In 1939 Keilin and Hartree (2) advanced the concept that there are two cytochrome components reacting between cytochrome c and oxygen; namely, cytochrome a and cytochrome a₃. They based their hypothesis principally on the action of carbon monoxide which caused the α-band of reduced cytochrome a to spread toward 590 mμ and the γ-band at 448 mμ to shift to 430 mμ and to be replaced by a very pale band at 452 mμ. They felt that their studies with other inhibitors, and especially with cyanide, strengthened their position. Under anaerobic conditions KCN broadened the α-band of reduced cytochrome a toward the blue end of the spectrum and broadened and slightly shifted the γ-band toward the red end of the spectrum. In other experiments, however, in which the succinate was added in the presence of air, the α-band remained unchanged, while the γ-band disappeared and was replaced by a pale band at 452 mμ.

The effect of cyanide on the spectrum of oxidized cytochrome oxidase has been noted by Smith (3) and by Ball and Cooper (4), whereas the effect on the spectrum of the reduced enzyme has been reported by a number of investigators (2–6).

The effect of CO on the spectrum of reduced cytochrome oxidase was studied in detail by Warburg and his associates (7, 8) when they determined the photochemical action spectrum of the CO compound of the enzyme in yeast. Other investigators have amply confirmed the action of CO (2, 5, 6, 9–11).

In this paper it is shown that NaCN modifies the spectra of both oxidized and reduced cytochrome oxidase.¹ CO is shown to affect the spec-
reactions of cytochrome oxidase in the manner previously reported by other investigators. NO has an action which is very similar to that of CO.

**EXPERIMENTAL**

Cytochrome oxidase was prepared by adding 2 per cent and then 3 per cent of sodium deoxycholate (termed Preparation 2-3) to a preparation of heart muscle particles as previously described by us (13, 14), or by adding 1.75 per cent of sodium deoxycholate, followed by 3 per cent (Preparation 1.75-3) or 2.25 per cent followed by 3 per cent (Preparation 2.25-3). The preparations were not lyophilized. The concentrated preparation was diluted with an equal volume of distilled water, and 2 ml. of the solu-

![Graph](https://www.jbc.org/)

**Fig. 1.** The α-peak of reduced cytochrome oxidase as developed by various agents. Curve 1, oxidized cytochrome oxidase; Curve 2, 1 × 10^-2 M hydroquinone; Curve 3, 5 × 10^-2 M potassium ferrocyanide; Curve 4, sodium dithionite; Curve 5, 1 × 10^-6 M cytochrome c and 5 × 10^-2 M sodium ascorbate.

...tion were pipetted into the spectrophotometer cuvette. The volume was brought to 3 ml. with other reagents or with water. The pH was that of the oxidase preparation which contained 0.1 M Na_2HPO_4·KH_2PO_4 buffer, i.e. 7.4.

Cytochrome c was purchased from Wyeth, Inc.

Reduction by Various Agents—In Fig. 1 it may be seen that a number of reducing agents produce a symmetrical peak which is centered at 603 to 605 m\(\mu\). Curve 1 is the spectrum of oxidized Preparation 2-3 without the addition of reducing agents. Curve 2 results when only hydroquinone is added (1 × 10^-2 M final concentration). Sodium ascorbate (5 × 10^-2 M final concentration) gives the same curve. Curve 3 is obtained with ferrocyanide (5 × 10^-2 M final concentration), whereas sodium dithionite produces the spectrum of Curve 4. If cytochrome c is added so that the final concentration is 1 × 10^-6 M when 5 × 10^-2 M ascorbate is the re-
ducing agent (Curve 5), the reduction equals that obtained with dithionite. The γ-peaks are presented in Fig. 2. The increase in absorption at 443 mμ parallels the increase in absorption at 604 mμ (Fig. 1), except that the absorption at 443 mμ obtained with ascorbate and cytochrome c (Curve 5) does not exceed that obtained with dithionite alone (Curve 4). The peak at 417 mμ on Curve 5 is largely due to the added cytochrome c. In none of these instances is there evidence for two cytochrome α components.

Reduction by Ferrocytochrome c in Air in Presence of Cyanide—We have previously demonstrated that cytochrome oxidase can be partially reduced by ferrocytochrome c under anaerobic conditions (15). It was also stated that the oxidation products from 5 mg. of sodium dithionite dissolved in water and aerated for 5 minutes would not reduce cytochrome oxidase, but no data were given.
If sodium cyanide is used at a final concentration of 0.1 M, 1.0 mg. of ferrocytochrome c will partially reduce Preparation 1.75-3 in air (Fig. 3, Curve 2). The ferrocytochrome c was prepared by adding 1 mg. of dithionite per mg. of cytochrome c. The solution was aerated for 5 minutes. Curve 1 is the spectrum of the oxidized preparation to which were added the oxidation products from 5 mg. of sodium dithionite (after aerating for 5 minutes) in the presence of 0.1 M NaCN. This curve is identical with that for the oxidized preparation alone. Curve 2 is a corrected spectrum. The mixture of partially reduced cytochrome oxidase and partially oxidized cytochrome c was corrected for the amounts of ferro- and ferricytochrome c present.

The following formula gives the amount of unoxidized ferrocytochrome c in a 1 cm. cell.

\[
\text{Mg. ferrocytochrome c per 3 ml.} = \frac{D_{560 \text{ experimental}} - D_{560 \text{ cytochrome oxidase}} - D_{560 \text{ ferricytochrome c}}}{\epsilon_{560 \text{ ferrocytochrome c}} - \epsilon_{560 \text{ ferricytochrome c}}} \times 3 \times 1.235 \times 10^7
\]

Fig. 4. Effect of sodium cyanide on the spectrum of oxidized cytochrome oxidase. The ordinate on the right is for the curve of the difference.
where $D_{550}$ cytochrome oxidase is an isosbestic point for cytochrome oxidase, $D_{550}$ ferricytochrome $c$ is the density when there is 1 mg. of ferricytochrome $c$ per 3 ml., $\epsilon_{550}$ ferrocytochrome $c = 0.264 \times 10^8$ sq. cm. per mole, $\epsilon_{550}$ ferricytochrome $c = 0.090 \times 10^8$ sq. cm. per mole, and $1.235 \times 10^7$ is the mg. molecular weight of cytochrome $c$. The corrections at each wave-length were obtained by multiplying the density of a solution of ferrocytochrome $c$ (1 mg. per 3 ml.) by the fraction of ferrocytochrome $c$ remaining and the density of a solution of ferricytochrome $c$ (1 mg. per 3 ml.) by the fraction of the ferrocytochrome $c$ oxidized. Curve 3 is also a corrected curve obtained by reducing the mixture of cytochrome $c$ and cytochrome oxidase with dithionite and then correcting for the ferrocytochrome $c$ present.

**Effect of Cyanide on Oxidized Cytochrome Oxidase**—The oxidase was Preparation 2.25-3. Sodium cyanide was added to a final concentration of 0.1 M. In Fig. 4 are presented the curves for the oxidized preparation with and without cyanide. The $\gamma$-peak is shifted from 418 to 424 m$\mu$. The difference spectrum has a maximum for oxidized cytochrome oxidase.
at 410 m\(\mu\) and a maximum for the cyanide complex of the oxidized compound at 438 m\(\mu\). In another instance the latter was at 435 m\(\mu\).

Effect of Cyanide on Reduced Cytochrome Oxidase—Preparation 2.25-3 of the previous experiment was reduced with sodium dithionite in the presence of 0.1 m NaCN (final concentration) under anaerobic conditions. Oxygen-free nitrogen was bubbled through 0.1 m NaCN and the special spectrophotometer cell for 10 minutes. The solution was then mixed with a pellet of Na\(_2\)S\(_2\)O\(_4\), which was contained in the side arm. The \(\gamma\)-peak (Fig. 5) shifted from 443 to 439 m\(\mu\) and the \(\alpha\)-peak shifted from 603 to 599 m\(\mu\). The difference spectrum has maxima for reduced cytochrome oxidase at 446, 555 to 560, and 607 m\(\mu\) and maxima for the cyanide complex of the reduced compound at 426 to 434, 461, and 593 m\(\mu\). The 555 to 560 m\(\mu\) maximum is not prominent. The 461 m\(\mu\) maximum has not been reported by other investigators.

Effect of Carbon Monoxide on Reduced Cytochrome Oxidase—The oxidase

![Image of graph showing spectral data](http://www.jbc.org)
was Preparation 2-3. The CO was generated by dropping concentrated 
$\text{H}_2\text{SO}_4$ on dry sodium formate and was passed through the solution in the 
closed cell for 30 minutes. The enzyme was then reduced with a pellet 
of $\text{Na}_2\text{S}_2\text{O}_4$ contained in the side arm. The $\gamma$-peak shifted from 443 to 
430 m$\mu$ (Fig. 6). The $\alpha$-peak at 605 m$\mu$ was shifted to 603 m$\mu$ and became 
asymmetrical on the lower wave-length side. The peak diminished in

![Graph showing the effect of nitric oxide on the spectrum of reduced cytochrome oxidase. The ordinate on the right is for the curve of the difference.](http://www.jbc.org/)

**Fig. 7.** Effect of nitric oxide on the spectrum of reduced cytochrome oxidase. The ordinate on the right is for the curve of the difference.

height by only 0.005 density unit. However, the absorption at 443 m$\mu$
was lowered by about 28 per cent. The $\gamma$-peak for the CO compound is
skewed somewhat toward the higher wave-lengths and would probably be
even more asymmetrical if the preparation were free of the small amount
of cytochrome c which it contains. Reduced cytochrome c has its $\gamma$-peak
at 415 m$\mu$. The difference spectrum has maxima for reduced cytochrome
oxidase at 446, 563, and 608 m$\mu$ and maxima for the CO complex of the
reduced compound at 427 to 429, 545, and 590 m$\mu$.

*Effect of Nitric Oxide on Reduced Cytochrome Oxidase*—The cytochrome
oxidase was the same Preparation 2-3 that was used in the previous experiment. NO was generated by dropping a mixture of 1 M NaNO₂ and 1 M K₄Fe(CN)₆ into a solution consisting of 1 part of glacial acetic acid and 2 parts of water. The gas was passed through the solution in the closed cell for 20 minutes. The oxidase was then reduced with a pellet of Na₂S₂O₄ contained in the side arm. The γ-peak of the reduced oxidase at 443 mμ (Fig. 7) was split into two maxima: one at 439 mμ and the other at 430 mμ. The α-peak was shifted from 605 to 603 mμ. The absorption at 443 mμ was reduced by about 28 per cent. The difference spectrum has maxima for reduced cytochrome oxidase at 446, 570, and 610 mμ and maxima for the NO complex of the reduced compound at 426, 545, and 597 mμ.

**DISCUSSION**

As a consequence of their studies with carbon monoxide and potassium cyanide, Keilin and Hartree (2) concluded that the α- and γ-bands of reduced cytochrome a were at 605 and 452 mμ, respectively, and that the corresponding bands for cytochrome a₃ were at 600 and 448 mμ, respectively. The unmasking of the weak 452 mμ band of cytochrome a was an important factor in their interpretation that there were two components.

The experiments reported herein reveal that the reduction by a number of agents, some of which do not require cytochrome c to mediate the reduction of the oxidase, always produces single peaks at 603 to 605 mμ and 443 to 445 mμ. There is no evidence for two α-peaks and two γ-peaks, such as would be required if there were two components.

The effect of cyanide on the oxidized spectrum of cytochrome oxidase has been previously reported by Ball and Cooper (Table I). The results reported here are essentially in agreement with those of Ball and Cooper, except that the γ-peak in these experiments is 5 to 8 mμ higher on the wave-length scale.

The effect of cyanide on the reduced spectrum of cytochrome oxidase has been previously reported by Ball and Cooper (Table I). Keilin and Hartree found that the 605 mμ band broadened toward the blue and formed a distinct band at about 595 mμ. The γ-band became more diffuse and lay at 450 mμ. The new band at 595 mμ is verified, and the 450 mμ band of Keilin and Hartree may be the unassigned peak at 461 mμ found in these experiments.

Carbon monoxide modifies the spectrum of reduced cytochrome oxidase, as noted by Keilin and Hartree (Table I). The new peaks are approximately at the wave-lengths assigned by Warburg and Negelein to the CO compound of the oxygen-transporting enzyme. However, the shift in the absorption is only a partial one in that the original absorption by the reduced compound persists, as previously reported by Keilin and Hartree.
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<th>( \beta )-Peak</th>
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REACTIONS OF CYTOCHROME OXIDASE

Kiese, and by Lundegardh. This partial shift in the spectrum could be interpreted as a reaction of the CO with some of the hemes in a polymeric molecule.

The effect of nitric oxide on the reduced spectrum of cytochrome oxidase verifies the conclusion that the effect of CO is a partial one, for NO forms a double peak in the Soret region. The absorption due to the reduced compound persists. The reduction in the absorption at 443 m\(\mu\) is only 28 per cent of the total absorption or about 46 per cent of the difference between the reduced and oxidized curves at this wave length.

CO and NO probably react in the same way with the reduced oxidase, since the effects on the spectrum of the reduced compound are identical. This becomes most apparent when the difference spectra are compared. The size of the peak representing the loss of absorption at 444 m\(\mu\) is exactly the same in both instances. The NO compound, however, has a much smaller specific absorption than does the CO compound, since the peak on the difference spectrum for the newly formed compound of NO with the reduced enzyme is very small.

It is interesting to propose that cytochrome oxidase might be a tetrapolymer consisting of four identical heme-protein submolecules, with each heme contributing an electron to the reduction of 1 molecule of oxygen, according to the thought expressed by Warburg (16) when he calculated the molar spectrum of the oxygen-transporting enzyme.

**SUMMARY**

1. Partially purified cytochrome oxidase has been reduced with hydroquinone, ferrocyanide, ascorbate in the presence of cytochrome c, dithionite, and ferrocytochrome c, and in no instance is there evidence for the existence of two cytochrome components reacting between cytochrome c and atmospheric oxygen.

2. Sodium cyanide at a final concentration of 0.1 M alters the spectra of both oxidized and reduced cytochrome oxidase to give the maxima previously reported by other investigators and, in addition, a peak is found at 461 m\(\mu\) in the difference spectrum of the cyanide complex of reduced enzyme. It is not possible to conclude whether the shifts are partial or complete.

3. The partial shift in the spectrum of reduced cytochrome oxidase which is known to occur on the addition of carbon monoxide has been reexamined, and the alternative interpretation is offered that it might be an effect involving some of the hemes in a polymeric cytochrome oxidase molecule.

4. The nitric oxide complex of reduced cytochrome oxidase has maxima in its spectrum at 426, 545, and 597 m\(\mu\) (obtained by difference). Nitric oxide also produces only a partial shift in the spectrum of reduced cytochrome oxidase, since the 443 to 445 and 603 to 605 m\(\mu\) peaks persist.
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