DIPHTHERIA TOXIN

V. A COMPARISON BETWEEN THE DIPHTHERIAL SUCCINOXIDASE SYSTEM AND THAT OF BEEF HEART MUSCLE

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(Received for publication, April 23, 1949)

The major iron-containing respiratory pigment of Corynebacterium diphtheriae is spectroscopically related to cytochrome b (1-3). In a previous communication (2) we presented evidence which suggests that diphtheria toxin may be closely related to the protein moiety of diphtherial cytochrome b. The tentative hypothesis was advanced that diphtheria toxin may act by interfering with the normal function of cytochrome b in the tissues of the susceptible host, possibly by blocking its synthesis.

Before attempting to verify this hypothesis, it has seemed necessary to investigate further the rôle of cytochrome b both in mammalian tissue respiration and in the bacterial cell. There seems to be little doubt that cytochrome b is concerned in the oxidation of succinate by heart muscle succinoxidase preparations in which it may serve as a link between succinidehydrogenase and the cytochrome c-cytochrome oxidase system (Keilin and Hartree (4); Ball et al. (5)). The diphtheria bacillus, on the other hand, contains very little cytochrome c or cytochrome oxidase, and cytochrome b appears to be the limiting factor concerned in succinate oxidation by these organisms (2).

In the present study we have investigated the succinoxidase systems of beef heart muscle and of the diphtheria bacillus. The principal difference between the bacterial and mammalian systems resides in the relative amounts of the various components of the succinoxidase system present, as noted above. Of particular interest is the exceptionally high succinidehydrogenase activity of the diphtheria bacillus, which parallels its high cytochrome b content. The evidence to be presented suggests that cytochrome b oxidation is the limiting factor in the oxidation of succinate by tissue and bacterial extracts in the presence of methylene blue and cyanide and that succinidehydrogenase and cytochrome b may be identical both in diphtherial extracts and beef heart muscle succinoxidase preparations.

EXPERIMENTAL

Hemin Analyses—1 to 2 cc. of crude diphtherial sonic extract containing 6 to 8 mg. of nitrogen per cc. (2 to 3 γ of hemin iron per cc.) are treated

*Supported by a grant from the Commonwealth Fund.
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with 1 cc. of 0.2 N NaOH and 2 cc. of reagent grade pyridine. The volume is then made up to 10 cc. with distilled water. In the case of crude sonic extracts this solution is always somewhat cloudy and must be clarified by centrifugation at high speed (1 hour at 10,000 R.P.M. in the International refrigerated centrifuge). The clear straw-colored supernatant is placed in two cuvettes and a little sodium hydrosulfite added to one of them. The difference in absorption between oxidized and reduced pyridine hemochromogens at 554 m\(^\lambda\) is then read in the Beckman spectrophotometer and compared with a standard pyridine hemochromogen containing 5.5 \(\gamma\) of hemin chloride per cc.

With crude bacterial extracts the results are fairly satisfactory, although there is some non-specific absorption even in the visible region. Moreover, the values are probably somewhat low because of some occlusion of hemochromogen in the precipitate. With purified cytochrome \(b\) preparations, however, no centrifugation is necessary, the 20 per cent pyridine solution is clear, and the absorption spectrum is superimposed on that of pure pyridine hemochromogen throughout the entire visible region. It must be realized that only the dissociable protohemin is determined by this method. The hemins of other iron enzymes, such as cytochrome \(c\) and catalase, do not form pyridine hemochromogen under these conditions.

The total iron content of diphtheria bacilli grown on a medium containing an excess of iron is about 0.85 \(\gamma\) per mg. of bacterial nitrogen (6). About 40 to 60 per cent of this iron can be accounted for as hemin iron by the above procedure.

Succindehydrogenase Determination—Succindehydrogenase activity was estimated in the Warburg apparatus at 36.35\(^\circ\) in 0.1 M phosphate buffer at pH 7.8. Each vessel contained 0.2 cc. of 20 per cent NaOH in the center cup, 0.2 cc. of 0.16 M sodium succinate, 0.1 cc. of 0.02 M KCN, and an appropriate amount of enzyme.\(^1\) After equilibration, 0.2 cc. of 0.1 per cent methylene blue was tipped in from the side arm. The total volume was 2 cc. per vessel. \(Q_{O_2}\) values were calculated from the oxygen uptake during the first 15 minutes and are expressed as c.mm. of \(O_2\) per mg. of N per hour.

Succindehydrogenase activity was occasionally checked by the ferricyanide method of Quastel and Wheatley (7). The results were in good agreement with those by the methylene blue method; i.e., \(Q_{Go_2}^N\) (ferricyanide) = 4\(Q_{O_2}\) (methylene blue).

Preparation of Crude Bacterial Extracts—The Toronto strain of Corynebacterium diphtheriae was grown in 5 to 10 liter lots on Mueller and Mil-

\(^1\) Low \(Q_{O_2}\) values are obtained if too much enzyme is present, because the rate of oxidation of reduced methylene blue becomes limiting. For this reason, more than one enzyme concentration was usually tested.
lor's casein hydrolysate medium (8) containing 25 mg. of FeSO₄·7H₂O per liter. The organisms were harvested after 6 to 7 days growth at 34°, collected by centrifugation, and washed three times with saline. The final pH of the culture filtrate was usually 6.8 to 7.0. The washed organisms from 5 liters of culture were suspended in 150 to 200 cc. of saline and the suspension disrupted for 30 minutes in the 9000 cycle sonic oscillator² in 25 cc. portions. The final crude extract usually contained 6 to 8 mg. of nitrogen per cc. at this stage.

Purification of Diphtherial Cytochrome b and Succindehydrogenase Activity by Differential Centrifugation—The purification and concentration procedure varied somewhat from lot to lot. The procedure used to obtain a typical cytochrome b concentrate was as follows:

Preparation 21—Washed organisms from 5 liters of culture were suspended in 180 cc. of saline and disrupted in the sonic oscillator as described above. The crude extract contained 7.58 mg. of nitrogen per cc. and 0.3 mg. of hemin iron per mg. of nitrogen. The Qₒ₂ (succinate-KCN) and Qₒ₂ (succinate-methylene blue-KCN) were 146 and 4070 c.mm. per mg. of N per hour respectively. The crude extract was centrifuged for 30 minutes at 10,000 r.p.m. in a refrigerated International centrifuge. The turbid supernatant was removed from bacterial débris and centrifuged in a Sorvall vacuum type angle centrifuge, model SS-2, at 12,000 r.p.m. (about 18,000 X g) for 30 minutes. After centrifuging, three layers were present. About 25 cc. of a fatty yellow upper layer of low activity were removed and discarded. 130 cc. of a clear red layer, containing more than 60 per cent of the total activity (Fraction B), was next removed. Its nitrogen content was 4.41 mg. per cc. and the Qₒ₂ (succinate) was 140. The sediment (Fraction C), containing 30 per cent of the total activity, was discarded in this particular experiment. Fraction B was then centrifuged at 16,000 r.p.m. (about 30,000 X g) for 3 hours. Once again three layers were obtained: an inactive yellow topmost layer, a clear reddish brown layer with considerable activity, and a dark wine-red transparent sediment at the bottom of the tubes. About 30 per cent of the total original activity was contained in the clear red sediment after the supernatant was poured off. This sediment was suspended in 0.02 M phosphate buffer and homogenized in the sonic vibrator for 20 minutes. 35 cc. of a dark red solution were obtained, which was clarified by centrifugation in the model SS-2 Sorvall centrifuge for 30 minutes at 12,000 r.p.m. The sediment was discarded and the supernatant dark red solution (Fraction B3) was clear by transmitted light but showed a marked Tyndall effect. The preparation showed strong cytochrome b bands at 560 and 529 mμ after 1:5 dilution and reduction with hydrosulfite. Frac-

² Raytheon Corporation, Waltham, Massachusetts.
tion B3 contained 3.90 mg. of N per cc. and 1.09 γ of hemin iron per mg. of nitrogen. The \( Q_{O_2} \) (succinate) was 440 and \( Q_{O_2} \) (succinate-methylene blue) was 14,000 c.mm. of \( O_2 \) per hour per mg. of N. The purification was thus 3- to 3.5-fold both on the basis of activity and of hemin content.

Purified cytochrome \( b \)-succinidehydrogenase preparations may be kept either in the frozen state at \(-70^\circ\) or may be lyophilized and stored in a desiccator over \( P_2O_5 \). The preparations keep for months without loss of activity, but reconstitution of the frozen or dried material yields solutions which are more turbid than the original preparation. Both cytochrome \( b \) and succinidehydrogenase activity may be precipitated at pH 4.8, washed with water, and resuspended in phosphate buffer at pH 7.8 without loss of potency. Little additional purification is obtained by acid precipitation.

**Heart Muscle Succinoxidase Preparations**—Succinoxidase was prepared from beef heart by the method of Keilin and Hartree (9) except that the defatted, minced, washed muscle pulp was suspended in chilled phosphate buffer (pH 7.0) by means of a Waring blender rather than by grinding with sand. The final precipitate obtained with acetate buffer of pH 4.5 was washed with chilled distilled water to remove excess cytochrome \( c \) before resuspending in 0.1 M phosphate buffer at pH 7.3. The sonic oscillator was used to obtain a uniform suspension. On reduction with hydrosulfite, the Keilin and Hartree preparation showed strong absorption bands characteristic of cytochromes \( a_0 \) and \( b \). The cytochrome \( c \) band was faint or absent. In a typical preparation, the final succinoxidase suspension obtained from 400 gm. of pressed, washed muscle pulp measured 70 cc. It contained 4.00 mg. of nitrogen per cc.

**Results**

**Succinoxidase Systems of Beef Heart Muscle and Corynebacterium Diphtheriae**—In Table I, Columns 1 and 3, the succinoxidase activity of an unfractionated homogenate of fresh beef heart muscle is compared with that of a crude sonic extract from freshly harvested diphtheria bacilli. The \( Q_{O_2} \) (succinate) of the heart muscle homogenate is about twice that of the bacterial extract. Addition of excess cytochrome \( c \) doubles the rate of oxygen uptake by the heart muscle preparation but has little effect on the \( Q_{O_2} \) of the diphtherial system. \( 2.5 \times 10^{-5} \) M cyanide inhibits oxygen consumption in heart muscle to the extent of 95 per cent but brings about only a 10 per cent reduction in the \( Q_{O_2} \) of the fresh diphtherial extract. The most striking differences between the mammalian and bacterial preparations are their relative "succinidehydrogenase" contents as measured by the methylene blue method and cytochrome oxidase contents as measured by oxidation of \( p \)-phenylenediamine in the presence of excess cytochrome \( c \). Succinate is oxidized more than 15 times as rapidly by the
diphtheria bacillus as by beef heart muscle in the presence of methylene blue and potassium cyanide. On the other hand, p-phenylenediamine is oxidized very slowly by the bacterial extracts as compared with its rapid oxidation by heart muscle.

These differences appear even more striking when the activity of purified diphtherial cytochrome $b$ is compared with the Keilin and Hartree succinoxidase preparation. As is seen from Columns 2 and 4 of Table I, cytochrome $c$ and KCN are without effect on the diphtherial preparation and cytochrome oxidase activity is completely absent. Addition of methylene blue (with or without cyanide) increases the $Q_0_2$ more than 30-fold. The Keilin and Hartree preparation, on the other hand, shows a 5-fold increase in $Q_0_2$ on adding excess cytochrome $c$ and almost complete inhibition by $2.5 \times 10^{-3} \text{ M }$ KCN. Activity in the presence of cyanide is restored by methylene blue but remains far inferior to that in the diphtherial extract. Phenylenediamine is oxidized several times as fast as is succinate.

**Effect of pH on Oxidation of Succinate by Diphtherial Cytochrome $b$ Preparations**—It has been shown previously (2) that the rate-limiting step in the oxidation of succinate by diphtherial extracts in the presence of cyanide is the autoxidation of cytochrome $b$. The $Q_0_2$ under these conditions is thus a measure of cytochrome $b$ concentration. The method is not a

### Table I

Comparison between Activity of Diphtherial and Mammalian Succinoxidase Systems at pH 7.8

<table>
<thead>
<tr>
<th>Substrate*</th>
<th>$Q_0_2$†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Beef heart homogenate</td>
</tr>
<tr>
<td>Succinate</td>
<td>312</td>
</tr>
<tr>
<td>&quot; + cytochrome $c$</td>
<td>646</td>
</tr>
<tr>
<td>&quot; + KCN</td>
<td>14</td>
</tr>
<tr>
<td>&quot; + &quot; + methylene blue</td>
<td>254</td>
</tr>
<tr>
<td>Phenylenediamine + cytochrome $c$</td>
<td>1470</td>
</tr>
</tbody>
</table>

* Final concentration of succinate and phenylenediamine, 0.02 $\text{ M }$; KCN, $10^{-3} \text{ M }$; cytochrome $c$, $5 \times 10^{-4} \text{ M }$; methylene blue, 0.01 per cent.

† C.mm. of $O_2$ per mg. of N per hour calculated from the first 15 minutes.
sensitive one, however, since reduced cytochrome b is oxidized at a relatively slow rate by molecular oxygen. Addition of an excess of methylene blue to diphtherial extracts or purified cytochrome b preparations increases the $Q_{\text{O}_2}$ some 25- to 30-fold even in the presence of cyanide.

The $Q_{\text{O}_2}$ under these conditions is generally considered to be a measure of succinidehydrogenase activity. In Fig. 1 we have compared the succinidehydrogenase activity measured in this way with the rate of oxidation of cytochrome b by molecular oxygen in the pH range 5.8 to 8.6. In both cases the optimum pH is 7.8 to 8.0. Although the $Q_{\text{O}_2}$ values at pH 5.8 are less than 25 per cent of the values at pH 7.8, the ratio of succinidehydrogenase activity to cytochrome b autoxidation remains practically constant between 25:1 and 30:1. The results suggest that oxidation of cytochrome b is the rate-controlling step not only when molecular oxygen is the oxidant but also when oxygen is replaced by methylene blue.

**Effect of pH on Succinidehydrogenase Activity of Keilin and Hartree's**
Preparation—As is shown in Table II, the optimum pH for oxidation of succinate by the Keilin and Hartree preparation in the presence of methylene blue and cyanide is also 7.8 to 8.0. The \( Q_{O_2} \) increases about 6-fold in the pH range 6 to 8. By analogy with the diphtherial system, it seems likely that here again oxidation of cytochrome \( b \) by methylene blue is the rate-controlling step. The Keilin-Hartree preparation differs from the bacterial preparation in that the former system is rapidly inactivated above pH 8. The \( Q_{O_2} \) at pH 8.5 and 8.8 was calculated from the oxygen consumption during the first 5 minutes after the enzyme was tipped in from the side arm of the vessel and cannot be regarded as significant.

Heat Inactivation of Succinoxidase Preparations—In a further attempt to differentiate between cytochrome \( b \) and succinidehydrogenase activity, the effect of different temperatures on the enzymic activity of purified diphtherial cytochrome \( b \) in phosphate buffer at pH 7.6 was studied. When Preparation 21B3 was heated for 30 minutes at 60° under these conditions, aggregation occurred and the solution became turbid, but actual coagulation did not occur below 75° and was not complete below 80°. As is shown in Table III, about three-fourths of the activity was lost after 30 minutes at 75°, and inactivation was practically complete at 80°. Once again it was found impossible to differentiate between cytochrome \( b \) and succinidehydrogenase activity.

The effect of heat on the succinidehydrogenase activity of the Keilin-Hartree preparation was irregular and seemed to depend somewhat on the purity of the preparation. In one case complete coagulation and inactivation occurred at 60°; in another some activity still remained after 30 minutes at 75°.

Reduction of Cytochrome \( c \) by Cytochrome \( b \) in Presence of Succinate—

### Table II

**Effect of pH on Succinidehydrogenase Activity of Keilin-Hartree Preparation**

<table>
<thead>
<tr>
<th>pH</th>
<th>( Q_{O_2} ) (methylen blue + KCN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>180, 254</td>
</tr>
<tr>
<td>6.5</td>
<td>370, 408</td>
</tr>
<tr>
<td>7.3</td>
<td>1270, 1350</td>
</tr>
<tr>
<td>7.7</td>
<td>1450, 1720</td>
</tr>
<tr>
<td>8.0</td>
<td>1580</td>
</tr>
<tr>
<td>8.5†</td>
<td>910, 946</td>
</tr>
<tr>
<td>8.8†</td>
<td>194, 347</td>
</tr>
</tbody>
</table>

* Figures are given for two successive experiments.
† Calculated from the first 5 minutes. Enzyme tipped in from the side arm of the Warburg vessel.
When purified diphtherial cytochrome b is added to a solution containing succinate and cytochrome c in excess, the cytochrome c is slowly reduced. This reduction may be followed at 550 mμ in the Beckman spectrophotometer, as shown in Fig. 2. Potassium cyanide and naphthoquinone SN-5949 (5) have no effect on the rate of reduction of cytochrome c by cytochrome b. The rate of reduction is very slow, however, and at pH 7.3 diphtherial cytochrome b is autoxidized faster than it is oxidized by cytochrome c. The turnover number for cytochrome c reduction at 25° under these conditions, calculated from the hemin iron content of purified cytochrome b, is only about 7. This may be compared with a turnover number of 1420 for added cytochrome c in the oxidation of succinate by the Keilin-Hartree preparation (10). In Table IV we have calculated the turnover numbers for diphtherial cytochrome b during the oxidation of succinate under various conditions.

From Fig. 2 it may be seen that cytochrome c was reduced about 60 times faster in the presence of succinate and cyanide by the Keilin-Hartree preparation than by the diphtherial system, despite the fact that the former preparation contained only 15 per cent as much succindehydrogenase activity. The mammalian preparation was almost completely inhibited by naphthoquinone SN-5949. Ball et al. (5) have suggested that SN-5949 blocks a factor which mediates the reduction of cytochrome c by cytochrome b. The existence of such a factor has also been suggested by Stoppani (11), Slater (12), and others. Because the bacterial cytochrome

<table>
<thead>
<tr>
<th>Heated 30 min. at</th>
<th>(Q_{O_2}) (autoxidation of cytochrome b)</th>
<th>(Q_{O_2}) (methylen blue)</th>
<th>Appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>400</td>
<td>14,000</td>
<td>Almost clear</td>
</tr>
<tr>
<td>60</td>
<td>300</td>
<td>9,000</td>
<td>Turbid</td>
</tr>
<tr>
<td>65</td>
<td>235</td>
<td>15,000</td>
<td>“</td>
</tr>
<tr>
<td>70</td>
<td>264</td>
<td>11,600</td>
<td>“</td>
</tr>
<tr>
<td>75</td>
<td>95</td>
<td>3,900</td>
<td>Partial coagulation</td>
</tr>
<tr>
<td>80</td>
<td>5</td>
<td>240</td>
<td>Complete “</td>
</tr>
</tbody>
</table>

4 Crude sonic extracts from the diphtheria bacillus invariably contain a substance which reduces cytochrome c even in the absence of added substrate. This reducing substance is removed during purification by differential centrifugation or by precipitation of cytochrome b at pH 4.8.
Reduction of cytochrome c in the presence of succinate at 25°. One cuvette contained 3 cc. of $3 \times 10^{-6}$ M cytochrome c and 0.04 M sodium succinate in 0.1 M phosphate buffer of pH 7.3. The control cuvette contained no succinate. 0.1 cc. of enzyme added to each cuvette and reduction followed at 550 mp in the Beckman spectrophotometer. •, 0.1 cc. of 1:50 Keilin-Hartree preparation, 5.37 mg. of N per cc. and $Q_{O_2}$ 1500 in the presence of methylene blue and KCN. ○, 0.1 cc. of undiluted diphtherial cytochrome b (Preparation 22B3) containing 5.96 mg. of N per cc. and $Q_{O_2}$ 10,100 in the presence of methylene blue.

**Table IV**

*Turnover Numbers* at 36.8° of Diphtherial Cytochrome b during Oxidation of Succinate under Varying Conditions

<table>
<thead>
<tr>
<th>Oxidant</th>
<th>pH 7.25</th>
<th>pH 7.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylene blue</td>
<td>1600</td>
<td>2120</td>
</tr>
<tr>
<td>Ferricyanide</td>
<td>1480</td>
<td></td>
</tr>
<tr>
<td>$O_2$</td>
<td>56</td>
<td>66</td>
</tr>
<tr>
<td>Cytochrome c (25°)</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

* Ratio of oxygen uptake per minute to oxygen equivalent of hemin iron added.

b preparation reduces cytochrome c so slowly, it should provide a good test system for the postulated factor acting between cytochromes b and
in the succinoxidase system. We have tested various muscle, yeast, and bacterial extracts as well as a rabbit liver preparation prepared according to Stoppani (11) and a highly active preparation of cytochrome reductase prepared from *Escherichia coli*, but have been completely unsuccessful in finding any material which will accelerate the reduction of cytochrome *c* by diphtherial cytochrome *b* in the presence of succinate and potassium cyanide.

DISCUSSION

The succinoxidase system in cell-free extracts of beef heart muscle, which oxidizes succinic acid to fumaric acid, is a complex one which is not as yet fully understood. Of the various carrier systems involved in the transfer of 2 hydrogen atoms from succinate to oxygen, only cytochrome *c* has been isolated as a soluble pure protein. The complete succinoxidase system is generally supposed to consist of the following steps.

\[
\text{Succinate} \rightarrow \text{succinidehydrogenase} \rightarrow \text{cytochrome b} \rightarrow \text{cytochrome c} \rightarrow \text{cytochrome oxidase} \rightarrow \text{O}_2
\]

There is evidence that a factor exists between cytochromes *b* and *c*, and it has been suggested that cytochrome *a* may act between cytochromes *c* and oxidase (*a*). On the other hand, there is little clear-cut evidence that succinidehydrogenase and cytochrome *b* exist as separate and distinct enzymes. The arrows indicate the direction of electron transfer and of increasing oxidation-reduction potential.

With the exception of cytochrome *c*, none of the components of the succinoxidase system has been separated from insoluble particles or from one another and much of the evidence for the various steps is derived from the action of inhibitors. However, the system can be simplified somewhat, since there are a number of substrates, such as *p*-phenylenediamine, cysteine, etc., which reduce cytochrome *c* directly. With *p*-phenylenediamine as substrate, for example, Steps 1 to 3 in the above scheme may be eliminated. In a similar way, the diphtherial system should prove useful for studying Steps 1 and 2, since, as we have shown (see Table I), purified diphtherial succinoxidase preparations are characterized by an exceptionally high succinidehydrogenase activity and a very high cytochrome *b* content, but contain neither cytochrome oxidase nor cytochrome *c*.

Our attempts to separate diphtherial cytochrome *b* from the succinidehydrogenase activity in bacterial extracts have been wholly unsuccessful. Both factors can be precipitated at pH 5 and cannot be separated by differential centrifugation. It was demonstrated previously (6) that the cellular iron content of the Toronto strain of *Corynebacterium diphe-
theriae can be varied over a 5- to 6-fold range by controlling the iron concentration of the media on which the organisms are grown. It has since been found that both succinidehydrogenase activity and cytochrome b content are directly proportional to the iron content of the cells.

There can no longer be any doubt that the rate of succinate oxidation by the intact diphtheria bacillus is mainly determined by the rate of autoxidation of cytochrome b. With purified cytochrome b preparations, its autoxidation is the sole limiting factor, since the reduction of cytochrome b in the presence of succinate is practically instantaneous and no cytochrome oxidase is present. Although it appears likely that reduced diphtherial cytochrome b is oxidized by molecular oxygen at a somewhat higher rate than the cytochrome b of beef heart muscle (perhaps 2 to 3 times), its rate of autoxidation is extremely slow when compared with that of cytochrome oxidase. It is for this reason that the diphtheria bacillus requires such a high concentration of cytochrome b.

In the presence of methylene blue or ferricyanide, the rate of succinate oxidation is increased 25- to 30-fold. The \( Q_{o_2} \) (succinate) in the presence of methylene blue and cyanide or the \( Q_{CO_2} \) in the presence of ferricyanide has generally been considered a measure of succinidehydrogenase activity. However, as we have shown, succinidehydrogenase activity measured in this way is always directly proportional to cytochrome b content, whether the latter is measured spectroscopically by its extinction at 560 nm, by its rate of autoxidation in the presence of succinate and cyanide, or even by the slow rate of reduction of cytochrome c in the presence of succinate. It seems quite possible that succinidehydrogenase is a hemin-containing enzyme which is in fact identical with cytochrome b. We are perhaps dealing with a system analogous to the lactic acid dehydrogenase from Delft yeast which Bach et al. (13) believe to be cytochrome b_2.

In an attempt to throw further light on the problem of whether one or two steps are involved in the oxidation of succinate by cytochrome b, we have studied the effect of pH on the system. As might be expected, the oxidation of succinate is very much slower at acid pH. Within the limits of experimental error, the ratio of \( Q_{o_2} \) (succinate-methylene blue)/\( Q_{o_2} \) (autoxidation of cytochrome b) or of \( 0.25Q_{CO_2} \) (succinate-ferricyanide)/\( Q_{o_2} \) (autoxidation of cytochrome b) is constant, i.e. about 25:1, at all pH values between 6 and 9. Optimum activity occurs at about pH 8 in all cases. The effect of pH on succinate oxidation by the Keilin-Hartree preparation in the presence of methylene blue and cyanide is similar, and again the maximum \( Q_{o_2} \) is obtained at about pH 8. At more alkaline pH values the \( Q_{o_2} \) of the heart muscle preparation falls off more rapidly than with the

\[\text{In 1931, Cook and Alcock (14) found that succinidehydrogenase activity of } E. \text{coli was optimum between pH 7.6 and 8.0 by the Thunberg technique.}\]
diphtherial system. Keilin and Hartree (10) have shown that exposure of their preparation to pH 9 for 1 hour at room temperature results in complete loss of succinidehydrogenase activity, while leaving the cytochrome oxidase system intact. At Dr. Keilin's suggestion, we have attempted to reactivate a succinoxidase preparation from beef heart, inactivated at pH 9, by addition of small amounts of diphtherial cytochrome b. No reactivation could be obtained. Apparently, the factor concerned in mediating the reduction of cytochrome c by cytochrome b is destroyed simultaneously with the succinidehydrogenase activity at pH 9. The diphtherial system is stable even in the presence of the Keilin-Hartree preparation at pH 9.

Diphtherial cytochrome b is relatively heat-stable and purified preparations are only partially destroyed after 30 minutes at 75°. After 4 hour at this temperature, 75 per cent of the activity against succinate is lost, whether measured by the rate of autoxidation of cytochrome b or in the presence of methylene blue.

In conclusion, it is our opinion that succinidehydrogenase may well be identical with cytochrome b both in diphtherial extracts and in beef heart muscle succinoxidase preparations. Alternatively, succinidehydrogenase and cytochrome b may be "intimately bound to the protein of a single colloidal particle forming thus a unit of a complete oxidizing system," as suggested by Keilin and Harpley (15) in considering the dehydrogenase-cytochrome system of Escherichia coli. In any event, it seems clear that by the usual methods for determination of succinidehydrogenase, whether by oxygen uptake in the presence of methylene blue and cyanide or anaerobically with ferricyanide (Quastel and Wheatley (7)), cytochrome b activity is actually measured. If a true succinidehydrogenase exists, separate and distinct from cytochrome b, which mediates the oxidation of succinic acid by cytochrome b, it must always be present in excess and so cannot be the rate-controlling step in the methods which have been used for its determination. In a recent note Slater (16) has arrived at a similar conclusion. From Slater's cytochrome b hemin analyses and succinidehydrogenase \( Q_{02} \) determinations in heart muscle, a turnover number of 1120 per iron atom per minute may be calculated. This is in good agreement with our estimate of 1300 for the turnover number of diphtherial cytochrome b at pH 7.0 in the presence of methylene blue.

SUMMARY

The succinoxidase system of beef heart muscle has been compared with that of the diphtheria bacillus. The principal differences between the mammalian and bacterial systems reside in the relative amounts of the various cytochrome components present. Thus, beef heart muscle con-
tains relatively large amounts of cytochrome c, cytochrome oxidase, and the factor which mediates reduction of cytochrome c by cytochrome b. The diphtheria bacillus possesses far more succinidehydrogenase activity and cytochrome b than does beef heart. On the other hand, the bacteria only possess traces of cytochrome c and cytochrome oxidase.

The evidence presented indicates that oxidation of cytochrome b is the rate-limiting step when succinidehydrogenase activity is measured by oxygen uptake in the presence of methylene blue and potassium cyanide or by anaerobic oxidation with ferricyanide according to the method of Quastel and Wheatley.

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