The Kinetics and Inhibition of Cytochrome Components of the Succinic Oxidase System

III. CYTOCHROME b *

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In the course of studies by Keilin and Hartree (1) on succinic oxidase preparation of heart muscle particles, it was observed that the kinetics of reduction of cytochrome b were not in accord with some rather fundamental criteria that must be met for an electron-transferring component in the respiratory chain (2). On the other hand, studies of the kinetics of cytochrome b in intact yeast cells showed its behavior to be in reasonably good agreement with that expected of an electron-transferring component (3). Studies of cytochrome b in isolated rat liver and heart mitochondria afforded an explanation for this apparent inconsistency, since in these efficiently phosphorylating particles cytochrome b has an active function (4, 5). At some point in the disruption of the heart sarcosomes occasioned by the Keilin and Hartree procedure, the function of cytochrome b is altered so that most of the electrons bypass it in their path from substrate to oxygen. Furthermore, at some stages in the disruption of the succinic-cytochrome c reductase activity of portions of the succinate-cytochrome c reductase activity of portions of the Keilin-Hartree particles. The results of such kinetic data now seem to have a real value in our understanding of what has occurred to the reactivity of cytochrome b in the preparation of the Keilin-Hartree particles. The results are also useful in evaluating the relationship of cytochrome b to the succinic-cytochrome c reductase activity of portions of the respiratory chain that have been isolated by various preparative procedures. Such studies may shed light on the mechanism of electron transfer in interactions between flavoproteins and cytochromes, even though the actual reactions involved are not the same as those that occur in the mitochondria during oxidative phosphorylation.

This paper describes methods for the spectroscopic study of cytochrome b, the kinetics of its reduction and oxidation by succinate and fumarate, and an unexpected activation of the succinate-cytochrome b reaction by antimycin A treatment. Antimycin A treatment also permits the identification of what appears, from its distinctive absorption band, to be a "modifiedocytochrome b."

METHODS

The succinic oxidase preparations were carried out by a modification of the Keilin-Hartree procedure (1), as discussed in a previous communication (6). The \((K_{i}a)_{n}\) values for the preparations were in the range of 70. The temperature was 26°C.

The experiments reported here cover a period of approximately 5 years, hence the spectroscopic recordings were made with several instruments as they were developed. Difference spectra were obtained with the split beam recorders of Yang and Legal-lais (7) and Chance (8), but the records actually presented here, such as those in Fig. 1, were obtained with an instrument of higher resolution (spectral interval of about 1.5 m\(\mu\)). Such split beam instruments automatically plot the spectrum representing the difference in absorbance between two samples that are identical except for the state of oxidation of their respiratory enzymes. Since we have been recording difference spectra for some time with an arrangement of end-on photocell and a cuvette that minimizes errors attributable to light scattering, the appreciable errors discussed by Keilin and Hartree (9) are not significant and their laborious procedure for cytochrome assay is therefore unnecessary.

The reaction kinetics illustrated by the subsequent figures in the paper were obtained with a double beam apparatus employing two quartz monochromators (8). This apparatus records, as a function of time, the difference between changes of absorbancy at two predetermined wave lengths.

RESULTS

Spectra—The spectrum representing the difference in absorbancy between the anaerobic, succinate-reduced forms and the oxidized forms of the heart muscle preparation is shown in Fig. 1A. The noteworthy features of this difference spectrum are:

1. The preparation represented is a "low cytochrome c" type.

1 The symbol \((K_{i}a)_{n}\) is related to the turnover number of cytochrome \(a_3\): \((K_{i}a)_{n} = \mu M O_2/sec./\Delta D_{ADP}\), where the respiratory rate is measured by the platinum microelectrode and \(\Delta D_{ADP}\) is the absorbancy change at 445 m\(\mu\) on reduction of cytochrome \(a_3\) (8).
The Soret band also lies at a slightly longer wave length than that of cytochrome b (Fig. 1B, solid trace). Dotted trace, a spectrum reduced by ascorbate, and the results are represented by the mit differentiation of absorption bands usually attributed to absorption bands due to cytochrome b and flavoprotein. The former set of components was reduced by ascorbate treatment of the cyanide-inhibited material, and the absorption bands of c + cl and a3 were obtained. It should be noted that the band of a3 corresponds to the difference between the oxidized and reduced forms of the cyanide-inhibited material. Absorption bands due to cytochrome b and flavoprotein were obtained by succinate treatment of the components already reduced by ascorbate, and the results are represented by the solid traces, which show the absorption bands of cytochrome b at 562 and 430 m\(\mu\).

C, chemical treatments of heart muscle preparations that permit differentiation of absorption bands usually attributed to cytochrome b. The solid trace represents the absorption bands that appeared upon addition of succinate to the material pre-treated with QO and shows the absorption bands attributable to cytochrome b (note that the absorption peak of cytochrome b lies at 564 m\(\mu\), as contrasted with 562 m\(\mu\) in Fig. 1B, solid trace). The Soret band also lies at a slightly longer wave length than that of cytochrome b (Fig. 1B, solid trace). Dotted trace, a spectrum of the pigment obtained when antimycin A was added to material already reduced by succinate; the absorption peaks are at 566 and 432 m\(\mu\). Dashed trace, the components that were not reduced by successive treatment with ascorbate, succinate, and antimycin A. Addition of dithionite resulted in a broad absorption band.

preparation in which the absorption band of cytochrome c at 554 m\(\mu\) is clearly recorded (10), whereas that of cytochrome c at 550 m\(\mu\) appears only as a broadened band of cytochrome c in the difference spectrum at room temperature. At low temperatures the spectrum clearly shows the presence of cytochrome c in such preparations (11).

2. The spectrum has been carried into the ultraviolet region in order that the content of ultraviolet-absorbing components in the DPN-free succinic oxidase preparation may be evaluated to show the extent to which such absorption interferes with pyridine nucleotide measurement in sarcosomes and mitochondria (6). The absorption maxima are 604, 592, 553, and 444.5 m\(\mu\) for cytochromes a, b, c1 (and c), and a3, respectively.²

The sequence of action of the cytochrome components of the nonphosphorylating succinic oxidase preparation previously presented (13) is enlarged to include cytochrome c1 (14),

\[
\text{sucinate} \rightarrow \text{dehydrogenase} \rightarrow f \rightarrow c_i \rightarrow c \rightarrow a \rightarrow a_1 \rightarrow O_2
\]

\[
\text{b}
\]

The cytochrome b component can be studied without interference from the other components by adding, first, a substrate (ascorbate) that chemically reduces the components between c1 and oxygen (Fig. 1B, dashed trace) and then a second substrate (succinate) (Fig. 1B, solid trace) that reduces the components of the chain lying between dehydrogenase and cytochrome c1. Alternatively, an inhibitor such as antimycin A or QO (15) (Fig. 1C, solid trace) intercepts electron transfer between dehydrogenase and cytochrome c1. In both of these cases the addition of succinate reveals absorption bands attributable to the flavoprotein and the cytochrome b components (Fig. 1B and C, solid traces). In Fig. 1B (solid trace) the absorption band is at 563 m\(\mu\), whereas in Fig. 1C (solid traces) it is at 564 m\(\mu\). This is a significant discrepancy (see below). Ascorbate treatment of the cyanide-inhibited material produces the absorption bands shown in Fig. 1B (dashed trace), in which the combined peaks of cytochromes c and c1 appear at 552 m\(\mu\). The characteristically small Soret band of cytochrome c + c1 is clearly distinguished at about 417 m\(\mu\). Inasmuch as cyanide is present the Soret band of cytochrome a is not as prominent as in Fig. 1A.

Fig. 1 illustrates the use of three successive chemical treatments to reveal different "b" compounds. The results may be compared with the results obtained with treatments used for liver mitochondria (16, 17). In the first step succinate is added to

² This spectrum is very similar to that of the "low cytochrome c" preparation discussed in Paper II of this series (12), in which the "cytochrome c" absorption band was displaced from that of purified cytochrome c at 550 m\(\mu\) to about 553 m\(\mu\) (Fig. 3C, (12)).

³ Actually two cuvettes are employed. Both are treated with ascorbate, and a base-line is plotted. Succinate is then added to one cuvette and the difference spectrum is plotted.

⁴ The abbreviation used is: QO, 2-n-heptyl-\(\alpha\)-hydroxyquinoline N-oxide.

with a peak at 556 to 566 m\(\mu\) and a very broad Soret band with a peak at 435 m\(\mu\).

The experiments were carried out in 0.15 M phosphate buffer, pH 7; the value of \(K_{400}\) for the preparation was 65. Cyanide concentration, 3 mM; ascorbate concentration, 1 mg. per ml.; succinate concentration, 8 mM; antimycin A concentration, approximately 10 \(\mu\)M; QO concentration, approximately 20 \(\mu\)M; dithionite concentration, 1 mg. per ml. (Experiment 687).
the ascorbate-reduced sample and the absorption bands of cytochrome b at 562 and 430 mp are clearly distinguished; a band of flavoprotein is also observed at 450 mp (Fig. 1B, solid trace). The second chemical treatment (Fig. 1C, dotted trace) consists of the addition of antimycin A to the sample to which succinate has already been added, revealing a new absorption band at 566 mp with a distinct shoulder at 560 mp and a Soret band at 432 mp (cf. 23). In the third step hydroxylamine is added to the solution already treated successively with ascorbate, succinate, and antimycin A (Fig. 1C, dashed trace), with the result that a broad absorption band is observed in the visible (12) and Soret regions at about 555 mp and at 435 mp, respectively. Examination of this pigment by a difference spectrum at liquid nitrogen temperatures does not show detectable amounts of cytochromes c or b. This three-step process makes possible an accurate assay of the pigments of the heart muscle preparation and is simpler than the "optical resolution" method (18, 19). Also, the first three steps are unaffected by hemoglobin contamination of the preparation.

The new pigment absorbing at 566 mp is not formed specifically by antimycin A; QO is equally effective. Ascorbate is not required for the reaction leading to the band at 566 mp. The peak at 564 mp, shown in Fig. 1B (solid trace), can be accounted for in terms of a mixture of compounds with peaks at 562 and 566 mp, respectively. The 566 mp absorption band may also be obtained as a component of the broad band caused by the addition of dithionite to succinate-reduced heart muscle preparation. These experiments identify the 566 mp pigment as a component of the heart muscle particles that can be reduced directly by dithionite or that becomes reducible by succinate in the presence of antimycin A or QO.

Kinetics and Equilibrium of Cytochrome b Reduction—Titration of cytochrome b with succinate in a cyanide-inhibited system is illustrated in Fig. 2A. The downward deflection of the trace after successive additions of succinate is attributable to increased absorption of the type indicated by Fig. 1C (solid trace). Under the conditions of the experiment, only 45 mp succinate is required to give half-maximal reduction of cytochrome b. The fact that the addition of dithionite results in a further absorbancy change after the end point of the succinate titration has been reached supports the data presented in Fig. 1C, which show the presence of an additional compound reduced in this reaction. The increased absorbancy induced by dithionite cannot be accounted for by an incomplete reduction of cytochrome b (562 mp) by succinate. The finding on dithionite is contrary to the assumption implicit in Ball's oxidation-reduction calculations (20) that cytochrome b is not completely reduced, even by a very large succinate to fumarate ratio, unless dithionite is added.

The fumarate oxidation of succinate-reduced cytochrome b in the cyanide-inhibited system is illustrated in Fig. 2B. As in Fig. 2A, measurements were made at 562 mp with reference to 575 mp. Cytochrome b is initially reduced by addition of 136 mp succinate. Successive additions of fumarate cause a decrease of absorbancy at 562 mp until a plateau is reached in the vicinity of 25 mp fumarate. Half-maximal oxidation is obtained with 5.8 mp fumarate. A 50 per cent reduction is obtained with a fumarate to succinate concentration ratio of 42; this corresponds to a much higher oxidation-reduction potential than that given by Ball (20) (see "Discussion").

The reactivity of cytochrome b towards succinate in the material treated with antimycin A (cf. Fig. 1C) is indicated by the experiments illustrated in Fig. 3. Since the absorption band is shifted somewhat to longer wave lengths, we have...
measured the absorbancy changes at 567 μm with reference to 575 μm. The first addition consists of antimycin A, which causes a small absorbancy change. Subsequent addition of 68 μM succinate causes a large and rapid absorbancy increase. In fact, a more detailed study (plotted in Curve A, lower portion) shows that only 7 μM succinate is required for half-maximal reduction as compared with 45 μM in the absence of antimycin A. Back-titration with reasonably low concentrations of fumarate is next carried out, the addition of 24 mM fumarate resulting in nearly a 50 per cent disappearance of the absorption caused by the addition of succinate. Further titration (Curve B) with succinate is begun, and an end point is reached with the addition of about 10 mM succinate. The concentrations of succinate and fumarate required to produce half-maximal reduction are 120 μM succinate in the presence of 24 mM fumarate, and 20 mM fumarate in the presence of 68 μM succinate. The two ratios of fumarate to succinate concentration that give 50 per cent oxidation-reduction of cytochrome b are 200 and 290, respectively.

To explain this significant change in the apparent equilibrium constant caused by treatment with antimycin A, we have measured the kinetics of cytochrome b reduction by succinate and of oxidation by fumarate in the presence and absence of antimycin A. The reduction of cytochrome b by the addition of 138 μM succinate to a cyanide-inhibited heart muscle preparation is illustrated in Fig. 4B. In agreement with results presented in Fig. 2 and with our findings in previous studies (2) concerning the sluggish response of cytochrome b of these particles, the reaction proceeds slowly to an end point. Even in the initial phases a maximal rate of only 4.5 μM cytochrome b per second is attained. Upon addition of fumarate, a relatively rapid oxidation takes place at an initial rate of 7.4 μM cytochrome b per second, and 50 per cent oxidation is obtained with a fumarate to succinate ratio of 54.

If, however, the heart muscle particles are pretreated with antimycin A (Fig. 4A), only 8 μM succinate is required for cytochrome b reduction at the rate of 10 μM per second, and the extent of reaction is even greater than that shown in Fig. 4B. In this case, oxidation of cytochrome b by fumarate is slow (1.7 μM cytochrome b per second), 14 μM fumarate being required to reach 70 per cent oxidation (6 μM for 50 per cent oxidation). It is clear from these two records that treatment with antimycin A has completely altered the equilibrium and kinetic properties of cytochrome b.

The effect of varying succinate and fumarate concentrations upon the rates of the reactions is illustrated by the graphs presented in Figs. 4C and 4D. In the absence of antimycin A the rate of increase in reaction speed with succinate concentration is small; the slope of the curve corresponds to a second order velocity constant of $2 \times 10^6 \text{M}^{-1} \text{X sec}^{-1}$ for the reduction of cytochrome b by succinate in the presence of cyanide. The slope of the line is about 65 times greater in the presence of antimycin A, and in its initial portion corresponds to a second order velocity constant of $10^5 \text{M}^{-1} \text{X sec}^{-1}$. As might be expected, the line is not straight, because at higher succinate concentrations the rate of reduction of succinic dehydrogenase by succinate becomes limiting. In Fig. 4D similar data are plotted for the oxidation of succinate-reduced cytochrome b by fumarate. In this case, however, the scales of the ordinate and abscissa have been changed, respectively, 10- and 80-fold, since the oxidation of re-

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**Fig. 4.** The kinetics of the reduction of cytochrome b by succinate and its oxidation by fumarate in the presence and absence of antimycin A.

A, recordings (obtained with the double beam spectrophotometer) of the kinetics of reduction of cytochrome b in material treated with antimycin A. The material pretreated with 14 μM antimycin A and 0.9 mM cyanide is reduced upon the addition of 8 μM succinate. After the reaction is complete, the oxidation by fumarate is initiated (Experiment 286-21). In the absence of antimycin A, recordings (obtained with the double beam spectrophotometer) of the kinetics of reduction of cytochrome b in material treated with antimycin A and 0.9 mM succinate is added. The oxidation reaction is caused by the addition of 7.1 mM fumarate. In both A and B the reaction rates are calculated from the slopes of the kinetics on the assumption that the molecular extinction coefficient of cytochrome b is 20 cm.$^{-1}$ M.$^{-1}$ (Experiment 286-10).

C, effect of antimycin A on the kinetics of reduction of cytochrome b in cyanide-inhibited heart muscle preparation (Experiment 286c).

D, effect of antimycin A upon the kinetics of oxidation of cytochrome b in the cyanide-inhibited system. In this case, the concentrations of succinate present are similar to those used in obtaining Traces A and B (Experiment 286e).
duced cytochrome b by fumarate is much slower than the reduction of oxidized cytochrome b by succinate. In the absence of antimycin A the rate of oxidation of reduced cytochrome b by fumarate has an initial slope of about $2 \times 10^5$ m$^{-1}$ X sec.$^{-1}$ whereas in the presence of antimycin A, the reaction is inhibited and the slope is only about $1 \times 10^9$ m$^{-1}$ X sec.$^{-1}$. In both cases there is a considerable inflection of the curves, which suggests that the fumarate-hydrogenase activity is rate-limiting at higher concentrations of fumarate. In summary, treatment with antimycin A increases the second order velocity constant for reduction of cytochrome b by a factor of 50, and it decreases the corresponding constant for fumarate oxidation of cytochrome b by a factor of about 20. It is of interest to note that antimycin A inhibition appears to limit the maximal rate of the fumarate oxidation reaction.

**DISCUSSION**

**General Features of Difference Spectra**—Three features of these data concerning oxidized minus reduced spectra are noteworthy:

1. They support the previously reported result (12) that the cytochrome c content of the heart muscle preparation varies considerably, and with little change of oxidase activity, because cytochrome c is not rate-limiting in these preparations. The displacement of the absorption band of cytochrome c of the heart muscle preparation to wave lengths of 550 to 551 mp, or higher, is attributable to cytochrome c, (10, 21). This preparation, together with the "low cytochrome c" preparation (Paper II, Fig. 3C (12)) and the washed mitochondrial preparations, shows clearly the cytochrome c band at 553 mp. Cytochrome c can scarcely be discerned at room temperature, but spectra measured at liquid air temperatures clearly identify cytochrome c as a component of the system.

2. The difference spectrum of the heart muscle preparation (Fig. 1A) shows absorbancy changes attributable to flavoprotein and, if the flavoprotein content is computed on the same basis as the analogous computation of the flavoprotein content of mitochondria (16), enough is present to provide at least 1 mole of flavoprotein per mole of cytochrome c, as was assumed previously by Slater (22). As in the case of the liver mitochondria, some flavoprotein is reduced by succinate on treatment with antimycin A, more is reduced in anaerobiosis, and an additional portion with dithionite. The first portion probably represents flavoprotein associated with succinic dehydrogenase. This flavoprotein is rapidly reduced upon the addition of succinate, in contrast to the rather slow response of flavin in some of the purified preparations (23). The flavoproteins of the heart muscle preparation have spectroscopic characteristics similar to those in mitochondria, the difference spectrum showing a pronounced trough at 455 mp and no corresponding trough in the vicinity of 370 mp, as is found with most isolated flavoproteins.

3. The absorption spectrum indicates that cytochromes and other pigments of the heart muscle preparation in the vicinity of 340 mp do not interfere with pyridine nucleotide measurement in the intact mitochondria or sarcosomes, although the absorption in the vicinity of 305 mp is appreciable and could be partly responsible for the results obtained by Holton et al. (24) with heart sarcosomes.

**The \"b\" Pigments**—By differential chemical treatments spectra are obtained that correspond to three \"b\" pigments. The first is cytochrome b which is completely, though slowly, reduced by succinate in the absence of oxidase activity. The second is a "modified cytochrome b" which is identified by the additional absorption band having a peak at 566 mp and a shoulder at 560 mp that is produced when antimycin A or QO is added to the succinate-reduced material. The third is identified by the fact that dithionite reduces additional pigment or pigments, as indicated by a very broad band extending from 556 to 566 mp. The dithionite-reduced material of the Keilin-Hartree preparation has a Soret band at a longer wavelength (485 mp) than that of the dithionite-reduced component of liver mitochondria (427 mp) (16).

The functions of these pigments are concluded to be the following: The pigment reduced by succinate in the absence of inhibitor is identified as cytochrome b and is characterized by \(\gamma\)- and \(\alpha\)-peaks at 430 and 562 mp, respectively. The positions of the bands are close to those reported by Sekuzu and Okumuki (25), but our data show a sharper \(\alpha\)-band than that obtained by them. Neither the second nor the third component fulfills the requirements for respiratory carriers in succinate oxidation, since they cannot be reduced by this substrate in the absence of oxidase activity. The amount of the 566-mp material relative to cytochrome b (562 mp), as assayed in the manner indicated in Fig. 1C, gives results in the range of 30 to 70 per cent for various Keilin and Hartree preparations. Since the 566-mp material is not a respiratory carrier, the succinic oxidase activity is not altered by preparations having a high content of it.

The clear-cut difference between the succinate- and dithionite-reduced pigments of the heart muscle preparation (cf. (12)) raises the question of the validity of studies of the oxidation reduction potential of "cytochrome b" in which the dithionite-reduced pigment is taken as the end point. The value found (20) (—40 mv.) is probably much lower than the true value (see below for further discussion).

In a preliminary report (2) it was noted that the absorption band in the region of 555 to 570 mp obtained in the anaerobic succinate-reduced preparation is more intense when antimycin A is present. The additional absorption was tentatively attributed to Slater's factor (26) or to a cytochrome b antimycin A compound. Subsequently, studies of mitochondria, fly sarcosomes, and so forth, have shown that the intensified absorption attributable to antimycin A is not regularly measurable; thus neither hypothesis seems applicable. It now appears that the additional absorption resulting from antimycin A treatment, here shown to have a 566-mp peak, is due to an inactive form of cytochrome b, i.e. one that can be activated only by the more rapid electron transfer to cytochrome b (562 mp) which we have shown to occur in the presence of antimycin A.

**Interaction of Cytochrome b with Flavoprotein and Cytochrome c**

A consideration of the two mechanisms for electron transfer in phosphorylating and nonphosphorylating systems provides a basis for the explanation of the effects of the kinetics and equilibrium of cytochrome b reduction. In considering the mechanisms below, it should be remembered that in the nonphosphorylating DPNH and succinic oxidase system, cytochrome b is not reduced rapidly enough to be considered an active participant in electron transport. On the other hand, in the phosphorylating system, the kinetics of cytochrome b justify its inclusion in the electron transport chain.
In the phosphorylating system (Equation 1), flavoprotein transfers electrons readily to cytochrome b and does not bypass it. Disruption of the mitochondria usually leads to a loss of phosphorylative activity and to a change in the pathway of electron transfer to one that bypasses cytochrome b (Equation 2). The system is antimycin A-sensitive, and the site of action of the inhibitor is between cytochrome c1 and a hypothetical factor (26). The Keilin and Hartree preparations behave in this way. Further purification leads insensitivity to antimycin A. The flavoprotein then interacts with cytochrome c without the intervention of cytochromes b or c1; this is the cytochrome c reductase activity of succinate or DPNH dehydrogenases (Equations 3 and 4). A related example of a change of flavoprotein specificity with purification is afforded by butyryl-CoA dehydrogenase (for a summary, see [27]). As an explanation for this stepwise loss of function of cytochrome b, we propose that the flavoprotein of the respiratory chain has a potentiality of cytochrome c-reductase activity which is not normally exhibited because cytochrome c is not directly accessible. During disruption of the mitochondria a series of structural alterations occurs in which portions of the chain, such as cytochromes c1 and c, become accessible to the flavoprotein, resulting in a bypassing of cytochrome b. Extraction of the flavoproteins from the respiratory chain allows their direct interaction with cytochrome c to be demonstrated.

Kinetic studies of the Keilin and Hartree preparation show that cytochrome b is somewhat more responsive to electrons donated by succinic dehydrogenase than to those donated by DPNH dehydrogenase. This result suggests a different sensitivity of these two dehydrogenases to structural alterations (28).

Although some increase in the rate of cytochrome b reduction would be expected in the material treated with Q0, because cytochrome c, no longer accepts electrons, the fact that the initial rate of cytochrome b reduction is greatly accelerated is of considerable importance. Since we have found experimentally that the rate of oxidation of cytochrome b is relatively slow (29), any great change in the initial rate, upon addition of the inhibitor, is unexpected. It seems necessary to postulate that the inhibitor alters the system so that the electrons that flow to cytochrome c, in the absence of the inhibitor are routed to cytochrome b in the presence of the inhibitor. Only by such a change can cytochrome b acquire such an increase over its initial rate of reduction in the presence of the inhibitor. A possible mechanism is suggested by Equation 2: that the $fp \rightleftharpoons b$ interaction is enhanced by treatment with an inhibitor, and that, insofar as cytochrome b is concerned, the system reacquires a rapid reduction of cytochrome b and simulates the intact system of Equation 1. It is possible that many degrees of inactivation of cytochrome b can be obtained by a variation of the $fp \rightleftharpoons b$ interaction in the absence of the inhibitor.

Jackson and Lightbown (30) have again taken up the proposal of Keilin (31) that cytochrome b is auto-oxidizable and, in the absence of these kinetic data, have proposed that a more rapid reduction of cytochrome b in the presence of Q0 would be the result of an inhibition of the auto-oxidation reaction. This simple "inhibition of auto-oxidation" theory is inadequate for the same reason as is the theory of inhibition of oxidation through the cytochrome chain, discussed above. Another objection to the explanation presented by these authors is the lack of evidence of the direct reaction of cytochrome b with oxygen at a significant rate.

The possibility that the inhibitor actually supplies a cofactor lacking in electron transfer from flavoprotein, however unlikely, is not to be ignored. This requires two modes of action for the inhibitor; first, to inhibit electron transfer between flavoprotein and cytochrome c1; and second, to supply a cofactor acting between flavoprotein and cytochrome b.

These results shed some light on the nature of the cytochrome b bypass in nonphosphorylating systems: (a) cytochrome b itself can still accept electrons at a rapid rate when Q0 is present, i.e. cytochrome b itself is not inactivated; (b) flavoprotein can still donate electrons to cytochrome b at a rapid rate when Q0 is present. It would appear that disintegration of the mitochondria and the consequent loss of phosphorylation enhance the interaction of flavoprotein and cytochromes c and c1 at the expense of the interaction of flavoprotein and cytochrome b.

Equilibrium and Kinetic Data—There are significant inconsistencies between the apparent equilibrium constants of the succinate-cytochrome b reaction computed from the titration data and those computed from the kinetic data. In the absence of antimycin A an apparent equilibrium constant (succinate to fumarate ratio) of 40 is suggested by the data presented in Fig. 2B, whereas the kinetic data in Fig. 4 show the ratio of second order velocity constants to be 10:1. In the material treated with antimycin A the discrepancy is much larger, for here the titration data (cf. Fig. 3B) give a ratio of 250:1, whereas the kinetic data give a ratio of 10,000:1, a 40-fold discrepancy.

The discrepancies between the titration and kinetic data suggest that we are not dealing with an equilibrium system and that different pathways exist for the succinate and fumarate reactions in the heart muscle preparation. The data of Singer et al. (25) have shown that the transfer of electrons from succinate to dyes is o-phenanthroline sensitive and that this is not the ease with the fumaric dehydrogenase activity. The alternative hypothesis, that an equilibrium does exist and that the addition of antimycin A raises the oxidation-reduction potential of cytochrome b by over 0.1 volt is unlikely, especially since cytochrome b does not become reducible by ascorbate in the antimycin A-treated system.

The possibility of a nonequilibrium system indicates that Ball's data on the oxidation-reduction potential of cytochrome b should be accepted with caution and that the ferri-ferro oxalate titration of Hill (32), which gives $E'_o = 0.0$ at pH 7.0 for the potential of cytochrome b, should be considered a more reliable value.

Slater (33) finds fault with our conclusions regarding the by-
passing of cytochrome b in nonphosphorylating preparations and attributes the slow reduction of this component in the anaerobic transition to a slow approach to equilibrium in the presence of fumarate accumulated during succinate oxidation. Here we find experimentally that the succinate-fumarate ratio that Slater assumes (0.15) would give a 150:1 ratio for the rate of reduction to rate of oxidation. Thus the oxidation rate is negligible and the objection has no foundation.

**SUMMARY**

The spectroscopic, equilibrium, and kinetic properties of cytochrome b of the Keilin and Hartree succinic oxidase preparations have been investigated. Two chemical treatments for obtaining the difference spectrum of this component are discussed, both involving addition of succinate to (a) the ascorbate-reduced material or (b) the material treated with antimycin A. In the latter case an absorption band in addition to that of cytochrome b is found. This new band has a peak at 566 mμ and a small shoulder at 560 mμ. Since this 500 mμ component is not reduced by succinate without antimycin A treatment, it is attributed to a modified cytochrome b. Although in some Keilin and Hartree preparations there is roughly as much modified cytochrome b with a peak at 566 mμ (α-band) as there is cytochrome b with a peak at 562 mμ, their succinic oxidase activity is not impaired because neither cytochrome b nor the modified component participates in the electron transport. Pretreatment of the heart muscle preparation with antimycin A or 2-n-heptyl-4-hydroxyquinoline N-oxide drastically alters the kinetics and equilibrium of the reactions of cytochrome b with succinate and fumarate, the reduction by succinate being greatly accelerated and the oxidation by fumarate being inhibited. This acceleration of electron transfer between flavoprotein and cytochrome b in the presence of antimycin A or 2-n-heptyl-4-hydroxyquinoline N-oxide simulates a reconstitution of the electron-transfer activity of cytochrome b in the particles. It appears that the disruption of mitochondria involving a loss of phosphorylative activities favors the interaction of flavoprotein with cytochromes c and c1 in preference to that with cytochrome b. Discrepancies between the kinetic and equilibrium data, especially in the presence of antimycin A, indicate that cytochrome b is not in reversible equilibrium with the succinate-fumarate system and that determinations of the oxidation-reduction potential of cytochrome b with this couple are open to question.

**REFERENCES**
