THE SPECTRAL AND ENZYMATIC CHARACTERIZATION OF CYTOCHROME $c_1$ OF LIVER MITOCHONDRIA

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The presence of cytochrome $c_1$ as a part of the respiratory chain was first reported in 1940 by Yakushiji and Okunuki (1). They concluded from visual spectroscopic observations of heart muscle preparations that this hemoprotein mediates the reactions between cytochromes $b$ and $c$, as well as between cytochromes $b$ and $a$. The validity of Yakushiji and Okunuki's work was questioned by Slater (2), who reported that cytochrome $c_1$ is merely a mixture of cytochrome $c$ and a denatured hemochromogen. Meanwhile, Keilin and Hartree (3) had developed a valuable technique for observing the absorption bands of hemoproteins when samples of these pigments were cooled to the temperature of liquid air. They observed by this method, in a variety of different cell types, a pigment distinct from cytochromes $c$ and $b$, which they called cytochrome $e$. Widmer et al. (4) have since reported a pigment with similar spectral properties in a preparation of a succinate-cytochrome $c$ reductase from heart muscle. Widmer et al. proposed that such a pigment, which they called "cytochrome 554," functions enzymatically between cytochromes $b$ and $c$. Keilin and Hartree (5) have extended their studies on cytochrome $e$ and concluded that it is identical to the cytochrome $c_1$ first observed by Yakushiji and Okunuki (1) and thus to that of Widmer et al. (4), "cytochrome 554." Recently Ball and Cooper (6) have published a paper dealing with observations on the function of cytochromes $c$ and $c_1$. Although they did not make direct measurements on the oxidation and reduction of cytochrome $c_1$, they were able to conclude that cytochrome $c_1$ cannot function in the place of cytochrome $c$ in the respiratory chain. They also suggested that if cytochrome $c_1$ is concerned in electron transport it works in series with cytochrome $c$ rather than in parallel.

The suggestion (1, 4–6) that cytochrome $c_1$ (or cytochrome 554) may

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† On leave of absence (1958) at Molteno Institute, Cambridge University, Cambridge, England.
mediate the transfer of electrons from cytochrome b to cytochrome c may be schematically represented as follows:

\[ \text{Substrate} \rightarrow \text{f} \rightarrow b \rightarrow c \rightarrow a \rightarrow a_3 \rightarrow O_2 \]

The following points, however, indicated that it would be desirable to investigate in detail the role of cytochrome c\textsubscript{1} in electron transport: (a) the proposed different loci of inhibition (4, 5) by using the inhibitors antimycin A and SN5949; (b) the report (1) that cytochrome c\textsubscript{1} can react directly with cytochrome a; (c) the experimental evidence (7) that cytochrome b does not participate in the main pathway of electron transport in Keilin and Hartree heart muscle preparations but does function in systems in which phosphorylation occurs concomitant with oxidation (8); and (d) the statements (9, 10) that cytochrome c may not be involved in DPNH\textsuperscript{1} oxidation.

During the course of experiments designed to determine the factors influencing electron transport in phosphorylating liver mitochondria (8), it was observed that saline washing of mitochondria, after treatment with water, revealed the presence of a respiratory pigment with an \( \alpha \) absorption band maximum at 554 m\( \mu \). The work reported here describes the results of a series of spectrophotometric studies of an enzymatically active cytochrome c\textsubscript{1} associated with these non-phosphorylating particles derived from liver mitochondria. In addition to the spectral studies, the present paper discusses the relative content of cytochrome c\textsubscript{1} to that of cytochrome c in guinea pig liver mitochondria.

\textit{Preparations and Materials}

Mitochondria were prepared from guinea pig livers in 0.25 M sucrose by the method of Schneider (11). This method provides mitochondria (Mw) which show a requirement for a phosphate acceptor (ADP) in order to obtain maximal respiration in the presence of various metabolic substrates (8).

The mitochondria (Mw) which result from 10 to 12 gm. wet weight of guinea pig liver were disrupted (lysed) by suspension in 40 ml. of cold, glass-distilled water. After remaining at 4\( ^\circ \) for 2 hours, these water-treated mitochondria (L) were centrifuged at 8500 r.p.m. for 30 minutes in a refrigerated Servall angle centrifuge. The yellow supernatant fluid (Ls) was decanted and the precipitate (Lp) was then suspended in 40 ml. of a 0.85 per cent NaCl solution. This material was centrifuged for 10 minutes at 8500 r.p.m. The supernatant fluid (Ss), which contained cyto-

\textsuperscript{1} The following abbreviations have been used in this paper: DPNH = reduced di-phosphopyridine nucleotide; BAL = British antilewisite; 2,3-dimercaptopropanol; SN5949 = 2-hydroxy-3(2-methyloctyl)-1,4-naphthoquinone; ADP, adenosine diphosphate.
chrome c (12), was decanted and the precipitate (Sp) washed again with isotonic saline. The final saline-washed particles (SWp) were suspended in 0.1 m phosphate buffer of pH 7.4 or in an isotonic medium² so that 1 ml. of the suspension represented the material from 5 gm. of wet weight of liver.

Cytochrome c and reduced diphosphopyridine nucleotide (DPNH, 90 per cent pure) were obtained from the Sigma Chemical Company. The cytochrome c was further purified by chromatography on the ion exchange resin Amberlite IRC-50 according to the method of Margoliash (13).

**Methods**

Oxygen uptake was determined polarographically with a silver wire and platinum electrode couple polarized at —600 mv. This method is similar to that employed by Chance (14) with the difference that Chance uses a vibrating platinum electrode while in these experiments the electrode remains stationary and the reaction vessel is rotated at 60 r.p.m.³

Spectrophotometric recordings were obtained either with the wave length scanning recording spectrophotometer described by Chance and his co-workers (14-17) or with the sensitive double beam monochromator technique developed by Chance (14). Recordings of samples cooled to the temperature of liquid air were obtained by the technique previously described (18). The oxidation or reduction of cytochrome c₁ was determined by measuring the change in optical density at 554 m\(\mu\), with 540 m\(\mu\) as a reference wave length.⁴ In a similar way, cytochromes b and a were measured at the wave lengths 562 or 564 m\(\mu\) minus 575 m\(\mu\) and 605 m\(\mu\) minus 625 m\(\mu\), respectively.

**Results**

**Difference Spectra at Room Temperature**—Chance and Williams (8, 19) have determined difference spectra for those pigments enzymatically reducible in liver mitochondria. They observed the large absorption bands at 605 and 444 m\(\mu\) which represent the contributions of cytochromes \(a + a_3\). An asymmetrical band with an absorption maximum at about 551 m\(\mu\) was ascribed to a composite of the \(\alpha\)-bands of reduced cytochromes c and b, cytochrome b being represented simply by a shoulder at about 562 m\(\mu\). The presence of any additional cytochrome, such as c₁, could not be

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² The isotonic medium contained 0.026 m Na⁺, 0.099 m K⁺, 0.006 m Mg⁺⁺, 0.108 m Cl⁻, 0.013 m HPO₄²⁻, and 0.003 m H₂PO₄⁻.
³ This modification was developed by Dr. G. R. Williams.
⁴ The convention adapted for designating the wave lengths employed during an experiment is that the wave length mentioned first is that of a maximum of an absorption band, while the second wave length represents a minimum or isosbestic reference point.
readily ascertained because of the superimposition of the absorption bands of cytochromes c and b.

Treatment of the mitochondria with water, followed by washing with isotonic saline, removed the cytochrome c, thus revealing the presence of the pigment, cytochrome c₁. The reduced minus oxidized difference spectrum (Fig. 1, solid line) of the resuspended particles (SWp) shows not only the absorption bands of cytochromes a + a₂ and b, but also the pigment with an α absorption band maximum at about 554 μ. This hemoprotein is considered to be the same as the pigment cytochrome c₁ or e described by Keilin and Hartree (5) and "cytochrome 554" reported by Widmer et al. (4). When a small amount of cytochrome c is added to the SWp preparation, the reduced minus oxidized difference spectrum (Fig. 1, dashed curve) is much like that seen with the "intact" mitochondria (8). It can be seen from Fig. 1 that cytochrome c₁ has α- and β-bands at about 554 and 523 μ, respectively, while the bands with maxima at about 551 and 521 μ, previously attributed to cytochrome c, are composites of the bands of these two hemoproteins. The summation of the absorption bands of cytochromes c and c₁ has been described in detail by Keilin and Hartree (5).
Other spectrophotometric experiments have shown that cytochrome c₁ is reduced by ascorbic acid (5) or cysteine (20) but is not reduced by so-

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

**Fig. 2.** Reduction of cytochrome c₁ by ascorbic acid. The reduced minus oxidized difference spectrum was obtained by placing 1.0 ml. of SW particles and 1.0 ml. of 0.1 M phosphate buffer, pH 7.4, in two cuvettes. A few crystals of solid ascorbic acid and 0.02 ml. of a 1 M KCN solution were then added to the sample cuvette to reduce the pigments. Other conditions were as those in Fig. 1.

**Fig. 2. A, the spectral distinction between cytochrome c and cytochrome c₁.** Two cuvettes, each containing 1.0 ml. of SWp particles and 1.0 ml. of 0.1 M phosphate buffer, pH 7.4, were employed; 0.01 ml. of 0.01 M potassium ferricyanide solution and 0.01 ml. of a 1.5 M potassium cyanide solution were added to both cuvettes. Curve A represents the base-line of equal light absorption by both cuvettes. To one cuvette were added a few crystals of ascorbic acid to give the difference spectrum of cytochrome c₁, as represented in Curve B. To this cuvette was then added 0.01 ml. of a 1.3 × 10⁻⁴ M solution of cytochrome c. The resulting spectrum, Curve D, represents the sum of absorption of the α-bands of cytochromes c and c₁. A few crystals of ascorbic acid were then added to the other cuvette (reference) which did not contain any cytochrome c so that cytochrome c₁ was reduced. The resulting difference spectrum, Curve C, therefore represents the spectrum of the exogenous cytochrome c added to the sample cuvette. Other conditions were as for Fig. 1.

dium succinate in the presence of antimycin A (5). The reduced minus oxidized difference spectrum of the ascorbic acid-reduced cytochromes is shown in Fig. 2. The maximum at 605 μ is the band of cytochrome α + α₃, while that at 554 μ is the band of reduced cyto-
chrome c\textsubscript{1}. Cytochrome b is not reduced by ascorbic acid under these conditions.

Fig. 2, A illustrates the spectra of cytochrome c\textsubscript{1}, reduced by ascorbic acid, in both the presence and the absence of cytochrome c. These spectra clearly show the 4 to 5 m\textmu difference in the location of the \( \alpha \) absorption band maxima between cytochromes c and c\textsubscript{1}. The spectra also show that mixtures of the two bands blend into a single absorption band with a maximum at about 552 m\textmu. This, as Keilin and Hartree have discussed (5), explains in part the failure of others to observe the presence of cytochrome c\textsubscript{1}. The fact that cytochrome c\textsubscript{1} is reduced by ascorbic acid while cytochrome b is not indicates an oxidation-reduction potential for cytochrome c\textsubscript{1} more positive than that of cytochrome b (5, 19).

Low Temperature Spectra—The \( \alpha \)- and \( \beta \)-bands of reduced cytochromes sharpen and intensify when samples of these pigments are cooled in liquid air (3, 18, 21). With use of a recording spectrophotometric technique, small amounts of samples can be cooled to the temperature of liquid air and the spectra of the pigments present in the samples recorded while the material is at about \(-190^\circ\). The spectral properties of the pigments present in SWp particles and mitochondria (Mw) were investigated by the low temperature technique in order to resolve the \( \alpha \) absorption bands of reduced cytochromes c, c\textsubscript{1}, and b. Typical results are presented in Fig. 3. Curve A shows the spectrum obtained when the pigments of the SWp preparation were reduced with sodium succinate in the presence of the inhibitor, potassium cyanide. The resulting spectrum represents a difference spectrum of the light absorbed by the enzyme preparation diluted in glycerol minus the light absorbed by a reference cell containing a mixture of glycerol and buffer (\textit{i.e.}, no enzyme is present in the reference cuvette). Such spectra have been termed “apparent absolute absorption spectra” (21). A sample of reduced, purified cytochrome c, treated in a similar manner, has its main \( \alpha \) absorption band at 548.5 m\textmu (18).\textsuperscript{5}

When cytochrome c was added to the SWp preparation and the spectrum recorded of the reduced pigments cooled in liquid air, the resolution of the absorption band maxima of cytochromes c, c\textsubscript{1}, and b was seen. Such a spectrum is shown in Fig. 3, Curve B, and should be compared with the room temperature spectrum in Fig. 1. In a similar way, it has been possible to resolve the maxima of cytochromes c and c\textsubscript{1} when the pigments were reduced with ascorbic acid (\textit{cf.} Fig. 2). The low temperature spectrum of the reduced hemoproteins of “intact” mitochondria show spectral maxima identical with those presented in Fig. 3, Curve B. The low temperature spectrum of

\textsuperscript{5} Repeated experiments have shown that these absorption bands can be located to an accuracy of \( \pm 0.3 \) m\textmu.
technique is the only satisfactory method of spectroscopically differentiating between these cytochromes. Indeed, it was found necessary to assess the extent of removal of cytochrome c from the mitochondrial fragments by the low temperature spectrophotometric technique before attempting any experiments designed to ascribe an enzymatic function to cytochrome c₁.

Concentration of Cytochrome c₁ in Mitochondria—The concentration of cytochrome c₁ in liver mitochondria was estimated by assuming that the
molar extinction coefficient\(^6\) of the \(\alpha\) absorption band of cytochrome \(c_1\) is identical with that of cytochrome \(c\). All of the other extinction coefficients employed are the same as those used by Chance (7). Since a large amount of protein remained in the supernatant fluid during the preparation of the SWp particles, it was necessary to choose a reference material which, it is assumed, remains with the particulate fraction. Cytochromes \(a + a_3\), as determined by the absorption at 605 \(m\mu\), was chosen as such a reference. The recovery of these cytochrome pigments is summarized in Table I, from which it may be seen that about 85 per cent of the pigment absorbing

### Table I

**Recovery and Distribution of Cytochrome Pigments**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Total optical density units*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytochrome (a)</td>
</tr>
<tr>
<td>Mw</td>
<td>1.75</td>
</tr>
<tr>
<td>Lp</td>
<td>1.63</td>
</tr>
<tr>
<td>SW supernatant</td>
<td>1.28†</td>
</tr>
<tr>
<td>SWp</td>
<td></td>
</tr>
</tbody>
</table>

* The total optical density units were obtained by multiplying the optical density difference between the maximum and a corresponding minimum of the absorption band of a cytochrome times the total volume of the sample represented. The optical density difference for reduced cytochrome \(a\) was determined by subtracting the optical density at 625 \(m\mu\) from that at 605 \(m\mu\). In a similar way, the concentrations of cytochromes \(c\) and \(c_1\) were determined by subtracting the optical density reading of the minimum at about 540 \(m\mu\) from the maximum at 550 to 554 \(m\mu\). Corrections for volume losses due to samples removed for spectrophotometric assay have been applied.

† Represents only cytochrome \(c\).

§ Represents only cytochrome \(c_1\).

at 605 \(m\mu\) (cytochrome \(a + a_3\)), originally present in intact mitochondria, can be accounted for in the SWp preparation. Of the material with absorption maxima around 550 \(m\mu\), the recovery experiment shows that 55 per cent is found in the saline washings (this fraction represents cytochrome \(c\)) while between 30 and 35 per cent of the original absorption at

\(^6\) Chance (14) has discussed the estimation of extinction coefficients for the various cytochromes. Studies with purified cytochrome \(c\) and the carbon monoxide dissociation spectrum of cytochrome \(a_3\) have permitted an accurate determination of their extinction coefficients. The similarity, spectrally, of cytochrome \(b\) to the diphtherial toxin pigment and cytochrome \(a\) to verdoperoxidase was the means of estimating the extinction coefficients of these cytochromes. Because of a lack of better data, similar reasoning has been applied in assuming the extinction coefficient of cytochrome \(c_1\) to be the same as cytochrome \(c\).
about 550 m\( \mu \) is recovered as cytochrome \( c_1 \) in the SW particles. Thus, if the above assumptions concerning extinction coefficients are correct, the absorption band at about 551 m\( \mu \) of intact mitochondria (Mw), which previously had been attributed to cytochrome \( c \) alone, is actually a composite, about two-thirds of which is due to cytochrome \( c \) and about one-third to cytochrome \( c_1 \).

**Oxygen Uptake**—When either DPNH or succinate is added to SWp particles, there is a very slow rate of oxygen uptake, unless cytochrome \( c \) is added to the system. As shown in Fig. 4, \( A \) and \( B \), the addition of cytochrome \( c \) can cause as much as a 30-fold increase in the rate of oxygen utilization. This rate of oxygen uptake is dependent upon the concentration of exogenous cytochrome \( c \), the enzyme concentration, and the substrate employed. The concentration of cytochrome \( c \) necessary to give half maximal velocity of oxygen utilization, when succinate is employed as substrate, is about \( 1.5 \times 10^{-6} \) mole per liter. It is evident from the data presented in Fig. 4 that the system not only has a high affinity for cytochrome \( c \) but also requires cytochrome \( c \) as a constituent of the electron transport system in order that such a system may mediate the reaction

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**Fig. 4.** Polarographic measurements of the rate of oxygen uptake by saline-washed particles. \( A \), the rate of oxygen uptake when sodium succinate is employed as substrate and the influence of exogenous cytochrome \( c \) upon this reaction. \( B \), the influence of exogenous cytochrome \( c \) on the rate of oxygen uptake when DPNH is used as substrate. The oxygen electrode was first equilibrated with isotonic buffer, after which SWp, substrate, and cytochrome \( c \) were added as indicated. The temperature of the reaction medium was 23\( ^\circ \).
between oxygen and substrate. One must conclude from these experiments, as well as those presented by Ball and Cooper (6), that cytochrome c participates in the oxidation of both succinate and DPNH (cf. Chance and Williams (8) and Green et al. (9, 10)). A more detailed study of the role of cytochrome c in electron transport will be discussed in a future communication.

Reduction of Cytochromes b and c₁—In an attempt to ascribe an enzymatic function to cytochrome c₁, spectrophotometric studies on the rates of reduction and oxidation of the pigments of the SWp particles were carried out. Fig. 5 shows that both cytochromes c₁ and b are reduced upon the addition of sodium succinate. Similar results are obtained when DPNH is employed as substrate. Since cytochrome c was not added in these experiments, one may envision the electron transport chain as being divided into a reductase and an oxidase (the pigments of the latter are not reduced by substrate under these conditions whereby cytochrome c has not been added to the system). Thus, one must conclude that, if cytochrome c₁ is a part of the respiratory chain, it is a part of the reductase and therefore is reacting between substrate dehydrogenase and cytochrome c. Fig. 5 also illustrates the more rapid reduction of cytochrome c₁ than cytochrome b; indeed, a comparison of the slopes for the initial rates of reduc-

![Fig. 5. The reduction of cytochromes b and c₁. The sample cuvette contained 1.0 ml. of SWp diluted with 1.0 ml. of 0.1 M phosphate buffer, pH 7.4. The reaction was initiated by the addition of 0.02 ml. of 0.1 M sodium succinate. The reduction of cytochrome b (Curve A) was determined by measuring the increase in optical density at 562 mč with 575 mč as a reference wave length. In a similar manner, the reduction of cytochrome c₁ (Curve B) was determined at 554 mč with 540 mč as a reference wave length. Photographic recordings were obtained with a Hathaway oscillograph recorder. The temperature of the reaction mixture was maintained at 50. An upward deflection indicates a reduction of the pigment.](http://www.jbc.org/fig5.png)
tion shows that cytochrome b is reduced only 10 to 15 per cent as fast as cytochrome c₁ (22). These differences in the rates of reduction of cytochromes b and c₁ afford a method of kinetically distinguishing between these two cytochromes. By investigating the rate of change of optical density at a variety of wavelengths near the α absorption bands of these pigments, in this case with succinate as substrate, it is possible to construct the spectrum shown in Fig. 6, which shows a plot of the extent of increase in optical density contributed by the fast reduction of cytochrome c₁ and the slower reduction of cytochrome b. These results confirm on a kinetic basis the assignment of 554 mµ as the α absorption band maximum for reduced cytochrome c₁.

Inhibition by Antimycin A and SN5949—Experiments in which the wavelength scanning recording spectrophotometer was employed indicated that antimycin A inhibits the reduction of cytochrome c₁. This was confirmed by measuring the reduction of cytochromes c₁ and b with the dual monochromator apparatus. The presence of antimycin A causes a marked decrease in the rate of reduction of cytochrome c₁ and a concomitant increase7 in the rate of reduction of cytochrome b (22). The influence of antimycin A on the extent of reduction of cytochrome c₁ can be shown in a second way (Fig. 7) by measuring the change in steady state reduction of the pigment in the presence of the inhibitor. If cytochrome c₁ is reduced with succinate and then antimycin A is added, there is no change in the extent of reduction of cytochrome c₁. The addition of a small amount of cytochrome c to such an inhibited system, however, causes an immediate and rapid oxidation of cytochrome c₁. Under such conditions the addition of a small amount of cytochrome c does not cause an oxidation of cytochrome b. Such experiments show that cytochrome c₁ is functioning between the antimycin A-sensitive portion of the respiratory chain and cytochrome c (i.e. antimycin apparently influences the velocity of reduction but not that of oxidation of cytochrome c₁). Similar experiments have been carried out with DPNH and essentially the same results were obtained as with succinate.

Widmer et al. (4) have reported that cytochrome c₁ is reduced by succinate in the presence of the inhibitor SN5949, although the reduction of cytochrome c is blocked by the same inhibitor. From these observations they have concluded that the inhibitor SN5949 acts between cytochromes c₁ and c. Experiments similar to those described above have been carried out with SN5949 as inhibitor. Results similar to that presented in Fig. 7 have been obtained, indicating that the site of inhibition by these two chemicals (i.e. antimycin A and SN5949) is the same. However, when one determines the influence of SN5949 on the rate of reduction of cyto-

7 Chance, B., in press.
chrome $c_1$, it is observed (Fig. 8) that the initial rate of reduction is not greatly influenced by the inhibitor and that the pigment is slowly reoxidized after a rapid phase of reduction. Such a reaction curve (Fig. 8) may be interpreted to mean that the SN5949 exerts its greatest influence on the electron transport chain only after some component between dehydrogenase and cytochrome $c_1$ is reduced. The mechanism of inhibition and the factors which influence the degree of inhibition by SN5949 are currently under investigation.

**Reaction with Cytochromes $a$ and $a_s$**—When one follows the optical density changes at 605 m$\mu$ (a wave length designated as the $\alpha$ absorption band maximum of cytochrome $a + a_s$), the addition of substrate causes little
or no reduction of these pigments. As shown in Fig. 9, the subsequent addition of potassium cyanide causes a very slow increase in optical density, indicative of a slow reduction of these pigments. The addition of a small amount of cytochrome c, however, causes a rapid reduction of cytochrome $a + a_3$. Such an experiment would indicate, if cytochrome $c_1$ were reacting with cytochrome $a$, that this is indeed a very slow reaction.
The rapidity of the reduction of cytochrome $a + a_3$ in the presence of cytochrome $c$ is certainly definitive evidence of the role of cytochrome $c$ in transferring electrons from cytochrome $c_1$ to cytochrome $a$.

**DISCUSSION**

The presence of the respiratory pigment, cytochrome $c_1$, has been shown in particles derived from liver mitochondria. The experiments presented here indicate that this pigment may function in the transfer of electrons from succinic dehydrogenase or DPNH dehydrogenase to cytochrome $c$. This may be schematically represented as shown in Fig. 10. A few comments on this scheme are appropriate. First, the function of cytochrome $b$ in such a system is questionable. In non-phosphorylating preparations, it is reduced enzymatically but at a rate much slower than that of cytochrome $c_1$. It has previously been proposed (7, 22) that under such a situation an alternative pathway from flavoprotein to cytochrome $c_1$ needs to be considered. Recently, Chance and Williams (8) observed that in phosphorylating mitochondria cytochrome $b$ did not lag in reduction and they have proposed that the role of cytochrome $b$ in electron transport is dependent upon the ability of the system to carry out oxidative phosphorylation. Secondly, the site of sensitivity of the system to antimycin A and SN5949 appears between cytochrome $c_1$ and cytochrome $b$ or flavoprotein. This is at variance with the interpretation of the results reported by Widmer et al. (4), who concluded that SN5949 inhibits between cytochromes $c_1$ and $c$. Thirdly, Yakushiji and Okunuki (1) indicated from their studies that cytochrome $c_1$ could react directly with cytochrome $a$. Similar experiments reported here concerning the dependency of the system upon cytochrome $c$ for oxygen uptake as well as the reduction of cytochrome $a + a_3$ indicate that cytochrome $c_1$ does not react rapidly with the oxidase portion of the system unless the reaction is mediated by cytochrome $c$. Lastly,
it is proper to question the relationship of cytochrome $c_1$ to Slater’s (23) “BAL-sensitive factor.” The position of cytochrome $c_1$ in the respiratory chain makes it an ideal candidate for this factor. The definition of the “BAL-sensitive factor,” as indicated by Slater, implies that it is a hemo-protein which is destroyed by BAL, and which functions between substrate dehydrogenase and cytochrome $c$. Although experiments have not been carried out with BAL, similar experiments with antimycin A and SN5949 indicate that these inhibitors affect the reduction of cytochrome $c_1$. Thus, according to the scheme proposed above, the labile reaction could be either the reduction of cytochrome $c_1$ or the oxidation of the carrier directly preceding cytochrome $c_1$ on the substrate side.

**SUMMARY**

1. The spectral properties of the respiratory pigment, cytochrome $c_1$, have been determined with the use of particles derived from water-treated, saline-washed guinea pig liver mitochondria. The maxima of reduced cytochrome $c_1$, as determined by difference spectra, are located at 554, 523, and 419 m$\mu$. The resolution of the $\alpha$ absorption band of cytochrome $c_1$ from that of cytochrome $c$ has been demonstrated by the low temperature spectrophotometric technique.

2. A comparison of the optical density changes in mitochondria with the changes observed with saline-washed particles derived from mitochondria has shown that the concentration of cytochrome $c_1$ is about two-thirds that of cytochrome $c$.

3. Oxygen uptake experiments have shown the dependency for cytochrome $c$ in the oxidation of both succinate and reduced diphosphopyridine nucleotide by saline-washed particles prepared from liver mitochondria.

4. Spectrophotometric studies disclose a method of kinetically distinguishing between cytochromes $b$ and $c_1$, cytochrome $b$ being reduced at a rate 10 to 15 per cent that of cytochrome $c_1$.

5. Other spectrophotometric experiments have shown that cytochrome $c_1$ is not reduced by sodium succinate in the presence of antimycin A but is reduced by ascorbic acid in the presence of cyanide. The influence of the inhibitor antimycin A on the extent of reduction of cytochrome $c_1$ has been demonstrated, showing that the position of cytochrome $c_1$ in the respiratory chain is between the antimycin-sensitive reaction and cytochrome $c$. Similar studies with SN5949 have been described.

6. The failure to obtain a rapid reduction of cytochrome $a + a_3$ in the absence of cytochrome $c$ shows that cytochrome $c_1$ does not mediate the reaction between dehydrogenase and cytochrome $a$.

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