The spectral properties of cytochrome c oxidase have been the subject of a number of studies (1-14). Although these studies have not, in themselves, convinced all investigators that more than one hemoprotein was involved, they have confirmed the original observations which formed the basis for distinguishing the cytochromes a and a3. These observations by Keilin and Hartree (1) indicated that the absorption at 605 and 444 nm was due to two components. One of these, which they termed cytochrome a, was autoxidizable and combined with carbon monoxide and cyanide, causing a spectral shift. The other, cytochrome a3, which was not autoxidizable and did not combine with these reagents, showed no spectral alteration.

Some investigators (15-19) have interpreted the spectral data obtained with purified cytochrome c oxidase as indicating that only a single cytochrome is present. Wainio (15, 16) has further suggested that the copper present in the purified preparations may account for the spectral changes observed with carbon monoxide, cyanide, and nitric oxide.

It has been held that only separation of the cytochromes a and a3 would offer satisfactory evidence for the existence of two cytochromes. However, in any study designed to isolate the cytochromes a and a3, it would be necessary to correlate the properties of isolated components with the properties of the cytochromes in the intact cytochrome c oxidase system. It is, therefore, essential to establish the properties of the cytochromes while they are still a part of that system. Yonetani (13) has recently made an effort to do this by detailed study of his purified preparation. By the use of difference spectra, he was able to distinguish quantitatively the absorption due to cytochrome a and that due to cytochrome a3.

In a continuation of our efforts to characterize the components (2, 3, 8, 20) of the cytochrome c oxidase, we have attempted to define further the spectral properties of cytochrome a and cytochrome a3. With this information, we should have added criteria useful in comparing preparations which contain only cytochrome a or cytochrome a3.

**EXPERIMENTAL PROCEDURE**

The cytochrome c oxidase was prepared as previously described (2). The heme and protein concentrations were determined as previously reported (2).

In most of the spectral studies, the concentration of cytochrome c oxidase contained 0.0123 mm heme a and 2 mg of protein per ml. The preparation was dissolved in 0.1 M phosphate buffer, pH 7.4, which contained 0.5% Tween 80. In experiments with cyanide, the concentration was 1.5 × 10^-3 M. In order to obtain the spectra of carbon monoxide derivatives, the solution was saturated with carbon monoxide by passing the gas through the solution for at least 3 minutes. The spectra were recorded on a Beckman DK-2 recording spectrophotometer.

The various forms of cytochrome c oxidase that were employed in the spectral studies are described below.

- **Oxidized cytochrome c oxidase (a+++, a3+++)** was considered to be the enzyme as prepared. The preparation was diluted with 0.1 M phosphate buffer containing 0.5% Tween 80 to the concentration indicated.

- The **cyanide derivative of the oxidized preparation (a+++ + a3++ + CN)**, was the solution described above, containing in addition 1.5 × 10^-2 M cyanide.

- **Reduced cyanide preparation (a++, a3++ + CN)** was the above preparation to which dithionite was added immediately before the recording of the spectrum.

- **Reduced cytochrome c oxidase (a++, a3++ + CN)** was the oxidized preparation to which 5 mg of solid sodium dithionite per 3 ml of sample were added 5 minutes before its spectrum was recorded.

- **Reduced cyanide preparation (a++, a3++ + CN)** was obtained by adding sufficient cyanide to make the reduced preparation 1.5 × 10^-4 M with respect to cyanide.

- The **carbon monoxide derivative of reduced cytochrome c oxidase (a++, a3++ + CO)** was prepared by passing carbon monoxide through the reduced preparation for 3 minutes while the sample was submersed in an ice bath.

In order to record spectra in which the oxidation state of cytochrome c differed from that of cytochrome a, the procedure outlined in Table I was employed. Two sets of matched cuvettes were placed in the reference and sample beams of a double beam spectrophotometer. Solutions were prepared as indicated and the spectra shown in the figures are those recorded.

The difference spectrum shown in Fig. 4, a base line was recorded with the cyanide derivative of the oxidized preparation in the B cuvettes of the reference and sample beam; the oxidized cytochrome c oxidase was placed in the A cuvette of both beams. Dithionite was then added to the A cuvette of this sample beam, and the solution was saturated with carbon monoxide, thus converting the material to the carbon monoxide derivative of reduced cytochrome c oxidase. Just before the spectrum was recorded, dithionite was also added to the B cuvette of the

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1 Purchased from the Nutritional Biochemical Corporation.
**Table I**

Outline of experimental procedures

<table>
<thead>
<tr>
<th>Figure No.</th>
<th>Reference cuvettes</th>
<th>Sample cuvettes</th>
<th>Algebraic sum of the recorded spectrum (sample and reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Reduced cyanide, (a^{++}), (a_{3}^{+++})CN</td>
<td>Reduced, (a^{++}), (a_{3}^{+++})</td>
<td>(a^{++} + a_{3}^{+++})</td>
</tr>
<tr>
<td>2</td>
<td>Oxidized cyanide, (a^{+++}), (a_{3}^{+++})CN</td>
<td>Oxidized, (a^{+++}), (a_{3}^{+++})</td>
<td>(a^{++} + a_{3}^{+++})</td>
</tr>
<tr>
<td>3</td>
<td>Reduced cyanide, (a^{++}), (a_{3}^{+++})CN</td>
<td>Carbon monoxide, (a^{++}), (a_{3}^{+++})CO</td>
<td>(a^{++} + a_{3}^{+++})CO</td>
</tr>
<tr>
<td>4</td>
<td>Oxidized, (a^{+++}, a_{3}^{+++})CN</td>
<td>Reduced cyanide, (a^{++}, a_{3}^{+++})CN</td>
<td>(a^{++} + a_{3}^{+++})</td>
</tr>
</tbody>
</table>

**Table II**

Outline of experimental procedures

<table>
<thead>
<tr>
<th>Figure No.</th>
<th>Percent reduction of cytochromes</th>
<th>Sample cuvettes</th>
<th>Reference cuvettes</th>
<th>Algebraic sum of the recorded spectrum (sample and reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>(100)</td>
<td>1</td>
<td>Oxidized, (a^{+++}) + (a_{3}^{+++})CN</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>(0)</td>
<td>0</td>
<td>Reduced, (a^{++}) + (a_{3}^{+++})CN</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>(100)</td>
<td>1</td>
<td>Reduced, (a^{++}) + (a_{3}^{+++})CN</td>
<td>1</td>
</tr>
</tbody>
</table>

* In this case three cuvettes are employed as indicated.

The concentration of the preparation was varied by accurate dilution with 0.1 M phosphate buffer containing 0.5% Tween 80. In Table II, a dilution factor of 1 indicates no dilution, whereas 1:4 indicates that 1 volume of the preparation was diluted to 4 volumes. Thus, on the assumption that the absorption of the preparation follows Beer's Law, the diluted material will have one-fourth the absorption of the

reference beam, converting the solution in this cuvette to the reduced cyanide preparation.

By varying the concentration of the cytochrome c oxidase preparation in the cuvettes in the reference and sample compartments, it was possible to obtain spectra of the cytochrome c oxidase preparation equivalent to partially reduced and partially oxidized cytochrome \(a\) and cytochrome \(a_{3}\).
original material. As shown in Table II, two sets of matched cuvettes were employed in most experiments. In one experiment, as indicated, three sets of cuvettes were employed.

RESULTS

The recorded spectrum is the algebraic sum of the absorbing materials in the system. In Fig. 1, for example, the sample beam contains $a^{+++} + a_{3}^{+++} + a^{++} + a_{3}^{++}$ in one cuvette and $a^{++} + a_{3}^{++}$ in the other. The reference beam contains only $a^{++} + a_{3}^{+++} + a^{++} + a_{3}^{++}$. The algebraic sum is thus

$$a^{+++} + a_{3}^{+++}CN + a^{++} + a_{3}^{++} - (a^{++} + a_{3}^{+++}CN) = a^{+++} + a_{3}^{++}$$

and the recorded spectrum in Fig. 1 is that of oxidized cytochrome $a$ and reduced cytochrome $a_{3}$.

In a similar manner, the spectrum recorded in Fig. 2 is equivalent to reduced cytochrome $a$ and oxidized cytochrome $a_{3}$. Fig. 3 presents the spectrum of oxidized cytochrome $a$ and the reduced cytochrome $a_{3}$-carbon monoxide complex.

In Fig. 1, where the spectrum is equivalent to a mixture in which only cytochrome $a_{3}$ is reduced, the greatest changes in the spectrum, as compared to the oxidized spectrum, are in the Soret region. The position of the absorption maxima in the visible region is at a slightly higher wave length (605 mp) than when both cytochromes $a$ and $a_{3}$ are totally reduced (603 mp). Fig. 5 shows a series of spectra in which the percentage of reduction of cytochrome $a_{3}$ is varied. The position of the visible absorption maximum is at 605 mp, whereas the Soret peaks are at 424 mp with a shoulder at approximately 442 mp when cytochrome $a_{3}$ is reduced. When both cytochromes are oxidized, the Soret peaks of the two cytochromes are indistinguishable with a maximum at 421 mp. When cytochrome $a_{3}$ is reduced, the oxidized Soret peak of cytochrome $a$ is at a longer wave length (423 mp).

Fig. 2 shows the spectrum of reduced cytochrome $a$ and oxidized cytochrome $a_{3}$. In contrast to the reverse situation as shown in Fig. 1, there is a marked change in the spectrum in both the visible and Soret regions upon reduction of cytochrome $a$. The peak in the visible region is at a slightly shorter wave length (603 mp) than that obtained in Fig. 1. There is a $\beta$-peak clearly present in Fig. 2, with a maximum at 518 mp. The spectral changes, which occur as the cytochrome $a$ is progressively reduced, can be seen in Fig. 6.

The spectra of cytochrome $a_{3}$-carbon monoxide complex and oxidized cytochrome $a$ are shown in Fig. 3. Also shown is the spectrum of cytochrome $a_{3}$-carbon monoxide complex in the presence of reduced cytochrome $a$. The position of the absorption maximum is shifted more than 10 mp to 591 mp when cytochrome $a$ is in the oxidized form. Compared to the usual spectrum in which both cytochromes are reduced, the $\beta$-peak is also more pronounced and the Soret peak is shifted to shorter wave lengths.

Fig. 4 shows the difference spectra between the oxidized and reduced cytochrome $a_{3}$ and the carbon monoxide complex. The position of the $\alpha$-absorption peaks in these spectra is at 605 mp for the reduced and at 589 mp for the carbon monoxide complex.

In both cases, a $\beta$-peak can be most clearly seen at 548 mp.
The absolute spectra of cytochrome c oxidase containing reduced cytochrome a-carbon monoxide complex and cytochrome a in reduced and oxidized forms. The conditions which were employed in obtaining the spectrum are outlined in the text and Table I. The extinction values are based on the total heme a content of the preparation. -----, Reduced cytochrome a-carbon monoxide complex and oxidized cytochrome a; --, reduced cytochrome a-carbon monoxide complex and reduced cytochrome a.

for the carbon monoxide complex, and although fused with the a-peak, there is clearly a shoulder in the 560 to 570 nm region of the reduced spectrum. In the Soret region, the maxima are at 430.5 nm for the carbon monoxide derivative and 443.5 nm for the reduced compound.

It is clear from Table II that spectra equivalent to partial reduction of either or both cytochromes can be obtained. The spectra are shown in Figs. 5, 6, and 7. In all cases, the absorption maxima and the shape of the curves are in complete agreement with those shown in Figs. 1 and 2.

The relatively small contribution of cytochrome a to the absorption at 605 nm, and its relatively large contribution in the Soret region, are apparent in Figs. 1 and 5. In contrast, cytochrome a has a much larger absorption in the visible region and also accounts for much of the absorption at 444 nm in the reduced form. The isosbestic points for the two cytochromes in the Soret region are not greatly separated, occurring at 430 and 462 nm for cytochrome a, with a very slight shift to a higher wavelength for cytochrome a.

**DISCUSSION**

It was the object of the present study to produce spectra of a cytochrome c oxidase preparation in which the cytochrome a and cytochrome a₃ each existed in a different valence state. In this way, it was hoped to establish that the cytochrome a and a₃ were distinct components and, further, to distinguish the spectral properties of each.

The fact that cyanide combined with both reduced and oxidized cytochrome a₃, as first indicated by Keilin and Hartree (1),
made it possible to produce cytochrome $a_2$ in a different valence state from the cytochrome $a$. Yonetani (13) had suggested that the oxidized cytochrome $a_2$-cyanide complex is not readily reduced, even by dithionite. In the present work, it was observed that the oxidized cyanide complex of cytochrome $a_2$ resisted reduction for some time after the addition of dithionite. Thus, it was possible to obtain a preparation with cytochrome $a$ in the reduced form and still maintain cytochrome $a_2$ in the oxidized form. Advantage was also taken of the fact that the recorded spectrum was the algebraic sum of the absorbing materials in the light path.

Yonetani (13) obtained a series of difference spectra of the cytochromes $a$ and $a_2$. He employed the oxidized cytochrome $a_2$-cyanide complex as oxidized cytochrome $a_2$, in cases where he could assume that the cyanide effect was small, and he corrected by calculation for the effect of cyanide in other experiments. The present study has, in most instances, been able to confirm Yonetani’s results, and we have been able to record directly all the difference spectra that he calculated.

Table III records the position of the maxima and the ratio of the extinction of the Soret and $a$-peaks of the difference spectra. The positions of the maxima are in complete agreement, but the ratio of the extinctions is not. The reason for these discrepancies is not apparent, but it is possible that the different techniques used to obtain the spectrum, or differences in the preparation employed, may be involved.

Although Yonetani (13) reported a ratio of 13 for the extinction of the $a$- and Soret maxima in the difference spectrum of reduced and oxidized cytochrome $a_2$, the present work shows this ratio to be significantly higher. In the case of the difference spectrum of reduced and oxidized cytochrome $a_2$, the ratio is lower than that of cytochrome $a_2$, and the value in the present work is lower than the value of 4.5 given by Yonetani (13). It is interesting to note that the reduced versus oxidized difference spectrum of the cytochrome $a_2$-cyanide complex has a Soret to $a$ ratio very similar to that of cytochrome $a$. The cyanide complex of cytochrome $a_2$ also resembles cytochrome $a$ in that it has a $\beta$-peak which is missing from the cytochrome $a_2$ spectra.

Nitrate can also be employed in the same manner as cyanide, which was used in this paper. Nitrate forms a complex with both the oxidized and reduced cytochrome $a_2$. The oxidized cytochrome $a_2$-nitrate complex, like the cyanide complex, is not readily reduced by dithionite. However, the oxidized nitrate does not appear to be as stable in the presence of dithionite as is the cyanide complex, and is converted to the reduced form more readily. Cyanide is, therefore, the reagent of choice.

Figs. 3 and 4 demonstrate that the cytochrome $a_2$-carbon monoxide complex has the characteristics attributed to the photochemical action spectrum (6, 7, 21). In addition, the spectrum of the reduced cytochrome $a_2$-carbon monoxide complex with oxidized cytochrome $a$ shown in Fig. 3 is identical with that obtained by treatment with ferricyanide of a reduced

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**Table III**

<table>
<thead>
<tr>
<th>Difference spectrum</th>
<th>Position maximum</th>
<th>Extinction ratio ($\Delta A$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$a_2^{++} - a_2^{++}$</td>
<td>600 (560-570)</td>
<td>443.5</td>
</tr>
<tr>
<td>$a_2^{++}CO - a_2^{++}$</td>
<td>580 (545-548)</td>
<td>430.5</td>
</tr>
<tr>
<td>$a_2^{++}CN - a_2^{++}CN$</td>
<td>500</td>
<td>514</td>
</tr>
<tr>
<td>$a^{++} - a^{++}$</td>
<td>603 (515-518)</td>
<td>445</td>
</tr>
</tbody>
</table>
The spectral properties of a purified cytochrome c oxidase preparation have been investigated. A procedure is outlined by which the difference spectra of any desired combination of reduced and oxidized cytochromes a and a3 can be obtained. The reduced versus oxidized difference spectrum of cytochrome a3 and the difference spectrum of oxidized versus reduced carbon monoxide-cytochrome a3 are presented.

The procedure can be modified to obtain absolute spectra of the cytochrome c oxidase preparation in which cytochromes a and a3 are present in different valence states. Thus, spectra are shown of the preparation with either oxidized cytochrome a or reduced cytochrome a3, or, conversely, reduced cytochrome a and oxidized cytochrome a3. It is also possible to obtain the spectrum of the preparation equivalent to partially reduced cytochrome a3 in the presence of oxidized cytochrome a or, conversely, the equivalent of partially reduced cytochrome a3 in the presence of oxidized cytochrome a3. Examples are also given of the spectra of various combinations of partially reduced cytochromes a and a3.

It is noted that most of the previous speculations concerning the contributions of the two cytochromes to the absorption at 444 and 605 μm in the reduced form are questionable. The most significant point in this regard is the high contribution of cytochrome a to the Soret peak of the reduced cytochrome c oxidase preparation. Cytochrome a accounts for at least twice as much absorption at 444 μm as does cytochrome a3.

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REFERENCES
