method. The rate constant was measured over the interval 4–7 msec. after initiation for the stopped-flow method, and over the interval 0·15–0·3 msec. for the flow–flash method. The lines were calculated from the scheme and with the rate constants described in the text. Other conditions were as in Fig. 5 for the stopped-flow experiments and as in Fig. 6 for the flow–flash experiments.

of oxygen concentrations (studied by the flow–flash method) the apparent rate constant trends steadily downwards with increasing oxygen concentration. The two methods give the same rates at low oxygen concentrations.

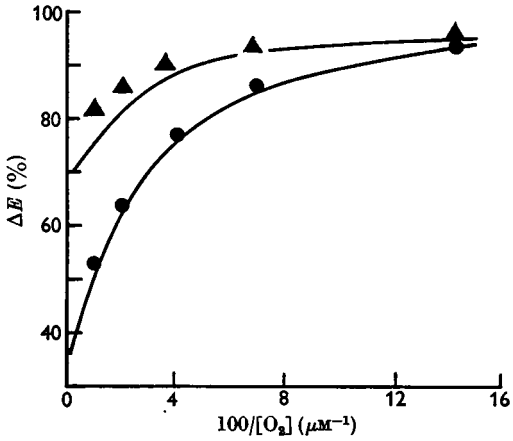

Fig. 8. Effect of O_2 concentration on the change in extinction observed by the flow–flash method at 605 mμ (▲), and at 445 mμ (●). The results are expressed as percentages of the change in extinction found at low concentrations of O_2 (less than 2 μM). The lines were obtained by calculation as described in the text. Other conditions were as for the experiments of Fig. 6.

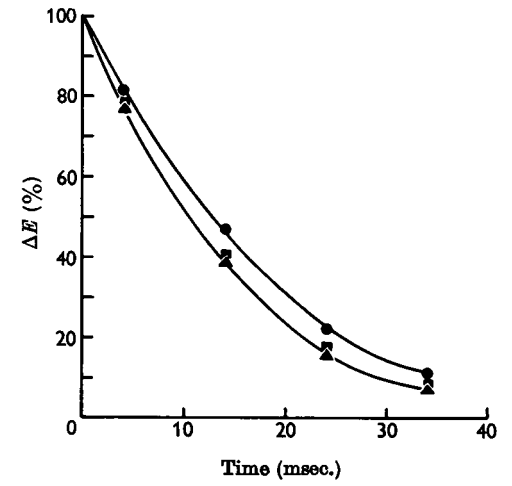

Fig. 9. Reaction of 2 μM-O_2 with an approximately equivalent amount of reduced cytochrome oxidase (Greenwood preparation) followed at 605 mμ (▲), 445 mμ (●) and 410 mμ (■). The enzyme was dissolved in tris–HCl buffer, pH 8·5. Other conditions were: temperature, 21°; light-path, 2 cm. The results are expressed as percentages of the total change in extinction between the first observation 4 msec. after mixing and 100 msec. after mixing.

(*d*) As the oxygen concentration is raised, the extinction changes take place faster, both at 605 mμ and at 445 mμ. Since the apparatus has a definite 'dead-time' between the initiation of the reaction and the time at which the first valid observation can be made, the proportion of the total change which can be observed decreases with increasing concentration of oxygen. The results plotted in Fig. 8 are based on observations with different oxygen concentrations at 605 and 445 mμ obtained with the flow–flash apparatus. These results suggest that, at infinitely high oxygen concentration, a considerable reaction would still be observed at both wavelengths. Extrapolation suggests that about 40 % would be seen at 445 mμ and about 70 % at 605 mμ.

(*e*) The Greenwood preparation shows similar effects: e.g. the rate of spectrophotometric change with low oxygen concentrations is greater at either 605 or 410 mμ than at 445 mμ (Fig. 9), but the difference is less striking than that with the Yonetani preparation. The actual rates of the change in extinction do not differ very much with the two preparations. Interpretation of the results obtained with the Greenwood preparation is, however, complicated by an additional factor. After the initial rapid change in extinction is complete, there follows a further slow change in extinction which is spread over a few tenths of a second. If the amount of oxygen added is insufficient to oxidize all the cytochrome oxidase, the slow change in extinction is in the opposite direction to the quick change; that is, at 445 mμ, where the extinction decreases during the quick change, it increases again in the slow phase (curve *A*, Fig. 10). When the amount of oxygen is large enough to oxidize all the cytochrome oxidase, the slow extinction change is in the same direction as the rapid one (curve *B*, Fig. 10).

Titration of reduced cytochrome oxidase with oxygen. Titrations were made in the stopped-flow apparatus by mixing reduced cytochrome oxidase with various concentrations of oxygen. With the Yonetani preparation the difference was measured between the least extinction (at 445 mμ) after adding oxygen and the value reached several minutes later after the reducing system (ascorbic acid–cytochrome *c*) had consumed the added oxygen and the enzyme had returned to the reduced form. An experiment of this kind (Fig. 11) shows that the extinction in a 1 cm. cell would change by 100 on the addition of oxygen (final concn. 1 mM) to excess of enzyme. With the Greenwood preparation the rate of reduction (by BAL) is too slow to permit the extinction of the fully reduced enzyme to be recorded after each addition of oxygen. The excursion in extinction observed with the stopped-flow apparatus was

measured and corrected for the change taking place in the 'dead-time' of the stopped-flow apparatus. The change at 445 mμ was 80/mM-oxygen.

Although the reproducibility of the titrations was good (the greatest deviation from the mean in eight titrations was 15 %), the interpretation of the results is difficult. The points lie on a straight line, but with a complex system this result may be fortuitous. Further, with the Greenwood preparation light-scattering prevented the concentration of enzyme being measured spectrophotometrically with precision. With these reservations, however, the results of the three experiments judged most satisfactory (with the Yonetani preparation) showed that 1 mol.prop. of oxygen oxidized 1·8 mol.prop. of haem. The value of $\Delta\epsilon_{Mm}$ [i.e. ϵ_{mM} (red.) $-\epsilon_{mM}$ (ox.)] per haem was 55, which compares with Yonetani's (1961) value of 67 for $\Delta\epsilon_{mM}$ at 445 mμ in static experiments.

Scheme to account for the changes in extinction during the reaction with oxygen. It is impossible to explain the changes observed on mixing oxygen with cytochrome oxidase by the oxidation of a single substance, but it is possible to do so in terms of two substances, one of which reacts with oxygen whereas the second reacts with the first. To give concreteness to this proposal and to relate the observations to other work, the substance reacting rapidly with oxygen is identified with cytochrome a_3, as defined in terms of absorption spectra by Yonetani (1960*a*), and the second substance is identified with cytochrome *a*, again defined spectrophotometrically by Yonetani (1960*a*). The ratio between the concentrations of these two compounds is given by the carbon monoxide:iron ratio, since it has been agreed, by authors from Keilin & Hartree (1938*a*, *b*) onwards who have accepted the separate existence of cytochromes a_3 and *a*, that a_3 reacts with carbon monoxide and that *a* does not. In making this assignment, it is supposed that cytochromes *a* and a_3 are iron porphyrins and that copper-containing compounds do not contribute significantly to light-absorption at 410, 445 and 605 mμ. On this basis the minimum functional unit becomes cytochrome (a_3 plus 2*a*) and its complete oxidation involves the formation of intermediate compounds with the intervention of

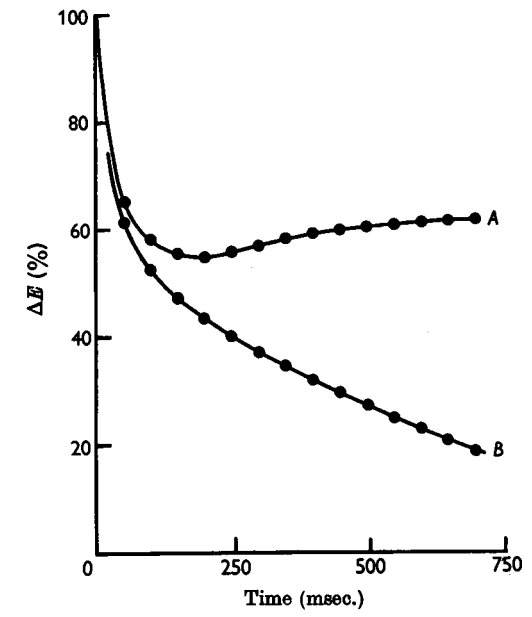

Fig. 10. Spectrophotometric changes at 445 mμ when 4 μM-cytochrome oxidase (Greenwood preparation) (concn. expressed as haem) was allowed to react with 0·67 μM-O_2 (*A*), and with 2·5 μM-O_2 (*B*). Other conditions were: temperature, 21°; 0·1 M-tris–HCl buffer, pH 8·5; light-path, 2 cm.

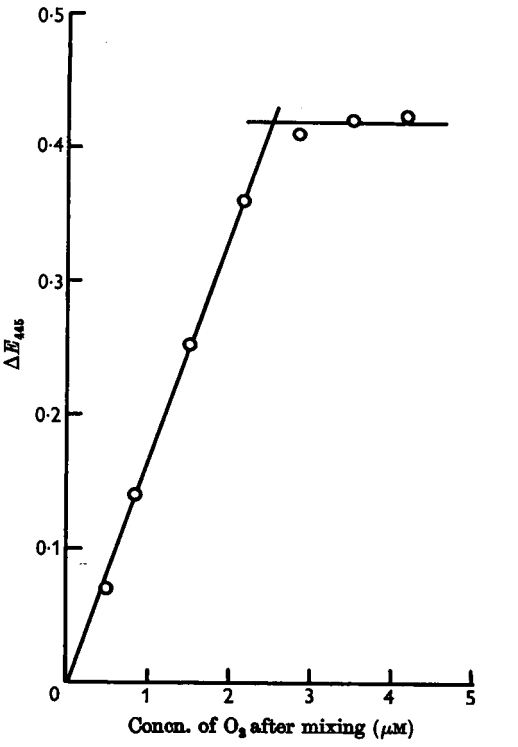

Fig. 11. Titration of reduced cytochrome oxidase (Yonetani preparation) with O_2. The ordinate gives the change in extinction at 445 mμ corresponding to the amount of O_2 shown on the abscissa. The enzyme concentration was 4·5 μM (expressed as haem) after mixing. Other conditions were: temperature, 21°; 0·1 M-phosphate buffer, pH 7·4; light-path, 2 cm.

oxygen three times in the complete process. The reactions are:

$$a_3^{2+}a^{2+}a^{2+} + \tfrac{1}{4}\,O_2 \xrightarrow{k_1} a_3^{3+}a^{2+}a^{2+}$$

$$a_3^{3+}a^{2+}a^{2+} \xrightarrow{k_2} a_3^{2+}a^{3+}a^{2+}$$

$$a_3^{2+}a^{3+}a^{2+} + \tfrac{1}{4}\,O_2 \xrightarrow{k_3} a_3^{3+}a^{3+}a^{2+}$$

$$a_3^{3+}a^{3+}a^{2+} \xrightarrow{k_4} a_3^{2+}a^{3+}a^{3+}$$

$$a_3^{2+}a^{3+}a^{3+} + \tfrac{1}{4}\,O_2 \xrightarrow{k_5} a_3^{3+}a^{3+}a^{3+}$$

This series of reactions can accommodate all the observations if (i) changes in extinction at 445 and 605 mμ are assigned to cytochromes a and a_3 in accordance with Yonetani (1960a), (ii) it is assumed that the rate of oxidation of cytochrome a_3 is independent of whether or not the associated cytochrome a is in the oxidized or reduced state, and (iii) it is assumed in addition that the rate of reduction of cytochrome a_3 by cytochrome a is the same if both the cytochrome a molecules are in the reduced state, or if one of them is oxidized. The curves in Figs. 5–8 were obtained by computation with a Solartron SCD 10 analogue computer by using the same values throughout of

$$k_1 = k_3 = k_5 = 1{\cdot}5 \times 10^8\,\text{M}^{-1}\,\text{sec.}^{-1}$$

and $$k_2 = k_4 = 1{\cdot}5 \times 10^3\,\text{sec.}^{-1}.$$

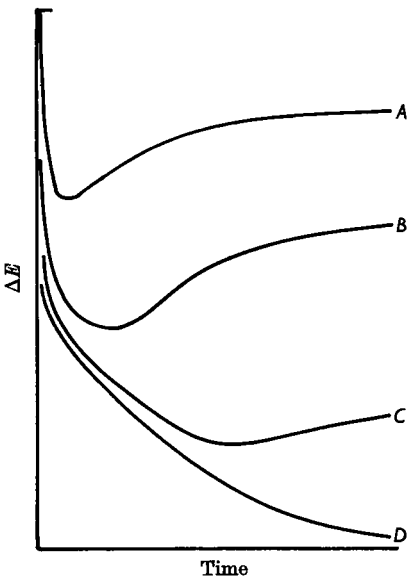

Fig. 12. Calculated curves to represent the spectrophotometric changes at 445 mμ on mixing cytochrome oxidase (Greenwood preparation) with various concentrations of O_2. The scheme used was that described in the text with $k_1 = k_3 = k_5 = 1{\cdot}5 \times 10^8\,\text{M}^{-1}\,\text{sec.}^{-1}$ and $k_2 = k_4 = 5\,\text{sec.}^{-1}$. The molar ratios of O_2 to enzyme were: A, 1:3·7; B, 1:1·9; C, 1:1·2; D, 1:0·73. The time-scale has not been indicated as the Figure is intended only for qualitative comparison with Fig. 10.

In effect, then, the full range of our experimental findings has been fitted by using only two freely adjustable constants. As the experiments of Figs. 5–8 were carried out at different times on several different preparations of cytochrome oxidase, the agreement between the experimental and calculated values is satisfactory. It is also possible to account for the results with the Greenwood preparation by using the same series of reactions but with the additional assumption that the values of k_2 and k_4 are much lower. Fig. 12 gives a family of calculated curves worked out on this assumption. The form of the experimental findings shown in Fig. 10 is satisfactorily reproduced.

It is possible formally to consider cytochrome a_3 as an enzyme with two substrates, oxygen and cytochrome a, which functions in this system by turning over three times in bringing about the oxidation of cytochrome a. The very marked effect of varying the concentration of oxygen on the spectrophotometric changes at 605 and 445 mμ may then be regarded as due to alteration in the 'steady-state' concentrations of oxidized and reduced cytochrome a_3. This may be shown by calculating the actual concentrations of cytochromes a_3^{2+} and a^{2+}, rather than the total extinction changes in the solutions. Figs. 13 and 14 show the changes in concentration of cytochromes

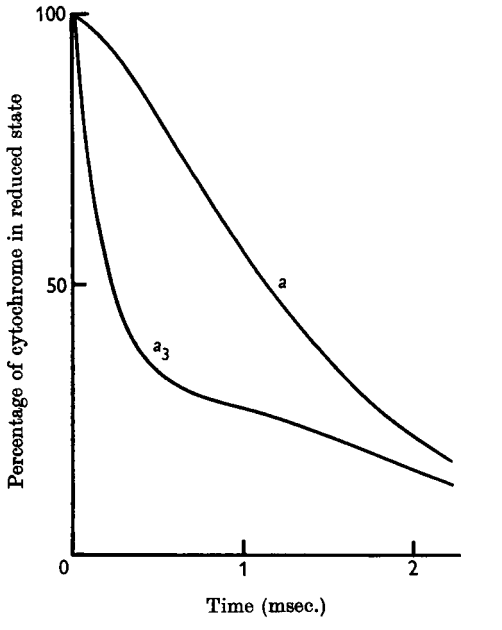

Fig. 13. Calculated time-course of the concentrations of cytochrome a_3^{2+} and cytochrome a^{2+} in the reaction of 1 μM-enzyme (concn. expressed as haem) with 25 μM-O_2. The initial concentration of both cytochromes has been set arbitrarily at 100. Rate constants were as for Figs. 5–8.

a_3^{2+} and a^{2+} with time for high and low concentrations of oxygen respectively. With high oxygen concentrations the reaction of oxygen with cytochrome a_3^{2+} is rapid as compared with its reduction by cytochrome a^{2+}, and the cytochrome a_3^{2+} is substantially converted into the oxidized form after a very short time. When the oxygen concentration is low, the rate of reaction of cytochrome a_3^{2+} with oxygen is much lower than the rate of reduction of cytochrome a_3^{3+} by cytochrome a^{2+}, so that much of the cytochrome a_3 remains in the reduced form until a considerable proportion of the total enzyme has reacted with oxygen. There is, in fact, a period near the beginning of the reaction when the concentration of cytochrome a_3^{2+} scarcely changes with time for several milliseconds.

Reaction of reduced cytochrome oxidase with cyanide

Wainio (1955) and Yonetani (1960*a*) have shown that the addition of cyanide to reduced cytochrome oxidase is associated with small changes in the spectrum, due to the formation of the cyanide–cytochrome a_3 complex. Although these changes are too small to allow the reaction with cyanide to be followed conveniently in the stopped-flow apparatus, it was possible to obtain the rate of combination, the rate of dissociation and also, independently, the equilibrium constant for the reaction of cyanide with the reduced cytochrome oxidase, by observing the effects of cyanide on the reactions of cytochrome oxidase with carbon monoxide.

When solutions of reduced cytochrome oxidase containing various concentrations of cyanide were mixed in the stopped-flow apparatus with solutions of carbon monoxide, the reaction observed depended on the concentration of cyanide. At zero cyanide concentration the accurately second-order reaction characteristic of the combination of carbon monoxide with reduced cytochrome oxidase was seen. At low cyanide concentrations, much of the total extinction change took place at the rate characteristic of the combination of carbon monoxide with the enzyme. This relatively rapid change was followed by a secondary slow change with a half-time of 9 sec. at 23° and pH 7·4. As the cyanide concentration was increased, the proportions of slow and rapid change altered until at high cyanide concentrations almost all the change took place slowly. The time-constant of the slow change was independent of the cyanide and carbon monoxide concentrations. This division of the reaction into two phases is shown in Fig. 15. If these observations are explained by supposing that the mixtures of cyanide and cytochrome oxidase contain originally a mixture of reduced cytochrome oxidase and reduced cytochrome oxidase–cyanide complex, then the proportions of slow and rapid

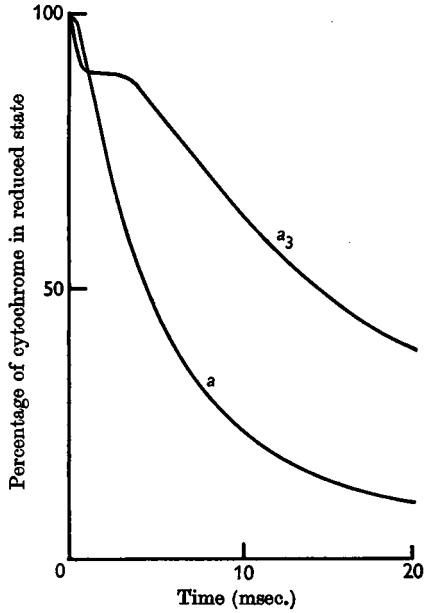

Fig. 14. Calculated time-course of the concentrations of cytochrome a_3^{2+} and cytochrome a^{2+} in the reaction of 5·0 μM-enzyme (concn. expressed as haem) with 2·3 μM-O_2. The initial concentration of both cytochromes has been set at 100. The rate constants were as for Figs. 5–8.

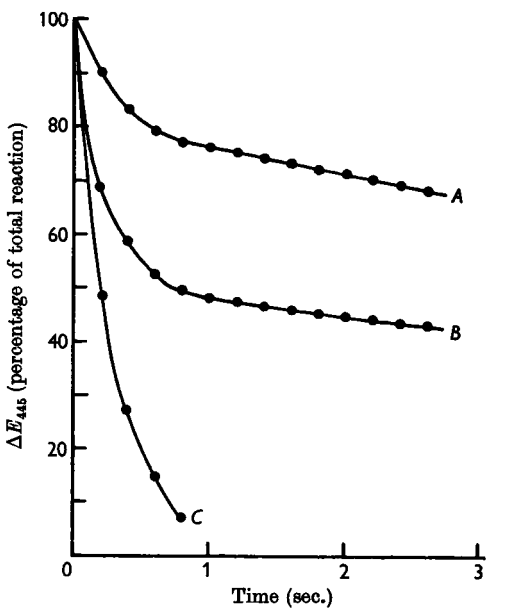

Fig. 15. Changes in extinction at 445 mμ when reduced enzyme (Yonetani preparation) [2·5 μM (expressed as haem) after mixing] was allowed to react with 50 μM-CO. The enzyme solution contained: *A*, 1·5 mM-KCN; *B*, 0·44 mM-KCN; *C*, no cyanide. Other conditions were: temperature, 23°; 0·1 M-phosphate buffer, pH 7·4.

change allow the equilibrium constant for the reaction between cyanide and cytochrome oxidase to be obtained. The experiment illustrated leads to a value of 0·7 mM. The observation that the slow change is first-order and independent of carbon monoxide and cyanide concentrations suggests that it is a measure of the velocity of dissociation of cyanide from the reduced cytochrome oxidase–cyanide complex. In the experiment quoted it was 0·08 sec.$^{-1}$ at 23°. The combination rate, which may be obtained by taking the equilibrium constant and dissociation velocity constants together, was $1{\cdot}2 \times 10^2$ M^{-1} sec.$^{-1}$. This value has been further confirmed by mixing reduced cytochrome oxidase in the stopped-flow apparatus with a solution of carbon monoxide dissolved in cyanide. As the combination velocity constant for carbon monoxide at 20° is about 500 times that for cyanide it would be expected that, when 1 mM-carbon monoxide dissolved in 0·5 M-cyanide (final concentrations) was mixed with reduced cytochrome oxidase, about one-half of the enzyme would combine directly with carbon monoxide at the usual characteristic rate, and that thereafter a slow reaction, the displacement of cyanide by carbon monoxide, would follow. This has been verified in several experiments with different total concentrations of carbon monoxide and cyanide. The agreement between the equilibrium constant and velocity constants is of importance in suggesting that cyanide and carbon monoxide combine with the same group on the enzyme. It would be possible to explain the biphasic reaction that is observed on mixing carbon monoxide with cytochrome oxidase in equilibrium with cyanide by the combination of cyanide with a group adjacent to the haem and by supposing that such combination altered the rate of reaction of the oxidase with carbon monoxide. It would, however, be a remarkable coincidence that in the experiments with carbon monoxide dissolved in cyanide solution such an effect should develop at a rate predicted by combining the apparent equilibrium constant for the reaction of cyanide with the cytochrome oxidase and the apparent rate of dissociation of cyanide from the cytochrome oxidase–cyanide complex. Because of the pH used in these experiments it is implied that hydrogen cyanide is the species active in combining with cytochrome oxidase.

Reproducibility of results

In general, reproducibility was satisfactory, and the results quoted are representative, the rates of reaction with oxygen and carbon monoxide showing little (± 20 %) variation from one preparation to another. This is true, however, only of fresh preparations: marked changes occurred during storage and on repeated freezing and thawing. These changes were not investigated in detail, but aged Yonetani preparations have a lower affinity for carbon monoxide and a biphasic reaction with oxygen similar to that observed with the Greenwood preparation.

DISCUSSION

In their classical study of a cytochrome-oxidase preparation from heart muscle, Keilin & Hartree (1938*a*, *b*, 1939) suggested that there are two forms of cytochrome containing haem *a*: cytochrome a_3, which reacts with carbon monoxide, cyanide and oxygen, and which has a weak α-band and strong γ-band, and cytochrome *a*, which does not react with inhibitors of cytochrome oxidase, and has a strong α-band but a weak γ-band. Keilin & Hartree (1939) also noted the presence of copper in their preparation and discussed the possibility that cytochrome oxidase might be a copper protein. Since their work the presence and function of copper and the separate existence and functions of cytochromes a_3 and *a* have been discussed by many authors. Griffiths & Wharton (1961*a*, *b*) have suggested that cytochrome oxidase is a copper haemoprotein, whereas Yonetani (1960*a*, *b*, 1961) has preferred to interpret his results in terms of cytochromes a_3 and *a*, and has attempted to show that the copper in his preparations does not undergo valency changes in enzyme action.

Our results have some bearing on these questions. Thus Griffiths & Wharton (1961*b*) write as a possible reaction scheme:

Cytochrome *c* → cytochrome *a* → copper → O_2

In the experiments by the flow–flash method carbon monoxide apparently protects the enzyme fully from oxidation until the ligand is removed photochemically, since the initial rate of reaction with low oxygen concentrations is the same by both the stopped-flow and flow–flash methods (Fig. 7). If the copper were not protected an initial rapid change in 'cytochrome *a*' would follow the removal of carbon monoxide inhibition by the firing of the photolysis flash. Although there are other possibilities, the simplest way of explaining such complete protection of the enzyme by carbon monoxide is that carbon monoxide combines with the same group as does oxygen. As is recognized by Griffiths & Wharton (1961*a*), the carbon monoxide compound of cytochrome oxidase behaves like a typical carbon monoxide–ferrous haemochromogen in showing sensitivity to light. In addition, our experiments on competition between cyanide and carbon monoxide, and between carbon monoxide and nitric oxide, give an order of stability of their complexes (nitric oxide, carbon monoxide, cyanide,

in decreasing order of stability) typical of ferrous haemoproteins, and the wavelengths of maximum absorption in the Soret region of these complexes are in the same order as those of typical haemoproteins (Yonetani, 1960*a*; Sekuzu, Takemori, Yonetani & Okunuki, 1959). Further, the strength of the absorption bands is high, ϵ_M being about 10^5 cm.^{2}/m-mole at the Soret absorption maxima for the three compounds. The proposal of Griffiths & Wharton (1961*b*) that copper reacts immediately with oxygen thus appears to be against the weight of the evidence, though they present convincing experiments to show that the enzyme contains copper and that this copper is associated with its activity. The experiments of Griffiths & Wharton (1961*b*) suggest that the ratio of enzymically active copper to iron is 1:1. If the ratio found by Kubowitz (1938) of carbon monoxide to copper, namely 1:2, for both phenol oxidase and haemocyanin applies to cytochrome oxidase, then the ratio of iron to carbon monoxide, namely 3:1, found by gasometric experiments requires either that copper be present in the enzyme in two forms, only one of which combines with carbon monoxide, or that carbon monoxide does not combine with enzyme copper, at least at low p_{CO}.

The kinetic results for the oxygen reaction, as followed at various wavelengths and over a range of oxygen concentrations, show that the reaction involves at least two spectroscopically distinguishable species. Further, the reaction records may be interpreted by supposing that the first of these species reacts directly with oxygen, and that the second can then pass an electron to the first. In addition, the results at different wavelengths can be fitted satisfactorily by identifying the species reacting with oxygen as cytochrome a_3, and the other species as cytochrome a, and applying the extinction coefficients derived by Yonetani (1960*a*). There is the difficulty, however, that the titration experiments with oxygen give the difference E_{445}(red.) $-\epsilon_{445}$(ox.) of only 80–100/mM-oxygen, or 20–25/m-electron equivalent, which is lower than would be expected for the γ-band of a haemoprotein. In all these experiments, the ΔE_{445} value has been within the range 78–120/mM-oxygen, both for the Greenwood preparation reduced with BAL and for the Yonetani preparation reduced with cytochrome *c* and ascorbic acid. Although it is difficult to make comparisons, this result seems to be rather lower than that of Chance & Yonetani (1959). The reason for the disagreement is not obvious, but, in general, contamination with oxygen is perhaps the most probable source of error, and would lead to too high rather than too low results for ΔE_{445}/mM-oxygen.

The method used for the oxygen titrations has been applied to titrations with carbon monoxide which give the reasonable value of 80/mM-carbon monoxide for E_{445}(red.) $-E_{445}$(CO). If, as is believed, only one haem out of three reacts with carbon monoxide, ΔE_{mM} per haem at 445 mμ is about 27. The oxygen and carbon monoxide titrations together yield the molar ratio of oxygen to carbon monoxide required to produce the full spectral change in a given quantity of enzyme. The result is a ratio of oxygen to carbon monoxide of 1·5:1, i.e. an amount of enzyme that can bind 1 molecule of carbon monoxide requires 6 electrons for its reduction. If it is assumed that cytochrome a_3 binds carbon monoxide whereas cytochrome *a* does not, and that both are iron porphyrins, the ratio of carbon monoxide to iron should be the same as the ratio of cytochrome a_3 to cytochrome (a_3 plus *a*). The gasometric determinations, made with a fourfold range of p_{CO}, give this ratio as 1:3. The iron content was estimated from spectrophotometric readings at several wavelengths by using the constants of Yonetani (1961), which are themselves in excellent agreement with those of Griffiths & Wharton (1961*a*).

To accommodate all these observations it is proposed that the functional unit of cytochrome oxidase consists of ($2a_3$ plus $4a$ plus 6Cu). The ratio of cytochrome *a* to cytochrome a_3 must be 2:1 to fit the ratio of oxygen to carbon monoxide found to give full spectral changes, as well as the ratio of iron to carbon monoxide, and also agrees with the ratio suggested by Ehrenberg & Yonetani (1961) from studies on the magnetic susceptibility of cytochrome oxidase. The 6Cu are available to donate electrons to oxygen, and, if it is assumed that the valency change of the copper is associated with negligible spectrophotometric changes, the value of $\Delta\epsilon_{mM}$[i.e. ϵ_{mM}(red.) $-\epsilon_{mM}$(ox.)] at 445 mμ will be doubled from 25 to 50. The findings of Griffiths & Wharton (1961*a*, *b*) are also accounted for, and possible roles for the enzyme copper are either as an intermediate between a_3 and *a* or as a source of electrons in equilibrium with them. The experiments described above do not allow a decision to be reached about the exact position of copper in the function of the enzyme, and both proposals raise difficulties in the interpretation of the spectrophotometric titrations of the enzyme with oxygen. It is possible (cf. Chance, 1961) that the reaction may begin:

$$2a_3^{2+} + O_2 + 4H^+ \rightarrow 2a_3^{4+} + 2H_2O$$

thus avoiding the need to postulate the formation of radicals such as $HO_2^{\cdot}$.

The reactions of cytochrome a_3 with carbon monoxide, cyanide and nitric oxide appear to be straightforward, though the affinity of the Yonetani preparation for carbon monoxide is unex-

pectedly high, because of a rapid rate of combination (8×10^4 M^{-1} sec.$^{-1}$) and a low rate of dissociation (0·023 sec.$^{-1}$) at 20° and pH 7·4. This high affinity would have interesting consequences in the application of Chance's (1953) method for the determination of the molecular extinction coefficient of the carbon monoxide compound of the cytochrome oxidase. The method depends on the fact that the return to equilibrium of a system that has been disturbed shows a rate constant equal to the sum of the rate constants for the forward and reverse reactions that determine the equilibrium. In the system cytochrome a_3, carbon monoxide and cytochrome a_3–carbon monoxide, equilibria are set up in the light and in the dark, and the rate constants for the transitions light-to-dark and dark-to-light are measured. For the light-to-dark transition:

$$k_{obs.} = k_{off}^{CO} + k_{on}^{CO}[CO]$$

For the dark-to-light transition:

$$k_{obs.} = k_{off}^{CO} + k_{on}^{CO}[CO] + k_d$$

where k_d is the rate of photochemical decomposition and is equal to the product $\epsilon_M \times$ quantum yield $\times$ light-intensity. The light-intensity is calibrated by using a compound of known extinction coefficient and quantum yield (myoglobin–carbon monoxide), and k_d for the cytochrome oxidase is evaluated by subtracting $k_{obs.}$ (dark) from $k_{obs.}$ (light). As Chance (1953) states, it is a condition of this method that the concentration of carbon monoxide remains effectively constant throughout; further, the most satisfactory changes in extinction are obtained when about half the haemoprotein is combined with carbon monoxide. For the Yonetani cytochrome-oxidase preparation at 20° the concentration of carbon monoxide at equilibrium in the dark is 0·23 μM, when the concentrations of cytochrome a_3 and of cytochrome a_3–carbon monoxide are equal, whereas to obtain a change in $E_{445}^{1\,cm.}$ of 0·06 would require the breakdown of 0·75 μM-cytochrome a_3–carbon monoxide, associated with a fourfold increase in the concentration of carbon monoxide, so that the condition of a constant concentration of carbon monoxide would be seriously violated. Very similar arguments apply to myoglobin, which has a somewhat higher affinity for carbon monoxide (Millikan, 1936; Q. H. Gibson, unpublished work). Thus, although the errors in the extinction coefficients are likely to be much smaller than the changes in the concentration of carbon monoxide, and although the similarity between myoglobin and cytochrome oxidase will give considerable compensation for errors, a re-examination of the photochemical extinction coefficient values may prove worth while.

A feature of the kinetic results is the constancy of the initial rapid reaction with oxygen, which has varied only about twofold over all the preparations made. The kinetic behaviour after the initial reaction is over has varied widely, but two extreme conditions can be distinguished. In most Yonetani preparations, with low concentrations of oxygen (less than 5 μM), the initial rapid spectrophotometric change continues until the whole reaction has been completed. With all Greenwood preparations and with Yonetani preparations that have been stored, the initial change is succeeded by slow changes extending over several seconds. In terms of the reaction scheme put forward above it seems that the reaction of cytochrome a_3 with oxygen is a very stable process, but that the flow of electrons from cytochrome a to cytochrome a_3 is more easily interrupted. The interpretation of the records of titration experiments at 445 mμ with the Greenwood preparation is complicated by the tendency of the extinction change after mixing with amounts of oxygen less than equivalent to the enzyme to reach a maximum quickly (less than 50 msec.), and then to decline slowly during the next 0·5–2 sec. With greater amounts of oxygen the first rapid phase is followed by a slow increase in extinction to a stable maximum. With small amounts of oxygen, the choice of the time-interval after which the measurements are made is necessarily arbitrary. These results are readily explained in terms of the scheme proposed above, in which the ratio of cytochrome a to cytochrome a_3 is 2:1, together with Yonetani's (1960a) observation that about a half of ΔE_{445} [i.e. E_{445}(red.) − E_{445}(ox.)] is to be attributed to the reaction $a_3^{2+} \rightarrow a_3^{3+}$, and the remainder to the reaction $a^{2+} \rightarrow a^{3+}$. It follows that ΔE_{445}/mM-cytochrome a is about half of ΔE_{445}/mM-cytochrome a_3. When less than 1 equiv. of oxygen is mixed with a reduced Greenwood preparation cytochrome a_3^{2+} is first oxidized, then, more slowly, some cytochrome a_3^{3+} is reduced by cytochrome a^{2+}, exchanging the larger ΔE_{445} for the smaller. The observed ΔE_{445} thus passes through a maximum. With larger amounts of oxygen almost all the cytochrome a_3 will be maintained in the oxidized form throughout and the oxidation of cytochrome a^{2+} will be observed as a slow increase in E_{445} after the initial rapid change.

The relation of the kinetic results for the oxidation of the reduced enzyme to its catalytic activity can be expressed very roughly by assuming a molecular weight and calculating the turnover number. Yonetani's (1961) analyses yield about $1{\cdot}5 \times 10^5$ g. of protein/g.atom of iron, and with the scheme proposed each iron atom must change valency twice for each molecule of oxygen reduced. A Q_{O_2} of 5000 μl./mg./hr. corresponds to the reduction of 0·125 μmole/sec. by 6·6 μm-moles of enzyme, giving a turnover number of about

40 sec.$^{-1}$. Thus the rate of reaction of the reduced enzyme with oxygen is amply sufficient to permit the turnover numbers observed. It would be useful to examine the highly active preparations of Griffiths & Wharton (1961*a*, *b*), who have obtained Q_{O_2} values of up to 30 000 at 25°. If the rate of the initial reaction is the same as for other preparations the concentration of oxygen required for half-maximal activity would be about 2·5 μM, corresponding to a p_{O_2} of about 1·2 mm. Hg. Unfortunately, however, the resources of this Laboratory do not permit the preparation of mitochondria in the quantity and of the quality required for the Griffiths & Wharton (1961*a*, *b*) preparation of the enzyme.

The autocatalytic time-course of the oxidation of cytochrome a_3–carbon monoxide by molecular oxygen deserves comment. The acceleration of the oxidation requires that some cytochrome a_3–carbon monoxide molecules be oxidized by cytochrome a^{3+} or Cu^{2+} ions produced in the earlier stages of the reaction. It appears that interactions between cytochrome a_3 and cytochrome a may involve considerable numbers of molecules of each kind.

Although Ambe & Venkataraman (1959) have succeeded in breaking down cytochrome oxidase to a single homogeneous monomeric but inactive protein, their result does not seem to be incompatible with the existence of two forms of binding of haem a in intact cytochrome oxidase, and may perhaps be correlated with the ready disturbance of the oxidation of cytochrome a found in the preparations used in the present study.

Work intended to explain the functioning of the cytochrome oxidase under physiological conditions requires unmodified enzyme preparations, but this does not apply to experiments with isolated enzyme systems, where the effect of alteration in the enzyme may give a clue to the mechanism of its action. For this reason it is satisfactory that all the spectrophotometric results with both enzyme preparations can be accommodated within the reaction scheme proposed with the alteration only of the value given to the constants k_2 and k_4. The participation of copper is optional in the scheme for describing the spectrophotometric results, which do not, in themselves, give chemical information about the enzyme. As Griffiths & Wharton (1961*a*, *b*) have pointed out, clarification of its function and definite correlation between copper and spectrophotometric changes must come from electron-spin-resonance studies within the time-scale used in the experiments described above. In an Addendum to the present paper, electron-spin-resonance work by the quick-freezing method of Bray (1961) is described, which shows that rapid changes in the valency of copper can occur.

SUMMARY

1. The reaction of the Yonetani (1960*a*, 1961) preparation of cytochrome oxidase with carbon monoxide is accurately second-order with a rate constant 8×10^4 M^{-1} sec.$^{-1}$ at 20° and activation energy of 6·4 kcal.

2. The dissociation velocity constant of carbon monoxide is 0·023 sec.$^{-1}$ at 20° and pH 7·4.

3. In the Yonetani (1960*a*, 1961) preparation, one-third of the total iron reacts with carbon monoxide to form the carbon monoxide compound as determined both by spectrophotometry and by gasometric methods.

4. The reaction of oxygen with reduced cytochrome oxidase is rapid and the course of the reaction is complex. At low oxygen concentrations the approximate second-order rate constant is 6×10^7 M^{-1} sec.$^{-1}$ for the Yonetani (1960*a*, 1961) preparation and 3×10^7 M^{-1} sec.$^{-1}$ for the Greenwood (1963) preparation.

5. When the reaction between reduced cytochrome oxidase and oxygen is followed at different wavelengths, the course of the reaction changes. At low oxygen concentrations (less than 5 μM) the reaction proceeds faster when followed at 605 mμ than when followed at 445 mμ. At concentrations of oxygen above 10 μM, the observed changes at 605 mμ are slower than those at 445 mμ. The apparent second-order rate constant at 445 mμ and at low oxygen concentrations increases as the reaction proceeds; at high oxygen concentrations it decreases.

6. These changes are attributed to consecutive reactions between oxygen and reduced cytochrome a_3, and between reduced cytochrome a_3 and oxidized cytochrome a. Satisfactory agreement between observation and calculation has been obtained by assuming that reduced cytochrome a_3 can react with oxygen with a velocity constant of $1·5 \times 10^8$ M^{-1} sec.$^{-1}$ and that the reaction between reduced cytochrome a and cytochrome a_3 is first-order with a rate constant of $1·5 \times 10^3$ sec.$^{-1}$. The spectrophotometric changes at different wavelengths may be reproduced by using the extinction coefficients given by Yonetani (1960*a*).

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ADDENDUM

Electron-Spin-Resonance Changes on Oxidation of Cytochrome Oxidase

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Sands & Beinert (1959) and Ehrenberg & Yonetani (1961) have shown that oxidized cytochrome oxidase gives an electron-spin-resonance signal, which can be identified with cupric copper in the enzyme. Sands & Beinert (1959) showed that the signal was smaller when the enzyme was reduced with cytochrome *c*–ascorbic acid, and this was confirmed by Ehrenberg & Yonetani (1961). Sands & Beinert (1959) obtained some reduction on the addition of cytochrome *c* alone, but Ehrenberg & Yonetani (1961) could not confirm this result. All the measurements so far reported have been on systems at equilibrium, or on systems in which the oxidation state of the enzyme was changing relatively slowly. Although they show that the valency state of the copper can change when cytochrome oxidase is oxidized and reduced they do not show that the change can take place at a rate compatible with the participation of the valency change of the copper in the functioning of the enzyme, nor do they help in establishing whether the iron–porphyrin or the copper-containing portions of the enzyme react with molecular oxygen. We have therefore made electron-spin-resonance measurements by using the quick-freezing technique of Bray (1961) in an attempt to answer this point.

EXPERIMENTAL

A rapid-freezing apparatus similar in basic construction to that described by Bray (1961) was used. Heptane at −80° was used to freeze the reaction mixture. The ice crystals so formed were subsequently tamped down to give a pack 3 cm. long in a quartz tube (3 mm. bore) suitable for use in the electron-spin-resonance spectrometer. Electron-spin-resonance measurements were made at liquid-nitrogen temperature in a Varian V 4501 100 kcyc./sec. spectrometer.

The electron-spin-resonance spectrum of the oxidized enzyme shown in Fig. 1 is in general agreement with that of Ehrenberg & Yonetani (1961) but shows less fine structure. In confirmation of the results of Ehrenberg & Yonetani (1961) and of Sands & Beinert (1959), the signal substantially disappears on reduction of the enzyme with ascorbic acid and cytochrome *c*. The signal obtained by the quick-freezing technique (Bray, 1961) is of the same form, but about half the intensity. For this experiment, equal volumes of 1 mM-reduced cytochrome oxidase (concn. expressed as haem) and 1·2 mM-O_2 dissolved in water were

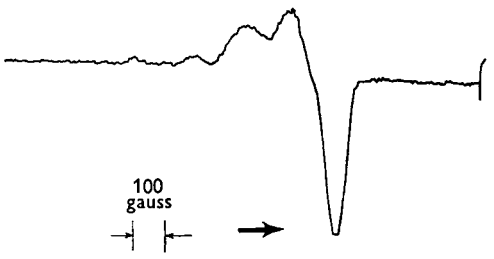

Fig. 1. Electron-spin-resonance spectrum of Yonetani's (1960, 1961) preparation of cytochrome oxidase in the oxidized form. The concentration of enzyme was 0·8 mM (expressed as haem), and observation was in a circular tube of 3 mm. diameter at 77° K. The arrow shows the direction of increase of magnetic field.

mixed. The solution was frozen as quickly as possible after leaving the mixer and the time was estimated to be approx. 10 msec. (Bray, 1961). The finely divided ice crystals, suspended in heptane, contain only about half as much enzyme per unit volume as do the undiluted preparations of enzyme. A substantial portion of the total enzyme had been converted into the oxidized form in the 10 msec. or so between mixing and the completion of freezing. When the cytochrome oxidase solution was first equilibrated with CO and then mixed with O_2, there was no signal due to oxidized copper.

DISCUSSION

Although the technique described by Bray (1961) represents a great improvement on the making of a mixture followed by freezing the tube containing it, the time of 10 msec. given by Bray for freezing the droplets is still rather long in the study of a rapidly reacting enzyme such as cytochrome oxidase. The satisfactory signals which we have obtained, however, certainly show that a large proportion of the enzyme copper can become oxidized in that time, so that the participation of copper in the enzyme reaction is in no way excluded, as it would have been if the electron-spin-resonance signal had failed to appear. The protection given by carbon monoxide helps in drawing conclusions about the order in which the haem and copper react with oxygen. For reasons which are given in the Discussion section of the main paper, it seems unlikely that carbon monoxide itself combines with cuprous copper of the enzyme; if this is accepted it follows that cuprous copper does not react directly with oxygen but is oxidized indirectly through the intervention of haem iron, a conclusion which fits well with the observation that the kinetics of the reaction of oxygen with reduced cytochrome oxidase are the same when studied by the stopped-flow method and by the flow–flash methods at similar oxygen concentrations.

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Studies on Dextrans and Dextranases

3. STRUCTURES OF OLIGOSACCHARIDES FROM *LEUCONOSTOC MESENTEROIDES* (BIRMINGHAM) DEXTRAN*

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It has been shown that the adaptively produced dextranases of *Penicillium lilacinum* (I.M.I. 79197; NRRL 896) and of *P. funiculosum* (I.M.I. 79195; NRRL 1132) hydrolyse the virtually unbranched dextran of *Streptococcus bovis* to give mainly isomaltose and that the degree of hydrolysis of dextrans possessing anomalous linkages depends on the percentage of such linkages in the dextrans (Bourne, Hutson & Weigel, 1962). This suggested that the dextranases could not hydrolyse the anomalous linkages of branched dextrans. Isolation of the oligosaccharides which cannot be hydrolysed by the dextranases should aid the structural analysis of dextrans and also elucidate further the mechanism of dextranase action. An analysis of the structures of 'branched' oligosaccharides produced by the action of the dextranase of *Lactobacillus bifidus* (Bailey & Clarke, 1959) on

* Part 2: Bourne, Hutson & Weigel (1962).