THE REACTION OF CYTOCHROME OXIDASE WITH CYANIDE*

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The ability of cyanide to inhibit the oxygen consumption of biological systems has long been known (cf. (4, 13)). Warburg (12, 13) appears to be the first to suggest that this action of cyanide was due to its combination with the ferric form of the enzyme, called by him the Atmungsferment and later called cytochrome oxidase by Keilin and Hartree (5). In this paper we make use of this reaction of cyanide with cytochrome oxidase to obtain some quantitative spectroscopic data on the reduced form of this enzyme and to draw certain conclusions concerning the nature of this enzyme whose positive identification has been somewhat elusive.

Methods

A preparation of beef heart muscle of the type described by Ball and Cooper (2) has been used as a source of cytochrome oxidase. This preparation contains all the components of the electron transmitter system (cf. Ball (1)). It was treated with sodium deoxycholate in the manner described by Ball, Strittmatter, and Cooper (3) in order to obtain a preparation suitable for spectroscopic observations. These observations were made at room temperature (23° ± 2°) with a Beckman DU model spectrophotometer, the wave-length scale of which had been calibrated against a series of lines of a mercury arc lamp. Optical cells with a 1 cm. light path were employed. When an inert atmosphere was desired, special cells fused to a Thunberg type of tube were employed. These were evacuated and flushed repeatedly with nitrogen and reducing agents added from the hollow stopper of the Thunberg tube. The potassium cyanide employed was recrystallized from water by the addition of 20 volumes of ethanol at a temperature of −27°.

Results

The procedure employed to reveal the spectrum of reduced cytochrome oxidase is based upon the following considerations. Since cyanide reacts

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with the oxidized form of cytochrome oxidase and so prevents its reaction with other members of the cytochrome chain, it should be possible, in the presence of air and cyanide, to reduce some or all of the cytochrome components in a preparation without reducing cytochrome oxidase. The spectrum of such a preparation may then be compared with that of a preparation in which cytochrome oxidase is also reduced. The difference between these two spectra will yield the absorption changes due to the conversion of the cyanide complex of oxidized cytochrome oxidase to reduced cytochrome oxidase. Additional corrections may then be made for the effect of cyanide and a spectrum obtained which represents the difference in absorption between oxidized and reduced cytochrome oxidase.

In Fig. 1 is shown a representative experiment in which cysteine has been employed as the reducing agent. To be suitable for this type of experiment, a reducing agent must not be able to reduce cytochrome oxidase directly (e.g., dithionite), but must act through cytochrome c. Cysteine, ascorbate, hydroquinone, and succinate have all been employed and yield similar results. The results observed in the ultraviolet region are plotted in Fig. 1, A. When all the components are in the oxidized state, a single absorption band centered at 415 m\(\mu\) is present. Upon the addition of cysteine in the absence of oxygen two bands appear. One, centered at 442 m\(\mu\), can be assigned to reduced cytochrome oxidase, as described previously (3). The other, at about 417 m\(\mu\), is due to reduced cytochrome a and c and the oxidized form of cytochrome b. Cysteine does not readily reduce cytochrome b, a fact which markedly aids the visualization of the cytochrome oxidase band at 442 m\(\mu\). When cysteine is added in the presence of cyanide and air, a different spectrum is obtained, as can be seen from the curve labeled “reduced + KCN” in Fig. 1, A. Now only one band, centered at 417 m\(\mu\), appears. The band at 442 m\(\mu\) is no longer in evidence. We interpret this to mean that cyanide has blocked the reduction of cytochrome oxidase and it remains in the oxidized state combined with cyanide.

The results obtained in the visible region are shown in Fig. 1, B. Here the addition of cysteine in the absence of air causes the appearance of a spectrum with three distinct peaks located at 523 m\(\mu\), 552 m\(\mu\) (with a shoulder at 562 m\(\mu\)), and 605 m\(\mu\). In the presence of cyanide and air the addition of cysteine still produces a three-banded spectrum with peaks at the same wave-lengths. There is, however, a decrease in the total absorption, which is marked at 605 m\(\mu\) and is somewhat less in the region 550 to 570 m\(\mu\). The shoulder at 562 m\(\mu\) has disappeared. This decrease in intensity of absorption we attribute again to the non-reduction of cytochrome oxidase in the presence of cyanide.

A similar result may be obtained by a somewhat different procedure.
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In the experiments described above two separate tubes were employed, one in air and the other in nitrogen. A single tube may be employed, in which air is present. Cyanide and cysteine are added to give a curve sim-

![Absorption Spectra](http://www.jbc.org/)

**Fig. 1.** The absorption spectra obtained by reduction with cysteine in the presence and absence of cyanide. Measurements were made on 3 cc. of a 2 per cent desoxycholate suspension of the electron transmitter system in 0.05 M glycylglycine buffer, pH 7.4. The light path was 1 cm. and the slit width 0.06 mm. from 630 to 660 m\(\mu\), 0.02 mm. from 413 to 620 m\(\mu\), and 0.10 mm. from 310 to 410 m\(\mu\). Readings were made at 5 m\(\mu\) intervals except at the points of inflection, at which 2 to 3 m\(\mu\) intervals were employed. The curve labeled “oxidized” was run in the presence of air. The curve labeled “reduced” was determined in the same solution after successive evacuations and flushings of the Thunberg type cell with N\(_2\). Cysteine, 0.1 cc. of a 0.1 M solution, was tipped in from the hollow stopper. Complete reduction requires about an hour in the absence of added cytochrome c. The curve labeled “reduced + KCN” was obtained on another portion of the same solution in the presence of air. The amount of cysteine added was the same as before. Solid KCN, 1.0 mg. weighed on a torsion balance, was added at the same time. The final concentration of total cyanide is thus of the order of 0.005 M. The activity of the original enzyme preparation used in this experiment was 327 c.mm. of O\(_2\) per hour per 0.1 cc. of enzyme at 37.2° and pH 7.4 with succinate as substrate and cytochrome c added in excess. With p-phenylenediamine as substrate the corresponding value was 436 c.mm. The desoxycholate suspension used in Fig. 1, B represents a 5-fold concentration of this original preparation. The results for the ultraviolet region, Fig. 1, A, were obtained on an aliquot of the same solution diluted 6-fold with the buffer-desoxycholate mixture. The results obtained were multiplied by 6 before plotting them in Fig. 1, A.

Similar to that labeled “reduced” in Fig. 1. Solid dithionite (Na\(_2\)S\(_2\)O\(_4\)) is then added. This reducing agent reacts directly with cytochrome oxidase to reduce it, even in the presence of cyanide. A redetermination of the absorption spectrum of the preparation now shows that there has been an increase in absorption at 605 m\(\mu\) and in the region 550 to 565 m\(\mu\). The increase
in the latter region is, however, very marked, owing to the reduction of cytochrome \(b\) by the dithionite. Therefore the changes in this region due to the reduction of cytochrome oxidase are not as obvious by direct inspection. The same procedure may also be used in the ultraviolet, but here again the reduction of cytochrome \(b\) interferes with a clear visualization of the changes due solely to cytochrome oxidase.

These experiments support the thesis that oxidized cytochrome oxidase combines with cyanide.\(^1\) Such a combination may be shown spectroscopically. The data in Fig. 2 show the alterations observed by us upon the addition of cyanide to an oxidized preparation. The change in the visible region is slight. In the ultraviolet an unmistakable shift in the absorption spectrum can be seen. There is a decrease in absorption at 415 \(\text{m} \mu\) and the peak is shifted slightly towards a longer wave-length. The difference between these two spectra is also plotted in Fig. 2. This difference spectrum (Curve 3) indicates that oxidized cytochrome oxidase

\(^1\) The term cyanide is used in a general sense. Our data do not permit us to differentiate between HCN and CN\(^-\) as the active agent, though the data of Stannard and Horrocker (10) suggest that HCN is involved.
absorbs maximally in the region of 410 m\(\mu\), while the cyanide complex absorbs maximally in the region of 430 m\(\mu\). This change is similar to that observed for ferrihemoglobin (7), wherein the addition of cyanide lowers the total absorption and shifts the peak some 10 m\(\mu\) towards longer wavelengths. Somewhat similar results are reported by Straub (11) for the reaction of cyanide with oxidized cytochrome oxidase. Keilin and Hartree (6) state, "No clear changes can be observed on addition of KCN to oxidized cytochrome which is due to the diffuse appearance of its spectrum." Smith (9) reports that a change in the absorption spectrum of the oxidized form of cytochrome oxidase may be observed, though no data are given in her preliminary report.

We have usually observed no shift in the absorption spectrum upon the addition of cyanide to a preparation freshly reduced with dithionite under aerobic or anaerobic conditions. However, when the reduced preparation is allowed to stand some time before the addition of cyanide, then a shift in the absorption spectrum is sometimes observed upon adding cyanide. A decrease in absorption at 605 m\(\mu\) occurs with a concomitant increase in the region of 590 m\(\mu\), a change not unlike that seen when CO is added to the reduced preparation (3). This phenomenon has not been reproducible in our hands and we therefore refrain at this time from attempting to interpret its meaning. It is apparently a phenomenon associated with a considerable time lag and does not interfere with the interpretation of the data given in Fig. 1. It should be noted, however, that Keilin and Hartree (6) report that the addition of KCN to a heart muscle preparation reduced anaerobically by succinate causes the appearance of new absorption bands centered at 590 and 450 m\(\mu\). Straub (11) has also added KCN to reduced heart muscle preparations. The reducing agent he employs for his spectroscopic studies is not clear to us, but it appears to be dithionite. He finds no clear cut shift in the position of the absorption bands of the reduced form upon the addition of cyanide. In the visible region he observed a general lowering of the absorption spectrum throughout the range of wave-lengths studied. Incidentally this experiment of Straub's should not be confused with the type of experiment we report here in Fig. 1. Smith (9) reports that cyanide does combine with reduced cytochrome oxidase. It is obvious that further investigations upon the reaction of cyanide with reduced cytochrome oxidase are desirable.

A spectrum representing the difference of absorption of the oxidized and reduced forms of cytochrome oxidase may now be derived. Such a spectrum will be termed a difference spectrum. If the data of Fig. 1 are used, one can obtain a spectrum which represents the difference between the absorption of reduced cytochrome oxidase and the cyanide complex of oxidized cytochrome oxidase. This difference spectrum may be corrected
for the effect of cyanide upon oxidized cytochrome oxidase by use of the data of Fig. 2. There is then obtained the difference spectrum between oxidized and reduced cytochrome oxidase plotted in Fig. 3. The portion of this curve above zero density is due to the absorption by reduced cytochrome oxidase. Three main peaks may be discerned in this spectrum. They are located at 605, 562, and 445 μ. There is also a small peak at 552 μ. Whether this peak is to be ascribed to cytochrome oxidase is a question. It may represent the result of a reaction of cyanide with cytochrome c of the type first observed spectroscopically by Potter (8).

The question might be raised as to whether the band at 562 μ is to be ascribed to a partial reduction of cytochrome b by cysteine. If so, then this reduction occurs only under strictly anaerobic conditions and not aerobically in the presence of cyanide. We believe this band at 562 μ is to be ascribed to reduced cytochrome oxidase, because we have observed in these experiments no indication of the band of reduced cytochrome b which lies at 430 μ (cf. (1)), and also because our studies with
CO (3) revealed an absorption peak for the reduced form of cytochrome oxidase at this same wave-length.

It will be observed that the main peak for the reduced form of cytochrome oxidase, as seen in the difference spectrum given in Fig. 3, lies at 445 m\(\mu\). In Fig. 1 the peak of reduced cytochrome oxidase appears at 442 m\(\mu\). We believe this latter position is nearer to the true value, since in the difference spectrum the cancellation of absorption by the oxidized and reduced forms of cytochrome oxidase is fairly great within the region 400 to 450 m\(\mu\). This factor tends to displace the apparent peak of reduced cytochrome oxidase slightly towards longer wave-lengths and the apparent peak of oxidized cytochrome oxidase towards shorter wave-lengths. In Fig. 3 the portion of the spectrum lying below zero density is to be ascribed to oxidized cytochrome oxidase. The main band to be seen here lies at 410 m\(\mu\).

**DISCUSSION**

We believe the results given here and those that we have presented previously on the reaction of CO with cytochrome oxidase (3) leave no doubt as to the position of the absorption bands of reduced cytochrome oxidase as found in beef heart tissue. The data presented here show this enzyme in the reduced state to be characterized by absorption bands at 605, 562, and 442 to 445 m\(\mu\). The ratio of the optical density readings at these wave-lengths is of the order of 1.0:0.7:12.0. The data obtained with carbon monoxide (3) indicated absorption bands for the reduced form of cytochrome oxidase in these same positions, their optical density values bearing a similar relationship to those found here. Thus in the visible region the primary band of cytochrome oxidase at 605 m\(\mu\) is fused with that of reduced cytochrome \(a\) and its secondary band at 562 m\(\mu\) overlaps that of reduced cytochrome \(b\). Keilin and Hartree (6) were the first to observe the band in the ultraviolet which is to be ascribed to reduced cytochrome oxidase. These workers observed a band at 448 m\(\mu\) which, however, they hesitated to identify with cytochrome oxidase. They assigned it to a compound they chose to call cytochrome \(a\)-3. They showed that this band did not appear if reduction was performed aerobically in the presence of cyanide. Under these conditions they state that no marked changes occurred in the \(a\)-bands of the cytochrome components. Since their observations were primarily of a qualitative nature, they presumably missed the changes at 605 and 562 m\(\mu\) which we describe here. They do, however, assign to reduced cytochrome \(a\)-3 an absorption peak at 600 m\(\mu\). Their reasons for this assignment are not at all obvious to us. Our data clearly indicate that the reduced forms of cytochrome \(a\) and cytochrome oxidase both absorb at 605 m\(\mu\). Absorption at 605 m\(\mu\)
and at 452 m\(\mu\) is assigned by Keilin and Hartree to reduced cytochrome \(a\). We have not been able to detect any band at 452 m\(\mu\) assignable to reduced cytochrome \(a\). It is possible our interpretation of Keilin and Hartree's results are at fault, because we find that the location of certain absorption bands as given in their tables and figures do not correspond. The interpretations made here are based on the values given in their tables.

The caution shown by Keilin and Hartree in hesitating to identify cytochrome oxidase with the compound they have labeled cytochrome \(a-3\) is admirable. We feel, however, that their initial experiments augmented by the quantitative data presented here and in our previous paper (3) leave little doubt that the cytochrome \(a-3\) and cytochrome oxidase of mammalian heart tissue are identical.

One fact which seems of great importance to us is that, of the total absorption at 605 m\(\mu\), three-fourths is due to cytochrome \(a\) and the other fourth is due to cytochrome oxidase. For example, in Fig. 1 the density readings at 605 m\(\mu\) for the three curves shown there are 0.188, 0.297, and 0.333. Thus the increase in density due to reduction of cytochrome \(a\) is 0.109 and that due to reduction of both cytochrome \(a\) and cytochrome oxidase is 0.145. Therefore the reduction due to cytochrome \(a\) in this experiment is exactly three-fourths of the total for both compounds. This relationship has held with surprising rigidity throughout a series of experiments with different reducing agents. If the extinction coefficients of cytochrome \(a\) and cytochrome oxidase are similar, this must mean that there is 1 cytochrome oxidase iron atom for each 3 iron atoms of cytochrome \(a\). We pointed out earlier (3) that the concentration of cytochrome \(c\) in heart muscle is 3 to 4 times that of cytochrome oxidase. At that same time we suggested that the absorption band at 605 m\(\mu\) might be due to a complex composed of four iron porphyrin rings in close juxtaposition, only one of which was cytochrome oxidase. The data presented here give quantitative support to this notion, and we therefore propose the following scheme as a working hypothesis of the manner in which cytochrome oxidase functions. The complex of cytochrome oxidase and cytochrome \(a\) as it exists in the reduced form is represented in Fig. 4 by \(\text{Fe}^{++}(\text{Fe}^{++})\text{c}(I)\).

Here the iron atom not enclosed in parentheses represents cytochrome oxidase and the 3 iron atoms within parentheses represent cytochrome \(a\).

The calculations of the cytochrome oxidase concentration were based on the density difference between reduced cytochrome oxidase and its CO complex at 590 m\(\mu\) and hence tended to be low. The data for reduced cytochrome oxidase presented here indicate that correction for the effect of reduced cytochrome oxidase at 590 m\(\mu\) alters such calculated values, however, by only about 25 per cent.
The cycle of events in the oxidation and reduction of this complex is visualized as follows: Molecular oxygen combines with the ferrous form of cytochrome oxidase (I) to form a compound analogous to oxyhemoglobin (II). Carbon monoxide can compete with oxygen for this same ferrous iron to form compound III and so block the cycle. However, when oxygen combines with the enzyme, it then rapidly strikes in to oxidize the ferrous iron of cytochrome oxidase and the ferrous iron of cytochrome a. Perhaps this occurs stepwise, with the formation of a peroxide intermediate of the type shown (IV). In this case the ferric cytochrome oxidase may be visualized as acting like a peroxidase to oxidize the remaining 2 ferrous iron atoms of cytochrome a. Hydrogen ions are picked up either from the environment or from the groups coordinated with the iron atoms and combine with oxygen to form water. There thus results the ferric form of the complex (V) which can again be reduced to the ferrous form (I), perhaps stepwise, by reaction with ferrous cytochrome c. In the presence of cyanide, however, the ferric form of cytochrome oxidase is converted into a derivative (VI) which is represented here as involving the HCN molecule according to the evidence of Stan- nard and Horecker (10). When this happens, ferrous cytochrome c can reduce only the ferrous iron belonging to cytochrome a to form compound VII and the cycle is blocked. It may be pointed out that in such a scheme cytochrome oxidase is assigned all the functions displayed by the various known hemochromogens. It reacts with \( \text{O}_2 \) like hemoglobin or myoglobin,
it reacts with hydrogen peroxide like peroxidase or catalase, and its iron undergoes reversible oxidation and reduction like the cytochromes. On this basis one could reason from an evolutionary standpoint that cytochrome oxidase is the most primitive hemochromogen and the one from which all others were later derived.

SUMMARY

The absorption spectrum of the cytochrome system in a beef heart preparation has been measured in the presence of air, potassium cyanide, and a reducing agent such as cysteine. This spectrum has been compared to that obtained anaerobically on the same preparation in the absence of cyanide but with the same reducing agent. Subtraction of one spectrum from the other has yielded a difference spectrum for reduced cytochrome oxidase. Such a spectrum reveals peaks located at 605, 562, and 445 μ, whose optical density values bear the relationship of 1.0:0.7:12.0 respectively. The reduced forms of cytochrome a and cytochrome oxidase both absorb at 605 μ, the latter contributing exactly one-fourth of the total absorption observed in this region. It is therefore proposed that three iron porphyrin rings from cytochrome a and one from cytochrome oxidase form a complex which acts as a unit to reduce O₂ to 2H₂O.

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