Preparation and Properties of Cardiac Cytochrome $c_1$*

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SUMMARY

In a continuation of our studies of the sequential fragmentation of the respiratory chain, we have worked out a method of isolating cytochrome $c_1$ from succinate cytochrome $c$ reductase. The purified cytochrome $c_1$ was stable in neutral solution and soluble in aqueous media. It contained 25 mmoles of heme per mg of protein and a small amount of carbohydrate. The absorption spectrum of the reduced cytochrome $c_1$ possessed maxima at 552.5, 530, 522.5, 512, 417, 317, and 276 nm, and the oxidized at 558, 522, 411, 355, and 276 nm. Neither carbon monoxide nor oxygen affected its spectral behavior. At $-160^\circ$, the $\alpha$-band of reduced cytochrome $c_1$ split into at least three bands and the $\beta$-band into nine. The low temperatures intensified the $\alpha$ and $\beta$-bands by 6- to 7-fold but did not cause any significant shift of the positions of the maxima. The extinction coefficient at $23^\circ$ for $A_{522}^{1000} - A_{410}^{1000}$ was found to be $17.5$ mm$^{-1}$ cm$^{-1}$ from the total iron assay and $18.5$ by titration with DPNH in the presence of phenazine methosulfate. The cytochrome $c_1$ can be used as an electron donor or acceptor in interaction with the electron transfer system in submitochondrial particles of bovine heart.

In a sequential fragmentation of the respiratory chain and systematic reconstitution from the components obtained we have reported the cleavage of succinate oxidase into two parts: cytochrome $c$ reductase and cytochrome oxidase (1). We have further separated succinate cytochrome $c$ reductase into succinate dehydrogenase and the cytochrome $b-c_1$ segment (2) and successfully reconstituted these isolated components into active succinate cytochrome $c$ reductase (3). However, further fragmentation to cytochromes $b$ and $c_1$ have been unsuccessful until recently.

Cytochrome $c_1$ has been unequivocally shown to be an indispensable component of electron transfer in intracellular respiration (e.g. see Reference 4). It was independently discovered by Yakushiji and Okunuki (5) and Keilin and Hartree (cytochrome $c$) (6). The Japanese group observed an absorption band at $552$ nm in addition to other cytochrome bands in a heart muscle preparation as well as a number of other samples examined. Since then, isolation of cytochrome $c_1$ from heart mince or mitochondria has been reported from several laboratories (3, 7-10). These methods for isolation generally involve a reaction with high concentrations of bile salts, with or without repeated heat treatment (7), proteolytic digestion, or reactions with such denaturing agents as urea, guanidine hydrochloride, and sodium dodecyl sulfate, singly or in combination (9, 11). The cytochrome $c_1$ thus obtained from these procedures is variable in composition with undefined activity.

In continuing our studies on sequential fragmentation of the respiratory chain, we have worked out a reproducible method utilizing a mild process for isolating active cytochrome $c_1$ with a relatively good yield. This communication reports the method and some properties which may be useful for those working in the field. Some of the work has been presented in a recent meeting (12).

EXPERIMENTAL PROCEDURES

Material—Crystalline cytochrome $c$ from bovine heart was prepared in this laboratory. Cytochrome $c$, type III, and bovine serum albumin were procured from Sigma and sodium cholate and ammonium sulfate were both "enzyme grade" from Mann. All other chemicals in the highest available purity were purchased commercially. The water used was doubly distilled in an all glass apparatus from deionized ordinary distilled water. Chromatographic calcium phosphate was prepared utilizing a mild process for isolating active cytochrome $c_1$ with a relatively good yield. This communication reports the method and some properties which may be useful for those working in the field. Some of the work has been presented in a recent meeting (12).

Methods—Cytochrome $c_1$ was determined spectrophotometrically with a millimolar extinction coefficient of 17.5 for $A_{522}^{1000} - A_{410}^{1000}$ which was calculated from the total iron content. The reduction was effected by dithionite. In the presence of cytochrome $b$, cytochrome $c_1$ was estimated from the difference spectra of the ascorbate reduced minus the ferricyanide-oxidized form, and cytochrome $b$ was estimated from the difference spec-

1 E. L. Jenner, personal communication.
tra of the dithionite reduced minus the ascorbate-reduced form. For cytochrome b, a millimolar extinction coefficient of 28.5 for $A_{562}^{285} - A_{470}^{285}$ was used (see Reference 10).

Total iron content was determined by atomic absorption spectroscopy. Platin was estimated by a fluorescence method previously reported (17). Total acid-extractable heme (18) and non-heme iron (19) were determined by methods previously used in this laboratory.

Protein in hemoproteins was assayed by a biuret method in the presence of hydrogen peroxide (20) with crystalline bovine serum albumin as a standard. The concentration of the bovine serum albumin solution was determined spectrophotometrically with $A_{380}^{250}$ = 6.6 (21). The protein concentration in the heart muscle preparation was estimated by the method described before (1). For routine purification, the protein concentration was estimated by the absorption at 276 nm; and an absorption index of $A_{276}^{1} = 14$ cm$^{-1}$ was used.

The spectroscopic measurements were carried out on a Cary spectrophotometer, model 14, at approximately 23°. The low temperature spectrophotometry was conducted in a Cary spectrophotometer model 14 equipped with Scattered Transmission Accessory, RCA 06217 Photomultiplier, and Sylvania DXL quartz halogen lamp. The sample was placed in a cuvette of 2- to 3-mm optical path (adjustable by spacers placed between the double observation windows) which was cooled by liquid nitrogen. The temperature was directly recorded on a Leeds-Northrop Speedomax W Strip-chart Recorder with a calibrated copper-constantan thermocouple; one end of the thermocouple was placed in the cuvette and the other in ice water. The setup was checked with crystalline cytochrome c and gave a somewhat higher intensification factor, but otherwise was essentially the same result as reported by Estabrook (22).

Total carbohydrate was estimated from the absorbance at 485 nm according to the method of Dubois et al. (23).

Enzymatic Activity—Succinate cytochrome c reductase and succinate dehydrogenase activities were assayed according to the methods described previously (2, 24) in a 1-ml reaction mixture. Enzymatic oxidation of cytochrome c1 was estimated in a reaction mixture which contained 0.1 M phosphate buffer, pH 7.4, usually 15 µg per ml of the heart muscle preparation, 0.75 µM cytochrome c, and various amounts of reduced cytochrome c. The reaction was started with the heart muscle preparation, and the oxidation rate was followed by the decrease of absorbance at 552.5 nm. For the enzymatic reduction of cytochrome c1, the reaction mixture contained 20 mM succinate, 1.5 mM cyanide, 0.1 M phosphate buffer, pH 7.4, 0.75 µM cytochrome c, the heart muscle preparation, and oxidized cytochrome c1. The reaction was started with succinate and cyanide. All assays were conducted at room temperature, approximately 23°.

Cleavage of Covalently Bound Heme—The cleavage of the heme covalently bound to the protein from cytochromes c or c1 was accomplished by the silver salt method essentially according to Margoliash et al. (25). A mixture of 5 ml of glacial acetic acid, 250 mg of silver acetate, and 120 mmole of cytochrome c was shaken at 60° for 60 min. At the end of the reaction, the heme was extracted three or four times with ether. The combined ethereal extracts were washed with 5% sodium chloride and then water. Since cytochrome c1, but not c, is insoluble in glacial acetic acid, a step of homogenization was used prior to the reaction at 60°.

### RESULTS

**Preparation of Succinate Cytochrome c Reductase**—The succinate cytochrome c reductase used for the isolation of cytochrome c1 was prepared according to the procedure described previously (2) with slight modifications. The Keilin-Hartree heart muscle preparation was finally suspended in 0.1 M phosphate-borate buffer (14), pH 7.8, to a protein concentration of 18 to 20 mg per ml. Subsequent operations for the preparation of the reductase and the isolation of cytochrome c1 were performed at 0-4° and the phosphate buffer used was the Sörensen type at 0.1 M concentration with respect to phosphate, unless otherwise specified. To 1 liter of heart muscle preparation were added 50 ml of sodium cholate solution, 20% w/v, and 265 g of pulverized ammonium sulfate. The mixture was adjusted to pH 8 with 1 N NaOH, usually 60 ml per liter of heart muscle preparations (the pH was taken after a 1:5 dilution at 23°). After stirring for 1 hour the mixture was centrifuged at 23,000 $\times$ g for 1 hour. The greenish brown residues were discarded. The reddish yellow supernatant solution was collected and pulverized ammonium sulfate was added at 125 g per liter of the solution. The decrease of the pH caused by the addition of ammonium sulfate was compensated for by the simultaneous addition of concentrated ammonium hydroxide; 5 µl per g of ammonium sulfate were usually employed. The mixture was centrifuged at 23,000 $\times$ g for 30 min after standing for 30 min. The supernatant solution was discarded. The reddish brown precipitate was dissolved in 0.1 M phosphate buffer, pH 7.4, containing 0.5% sodium cholate with a volume of approximately one-sixth of that of the heart muscle preparation used. If necessary, the precipitate could be stored overnight in the refrigerator without losing activity.

The solution was brought to 40% ammonium sulfate saturation1 by adding pulverized ammonium sulfate and the pH was adjusted with ammonium hydroxide. The mixture was then allowed to stand at least 1 hour before centrifugation at 48,000 $\times$ g for 20 min. The yellowish supernatant solution (S0), which contained only a small amount of cytochromes b and c1 and yet possessed an ability to greatly increase the succinate cytochrome c reductase activity, was discarded or saved for further investigation. The precipitate was again dissolved in 0.1 M phosphate buffer containing 0.5% sodium cholate in the same volume as used in the previous step. The solution was then brought to 25% saturation of ammonium sulfate by adding pulverized ammonium sulfate, 138 g per liter, in the same manner as in the previous step.

### Notes

1 In this paper when the solid reagent was used for adjusting the saturation of ammonium sulfate the following equation was employed (26):

$$X = \frac{0.506 (S_0 - S_i)}{1 - 0.286 S_i}$$

where $X$ is grams of ammonium sulfate required to be added to a 1-ml solution of the initial ammonium sulfate saturation $S_i$ to the final saturation $S_f$. When the saturated ammonium sulfate solution was used for adjusting the saturation of ammonium sulfate, the following equation was employed (2):

$$Y = \frac{(S_0 - S_i)}{1 - S_i}$$

where $Y$ is the volume in milliliters of saturated ammonium sulfate to be added to a 1-ml solution of initial saturation $S_i$ to the final saturation $S_f$. 

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After standing for 20 min, the precipitate was removed by centrifugation, and the supernatant solution was then further brought to 50% ammonium sulfate saturation. The precipitate obtained by centrifugation after standing for 20 min was dissolved in 0.1 M phosphate buffer, pH 7.4, to a protein concentration of approximately 10 mg per ml. The reddish solution at this stage was completely clear and could be stored (2). The yield of cytochrome c1 up to this step was 70 to 80% of that present in the heart muscle preparation.

**Isolation of Cytochrome c1 from Succinate Cytochrome c Reductase**—Cytochrome c1 was isolated and purified by extraction with β-mercaptoethanol, fractionations with ammonium sulfate, and chromatography on a calcium phosphate column. β-Mercaptoethanol and neutralized saturated ammonium sulfate solution, 10 ml each, were added to 80 ml of succinate cytochrome c reductase solution (approximately 10 mg per ml). The solution was constantly stirred for 1 hour. Brown precipitate was formed during the incubation. The mixture was then diluted with an equal volume of cold 50 mM phosphate buffer, pH 7.4, and centrifuged at 48,000 × g for 10 min to remove the precipitate. The supernatant solution was dialyzed against at least 20 volumes of 50 mM phosphate buffer, pH 7.4, overnight with two changes of buffer. The small amount of precipitate formed during dialysis was removed by centrifugation. The dialyzed solution was concentrated by ammonium sulfate precipitation. The solution (in 50 mM phosphate) was brought to 58% ammonium sulfate saturation, and the precipitate thus formed was collected by centrifugation after standing for 10 min. It was dissolved in 0.2 M potassium phosphate buffer, pH 7.4, to a protein concentration equivalent to an A₆/e = 6 (or approximately 4.5 mg per ml). The yield up to this stage was about 60%.

To the above solution were added a sodium cholate (20 mg per ml) solution to 1.8% final concentration and neutralized saturated ammonium sulfate to 20% saturation. The solution was then frozen overnight at about −100°C. The mixture was thawed and the precipitate, if any, removed by centrifugation. Sodium dithionite was added under nitrogen to 3 mg per ml. The solution was incubated under nitrogen for 20 min before carrying on the subsequent anaerobic ammonium sulfate fractionation. Saturated neutralized ammonium sulfate solution was added dropwise under nitrogen to 28% saturation. The precipitate was removed by centrifugation after standing for a few minutes and the supernatant solution was further brought to 32% saturation. The precipitate was collected by centrifugation and then dissolved in 80 mM phosphate buffer, pH 7.4, to a protein concentration of approximately 2 mg per ml. The preparation up to this stage showed A₄₃₃/A₅₄₈ higher than 0.8. During the anaerobic fractionation care had to be taken to maintain the solution at neutrality; the decrease of pH, apparently due to the oxidation of dithionite, could be best prevented by elimination of air, or, less satisfactorily, compensated for by the addition of ammonium hydroxide. If the preparation showed a purity of A₄₃₃/A₅₄₈ less than 0.8, the procedure from the freezing step on might be repeated in order to get a satisfactory result for the subsequent steps. A sacrifice of the yield at this step to ensure a better quality was sometimes advised.

The fraction obtained from the ammonium sulfate fractionation with A₄₃₃/A₅₄₈ higher than 0.8 was chromatographed on a calcium phosphate cellulose column (2 × 7 cm) equilibrated with 80 mM phosphated buffer, pH 7.4. The loading capacity of the column was 3 mg of protein per ml of the column bed volume. The column was eluted stepwise in a sequence with 0.1, 0.15, 0.2, and 0.3 M phosphate buffer at a flow rate of approximately 1 ml per min. Two column bed volumes of each concentration of buffer were used. The highest purity of cytochrome c1 was eluted between 0.2 and 0.3 M phosphate buffer. Fractions which contained cytochrome c1, with a ratio of A₄₃₃/A₅₄₈ over 2.3 were com-

| Table 1 |
| Isolation and purification of cytochrome c1 from succinate cytochrome c reductase |

The data given in this table were average values from five batches of succinate cytochrome c reductase. No absorbance ratio of A₄₃₃/A₅₄₈ was given in the reductase preparation because cytochrome b also showed Soret absorption.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Volume</th>
<th>Protein Concentration</th>
<th>Cytochrome c1 Purity</th>
<th>Cytochrome c1 Recovery</th>
<th>Ratio of A₄₃₃/A₅₄₈</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reductase</td>
<td>ml</td>
<td>mg/ml</td>
<td>ug/μmol/ug</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>β-Mercaptoethanol</td>
<td>100</td>
<td>10.0</td>
<td>1000</td>
<td>1.55</td>
<td>1.55</td>
</tr>
<tr>
<td>extract</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fractionation</td>
<td>50</td>
<td>4.1</td>
<td>207</td>
<td>4.30</td>
<td>0.89</td>
</tr>
<tr>
<td>from ammonium sulfate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>58</td>
</tr>
<tr>
<td>sulfate</td>
<td>20</td>
<td>3.4</td>
<td>68</td>
<td>7.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Chromatography</td>
<td>26</td>
<td>0.5</td>
<td>13</td>
<td>25.0</td>
<td>0.22</td>
</tr>
</tbody>
</table>

**Fig. 1.** The effect of β-mercaptoethanol and ammonium sulfate concentration on the extraction of cytochrome c1 (Cyt C). The abscissa for β-mercaptoethanol concentration (βME) is in per cent and that for ammonium sulfate the percentage of saturation; both are in the numerical scale. The spectral purity of cytochrome c1 was expressed as the percentage of cytochrome c1 present in the extract. The extract was obtained after incubation of succinate cytochrome c reductase at two protein concentrations, 18 mg per ml (X—X), and 9 mg per ml (□—□), in the presence of 10% ammonium sulfate saturation and the concentrations indicated of β-mercaptoethanol at 4°C for 1 hour. The mixture was finally diluted with an equal volume of 50 mM phosphate buffer and centrifuged at 48,000 × g for 10 min. To observe the effect of ammonium sulfate concentration on the extraction (□—□), the experiment was carried out at a protein concentration of 18 mg per ml and 10% β-mercaptoethanol. The concentrations of ammonium sulfate, in percentage of saturation, were varied as indicated.
bined, if so desired; otherwise samples of different purity might be separately stored in a deep freeze. If preparations of higher concentrations were required, the combined eluate was concentrated by membrane filtration. A summary for the purification is given in Table I.

**Effect of β-Mercaptoethanol and Ammonium Sulfate Concentration on Extraction of Cytochrome c**—As shown in Fig. 1 the purity of the cytochrome c₁ preparation extracted from the reductase was very much dependent on the concentration of β-mercaptoethanol and ammonium sulfate. Low concentrations of β-mercaptoethanol or ammonium sulfate resulted in incomplete cleavage of the cytochrome b₁-c₁ segment. Although sometimes a small amount of cytochrome b present in the soluble extract could be removed by the subsequent purification steps, a drastic reduction in the yield was always observed. On the other hand, high concentrations of β-mercaptoethanol caused apparent "denaturation" of cytochrome c₁ and also resulted in the low recovery. High concentrations of ammonium sulfate precipitated the uncleaved b₁-c₁ segment and also reduced the recovery of cytochrome c₁. A final concentration of 10% β-mercaptoethanol and 10% ammonium sulfate saturation was found to give a satisfactory result. Under these conditions cytochrome b was split from cytochrome c₁ and precipitated.

**Purity and Composition of Cytochrome c₁**—Cytochrome c₁ thus prepared was freely soluble in aqueous media. Electrophoresis on cellulose acetate strips at pH 6.1, 6.6, 7.4, and 8.6, showed the preparations of A₄₃₇:A₂₇₆ of 2.5 to be at least 95% pure. Only one major band with a very minor band of less than 5% of the total protein was detected on the strips.

Gel electrophoresis in a dissociating solvent system was conducted according to the method developed by Weber and Osborn (27). Cytochrome c₁ at about 1.4 mg of protein per ml was incubated at 37° for 2 hours in 1% β-mercaptoethanol, 1% sodium dodecyl sulfate, and 10 mM sodium phosphate buffer, pH 7.0, in the presence and absence of 8 M urea. After incubation, the solution was dialyzed against 500 volumes of 10 mM sodium phosphate buffer containing 0.1% dodecyl sulfate and 0.1% β-mercaptoethanol for 8 hours. Aliquots of 15 and 50 μl of dialyzed samples were used for the electrophoresis on 10% polyacrylamide gel. One hemoprotein band was discernible before staining in all cases. Another protein band containing apparently no heme was visible after staining with Coomassie brilliant blue for 10 hours. The same results were obtained in the presence and absence of 8 M urea. The ratio of these two bands was about 2:1 with molecular weights of approximately 2.9 × 10⁴ and 1.5 × 10⁴, respectively, calculated according to the mobility values reported by Weber and Osborn (27). It cannot be conclusively ruled out that the protein component without heme is not alien to cytochrome c₁. However, that this component is most probably indigenous to the cytochrome is borne out by results from experiments of electrophoresis without sodium dodecyl sulfate, density gradients, chromatography in various systems. It may be of interest that the ratio of the hemoprotein to other protein bond (70 to 30%) is similar to the ratio of their molecular weights. Ex-

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### Table II

**Composition of purified cytochrome c₁**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>nmoles/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total, by atomic absorption</td>
<td>25.4</td>
<td></td>
</tr>
<tr>
<td>Heme, by hemochrome spectra</td>
<td>25.2</td>
<td></td>
</tr>
<tr>
<td>Nonheme</td>
<td></td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>Flavin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acid nonextractable</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Acid extractable</td>
<td>(0.67)</td>
<td>&lt;2% w/w</td>
</tr>
<tr>
<td>Carbohydrate, total</td>
<td></td>
<td>Not determined</td>
</tr>
<tr>
<td>Lipids</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The concentration of acid-extractable flavin was probably due to the released heme under the experimental condition (see text).*

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**Fig. 2. Absorption spectra of cytochrome c₁.** Purified cytochrome c₁ had 0.28 mg of protein per ml of 50 mM phosphate buffer, pH 7.4; optical path, 1 cm; temperature approximately 23°. Solid line, as prepared, reduced; broken line, oxidized with a minimum amount of potassium ferricyanide.
samples are available that monodisperse proteins can yield multiple bands on electrophoresis in dissociating media (e.g. References 27 and 28). On the other hand, these results may suggest cytochrome c\textsubscript{1} contains two classes of nonidentical subunits; one of them may be related to the carbohydrate component detected.

A small amount (less than 0.7 nmole per mg of protein) of acid-extractable flavin (cf. Table II) but no acid nonextractable flavin or nonheme iron was detected in the purified preparations. However, the so-called acid-extractable flavin found most probably resulted from the fluorescence of cytochrome c\textsubscript{1} which remained in the 10% trichloroacetic acid extract (17, 29). The preparation also showed an apparent carbohydrate content of approximately 20 µg (in terms of galactose) per mg of protein; its significance is not known at present.

The cytochrome c\textsubscript{1} as prepared contained 25 nmoles heme per mg of protein assayed as total iron or as heme. No hemoproteins other than cytochrome c\textsubscript{1} or acid-acetone-extractable heme were found. Ultracentrifugal studies suggested the isolated cytochrome c\textsubscript{1} was in an aggregated form. The molecular weight determined by the sedimentation equilibrium method with an assumed partial specific volume of 0.71 was found to be 210,000 and 93,000 at pH 7.4 and 11, respectively. The minimum molecular weight calculated from the heme content was about 40,000. It seemed that even at pH 11 the cytochrome was still in a polymeric form.

Absorption Spectra and Extinction Coefficients—The oxidized and reduced spectra of cytochrome c\textsubscript{1} are depicted in Fig. 2. The reduced form possessed maxima at 552.5, 530, 522.5, 512, 417, 317, and 276 nm, and the oxidized at 558, 522, 411, 355, and 276 nm. Carbon monoxide did not affect cytochrome c\textsubscript{1} spectrally.

It is well known that cytochrome c shows a broad maximum at 695 nm with a millimolar extinction coefficient of about 0.7 (cf. Fig. 3B). Cytochrome c\textsubscript{1} likewise exhibited an even broader band in this region (Fig. 3A). Upon reduction, the absorption of c\textsubscript{1} between 570 and 770 nm decreased greatly and the broad band at 700 nm was no longer discernible. However, a very broad maximum around 670 nm appeared.

At -160° reduced cytochrome c\textsubscript{1} exhibited a very colorful spectrum as shown in Fig. 4. The α-band, which was relatively sym-

\footnote{It should be noted that the scale ratio for the low temperature spectra is different from that for the spectra at room temperature; the wavelength axis is expanded two times.}

![Fig. 3. Absorption spectra of cytochrome c\textsubscript{1} and c in the near-infrared region. A, cytochrome c\textsubscript{1}, 138 µM in 0.1 M phosphate buffer, and B, bovine heart cytochrome c, 194 µM in 0.1 M phosphate buffer.](http://www.jbc.org/)

![Fig. 4. Low temperature spectra of cytochrome c\textsubscript{1}, 61 µM in 50 mM phosphate buffer, pH 7.4. Spectra were recorded at about -160°. Sample was cooled with liquid nitrogen in a gold-plated cuvette. The slit opening was adjusted from 0.02 to 0.05 mm. Oxidized form was made directly by the addition of a small amount of potassium ferricyanide.](http://www.jbc.org/)
metrical at room temperature, split into at least three absorption bands with discernible maxima at 552.5, 550, and 549 nm. The \( \beta \)-band of cytochrome \( c_1 \), which showed some fine structure even at room temperature, split into a minimum of nine bands. Those which were easily discernible included maxima at 528, 523, 521, 514, 510, and 506 nm, and shoulders at 541, 452, 524, and 516 nm. The intensification of the \( \alpha \)- and \( \beta \)-bands at low temperatures was about 6- to 7-fold. In contrast, the Soret maximum was not so intensified nor clearly split. In contrast to cytochrome \( c \), no blue shift of these bands at low temperature was found in cytochrome \( c_1 \). This finding is in accordance with that of Keilin and Hartree (6, 30). They observed a sharp and narrow band at 552 nm between the cytochromes \( b \) and \( c \) in the heart muscle preparation at these low temperatures.

The heme prosthetic group of cytochrome \( c_1 \) was not acid-acetone extractable. After the cleavage of the covalent bonds by silver salt, the heme became soluble in acetone or ether. The reduced pyridine hemochromogen spectrum of the cleaved heme is shown in Fig. 5A; for comparison, the cleaved heme from horse cytochrome \( c \) (Fig. 5B) is also presented. The maxima were

![Fig. 5. Comparison of reduced pyridine hemochromogen spectra of the heme isolated from cytochrome \( c_1 \) (A) and cytochrome \( c \) (B). See text for the details of experimental conditions.](image-url)

![Fig. 6 (left). Titration of cytochrome \( c_1 \) with DPNH in the presence of phenazine methosulfate (PMS). Twenty-six nanomoles of oxidized cytochrome \( c_1 \) in 1 ml of 50 mM phosphate buffer, pH 7.4, containing 0.36 nmole of PMS was titrated with DPNH. Two microliters of 0.87 mM DPNH were used in each of eight additions. The spectra were recorded in a Cary 14 right after addition of DPNH. No volume correction was made.](image-url)

![Fig. 7 (center). Autoxidizability of cytochrome \( c_1 \). Purified cytochrome \( c_1 \), 12 \( \mu \)g, in 0.2 M acetate or phosphate buffer at indicated pH was incubated at 0°. The absorbance change at 552.5 and 540 nm was recorded at 1 hour, O——O; 4 hours, X——X; and 24 hours, Δ——Δ. The autoxidizability was defined as \( (\Delta A_0 - \Delta A_t)/\Delta A_0 \), \( \Delta A = A_{552.5} - A_{540} \), and subscripts 0 and t are for time at zero and t, respectively.](image-url)

![Fig. 8 (right). Enzymatic reduction and oxidation of cytochrome \( c_1 \) catalyzed by the heart muscle preparation. In a 1-ml reaction mixture for the oxidation experiment (△△) were contained 0.1 M phosphate buffer, pH 7.4, 17.4 \( \mu \)g of the heart muscle preparation, 0.75 \( \mu \)M cytochrome \( c_1 \), and the indicated concentrations of reduced cytochrome \( c_1 \). In the 0.7-ml reaction mixture for the reduction experiment (○——○) were contained 0.1 M phosphate buffer, pH 7.4, 20 \( \mu \)M succinate, 1.5 \( \mu \)M cyanide, 0.75 \( \mu \)M cytochrome \( c \), 15.4 \( \mu \)g of the heart muscle preparation, and the indicated concentrations of oxidized cytochrome \( c_1 \).](image-url)
found to be at 547, 517, and 407 nm the same as those in the spectrum from cytochrome c. Reduced pyridine hemochromogen of uncropped cytochrome c1 showed an a-band at 550 and a Soret band at 413 nm.

Since the purified preparation did not contain nonheme iron or other acid-acetone-extractable heme, it might be safe to use the total iron content for the computation of the absorbance index. By this calculation, we obtained an extinction coefficient of 17.5 mM⁻¹ cm⁻¹ for A₄₃₅₂;₆ - A₂₅₄₀. However, we found an extinction coefficient of 18.5 by the titration of the cytochrome with DPNH in the presence of phenazine methosulfate (cf. Fig. 6) as used for cytochrome c (31). The higher value might be due to the side reaction of the reduced phenazine methosulfate with oxygen since the titration was conducted aerobically.

Autoxidation of Cytochrome c₁—The rate of autoxidation of the purified cytochrome c₁ was very slow between pH 5.5 and 11; indeed it was slower than that of cytochrome c. At pH 12 or higher, the autoxidation became very fast as shown in Fig. 7. Moreover, the cytochrome was apparently thus denatured. On the acidic side, the solution gradually became turbid and the cytochrome oxidized when stored at pH lower than 5.5.

The oxidized cytochrome c₁ in the purified form showed slow reduction in air upon irradiation. However, in the absence of light, the cytochrome was completely stable in contrast to the impure form which always became reduced upon standing. Oxidized cytochrome c₁ was rapidly reduced by β-mercaptoethanol, ascorbate, or dithionite to the same extent; no difference in the reduced spectra was observed.

Enzymatic Oxidation and Reduction of Cytochrome c₁—Both the enzymatic oxidation and reduction of cytochrome c₁ were determined with the heart muscle preparation or succinate cytochrome c reductase as the electron carrier. In other words, cytochrome c₁ in the reduced form was used as an electron donor and that in the oxidized form as an acceptor with succinate as a substrate. The purified cytochrome c₁ did not show any succinate dehydrogenase activity with phenazine methosulfate as the acceptor.

As shown in Fig. 8, the oxidation of reduced cytochrome c₁ by oxygen in the presence of the heart muscle preparation was dependent on the concentration of cytochrome c₁. Under these conditions, the Kₘ for cytochrome c₁ was found to be 1.3 μM and the V_max to be 137 nmoles of cytochrome c₁ per min per mg of the heart muscle preparation at 23°. The reaction was sensitive to cyanide.

Antimycin A inhibited practically completely, as expected, the reduction of oxidized cytochrome c₁ by succinate catalyzed by either succinate cytochrome c reductase or the heart muscle preparation. Under the conditions detailed in the legend of Fig. 8, the Kₘ for cytochrome c₁ was found to be 6.5 μM with a V_max of 171 nmoles of cytochrome c₁ reduced per min per mg of the heart muscle preparation.

The enzymatic activity for the oxidation of reduced cytochrome c₁ was found to be relatively constant at the various levels of purity in isolation as shown in Table III. Likewise, the Kₘ values were practically the same. No similar experiments for the reduction of oxidized cytochrome c₁ were conducted.

**DISCUSSION**

The results described show that the cytochrome c₁ obtained is probably the purest so far reported in the literature based on the ratio of A₄₁₇:A₂₅₄₀ and on the direct iron or heme assay per mg of protein. That Sekuzu et al. (8) have reported 27.3 nmoles of heme per mg of protein of their sample is difficult to understand in view of the fact that the ratio A₄₁₇:A₂₅₄₀ is less than 1.5. Bomstein et al. (7) did not report the absorption spectrum in the ultraviolet region, and thus the ratio A₄₁₇:A₂₅₄₀ cannot be computed. Their claim of 27.2 nmoles of protoporphyrin per mg of protein for their cytochrome c₁ remains to be confirmed. However, several points in the Bomstein et al. (7) paper are not obvious to us. For example, in their studies of the molecular weight they used a sample containing only 19 nmoles of heme per mg of protein in spite of the availability in their laboratory of much purer preparations. It is also interesting to note that they were able to use less than 0.015% sodium dodecyl sulfate to degenerate a polymer of about 320,000 to the monomeric cytochrome c₁ of 53,000 determined by the Archibald approach to sedimentation equilibrium method, although the calculated molecular weight for the purest preparation is less than 37,000. Equally puzzling is the absorption of the cytochrome in the 600 nm region as shown in their Fig. 2 (7); an absorbance of about 0.05 was reported for 0.17 mg of protein per ml, but this sample showed less than 0.03 absorbance when the protein concentration is more than quadrupled. In the last 6 years, on many occasions, our laboratory was not able to obtain samples of more than 10 nmoles per mg by this published method (7); or a similar version of the method (11). Moreover, the enzymatic activity of the samples thus made decreased drastically with the increase of the purity.

In contrast, the method described here is simple and reproducible. Actually, these advantages should be expected because, being taxed for many years by lacking a good simple method for cytochrome c₁, we have tried all published methods and searched for possible procedures. In the sequential fragmentation we have naturally selected succinate cytochrome c reductase instead of the heart muscle preparation, mitochondria, or heart mince as the starting material. On the basis of the unit weight the active component is several times higher in the reductase than in mitochondria or tissue mince. In addition, the method relies on two key steps, the β-mercaptoethanol extraction and anaerobic ammonium sulfite fractionation.

It may be mentioned that the a-band we have found in the isolated cytochrome c₁ possesses a maximum at 552.5 nm at about -160° and room temperature rather than 554 nm as other reported (7–9). Our value is very close to that originally reported at 552 nm at room temperature (5) and at 552 to 553 nm at room or low temperatures (6, 30) for cytochrome c₁ in heart muscle preparations.

Although cytochrome c₁ possesses a very similar visible spectrum
to that of cytochrome c at both room and liquid nitrogen temperatures the near infrared spectra of the reduced cytochromes are different. If the long wave length absorption is some reflection of conformation then the conformation of both cytochromes should be expected to be different. Indeed the studies of circular dichroism spectra (32) have confirmed this deduction.

We think that our cytochrome cI is probably an active preparation by the following considerations. (a) It is capable of serving as an electron carrier through native electron transfer systems, although the values summarized in Table III may be apparent rather than true activities. Since particles of the heart muscle preparations are in a vesicle form and cytochrome cI presumably exists in the inner phase (33), the transfer of electrons from whatever the component to cytochrome cI in solution may not measure the actual activity. Also, the possibility that the rapid equilibration which exists between cytochromes c and cI electronically renders the reported values of the enzymatic property apparent rather than "genuine." (b) The cytochrome cI does not react with carbon monoxide. (c) The reduced cytochrome cI is not autoxidizable. (d) The electron paramagnetic resonance behavior of the isolated cytochrome cI is practically the same as that in submitochondrial particles. (e) Unpublished observation from this laboratory showed our particulate cytochrome b preparation can take up the soluble cytochrome cI to yield a particle with the cytochrome b to cytochrome cI ratio similar to that in the intact segment.

REFERENCES

6. Preliminary results show that indeed these two cytochromes equilibrate rapidly, within the limit of the equipment used, i.e., in 2- or 3-msec range.
7. Collaborative experiments with Dr. H. Beinert to be published.

Preparation and Properties of Cardiac Cytochrome \( c_1 \)
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