The classical work of Keilin and Hartree (1), based primarily on microspectroscopic and enzymatic studies, has served as a foundation for our present understanding of terminal electron transport. The application of spectrophotometric techniques was generally limited to studies of soluble purified pigments such as cytochrome c, or of preparations treated with clarifying agents such as sodium cholate, until the development of rapid and sensitive spectrophotometric methods by Chance (2). By means of such techniques, which permit the measurement of small spectral changes in turbid solutions, Chance has been able to deal quantitatively with the functional relationships of the respiratory pigments. Recently the wavelength-scanning spectrophotometer, devised by Chance and his coworkers (3–6), has been modified to permit the recording of spectra of samples cooled to low temperature (7), simulating the technique first applied by Keilin and Hartree to the microspectroscope (8). Spectrophotometric studies at low temperatures make it possible to identify clearly those pigments of the respiratory chain which at room temperature are indistinguishable one from another. This is best exemplified by the resolution of the absorption bands of cytochromes c, c1, and b. In addition, the visible absorption bands of hemoproteins are greatly intensified at low temperatures, permitting measurements of pigments which are of such low concentration that they cannot be determined by measurement at room temperature.

In addition to the recent advances in spectrophotometric techniques, the electron transfer systems have been isolated in highly active form. A particulate enzyme (ETP) isolated by Green et al. (9) catalyzes the oxidation of succinate and reduced diphosphopyridine nucleotide (DPNH) by molecular oxygen without the addition of external cytochrome c. DPNH oxidase, a derivative form of ETP without succinoxidase activity, has been

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prepared in two forms (10, 11), one catalyzing the reaction without added cytochrome c (closed form) and the second needing added cytochrome c for maximal activity (open form). Succinic dehydrogenase complex (SDC) prepared by Green et al. (10) catalyzes the oxidation of succinate by cytochrome c and a number of other oxidizing agents. The present communication describes the results of spectrophotometric studies at room temperature and at low temperature, of Keilin and Hartree heart muscle preparations, and of preparations of ETP, SDC, and DPNH oxidase. The enzymatic activities of the preparations studied are discussed in terms of the differences in the absorption spectra as determined for each type of preparation.

Methods

The rapid and sensitive wave length-scanning spectrophotometer has been modified to obtain low temperature spectra as previously described (7). Briefly, the method consists of dilution and reduction of the heart muscle homogenates with appropriate reagents, after which an equal volume of glycerol is added. These samples, together with appropriate reference samples, are cooled rapidly by being plunged in liquid air. The solidified contents of the cuvettes are permitted to devitrify, at which time the contents of both the sample and reference cuvette become turbid (Condition II). The cuvettes are then recooled to −190°, and the difference of optical density between the turbid sample and the turbid reference cell is automatically recorded. Since it is not necessary for the reference cuvette to contain any enzyme preparation (see below), the resulting spectrum can be a measure of the “apparent absolute absorption spectrum” of the pigments present in the sample. The magnitude of the absorption bands may be determined from the “absorbancy or optical density increment scale.” The band of purified reduced cytochrome c, which at room temperature has a maximum at 550 μm, was used as the reference wave length for locating the absorption maxima of the pigments described below.

Modified Keilin and Hartree heart muscle homogenates (12) were prepared as described by Slater (13) and Chance (2). Heart muscle preparations called SDC, ETP, and “open” DPNH oxidase (14) were kindly supplied by Dr. David E. Green of the University of Wisconsin. “Closed” DPNH oxidase preparations were prepared by methods previously described (14).

1 The term “Condition II” implies the turbid state resulting upon devitrification of the precooled solvent mixture by warming to about −50°. After this state is attained, samples are recooled in liquid air to −190°, and the spectrum is recorded.
RESULTS AND DISCUSSION

Difference Spectra at Room Temperature—The difference spectra (anaerobic reduced minus oxidized), as recorded at room temperature for preparations of "open" and "closed" DPNH oxidase and a Keilin and Hartree heart muscle preparation, are given in Fig. 1. The three preparations show similar absorption maxima at 605 m\( \mu \), demonstrating the presence of cytochromes \( \alpha \) and \( \alpha_3 \) in the preparations. The rather broad absorption bands extending from 550 to 565 m\( \mu \) are different in shape and configuration for each of the preparations. These bands represent a summation of the \( \alpha \) absorption bands of cytochromes \( c, c_1 \), and \( b \). In the Keilin and Hartree preparation the band of \( c \) plus \( c_1 \) dominates the absorption with
the band of cytochrome $b$ appearing merely as a shoulder at about 564 m.$\mu$. The "closed" and "open" DPNH oxidase preparations show more clearly the band of cytochrome $b$ and indicate a decreased content of either cytochrome $c$ or $c_1$. Indeed, previous attempts to determine cytochrome $c$ chemically in the DPNH oxidase preparations were not successful (14). The broad absorption bands, with maxima at about 520 m.$\mu$, represent a mixture of the $\beta$-bands of cytochromes $c$, $c_1$, and $b$.

Low Temperature Difference and Absolute Spectra—Fig. 2 shows the spectra obtained when a Keilin and Hartree heart muscle preparation is cooled in liquid air to $-190^\circ$. Two types of spectra are presented in Fig. 2. One is a reduced minus oxidized difference spectrum, as obtained when the sample cuvette contains a mixture of the enzyme in which the pigments have been reduced and the reference cuvette contains a mixture of the enzyme in which the pigments are oxidized. The other is an "apparent absolute absorption spectrum" in which the contents of the sample cuvette are the same as that described for the difference spectrum, but the reference cuvette now contains only a mixture of glycerol and phosphate buffer; i.e., no enzyme preparation is present in the reference cuvette. Certain differences and similarities are obvious. Both types of spectra show the absorption bands of cytochromes $a$, $b$, $a_1$, and $c$. The "apparent absolute absorption spectrum," however, differs from the "difference" spectrum in an increase in light absorption as shorter wave lengths are approached (this is represented by Curve AA in Fig. 2). This is presumed to be due to the difference in turbidity of the two cuvettes because of the absence of enzyme from the reference cuvette. When the concentration of a pigment is estimated by determining the maximal to minimal optical density difference for a specific absorption band, it is necessary to correct for this non-specific increase in absorption observed with the "apparent absolute absorption spectrum." Since it is often difficult to obtain material in the oxidized state, viz. bacteria and yeast, we have chosen to present most spectra as "apparent absolute absorption spectra."

Fig. 3 shows the "apparent absolute absorption spectra" for the three types of preparations shown in Fig. 1. The pigments were reduced either by DPNH or by succinate in the presence of a respiratory inhibitor such as cyanide. The absorption maximum at about 601 m.$\mu$, corresponding to cytochromes $a$ and $a_3$, is similar for the three preparations. The absorption maxima at about 549, 554, and 560 m.$\mu$, corresponding, respectively, to reduced cytochromes $c$, $c_1$, and $b$, are clearly defined. The amount of cytochrome $c$, however, varies in each of the preparations; the "open" DPNH oxidase has very little cytochrome $c$, the "closed" DPNH oxidase has a greater amount, and the Keilin and Hartree preparation the largest amount. The failure of the "open" DPNH oxidase to operate maximally
with oxygen as terminal electron acceptor may be related to the low cytochrome c content of the preparation. The "open" DPNH oxidase contains

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

**Fig. 2.** Low temperature difference and "absolute" spectra of a Keilin and Hartree heart muscle preparation. Curve A shows the "apparent absolute absorption spectrum." The sample cuvette contained a mixture of 0.2 ml. of heart muscle preparation, 0.2 ml. of 0.1 M phosphate buffer at pH 7.4, a few crystals of sodium dithionite, and 0.4 ml. of glycerol. The reference cuvette contained a mixture of equal volumes of glycerol and phosphate buffer. Curve AA indicates the estimated increase in absorption due to unequal turbidity of the two cuvettes. Curve B is the reduced minus oxidized difference spectrum. The sample cuvette contained the same mixture as described above for the "absolute spectrum." The reference cuvette contained a mixture similar to that in the sample cuvette, but sodium dithionite was omitted. Analogous to the room temperature difference spectra, isosbestic points are considered to be at about 625 and 510 m\(\mu\), permitting one to estimate a base line, Curve BB. Optical depth of cuvettes was 1 mm.; effective band width was 0.6 m\(\mu\); temperature, \(-190^\circ\); Condition II.

succinic dehydrogenase activity as described previously (14), and the pigments are therefore reducible by either succinate or DPNH. The \(\beta\)-bands may also be seen for each component. These \(\beta\)-bands have maxima at 529, 523, and 519 m\(\mu\) for cytochromes \(b\), \(c_1\), and \(c\), respectively. The \(\gamma\)-(Soret) bands, however, are not clearly defined by the low temperature
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technique. It is possible to assign approximate wavelengths for the
γ-bands representing the various cytochromes. Those bands which have
maxima at about 444, 430, 420, and 415 mμ correspond to cytochromes

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\lambda(nm)
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**Fig. 3.** Low temperature spectra of enzyme preparations. Curve A, "open" DPNH oxidase preparation. The cuvette contained a mixture of 0.2 ml. of preparation (3.2 mg. of protein), 0.2 ml. of 0.1 M phosphate buffer at pH 7.4, 0.01 ml. of 0.1 M cyanide, 0.01 ml. of 1 M succinate, and 0.4 ml. of glycerol. Curve B, "closed" DPNH oxidase preparation. The cuvette contained a mixture of 0.2 ml. of preparation (5.5 mg. of protein), 0.2 ml. of 0.1 M phosphate buffer at pH 7.4, 0.01 ml. of 0.1 M cyanide, sufficient solid DPNH to reduce the pigments, and 0.4 ml. of glycerol. Curve C, Keilin and Hartree preparation. The sample cuvette contained a mixture of 0.2 ml. of enzyme, 0.3 ml. of 0.1 M phosphate buffer, pH 7.4, 0.01 ml. of 0.1 M cyanide, 0.02 ml. of 1 M sodium succinate, and 0.5 ml. of glycerol. The reference cuvette contained a mixture of 50 per cent glycerol and 50 per cent 0.1 M phosphate buffer, pH 7.4. Optical depth of cuvettes was 1 mm.; effective band width was 1 mμ; temperature, -190°; Condition II.

\(a_3, b, c_1, \text{ and } c\), respectively. Since the α-bands are most clearly resolved, the following discussion will place most emphasis on the nature of these bands, rather than the β- or γ-bands of the various cytochromes. The differences in cytochrome c content, however, may be seen by comparing the magnitude of the 519 and 415 mμ bands for the various preparations.

The intensification of the absorption bands, as determined by comparing
the optical density difference between an absorption band maximum and a corresponding minimum of a reduced cytochrome, when the sample is at room temperature and at the temperature of liquid air, shows that the α-bands are magnified about fifteen times by treatment in liquid air. This intensification, as well as the resolution of the absorption bands, is the principal advantage of the low temperature technique for the recognition and characterization of hemoproteins.

Keilin and Hartree (15) have shown by room temperature spectra the variability of the position of the α-band of cytochrome c in heart muscle extracts. They attribute the displacement of the absorption maximum of cytochrome c to the presence of the pigment, cytochrome c₁. Reduced cytochrome c₁ was thus described as a pigment with an α-band maximum at 553 to 554 mπ, as observed in a cytochrome c-deficient heart muscle preparation. Keilin and Hartree also found that, when exogenous cytochrome c was added to a preparation deficient in cytochrome c, a band representing the summation of the bands of cytochrome c and c₁ was observed at about 552 mπ. Experiments similar to those of Keilin and Hartree have been carried out with the “open” DPNH oxidase preparation, and the low temperature spectra of the samples were recorded (Fig. 4). The resolution of the absorption bands of a mixture of reduced cytochromes c and c₁ under such conditions is clearly demonstrated by the low temperature spectra. It can be seen from these curves that, when cytochrome c is bound to particles, it retains the characteristic spectral maxima of cytochrome c and does not become incorporated as cytochrome c₁. The difference between the two pigments, although they appear to possess similar prosthetic groups, is further exemplified by studies of their enzymatic function (16). The experiments present in Fig. 4 also indicate the rather high affinity of the preparation for exogenous cytochrome c.

Low Temperature Spectra of Keilin and Hartree Preparations—The low temperature absolute spectra obtained when the Keilin and Hartree preparation is either oxidized or reduced with sodium succinate or sodium dithionite are shown in Fig. 5. The spectrum of the oxidized preparation shows the presence of a rather broad visible absorption band with a maximum at about 530 mπ and indications of a second band with a maximum at about 595 mπ. The Soret band of the oxidized cytochromes appears as a single broad band with a maximum located at about 412 mπ. In the

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2 Exogenous cytochrome c was incorporated in the “open” DPNH oxidase preparation by adding 0.2 ml. of $2 \times 10^{-4} \text{M}$ cytochrome c to 10 ml. of DPNH oxidase preparation (11 mg. of protein per ml.). The mixture was centrifuged at 40,000 × g for 20 minutes. The resulting precipitate was washed twice by resuspension and homogenization in 5 per cent sucrose and subsequent centrifugation. The twice washed precipitate was finally suspended in 5 per cent sucrose, and the spectra were recorded as described.
Fig. 4. Low temperature spectra of "open" DPNH oxidase with incorporated cytochrome c. Curve A, "open" DPNH oxidase. The cuvette contained a mixture of 0.3 ml. of enzyme preparation, 0.3 ml. of 0.1 M phosphate buffer, pH 7.4, 0.01 ml. of 1 M cyanide, and 0.6 ml. of glycerol. Curve B, "open" DPNH oxidase with bound cytochrome c. The cuvette contained a mixture similar to Curve A except for the substitution of the preparation treated with cytochrome c. Curve C, purified, reduced cytochrome c. The cuvette contained a mixture of 0.1 ml. of 2 × 10^{-4} M heart muscle cytochrome c, 0.4 ml. of 0.1 M phosphate buffer, pH 7.4, a few crystals of sodium dithionite, and 0.5 ml. of glycerol. The optical density increment was 0.01 for Curves A and B and 0.1 for Curve C. The reference cuvette and other conditions are as described in Fig. 3.
succinate-reduced sample the absorption maxima for reduced cytochromes c, c₁, b, and a are located as stated above at about 549, 554, 560, and 601 μm, respectively. Indeed, it is possible to differentiate between the two absorption bands of reduced cytochrome c with such preparations, i.e. the

![Graph of absorption spectra](image)

**Fig. 5.** Low temperature spectra of Keilin and Hartree heart muscle preparation

Curve A, the cuvette contained a mixture of 0.2 ml. of enzyme preparation (4.3 mg of protein), 0.3 ml. of 0.1 M phosphate buffer at pH 7.4, and 0.5 ml. of glycerol. Curve B, the cuvette contained the same constituents as in Curve A in addition to 0.01 ml. of 0.1 M cyanide and 0.01 ml. of 1 M succinate. Curve C, the cuvette contained the same constituents as in Curve A, but enough solid dithionite was added to reduce the pigments. The contents of the reference cuvette, and other experimental conditions, are as described in Fig. 3.

bands c₀₁ and c₀₂ with maxima at 549 and 546 μm, respectively (7). When the preparation is reduced by sodium dithionite, however, the amount of absorption in the region 561 μm is much greater than when the preparation is reduced enzymatically with succinate. This increase in the band at 561 μm also causes a slight shift of the band of cytochrome c to about 555 μm. The increased absorption at about 560 to 565 μm, when a chemical reducing agent such as sodium dithionite is added to an enzymatically reduced
system, has been noted previously by Chance and Estabrook\(^3\) with difference spectra recorded at room temperature, indicating the complexity and possible heterogeneity of absorption pigments with maxima in the vicinity of cytochrome \(b\). The \(\beta\)- and \(\gamma\)-bands for the pigments of the Keilin and Hartree preparation are the same as those described above for the DPNH oxidase preparations.

Chance (17) has shown that the cytochrome \(c\) content of Keilin and Hartree preparations varies considerably from preparation to preparation. A comparison of the low temperature spectra of the Keilin and Hartree preparations presented in Figs. 2, 3, and 5 shows the variability of the cytochrome \(c\) content of such preparations. The reason for this variation in cytochrome \(c\) content cannot be explained in terms of differences in enzymatic activity, for all the preparations have about the same succinoxidase activity in the absence of added cytochrome \(c\).

**Low Temperature Spectra of SDC and ETP**—The low temperature absolute spectra of ETP and SDC are presented in Fig. 6. As shown in Fig. 6, cytochrome \(c\) is present in the ETP preparation in somewhat larger amounts than in SDC, while cytochromes \(a\) and \(a_s\) (601 and 444 mp) are present in SDC in much lower amounts than in ETP or preparations of DPNH oxidase. The presence of the additional absorption in the region of 561 mp is shown with the ETP preparation when such preparations are reduced by sodium dithionite rather than sodium succinate. This additional absorption is presumed to be due to an inactive cytochrome \(b\). It is of interest that preparations of DPNH oxidase do not show such a large increase in absorption at about 560 to 565 mp when the spectra of the enzymatically and chemically reduced samples are compared.

The spectra presented have shown qualitatively that all the preparations studied have the same complement of cytochromes. The quantitative differences in the cytochrome content may be correlated to the differences in the enzymatic activity of the various preparations. The Keilin and Hartree preparation, which is capable of functioning as both a DPNH oxidase and succinoxidase, has cytochromes \(a\), \(a_s\), \(b\), \(c_1\), and \(c\), as well as an additional pigment at about 561 mp which is reduced by sodium dithionite but not sodium succinate (inactive cytochrome \(b\)).\(^4\) The "closed" DPNH oxidase, although lacking in succinoxidase activity, appears to have a similar spectrum. The lower content of cytochrome \(c\) and the apparent absence of inactive cytochrome \(b\)\(^4\) are points of difference. The "open" DPNH oxidase preparation is very low in cytochrome \(c\), which may explain the inability of the preparation to catalyze directly the oxidation of DPNH by oxygen. The "open" DPNH oxidase is similar spectrophotometrically to a preparation derived from lysis and salt extraction of liver

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*Unpublished data.

\(^4\) Or a denatured hemochromogen.
mitochondria. The dependency of the respiratory system upon added cytochrome c can be demonstrated with both types of preparations. The low content of cytochromes $a$ and $a_3$ in the SDC preparation indicates why this preparation acts as a poor succinoxidase system even in the presence of exogenous cytochrome $c$. The ETP appears to be very similar spectrally to the Keilin and Hartree preparation, although it has been prepared by a different method of fractionation.

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SUMMARY

1. The application of low temperature spectrophotometry to the resolution of absorption bands of hemoproteins, the maxima of which cannot be distinguished at room temperature, has been illustrated by spectra of various heart muscle preparations. These preparations have been studied spectrophotometrically at both room temperature (23°) and at liquid air temperatures (−190°).

2. Cytochromes a, a₃, b, c₁, and c were shown to be present in all the preparations studied.

3. The quantitative differences in the cytochrome content of the preparations explains in part the differences in enzymatic activity as described for the various preparations.

4. The incorporation of exogenous cytochrome c by a preparation having a low endogenous concentration of this pigment indicates from the spectral studies that “bound” cytochrome c is not identical to cytochrome c₁.

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