Kinetic Observations on the Near Infrared Band of Cytochrome c Oxidase*

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Although it has been more than 25 years since Keilin and Hartree (1, 2) noted that their preparations of cytochrome oxidase contained copper, its relation to the function of the enzyme continues to be a matter for discussion. The first titrations of the reduced enzyme with oxygen, performed by Chance and Yonetani (3), seemed to exclude copper as an electron donor since 1 molecule of oxygen was able to oxidize 4 molecules of heme a. Gibson and Greenwood (4), however, found that 1 molecule of oxygen oxidized only 2 molecules of heme a, and they suggested that copper might indeed function as an electron donor; their results have recently been confirmed and extended by Van Gelder and Muijsers (5), who have titrated oxidized cytochrome oxidase with reduced diphosphopyridine nucleotide with the use of phenazine methosulfate as a mediator and have found that their oxidase preparations could accept 2 electrons per heme group. Further, the numbers of nonheme acceptor groups accessible to titration with DPNH may be altered by copper-binding agents such as cyanide, ethylenediaminetetraacetate, and salicylaldoxime (6). There is thus little doubt that copper can undergo reversible oxidation and reduction, and this has also been confirmed by electron paramagnetic resonance spectroscopy (7).

If copper is to be accepted as a functional constituent of the enzyme, it must also be shown to undergo oxidation and reduction at a rate consistent with the turnover numbers for heme a observed in the system, but, because of the rapid rates of reaction of the enzyme and the relative insensitivity of electron paramagnetic resonance spectroscopy, correlation between the behavior of heme a and of copper has been difficult to establish. Thus, although the results are consistent with the participation of copper in enzyme function (7, 8), it has not yet been shown to react as rapidly as heme a.

Another approach to the problem is offered by the discovery of Griffiths and Wharton (9) that the enzyme has a broad absorption band centered around 820 mJ. They suggested that this band was due to copper, and their proposal has since been supported by Van Gelder and Muijsers (6) and by Wharton and Taagoloff (10). This paper reports experiments on the kinetic behavior of the long wave length band and shows that the species producing it is kinetically distinct from both cytochrome a and cytochrome a3.

EXPERIMENTAL PROCEDURE

Cytochrome Oxidase—Cytochrome oxidase prepared by the method of Yonetani (11) was stored at 12°. In no case was a preparation used more than 1 week after it was made. The concentrations given in this paper are based on heme a content with the use of the extinction coefficients of Yonetani (12).

Cytochrome c—Cytochrome c monomer prepared from beef heart was a gift from Dr. E. Margoliash.

Stopped Flow Kinetic Experiments—These were performed with the apparatus of Gibson and Milnes (13) with the use of an RCA photomultiplier, type 7102, for determinations at 820 mJ. The Bauch and Lomb small grating monochromator was supplemented by a red filter glass (Corning No. 2434) to remove stray and second order light.

Flash Photochemical Kinetic Experiments—These were performed with the apparatus of Gibson and Greenwood (4), modified by surrounding the 8-cm path observation tube with a cylindrical liquid filter 0.75 cm deep. For experiments at 820 mJ the filter contained a saturated solution of cuprammonium sulfate with an absorbance at that wave length of about 15 cmJ. The photochemically effective light passed by the cuprammonium solution was then screened by the monochromator and photomultiplier with a red filter (Corning No. 2434). With due care to exclude stray light leaking from the flash box and fluorescent light excited by the flash, observation was possible during the photolysis flash itself. The discharge energy was up to 750 joules, divided between two 4-microfarad condensers, and was released with a half-time of 6.5 microseconds. Energies of the order of 100 joules were sufficient to decompose more than 90% of the cytochrome oxidase carbon monoxide compound. For experiments at 445 mJ, the cuprammonium solution was replaced by a saturated solution of K2Cr2O7. With this filter flash energies of the order of 500 joules were regularly used.

RESULTS

Reaction of Reduced Cytochrome Oxidase with Oxygen by the Stopped Flow Method—Experiments performed by the stopped flow method allow comparisons between the results at 605 mJ and at 820 mJ to be made with half-times for the reaction down to about 5 milliseconds, and an example is given in Fig. 1. This shows that the rate of change at the two wave lengths is similar. The experiment strains the stopped flow method because of the combination of a small change in absorbance with a rapid rate, and the difference between the two curves (maximally 0.002 in absorbance at 820 mJ) is not considered significant.

Control Experiments with Flash Photochemical Method—The proportion of the cytochrome oxidase-CO compound decomposed by the photolysis flash was determined by varying the energy input to the flash tubes over a 25-fold range. The results shown in Fig. 2 indicate that, although the cuprammonium filter in-
increased the flash energy required to break down the oxidase-CO compound by about 4 to 5 times as compared with the unscreened flash (4), there was no difficulty in working with flash energies sufficient to break down virtually all of the CO compound.

The absorbance changes which can be measured at 820 mμ, even with an 8-cm optical path, are small compared with those employed in earlier work with the oxidase (4). As a result, appreciable slow changes were observed in the solutions after flow, which sometimes amounted to as much as 10% of the total change in absorbance measured. These changes did not seem to depend on the chemical reaction taking place in the solution and were attributed to changes in scattering of the enzyme solution. It should be stressed that their absolute size was small and was always less than 0.001 absorbance unit per cm. Because of this difficulty all absorbance changes at 820 mμ were measured from the time of first observation, and the rapid changes occurring in the first 5 milliseconds after initiation of the reaction were correlated with absorbance changes measured over the corresponding time interval at other wave lengths.

As an over-all check the reaction of dimethyl deuteroheme-disulfonate with CO was followed at 820 mμ and at 605 mμ, and the changes in absorbance at the two wave lengths were correlated. With a pseudo-first order constant of 5.6 \times 10^3 sec^{-1}, the greatest difference between the results at the two wave length, expressed as the percentage of the total absorbance change, was 5% with a mean difference of 2% for six points. The change in

![Fig. 1 (left). The reaction of reduced cytochrome oxidase with oxygen followed in a stopped flow apparatus at 605 mμ (O) and at 820 mμ (•). The point at zero time was obtained by waiting for re-reduction of the enzyme to occur after completion of the rapid reaction. The results at the two wave lengths have been normalized to coincide at zero time and at maximum reaction. The concentrations were: enzyme, 2.6 \times 10^{-5} M (heme a); O_2, 1.2 \times 10^{-5} M; cytochrome c, 2 \times 10^{-5} M; and sodium ascorbate, 1 \times 10^{-4} M, all before mixing in 0.1 M phosphate buffer, pH 7.4, at 20°. The reaction was followed with an RCA photomultiplier, type 7102, and the Bausch and Lomb small grating monochromator and a 2-mm Corning glass filter, No. 2434. The nominal band width was 3 mμ at 605 mμ and 6 mμ at 820 mμ, with an optical path length of 2 cm.

Fig. 2 (right). The change in absorbance at 620 mμ observed when the carbon monoxide compound of cytochrome oxidase was exposed to photolysis flashes with variable input energies. The four left-hand points were obtained with two 1-μicrofarad condensers, and the two right-hand points with two 4-μicrofarad condensers. The enzyme concentration was 5 \times 10^{-6} M heme a, in 0.1 M phosphate buffer, pH 7.4; \text{pHCO} 100 mm of Hg, at 22°. Optical path length was 8 cm, and the photolysis flash was filtered through 0.75 cm of a saturated solution of cuprammonium sulfate.

![Fig. 3 (left). The reaction of reduced cytochrome oxidase with oxygen followed by the flow flash method. Curve A shows the changes in absorbance at 605 mμ (O) and at 820 mμ (•) when 5.7 \times 10^{-6} M oxygen reacts with approximately 5 \times 10^{-5} M cytochrome oxidase. Curve B shows the changes with an oxygen concentration of 1.1 \times 10^{-5} M. Other conditions were: cytochrome c, 1 \times 10^{-7} M; sodium ascorbate, 1 \times 10^{-5} M; 0.1 M phosphate buffer, pH 7.4, at 28°. The reaction was followed with optical components as described in the legend for Fig. 1 for multiplier and monochromator. The observation tube and flash equipment were those described in the legend for Fig. 2.

Fig. 4 (right). Oxidation of reduced cytochrome oxidase by molecular oxygen followed at 820 mμ (•) and at 605 mμ (O) in the flow flash apparatus. Cytochrome oxidase, 2 \times 10^{-5} M heme a, with 5 \times 10^{-3} M cytochrome c and 0.01 M sodium ascorbate in 0.1 M phosphate buffer, pH 7.4, equilibrated with 110 mm of Hg \text{pHCO}, was used for observations at 820 mμ. The enzyme solution was diluted 1:1 with deoxygenated buffer for the observations at 605 mμ. The reaction concentration was 1.3 \times 10^{-5} M after mixing with the enzyme, and the temperature 28°. The reaction was followed with an RCA photomultiplier, type 7102, at 820 mμ with a Bausch and Lomb large grating monochromator and a 2-mm Corning glass filter, No. 2434. At 605 mμ an EMI photomultiplier, type 9520, and a Bausch and Lomb large grating monochromator were used with a nominal band width of 2.5 mμ. The optical path was 8 cm, and the photolysis flash of 150 joules was filtered through 0.75 cm of saturated cuprammonium solution. The absorbance change measured was a decrease at 605 mμ and an increase at 820 mμ.

absorbance measured at 820 mμ was 0.0029 cm\(^{-1}\), of the same order as the change in absorbance in experiments with the cytochrome oxidase. The agreement between the two wave lengths was regarded as satisfactory.

**Reaction of Reduced Cytochrome Oxidase with Oxygen by Flash Photophysical Method**—With the regenerative method, in which oxygen was added from a much smaller syringe than that used for the enzyme (the ratio of volumes delivered was usually 21:1), the absorbance changes at 820 mμ and at 605 mμ were compared for a range of oxygen concentrations. It was found, as shown in Fig. 3, that there is a fair correlation between the absorbance changes at the two wave lengths down to half-times of the order of 0.5 millisecond. At the highest oxygen concentration which could be reached with the particular experimental arrangement (5.7 \times 10^{-5} M), however, the form of the reaction record appeared somewhat different at the two wave lengths. Thus, at 605 mμ the rate of change of absorbance was higher initially than at 820 mμ, while in the later stages of the reaction the converse was true. The reality of the differences shown in Fig. 3 was supported by the results obtained with a rearranged flow apparatus in which equal volumes of oxygenated buffer and enzyme solution were mixed in the observation tube. With 1.3 \times 10^{-4} M oxygen (Fig. 4) the initial rapid change at 605 mμ was half complete in
Fig. 5 (left). The reaction of reduced cytochrome oxidase with oxygen followed at 610 m\(\mu\) by the flow flash method. Cytochrome oxidase, 1.1 \(\times\) \(10^{-2}\) m heme \(a\); 4 \(\times\) \(10^{-2}\) m cytochrome \(c\); and 0.01 m sodium ascorbate in 0.1 m phosphate buffer, pH 7.4; equilibrated with 80 mm of H\(_2\) \(pK_0\), were used. The concentration of oxygen was 6 \(\times\) \(10^{-2}\) m after mixing. Other conditions were the same as those for Fig. 4.

Fig. 6 (right). The reduction of cytochrome oxidase by dithionite followed at different wave lengths. The ordinate is the difference between the final absorbance and the absorbance at the time given by the abscissa. The change measured after mixing was an increase in absorbance at 605 m\(\mu\) and at 445 m\(\mu\) and a decrease at 820 m\(\mu\); \(\bullet\), in the presence of 1 \(\times\) \(10^{-3}\) m ethylenediaminetetraacetate sodium (EDTA) in its absence. The cytochrome oxidase contained 3.3 \(\times\) \(10^{-4}\) m heme \(a\) and was mixed with an equal volume of 0.01 m NaaS\(_2\)O. All of the reagents were dissolved in 0.1 m phosphate buffer, pH 7.4, and the temperature was 10.5\(^\circ\) C. The optical path was 2 cm for observations at 605 m\(\mu\) and 820 m\(\mu\), and 2 mm at 445 m\(\mu\). The nominal band width was 3.3 m\(\mu\) for observations at 605 m\(\mu\) and 445 m\(\mu\), and 0.6 m\(\mu\) for those at 820 m\(\mu\).

An important feature of the record at 605 m\(\mu\) is the "plateau" at about 300 microseconds, where the rate of change of absorbance passes through a minimum. At the highest oxygen concentrations which could be reached with the apparatus (6 \(\times\) \(10^{-4}\) m after mixing) the pattern of the absorbance changes was similar to that shown in Fig. 4. The only difference was that the rate of the initial change at 605 m\(\mu\) was approximately doubled so that the plateau was more striking than that with the lower oxygen concentration. The time course at 820 m\(\mu\) with 6 \(\times\) \(10^{-4}\) m oxygen was the same as that shown in Fig. 4.

**Correlation of Absorbance Changes at Various Wave Lengths**

The absorbance changes due to oxidation of cytochromes \(a_3\) and \(a\) in the records made at 605 m\(\mu\) were quite distinct at the highest concentrations of oxygen and could be identified with change in absorbance from 0 to 0.16 and 0.16 to 0.35, respectively, in Fig. 4, in accordance with earlier kinetic work (4). At 820 m\(\mu\), although the change from 0.035 to 0.060 took place at the same rate as the oxidation of cytochrome \(a\), the earlier and more rapid phase of the change in absorbance could not be correlated with the oxidation of either cytochrome \(a_3\) or cytochrome \(a\). It was also noticed, as Fig. 4 shows, that the plateau in the absorbance change at 605 m\(\mu\) ended about 300 microseconds after initiation of the reaction, at about the same time as the completion of the rapid phase of the 820 m\(\mu\) change. A natural explanation of the plateau at 605 m\(\mu\) and of the rapid phase at 820 m\(\mu\) is that both are the spectrophotometric reflection of a kinetic process distinct from the oxidation of \(a_3\) and \(a\). If so, the spectrophotometric changes at any wave length should be generated by combining the time course of \(a_3\) oxidation and of \(a\) oxidation with the estimated course of the third reaction as derived from the observations at 820 m\(\mu\), giving to each component a weight proportional to its extinction coefficient. As it was not known whether the scheme

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a_3 \rightarrow x \rightarrow a
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or the scheme

\[
a_3 \rightarrow a
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should be used in calculating the time course of cytochrome \(a\) concentration, both were examined, and the extinction coefficients were assigned by the method of least squares. It was found that at 605 m\(\mu\) a good fit was obtained with either of the two kinetic schemes, with a root mean square residual of 0.003 (rather less than 1% of the total absorbance change observed in Fig. 4). If the terms corresponding to the reaction followed at 820 m\(\mu\) were omitted and the curve fitted in terms of \(a_3\) and \(a\) oxidation alone, not only was the mean residual more than doubled, but the distribution of residuals was nonrandom, and the plateau of Fig. 4 was not reproduced.

An attempt was made to obtain a difference spectrum in the \(\alpha\)-band region for the species associated with the rapid phase of the absorbance change at 820 m\(\mu\). The difficulties of assigning three spectrophotometric constants from a single kinetic curve led to somewhat erratic results: qualitatively it was found that at wave lengths between 620 and 565 m\(\mu\) the change associated with the 820 m\(\mu\) component was an increase in absorbance on oxidation of the enzyme. This change was maximal at about 610 m\(\mu\), where it was about one-quarter of the change associated with the oxidation of cytochrome \(a\) at the same wave length. At wave lengths shorter than 580 m\(\mu\), the change was equal to or greater than that associated with the oxidation of cytochrome \(a_3\) and the two spectrophotometric contributions continued to be in opposite directions from each other. The difference spectrum for the new species in the \(\alpha\) region thus appears to be a broad band extending over approximately 60 m\(\mu\) with a maximum at around 610 m\(\mu\). These results predicted that the greatest spectrophotometric effect of the new species should be observed by using maximal oxygen and making measurements at 610 m\(\mu\). Under these conditions an actual reversal of the direction of absorbance change occurred (Fig. 5) rather than a plateau as shown in Fig. 4. Such a result cannot be explained in terms of the oxidation of \(a\) and \(a_3\), both of which have a lower absorbance in the oxidized than in the reduced form, even if an additional step not associated with a spectrophotometric change is introduced.

**Oxidation of Cytochrome Oxidase-Carbon Monoxide Compound**

In the experiments described in the last section no very rapid changes were observed at 820 m\(\mu\) which could be correlated with the oxidation of cytochrome \(a_3\) as followed, for example, at 445
the near infrared spectrum, it was necessary to use much higher
chelator concentration in order to achieve the absorbance change at 820 μm (cf. Fig. 7) (14). Although earlier work has shown that carbon monoxide can protect cytochrome a₃ from oxidation by molecular oxygen (15), such protection need not necessarily extend to copper. In the flow flash method the enzyme-carbon monoxide compound is mixed with oxygen and remains in contact with it for up to about 0.5 second before the photolysis flash is fired, and it seemed possible that oxidation of copper might occur in this period. This was excluded by control experiments in which both the oxidation of the CO compound of the enzyme by oxygen and its re-formation after the oxygen had been consumed were followed in the stopped flow apparatus. It was found that the absorbance returned to its starting point when the oxygen had been exhausted. If there had been an initial rapid reaction on mixing the CO compound of the enzyme with oxygen, resulting in an increase of absorbance at 820 μm, either the reaction would have been observed, or, if it had been too fast to be followed by the stopped flow method, the final absorbance level after the oxygen had been consumed would have lain beyond the initial point. In the course of these experiments the finding of Gibson and Greenwood (15) that about 80% of the total change in absorbance at 820 μm was correlated in time with the oxidation of cytochrome a and about 20% with the change a₄⁻−CO → a₄⁺ was confirmed.

Effect of Chelating Agents on Reactions of Cytochrome Oxidase as Observed at 820 μm—Side by side comparisons have been made between samples of enzyme made up in the presence and absence of 5 × 10⁻³ M salicylaldoxime and 1 × 10⁻³ M ethylenediamine-tetraacetate. These reagents were not observed to affect the time course of (a) the reaction of reduced enzyme with oxygen, (b) the reaction of the enzyme-CO compound with oxygen, or (c) the reaction of the oxidized enzyme with 0.01 M Na₂S₂O₄. In all cases the enzyme was incubated with the chelating agent for at least 30 minutes before the kinetic experiments were performed. In many cases observations were made at wave lengths other than 820 μm, but in no experiment was any effect of the chelator observed which was regarded as significant. The results of one experiment with dithionite are presented in Fig. 6. The time course was somewhat different at the various wave lengths; in agreement with the observation of Lemberg et al. (16) that cytochrome a is reduced faster than cytochrome a₃, the initial rate of change at 605 μm was greater than that at 445 μm. The time course at 820 μm did not agree with that at either 605 or 445 μm, but showed a lag period. Such an effect would be expected if reduction of cytochrome a preceded reduction of the species chiefly responsible for the absorbance change at 820 μm.

Effect of Concentration of Oxidase on Rate of Reaction with Oxygen—In order to follow the spectrophotometric changes in the near infrared spectrum, it was necessary to use much higher concentrations of enzyme than those employed in earlier work (4). To test the effect of enzyme concentration an observation cell with a path length of 1 mm was constructed, and experiments were performed to compare the results with this cell and with the usual 8-mm path cell at the same concentration of oxygen but at different concentrations of enzyme. The comparisons could only be made fruitfully at 445 μm, but at this wave length it appeared that the results were independent of enzyme concentration. This result is important not only in justifying the use of various enzyme concentrations, but also in demonstrating that oxidation of a by a₃ is independent of concentration over a much wider range than has previously been examined, and in validating its treatment in earlier work (4) as a first order reaction.

DISCUSSION

As a preliminary it should be pointed out that, although figures for the concentration of enzyme in terms of heme a have been given, their application to the reaction records requires discussion. First, in the regenerative stopped flow method, the oxygen introduced is mixed not with the total volume of enzyme in the system but with a fixed volume which forms a variable portion of the whole as the enzyme becomes diluted by the successive injections of oxygen-containing buffer. The concentration of oxygen is thus fixed, but the enzyme concentration varies and is not necessarily uniform throughout the apparatus. Second, it has become clear that purified cytochrome oxidase preparations vary in their carbon monoxide-binding capacity over a range of as much as 2:1 (17). In the flow flash method, only that portion of the enzyme preparation able to bind CO can take part in the reaction, and the change in absorbance per total heme a will be reduced in proportion to the CO-binding capacity. The enzyme which does react, moreover, may show a different distribution of absorbance change between a and a₃ from that deduced from static experiments; in Fig. 7, for example, it is clear that the rapid change (attributed to a₃) is about two-thirds of the whole, confirming the suggestion of (4, 17) that change in absorbance of the reduced minus oxidized forms at 445 μm is about twice as great for a₃ on a molar basis as for a, whereas static experiments have suggested that the two were about equal (11).

The experiments described here seem to establish (a) that under suitable conditions, the greater part (about two-thirds, cf. Fig. 4) of the increase in absorbance observed at 820 μm on the oxidation of reduced cytochrome oxidase may occur at a rate different from that of the oxidation of either cytochrome a₃ or cytochrome a, and (b) that the changes in absorbance in the β-band region cannot be explained by the oxidation of cytochromes a and a₃ only. An additional process giving rise to an
increase in absorbance in the $a$ region on oxidation is required and is correlated in time with the major change in absorbance at 820 m.$\mu$. Although these experiments do not contribute directly to the chemical identification of the species responsible for the absorption band at 820 m.$\mu$ in the oxidized oxidase, they may be discussed briefly in the light of three chemical possibilities. The first is that the band is due to copper. The titrations of Van Gelder and Muijsers (6) may be interpreted to mean that there are two forms of copper in cytochrome oxidase: one, associated with $a_3$, is accessible to chelating agents; the other, associated with $a$, is not. As chelating agents have not influenced any reaction so far examined either in rate or in extent of absorbance change, the 820 m.$\mu$ band must be attributed mainly to the form of copper associated with $a$. In this connection it is interesting to note that Beinert and Palmer (18) have recently pointed out that only a part of the total enzyme copper contributes to the electron spin resonance signal, and Wharton (19) has shown that oxidation of $a$ alone, achieved by the use of K$_3$Fe(CN)$_6$ in the presence of CO, is associated with the appearance of the same electron spin resonance signal as is given by the enzyme when both $a$ and $a_3$ are in the oxidized form. From a kinetic point of view the spectrophotometrically visible copper is a feasible member of any scheme for cytochrome oxidase action, as it can react at a rate comparable with that of the other components of the enzyme. Although there is as yet insufficient evidence to establish the sequence of the reactions with certainty, the simplest linear scheme with copper as an obligatory intermediate between $a$ and $a_3$ is consistent both with the experiments on the oxidation of the reduced enzyme by oxygen and with those on its reduction by dithionite. The present experiments give no hint whatever of a possible role for the spectrophotometrically invisible copper associated with cytochrome $a_3$.

The two main alternatives to correlating the appearance of the band at 820 m.$\mu$ with the oxidation of copper are polymerization of the enzyme on oxidation and an intramolecular rearrangement with wider separation of heme $a$ and $a_3$ for example. While polymerization would require a very large second order rate constant of $10^5$ $\text{m}^{-1} \text{sec}^{-1}$ or more to permit the observed rate of appearance of the band and so is perhaps improbable, there is no kinetic objection to postulating an intramolecular change which would be associated with a first order rate constant. While these experiments were being carried out the opportunity was taken to confirm the observation (9) that the band at 820 m.$\mu$ is selectively diminished by the action of urea.

Last, it is clear that the demonstration of a kinetically distinct absorbing species other than cytochromes $a_3$ and $a$ in soluble preparations must complicate the problem of obtaining satisfactory difference spectra for these components. It will certainly be necessary, in the future, to take into account the oxidation state not only of the cytochromes $a_3$ and $a$, but also of the third absorbing species. For example, in resolving the components kinetically as described here, at high oxygen concentrations the third species would tend to be reckoned in with cytochrome $a$, since the $a_3$-oxygen reaction can readily be made to take place up to 5 times faster than the limiting rate for the oxidation of the third species. At lower oxygen, with an $a_3$-oxygen rate of, say, $2 \times 10^4$ sec$^{-1}$ (pseudo-first order), the third component would mostly be reckoned with $a_3$.

SUMMARY

1. In the reaction of reduced cytochrome oxidase with oxygen the rate of appearance of the near infrared band of the oxidized form at 820 m.$\mu$ increases with oxygen concentration up to a limiting rate of 6 $\times 10^4$ sec$^{-1}$.

2. This rate is greater than the rate of oxidation of cytochrome $a$ ($1 \times 10^3$) but below the maximal rate of oxidation of cytochrome $a_3$ ($30 \times 10^3$ sec$^{-1}$) which can be reached with these preparations.

3. Appearance of the band at 820 m.$\mu$ must be attributed to a change other than the oxidation of cytochromes $a$ and $a_3$.

4. The species chiefly responsible for the band at 820 m.$\mu$ also contributes to the absorbance changes in the $a$ region which occur on oxidation or reduction of cytochrome oxidase preparations.

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