The Interaction of Energy and Electron Transfer
Reactions in Mitochondria*

IV. THE PATHWAY OF ELECTRON TRANSFER

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Two types of reaction have been described in which a reversal of electron transfer may occur: (a) energy-linked reduction of pyridine nucleotide in the presence of a flavin-linked substrate (1) and (b) energy-linked oxidation of cytochrome and reduction of pyridine nucleotide (2).

In the latter, the presence of both oxidized and reduced forms of the respiratory carriers suggests that the system may not require additional reducing equivalents from an external substrate, provided an initial state is achieved in which some carriers are reduced and some are oxidized. In this reaction, electron transfer from oxidized carriers to pyridine nucleotide may suffice.

The first reaction, however, is usually observed only in the presence of oxidized carriers, and therefore electrons must be transferred into the respiratory chain from an external substrate to provide the necessary reducing equivalents for reduction of pyridine nucleotide. This paper considers the pathway of electron transfer in an energy-linked reaction involving certain carriers of the respiratory chain. Kinetic and inhibitor studies are performed to determine which components are so involved.

EXPERIMENTAL PROCEDURE

The mitochondrial preparations and physical techniques are described in Paper I (1).

RESULTS

Effect of Amytal on Interaction of DPNH and Flavoprotein

Because ~1 mM Amytal causes simultaneous reduction of mitochondrial pyridine nucleotide and oxidation of flavoprotein, it has been concluded that Amytal interferes with electron transfer between DPNH and flavoprotein (4). Succinate-linked reduction of flavoprotein is not, however, inhibited by the same concentrations of Amytal, and it is therefore assumed that some component of the DPNH-flavoprotein interaction is inhibited (4, 5). Such an inhibition might reasonably affect both the oxidation and the reduction reactions between DPNH and flavoprotein, a possibility that can be tested by observing the kinetics of oxidation and reduction of succinate-linked pyridine nucleotide in the presence of ADP. In the absence of Amytal (Fig. 1B of Paper I (1)), the rate of oxidation of pyridine nucleotide upon addition of 220 \( \mu \text{M ADP} \) is 0.56 \( \mu \text{M DPN per second} \), and the rate of oxygen utilization is 1.0 \( \mu \text{M O}_2 \) per second. As the ADP is exhausted, the reduction is 30% complete at the time the steady state of oxygen utilization is achieved (state 4) and is complete 5 seconds thereafter, suggesting synchronization of the activation and inhibition of respiration with the oxidation and reduction of succinate-linked pyridine nucleotide.

In mitochondria pretreated with succinate and 0.8 mM Amytal (Fig. 1), addition of 220 \( \mu \text{M ADP} \) activates respiration to approximately the same extent (0.9 \( \mu \text{M O}_2 \) per second). However, the initial rate of oxidation of pyridine nucleotide is 8-fold slower (0.07 \( \mu \text{M DPN per second} \)) than in the absence of Amytal. In fact, a steady state is barely achieved by the time ADP is exhausted. The reduction is also inhibited and is only 10% complete by the time the slow state of oxygen utilization (state 4) is reached. Half-maximal reduction does not occur until 25 seconds later. It is apparent from this experiment that Amytal, in addition to partially inhibiting the oxidation of DPNH by flavoprotein, also inhibits the reduction of DPN by succinate.

If the mitochondria are pretreated with 0.8 mM Amytal but no succinate, addition of 4 mM glutamate then elicits a slow electron transfer (Fig. 2), permitting accumulation of high energy intermediates for reducing succinate-linked pyridine nucleotide (1). The slow reduction of pyridine nucleotide reaches a steady state in about a minute and a half, during which time there is no measurable respiration (see „Discussion”). Addition of 4 mM succinate then initiates respiration at 0.8 \( \mu \text{M O}_2 \) per second, a rate comparable to that in Fig. 1. The rate of pyridine nucleotide reduction is, however, only 0.042 \( \mu \text{M DPN per second} \), which is 7-fold less than that obtained in mitochondria in the absence of Amytal (0.28 \( \mu \text{M DPN per second} \). See Fig. 1B of Paper I (1)) This may be compared with the 8-fold inhibition of oxidation in Fig. 1. Thus, the concentration of Amytal that inhibits both the oxidation and reduction of pyridine nucleotide in the succinate-linked reaction is about the same as that which inhibits the oxidation reaction in the presence of glutamate (4, 5).†

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FIG. 1. Inhibition of oxidation and reduction of succinate-linked pyridine nucleotide by pretreatment with 0.8 mM Amytal. A control experiment appears in Fig. 1B of Paper I (1). Guinea pig kidney mitochondria pretreated with 4 mM glutamate and 4 mM succinate; sucrose-phosphate-magnesium medium (pH 7.4); 0.6 mg of protein per ml; 3.7 μM pyridine nucleotide; temperature, 26°. Pyridine nucleotide (PN) is recorded spectrophotometrically and oxygen utilization polarographically (Experiment 683-3).

nucleotide provides: (a) a key to identification of the pathway of electron transfer for this reaction with components of the respiratory chain; and (b) evidence against the possibility that Amytal-insensitive DPN-linked dehydrogenases participate, it is of interest to observe its effect in the presence of ATP. Fig. 3A and B illustrates effects observable in pigeon heart mitochondrial preparations that require ATP for succinate-linked pyridine nucleotide reduction (6). In both experiments, mitochondria are suspended in mannitol-sucrose-Tris medium (Pi- and Mg++-free) (1) and are treated with succinate and ATP, the latter addition being followed immediately by a reduction of pyridine nucleotide. In Fig. 3A, the initial rate of the reduction is 0.16 μM DPNH per second. After 30 seconds, 2 mM Amytal is added and abruptly inhibits the reduction. In Fig. 3B, where two records are superimposed, the Amytal-free sample shows a rapid and large reduction of pyridine nucleotide, whereas the Amytal-treated sample shows a 5-second induction period after addition of ATP and a maximal rate of reduction one-half that in the untreated material.

Effect of Amytal on Oxidation and Reduction of Cytochrome b—
The effect of Amytal upon the kinetics of reduction and oxidation of cytochrome b substantiates the supposition that the succinate-linked reduction of DPN involves electron transfer through the respiratory chain. Fig. 4 (a control experiment in which succinate is added to Amytal-free guinea pig kidney mitochondria) indicates reduction of cytochrome b, measured at 430 to 410 μm, at a rate of 0.0034 μM Fe per second. The kinetics of this reaction approach zero order, and completion is not obtained until a time comparable to that required for DPN reduction has elapsed (see Fig. 1B of Paper I (1)). Addition of 200 μM ADP causes a typical oxidation-reduction cycle of cytochrome b and acceleration and deceleration of respiratory activity. The rate of oxidation of cytochrome b is ~0.07 μM Fe per second.

The kinetics of cytochrome b in Amytal-treated mitochondria are illustrated by Fig. 5. In this case, sufficient Amytal (1.6 mM) is used to block electron transfer through DPN almost completely. Addition of 4 mM succinate causes a reduction of

FIG. 2. Effect of Amytal on glutamate and succinate reduction of mitochondrial pyridine nucleotide. Guinea pig kidney mitochondria pretreated with 0.8 mM Amytal and subsequently treated with glutamate and succinate. Experimental conditions as in Fig. 1 (Experiment 638-5).

FIG. 3. Inhibition of energy-linked pyridine nucleotide reduction by Amytal. A. Amytal, 2 mM, added after succinate and ATP. Pigeon heart mitochondria; mannitol-sucrose-Tris (pH 7.4) medium; 2.6 mg of protein per ml; temperature, 26° (Experiment 165d). B. Mitochondria pretreated with succinate and Amytal, 1.4 mM (—) and with succinate alone (—), 1.1 mg of protein per ml. Other conditions as in 3A (Experiment 178f).

FIG. 4. Kinetics of reduction of cytochrome b on addition of 4 mM succinate to glutamate-treated mitochondria and those of oxidation on addition of 220 μM ADP. Experimental conditions as in Fig. 1 (Experiment 684-5).

FIG. 5. Effects of Amytal, succinate, and ADP on the kinetics of cytochrome b in the absence of glutamate. Experimental conditions as in Fig. 1 (Experiment 684-10).
**Table I**

Effect of Amytal on kinetics of oxidation and reduction of pyridine nucleotide, flavoprotein, and cytochrome b of guinea pig kidney mitochondria

<table>
<thead>
<tr>
<th>[Amytal]</th>
<th>Reduction by succinate (State 4&lt;sub&gt;a&lt;/sub&gt; to 4&lt;sub&gt;a&lt;/sub&gt;)</th>
<th>Oxidation by ADP (State 4&lt;sub&gt;a&lt;/sub&gt; to 3&lt;sub&gt;a&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pyridine nucleotide</td>
<td>Flavoprotein</td>
</tr>
<tr>
<td>0</td>
<td>0.32</td>
<td>0.015</td>
</tr>
<tr>
<td>0.8</td>
<td>0.10</td>
<td>0.055</td>
</tr>
<tr>
<td>1.6</td>
<td>0.035</td>
<td>0.055</td>
</tr>
</tbody>
</table>

* Not measured.

The rate of reduction of flavoprotein has increased 4-fold. The rate of oxidation of pyridine nucleotide is inhibited 10-fold, and the rate of reduction of flavoprotein is no faster; cytochrome b reduction is 10 times more rapid than in the Amytal-free material. The rate of oxidation of DPNH is not measurable at the highest Amytal concentration whereas the oxidation of flavoprotein remains increased 3-fold. Cytochrome b oxidation is twice as slow as in the Amytal-free material.

It can be concluded that DPN acts as an oxidant for flavoprotein and cytochrome b in the reduction reaction caused by succinate addition (state 4<sub>a</sub> to 4<sub>a</sub>). Similarly, DPNH appears to act as a reductant for cytochrome b and flavoprotein in the oxidation reaction caused by ADP addition (state 4<sub>a</sub> to 3<sub>a</sub>).

**Effect of Antimycin A and HQO**—In studies of pigeon heart mitochondria that are essentially free of substrate, it has been observed that addition of succinate causes no measurable reduction of pyridine nucleotide under anaerobic and terminally inhibited conditions (e.g., cyanide- or hydrogen sulfide-treated (2)). However, addition of ATP is followed by rapid reduction of pyridine nucleotide. Thus, neither of these conditions itself blocks electron transfer into succinate-linked pyridine nucleotide; indeed the same concentration of ATP causes a somewhat more rapid reduction in sulfide-inhibited mitochondria than in aerobic, uninhibited mitochondria (2). The effects of terminal inhibitors are also not inconsistent with the effects of Amytal that indicate participation of the respiratory carriers in the succinate-linked reduction of pyridine nucleotide. However, a study of the effects of antimycin A is essential to determine the extent to which the respiratory chain may be involved in both the aerobic and anaerobic (terminally inhibited) reactions.

Fig. 6 illustrates the remarkable effect of low concentrations of antimycin A and HQO on ATP-activated reduction of pyridine nucleotide by succinate. As in Fig. 3B, two experiments are represented, each having slightly different initial levels of fluorescence. Upon addition of ATP, both traces coincide during the reduction of pyridine nucleotide. One sample is now treated with 3.6 μg per ml of antimycin A and one with 3.6 μg per ml of HQO, the moment of addition being indicated by the break in the traces caused by stirring. Pyridine nucleotide is now rapidly oxidized ~100% in the antimycin-treated sample and ~75% in the HQO-treated sample. (In control experiments without HQO, pyridine nucleotide remains reduced for several minutes.) To determine whether the oxidation is caused by rapid hydrolysis of ATP or by some other effect, double the initial amount of ATP is added. The reduction of pyridine nucleotide now obtained is: (a) much slower than that obtained with half as much ATP before treatment with the inhibitors; (b) transitory, considerable reoxidation of pyridine nucleotide occurring within 3 minutes.

To determine whether antimycin A interferes in some hitherto unknown way with the reduction of pyridine nucleotide by directly linked dehydrogenases, we can compare the kinetics of pyridine nucleotide with those of flavoprotein, the latter serving as an indicator of the transfer of reducing equivalents from such a dehydrogenase into the respiratory chain. In the experiment of Fig. 7, aerobic pigeon heart mitochondria are pretreated with 0.4 μM antimycin A. This elicits a slow reduction of flavoprotein, recorded spectrophotometrically at 465 to 510 μm, which is

![Diagram](https://example.com/diagram.png)

Fig. 6. Inhibitory effects of 3.6 μg(α) per ml of antimycin A and 3.0 μg per ml of hydroxyquinoline oxide (HQNO) on succinate-linked reduction of pyridine nucleotide activated by ATP. Pigeon heart mitochondria; 1.2 mg of protein per ml; other conditions as in Fig. 3A (Experiment 178c).
attributed to a small amount of endogenous substrate present in the preparation. About 40 seconds later, 4 mM succinate is added, causing no reduction of pyridine nucleotide (recorded fluorometrically at 365 mμ excitation and 440 mμ measurement) and slight acceleration of reduction of flavoprotein. About 90 seconds thereafter, 4 mM glutamate is added, causing, as indicated by the abrupt downward deflection of the spectrophoto-
metric trace, a rapid transfer of reducing equivalents into the respiratory chain. The smaller deflection of the fluorescence trace corresponds to the reduction of a small portion of mitochondrial pyridine nucleotide (cf. Paper I (1)).

In another experiment with succinate- and glutamate-treated mitochondria, sufficient antimony A was added to the mitochondria in state 3 to block respiration within a few seconds; under these conditions, the rate of pyridine nucleotide reduction proceeds very slowly. These results support the view that pyridine nucleotide is not on the main pathway of electron transfer.

**DISCUSSION**

**Pathways for Pyridine Nucleotide Reduction**—The equations below indicate the range of possibilities that can be considered for the pathway of succinate-linked reduction of pyridine nucleotide. The first three pathways would not involve the carriers of the respiratory chain and would consist of:

Pathway 1. A “switch” mechanism dependent upon diverting oxidizing equivalents from the pyridine nucleotide-linked substrates at a rate compatible with the speeds of oxidation of succinate and DPNH. Several objections to this hypothesis, which is essentially that of Birt and Bartley (7) and Kulka et al. (8), have been advanced (1–3) and we now can add to them the antimony and Amytal sensitivity of the reduction. Although the “switch” mechanism appears to be a very simple one, as yet it has not been demonstrated to occur even in model systems.

\[
\text{DPNH} \xrightarrow{O_{2}} \text{succinate} \quad (1)
\]

Pathway 2. Formation of a pyridine nucleotide-linked oxidation product of succinate by the reactions of the citric acid cycle, for example malate. Such a reaction has been considered (3), and it is concluded that much of the reduction observed in the presence of malate and glutamate is due to succinate formation.

In this case also, sensitivity to Amytal and antimony A would not be expected.

\[
\text{sucinate} \rightarrow \text{fumarate} \rightarrow \text{malate} \rightarrow \text{oxaloacetate} \quad (2)
\]

Pathway 3. An energy-linked pathway in the presence of succinate, in which the activated succinate* would be suitable for reducing pyridine nucleotide by means of an ordinary dehydrogenase mechanism. Alternatively DPNH could be activated so as to be reducible by succinate.

\[
\text{sucinate} \xrightarrow{X \sim 1} \text{sucinate}^{*} \xrightarrow{\text{DPN}} \text{fumarate} + \text{DPNH} + \text{H}^{+} \quad (3)
\]

Pathway 4. Finally, there is a possible energy-linked pathway differing from that of Pathway 3, in that the respiratory carriers would be involved.

\[
\text{sucinate} \xrightarrow{\text{DPN}} \xrightarrow{X \sim 1, \text{carriers}} \text{DPNH} + \text{H}^{+} + \text{fumarate} \quad (4)
\]

The sensitivity to Amytal and antimony A is decisively in favor of Pathway 4, particularly in Fig. 3A where the effects of succinate and ATP are blocked by Amytal. As discussed before (3), a combination of Pathways 3 and 4 is possible; in such a combined pathway, activated succinate would be produced by a reaction involving the carriers of the respiratory chain. Such a mechanism cannot be distinguished at present from Pathway 4.

In view of the foregoing, we may consider pathways of electron transfer of varying complexity consistent with the response of succinate-linked pyridine nucleotide reduction in the experiments presented here.

**Pathways Involving Interaction of Respiratory Components at Level of Flavoprotein**—The simplest pathway of reducing equivalents which would account for the effects of malonate (1) and Amytal upon the kinetics of oxidation and reduction of pyridine nucleotide in kidney mitochondria is summarized by Fig. 8.

The malonate-sensitive point is indicated to be between succinate and succinic dehydrogenase (flavoprotein) and the Amytal-sensitive point between flavoprotein and DPN. Thus, the malonate block would cause the oxidation of pyridine nucleotide, and the Amytal block would cause its reduction.

The fact that the oxidation and reduction of succinate-linked pyridine nucleotide closely follow the increases and decreases of respiratory activity accompanying the state 4-3-4 transitions suggests that there is an active oxidative pathway for this material. The demonstrated sensitivity of the pathway to Amytal, malonate, and antimony A supports this view. Possible pathways for this oxidation are a reverse of the reduction reaction or a separate chain (---). For the purposes of this discussion, the

**Fig. 7.** Site of action of antimony A, 0.4 μM, in inhibiting succinate-linked reduction of pyridine nucleotide. Combined spectrophotometric record of flavoprotein reduction and fluorescence record of pyridine nucleotide reduction. Pigeon heart mitochondria; mannitol-sucrose-Tris medium (pH 7.4); temperature, 26° (Experiment 129).

**Fig. 8.** Pathway of electron transfer in succinate-linked pyridine nucleotide reduction (MD-92).
two can be considered equivalent, since they would both show the same inhibitor sensitivity.

In phosphorylating mitochondria inhibited by Amytal, flavoprotein becomes more oxidized and pyridine nucleotide more reduced; therefore, it is logical that a site of Amytal inhibition lies between these two components (4, 5). This observation can now be supplemented by that reported here, that Amytal inhibits the rate of energy-linked reduction of pyridine nucleotide, although the reduction eventually proceeds to completion. In nonphosphorylating preparations on the other hand, flavoprotein becomes more reduced (9) whereas ubiquinone is not reduced (10).

Thus, it is evident that the principal site of action of Amytal differs in phosphorylating and nonphosphorylating mitochondria. In the former, we postulate that Amytal can interact with energy transfer reactions themselves in a way similar to guanidine (5) with the result that the DPNH-flavoprotein couple is inhibited for forward and for reversed energy transfer as well. This view of Amytal inhibition is consistent with thermodynamic considerations (11).

Thus, in the mechanism of Fig. 8, the site of Amytal inhibition is identified with flavoprotein1 and flavoprotein2, implying that the flavin component of the respiratory chain is affected by the inhibitor in such a way that its ability to transfer reducing equivalents to pyridine nucleotide or to accept oxidizing equivalents from the adjacent member of the respiratory chain is inhibited. Actually, the site of inhibition could be between pyridine nucleotide and flavoprotein. However, identification of the electron transfer pathways would be unaffected by this change.

The mechanism of Fig. 8 is also consistent with the accelerated reduction of cytochrome b in the absence of Amytal, inasmuch as electrons can be transferred from succinate through flavoprotein1 directly to cytochrome b without a bypass into flavoprotein2 and DPN.

The pathway of electron transfer from flavoprotein1 to flavoprotein2 is in accord with the data of Beinert and Crane (12) who favor the idea of direct transfer between flavoproteins. The reduction of DPN by diaphorase preparation has recently been studied by Massey and Searls (13) and Searls and Sanadi (14).

Although the function of ubiquinone (coenzyme Q) in phosphorylating mitochondria has not been clearly defined, there is nothing in the current experimentation to rule out the possibility that ubiquinone could be the transfer intermediate between flavoprotein1 and flavoprotein2. The arguments presented so far would not be affected, and the objections to Fig. 8 brought up in the following paragraph would be equally applicable, inasmuch as the reduction of ubiquinone in nonphosphorylating preparations (15) and in phosphorylating preparations (16) has been shown to be unaffected by antimycin A.

Pathways Involving Interaction of Respiratory Components at Level of Cytochrome—The fact that the reduction of pyridine nucleotide is inhibited by antimycin A rules against the mechanism of Fig. 8 which should give more rapid reduction of DPN in the presence of antimycin A, particularly in the presence of added ATP. For this reason, the mechanism of Fig. 9 is proposed, in which reducing equivalents for DPN pass through the respiratory chain at the level of cytochrome b. In this way, the system acquires antimycin A sensitivity of electron transfer to oxygen and to DPN. A current theory on the site of action of antimycin A (16) is that it lies between cytochromes b and c1 and represents an inhibition of the ability of cytochrome b to transfer reducing equivalents along the chain. The interesting possibility that antimycin A is blocking energy transfer, not electron transfer, is unlikely in view of the clear-cut inhibition observed between cytochromes b and c + c1 in nonphosphorylating preparations (17). (See Note, p. 1568.)

At the present time there is no evidence for or against the transfer of electrons to a point higher than cytochrome b in the respiratory chain, such as cytochromes c or c1. A study of this point requires an inhibitor that would act between cytochromes c1 and c or between c and a + a3.

Cytochrome b responds to Amytal treatment in accordance with the mechanism of Fig. 9; it is much more rapidly reduced and somewhat more slowly oxidized in the Amytal-inhibited material. The fact that this increase in the velocity of reduction is 10-fold for cytochrome b and only 3-fold for flavoprotein also suggests that the mechanism of Fig. 9 is the more probable.

In the experiment of Fig. 6, DPNH is oxidized by the addition of antimycin A or H2O2. To explain this result, we postulate as a general phenomenon that antimycin A inhibits the ability of cytochrome b to transfer electrons to the adjacent component which, as seen in the mechanism of Fig. 9, would occur at a site between cytochromes b and c1 or between cytochrome b and flavoprotein2. However, the sensitivity of the two reactions to antimycin A would necessarily differ, that between cytochrome b and flavoprotein being the more sensitive.

In summary, the proposed pathway of electron transfer from succinate to pyridine nucleotide involves the ordinary carriers of the respiratory chain up to and including the antimycin-sensitive point, at which electron transfer is reversed in such a way as to activate a DPNH-linked reductase (cf. 13, 14) designated flavoprotein.

Schematic Diagram of Electron and Energy Transfer Pathways—These considerations of the pathway of succinate-linked reduction of pyridine nucleotide have been incorporated into the schematic diagram of Fig. 10 which shows the complete respiratory chain and the various sites of energy conservation. The pathways shown here are identical to those described above except for the possible participation of ubiquinone in electron transfer to the pool of succinate-linked pyridine nucleotide (18). Inasmuch as transfer via ubiquinone is not firmly established, the mechanism provides for electron transfer directly from cytochrome b to flavoprotein1 as indicated in Fig. 9. It also includes a pathway from succinate through flavoprotein1 and the cytochromes to oxygen and from flavoprotein2 to cytochrome b via ubiquinone. Similarly, the pathway for oxidation of DPNH through flavoprotein may or may not include ubiquinone.

4 B. Chance and Y. Hatefi, unpublished observations.

5 B. Chance and E. R. Redfearn, unpublished observations.
Electron transfer from flavoprotein$_d$ to DPN in the energy-linked pathway would proceed via cytochrome $b$ and flavoprotein$_e$ with or without mediation of ubiquinone. The utilization of high energy intermediates in reduction of pyridine nucleotide is indicated at only one site, but others may be involved.

The electron transfer chain participating in ATP-activated oxidation of cytochrome and reduction of pyridine nucleotide in anaerobiosis or in the presence of sulfide can also be indicated by Fig. 10. The observed sensitivity of the chain to antimycin $A$ suggests that some of the electrons donated in oxidation of cytochromes passes through the antimycin-sensitive point in the chain in accordance with Fig. 9. The question to be considered is whether these electrons pass into the succinate-linked pool of pyridine nucleotide via flavoprotein$_d$ or into the direct dehydrogenase-linked pool via flavoprotein$_e$. No definite conclusion can now be reached.

Electron Flux through Succinate-linked Pool—The diagram of Fig. 10 also provides a framework for a more critical discussion of the amount of electron transfer that may pass into the succinate-linked pool and its function in respiration. It is apparent that the initial rates of oxidation and reduction of this material in aerobic mitochondria, as measured in the state 4-to-4, and 4-to-3, transitions are slow compared to the rate of oxygen utilization (1). However, in anaerobic or terminally inhibited systems, the rates of pyridine nucleotide reduction are much faster. The determining factor in these kinetics, as indicated in Fig. 10, is the actual concentration of $X \sim I$ at the energy-requiring site. Thus, in anaerobic or terminally inhibited systems, high rates of pyridine nucleotide reduction are obtained when all electrons from succinate are available and when the concentration of $X \sim I$ is high. Similarly, the aerobic state 4 affords conditions for a high degree of pyridine nucleotide reduction. However, under none of these conditions is the rate of reduction as slow as that obtained in state 3, in which ADP and $P_i$ block the reverse pathway, for example by diminishing the concentration of $X \sim I$. In this state, it is unlikely that any significant electron transfer passes through the pool of succinate-linked pyridine nucleotide. In fact, the level of pyridine nucleotide in the pool indicates the content of high energy intermediates in the mitochondria.

Why Don't Directly Linked DPN-reducing Substrates Act as Succinate?—Any mechanism in which electrons pass from DPN-linked substrates through the respiratory chain to a point common to those involved in succinate-linked pyridine nucleotide reduction would appear to allow the energy-linked process to occur with either a DPN-linked substrate or a flavin-linked substrate. This is true of the mechanism of Fig. 10. Three explanations can be considered at the present time; one mentioned earlier (1) implies that electron transfer from succinate has a special activating effect on the energy-linked pathway. This in turn implies that succinate affects the mitochondria in some way that dehydrogenase-reduced pyridine nucleotide cannot. The other two explanations involve actual differences in the pathways for oxidation of succinate and DPNH. One directly applicable to Fig. 10 is that electron flow from dehydrogenase-linked pyridine nucleotide can somehow be distinguished from that obtained from flavin-linked substrates at the antimycin-sensitive point, succinate being for this reason essential for the energy-linked reaction; only the electron flow from the flavin-linked substrate connects with the energy-linked pathway. Separation of the electron flow from DPN-linked dehydrogenase and that from direct flavin-linked dehydrogenase on a larger scale would involve separate assemblies of respiratory enzymes for oxidation of succinate and DPNH. This is very unlikely in both the nonphosphorylating and the phosphorylating respiratory chain since the extent of reduction of cytochrome $c$ in anaerobiosis or upon addition of a terminal respiratory inhibitor is the same, regardless of whether DPNH or succinate is the substrate. Moreover, the kinetics of reduction of cytochrome $c$ do not appear to be biphasic with either substrate. The possibility that such chains could be interconnected at the level of cytochrome $c$ has been disproved (19) but connections at the antimycin-sensitive site require further study.

Degree of Reduction of Pyridine Nucleotide in Anaerobiosis—Finally, the observation that, in mitochondria treated with a pyridine nucleotide-linked substrate, pyridine nucleotide becomes fully reduced in the transition from state 4 to anaerobiosis should be clarified (for the energy requirement for this transition, see Paper I (1)). The preferred explanation for this phenomenon is that a small concentration of succinate is present during the oxidation of the pyridine nucleotide-linked substrate and that this concentration is responsible for the anaerobic reduction of pyridine nucleotide. This hypothesis is supported by the fact that the anaerobic reduction by succinate is malonate-sensitive (2) and that this sensitivity is not overcome by the addition of a pyridine nucleotide-linked substrate. Although it is possible that transhydrogenase activity, for which high energy intermediates are not required, could cause anaerobic reduction of pyridine nucleotide, other experiments (1) show that the actual effect is sensitive to ADP and $P_i$. For this reason, it seems that the role of transhydrogenase in the anaerobic reduction of pyridine nucleotide is a minor one. In this connection, it is relevant to point out again (20) that succinate-linked pyridine nucleotide reduction is itself a type of transhydrogenase activity in which a portion of the energy conserved in the oxidation of one molecule of pyridine nucleotide may be used in the reduction of another.

**SUMMARY**

1. The pathway of electron transfer from succinate to pyridine nucleotide shows a sensitivity to antimycin $A$, suggesting that carriers of the respiratory chain up to and including the antimycin-sensitive point are involved in succinate-linked reduction of pyridine nucleotide.
2. The sensitivity of succinate-linked reduction of pyridine nucleotide to Amytal suggests that a reverse of the flavoprotein-pyridine nucleotide interaction observed in the oxidation of pyridine nucleotide in phosphorylating mitochondria is also part of the electron transfer pathway.

3. Mechanisms indicating the interconnection of electrons from the antimycin-sensitive point to this flavoprotein via electron carriers such as cytochrome b and ubiquinone are considered. These mechanisms appear to apply to both aerobic and anaerobic (terminally inhibited) energy-linked reduction of pyridine nucleotide.

4. Three mechanisms for increased reduction of pyridine nucleotide in succinate-treated mitochondria that do not involve the above pathway fail to show responses of the experimentally observed sensitivity to Amytal or to antimycin A.

5. The properties of the energy-linked pool of pyridine nucleotide in metabolism are considered. Its participation is likely to be small in state 3 and of some consequence in state 4.

REFERENCES

Noted Added in Proof—Löw et al. (Federation Proc., 20, 481 (1961)) have reported verbally the reduction of exogenous DPN on addition of ATP and succinate to phosphorylating particles prepared from beef heart (ETPH), supplementing the previous demonstration by Chance and Fugmann (Biochem. and Biophys. Research Communs., 4, 317 (1961)) that exogenous cytochrome c can be oxidized in reversed electron transfer. The reaction reported by Löw and his co-workers was highly inhibited by Amytal (2 x 10^-1 M) and partially inhibited by antimycin A (2 µg per mg of protein), thus confirming the existence of an energy-linked reduction of pyridine nucleotide that passes through carriers of the respiratory chain. Since they consider that a relatively high concentration of antimycin A is required, they have chosen to attribute its effect to an activation of ATPase instead of to an inhibition of electron transfer. The data presented in Fig. 8 and Table IV of Paper II (1) and in Fig. 6 of Paper IV above show that both antimycin A and H2O have a marked effect on the rate of ATP-activated reduction of DPN. The simplest explanation is that electron transfer has been partially inhibited. It is not to be expected that the quantitative effect of inhibitors of reversed electron transfer would be identical to that on forward electron transfer since the rates of electron flow differ by about 10-fold (cf. Paper IV). The relation between inhibitor sensitivity and the rate of electron flow has been studied in skunk cabbage mitochondria (cf. Fig. 8 of Chance and Hackett, Plant Physiol., 34, 33 (1959)).
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