Demonstration of a Requirement of High Energy Phosphate for the Aerobic Oxidation of Succinate in Liver Mitochondria

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It was concluded in a previous paper (1) that the enhancement of respiration by inorganic orthophosphate, occurring in rat liver mitochondria oxidizing a number of Krebs cycle metabolites or glutamate in the presence of 2,4-dinitrophenol or Dicumarol, is due to the substrate level phosphorylation linked to the oxidation of α-ketoglutarate which is not uncoupled by these agents. Yet, no requirement of inorganic orthophosphate under these conditions was observed when succinate was used as a substrate. It was observed in the course of these studies, however, that when the mitochondria were pretreated1 with dinitrophenol plus adenosine 5'-phosphate, in order to remove endogenous inorganic orthophosphate, and the initial rate of respiration after incubation with succinate and dinitrophenol was measured with the oxygen electrode, this was markedly low, unless inorganic orthophosphate was included in the incubating medium. Moreover, it was found necessary to add the inorganic orthophosphate together with the succinate some time before the addition of dinitrophenol, in order to obtain a high rate of respiration. Alternatively, succinate oxidation could be brought to a high rate by adding adenosine triphosphate to the system, and in this case even after the addition of dinitrophenol. Similar effects were subsequently obtained, and even in a more striking manner, when the mitochondria were preincubated with arsenate, instead of the pretreatment with dinitrophenol plus adenosine 5'-phosphate.

Studies with P32-labeled mitochondrial preparations have revealed that treatment of mitochondria both with dinitrophenol plus adenosine 5'-phosphate (1) and with arsenate (2) results in an extensive depletion of not only endogenous inorganic orthophosphate but also endogenous high energy phosphate. To the above findings were therefore interpreted to suggest that the mitochondrial oxidation of succinate might require a supply of high energy phosphate. Experimental evidence in favor of this concept has been reported briefly (8), and is presented here in detail.

Parallel to the accomplishment of the present work an investigation was undertaken, in collaboration with Dr. M. Klingenberg at Marburg, about a possible correlation between the present activation mechanism and the endergonic reduction of the mitochondrial pyridine nucleotides by succinate, earlier described by Chance and Hollunger (4, 5) and by Klingenberg et al. (6, 7). The results of these studies are reported elsewhere (8).

From the experimental evidence collected, the concept is developed that the aerobic oxidation of succinate in intact liver mitochondria involves an adenosine triphosphate-dependent activation mechanism with the formation of a phosphorylated, reduced electron carrier; this high energy intermediate can subsequently be oxidized either by way of the terminal respiratory chain, with the regeneration of adenosine triphosphate, or by diphosphopyridine nucleotide, with the liberation of inorganic orthophosphate.

EXPERIMENTAL PROCEDURE

Rat liver mitochondria were prepared as previously described (9). Oxygen uptake was measured in a rotating cuvette with a stationary platinum electrode following the procedure described by Chance and Williams (10). The concentrations of the reagents in a final volume of 1.5 ml were as follows (unless otherwise stated): 50 mM KCl, 33 mM Tris buffer (pH 7.5), 8 mM MgCl2, 50 mM sucrose. Of the incubation medium, 1.3 ml were added to the cuvette before the experiment was started. Other substances were added during the incubations at the points indicated in the figures. Unless otherwise indicated, 0.2 ml of mitochondrial suspension in 0.25 M sucrose (corresponding to 400 mg of rat liver, wet weight, or to about 8 mg of mitochondrial protein) was used in each experiment. Pretreatment with dinitrophenol or with dinitrophenol plus AMP was performed as described previously (1).

Cysteine sulfinate was a kind gift of Professor D. Cavallini, University of Modena, Italy. ATP, CTP, UTP, ITP, and GTP were products of the Sigma Chemical Company.

RESULTS

Depression of Capacity for Succinate Oxidation by Preincubation with Arsenate and Dicumarol, and Restoration by ATP

The main findings are illustrated by Experiments a to g in Fig. 1. Experiments a, b, and c show the effect of Dicumarol, arsenate, and arsenate plus Dicumarol, respectively, when added
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Fig. 1. Stimulation of succinate oxidation by ATP in liver mitochondria preincubated with arsenate and dicumarol. Concentrations of the reagents in a final volume of 1.5 ml were as follows: 50 mM KCl, 33 mM Tris buffer (pH 7.5), 8 mM MgCl₂, 50 mM sucrose, 1 mM ATP, 13 mM succinate (succ), 2 mM arsenate (Arsen), 0.06 mM dicumarol (Dic). Mitochondria (mit) from 400 mg of rat liver, wet weight (about 8 mg of protein). The substances were added at the points indicated. The term "1.3 ml Medium" represents KCl, Tris, MgCl₂, and sucrose, added in a volume of 1.3 ml. Oxidation rate of succinate is given in milli-microatoms of oxygen per minute.

to mitochondria in the presence of succinate. Dicumarol, 0.05 mM, alone was able to elicit a maximal rate of respiration (Experiment a), whereas the respiratory rate was only about half-maximal when 2 mM arsenate alone was added (Experiment b); this difference in rate was probably due to the fact that whereas Dicumarol was able to release respiratory control completely, arsenate caused only a partial release. In fact, addition of Dicumarol to the arsenate-supplemented system brought the respiratory rate up to the maximum (Experiment c).

In Experiments d, e, and f, the same three sets of conditions were tested, but here, the Dicumarol, the arsenate, or both, were added 3 to 4 minutes before, rather than after, the addition of succinate. In all three cases, the mitochondria exhibited a moderate rate of endogenous respiration during the preincubation. When succinate was added to the system preincubated with Dicumarol alone, the ensuing respiratory rate was about half-maximal and fairly constant (Experiment d).² After preincubation with arsenate alone, again a half-maximal rate of succinate oxidation was eventually attained, but in this case only after an initial lag phase (Experiment e). Finally, when the preincubation was carried out in the presence of both arsenate and Dicumarol, the resulting rate of succinate oxidation was very low, only slightly more than 10% of the maximum,

² In later experiments, performed in connection with spectrophotometric studies (8), a strong inhibition of succinate oxidation could also be obtained by preincubation with 0.04 mM Dicumarol or 0.1 mM dimethylphenol alone. Also in this case, added ATP was able to stimulate the oxidation of succinate several-fold. The time and conditions of preincubation required for inhibiting succinate oxidation appear to be dependent on the complement of endogenous substrates initially present in the mitochondria.
and in this case it remained low upon prolonged incubation (Experiment f).

It was found previously (2) that treatment of mitochondria with arsenate may lead to a rapid depletion of endogenous high energy phosphate. It therefore seemed possible that the initial low respiration in Experiment e might be a reflection of this depleted state and that the gradual increase of the respiratory rate was due to a slow regeneration of high energy phosphate which occurred during the oxidation of succinate. That high energy phosphate was involved in the observed phenomenon was also indicated by Experiment f, in which the addition of an uncoupling concentration of Dicumarol was able to prevent the successive recovery of the respiratory rate, previously observed in Experiment e. This reasoning could be verified in Experiment g, by adding ATP to the arsenate-Dicumarol-preincubated system and showing that this addition resulted in an increase of the rate of succinate oxidation by nearly 5-fold. This rate was equal to, or even slightly exceeded, that previously found after preincubation with Dicumarol alone (cf. Experiment d). In other words, ATP was able to repair completely the inhibitory effect of arsenate.

Factors Influencing Arsenate Effect

Time of Preincubation—The extent of depression of the capacity for succinate oxidation was dependent on the length of preincubation in the presence of arsenate. In the prevailing experimental conditions were needed to obtain maximal depression, the extent of which was about 90%. On the other hand, it was found to be of no major significance for the extent of inhibition at what time the Dicumarol was added in relation to the addition of arsenate, or whether Dicumarol was replaced by 0.1 mM dinitrophenol. As could be expected, also in this latter case, added ATP was able to stimulate the rate of succinate oxidation several-fold.

Effect of ATP—In Fig. 2, it is demonstrated that the effect of arsenate could be eliminated if the preincubation was performed in the presence of a 5-fold molar excess of ATP, the latter probably abolishing the effect of arsenate on the mitochondrial endogenous phosphate. If ATP was added after the preincubation with arsenate, no effect was found and ATP was required for stimulating the respiration.

Effect of Amytal—It was shown previously (2) that Amytal is able to prevent the depletion of the mitochondrial high energy phosphate by arsenate. It could therefore be expected that Amytal should also be able to prevent the arsenate-induced depression of succinate oxidation. In fact, when the preincubation with arsenate and Dicumarol was carried out in the presence of 2 mM Amytal (Fig. 3A) a high rate of succinate oxidation ensued. If, on the other hand, Amytal was added after a period of preincubation with arsenate and Dicumarol (Fig. 3B), the rate of oxidation of succinate was low, and could be greatly enhanced by added ATP.

Effect of Cysteine Sulfinate—A partial protection against the arsenate-induced depletion of mitochondrial endogenous phosphate was previously also noticed when cysteine sulfinate was present in the preincubating medium (2). In accordance with this, a high rate of succinate oxidation ensued if the preincubation with arsenate and Dicumarol was performed in the presence of cysteine sulfinate (Fig. 4A). When, on the other hand, cysteine sulfinate was added after the preincubation, the ensuing rate of succinate oxidation was low, and could be stimulated several-fold by the addition of ATP (Fig. 4B).

This latter finding was of particular importance, inasmuch as it seemed to exclude the possibility that the observed inhibition of succinate oxidation was due to an accumulation of oxaloacetate. Cysteine sulfinate, as has been shown by Singer and Kearney (11), efficiently removes oxaloacetate from mitochondria, converting it to aspartate by way of a transaminase reaction. Therefore, if oxaloacetate were the inhibitory agent, addition of cysteine sulfinate should eliminate the inhibition, regardless of whether it is added before or after the preincubation of the mitochondria with arsenate and Dicumarol. This clearly was not the case (Fig. 4), cysteine sulfinate giving an effect only if added before, but not if added after, the preincubation.

To obtain this differential effect of cysteine sulfinate in a consistent manner, it was found advisable, however, to include Amy-
FIG. 4. Effect of cysteine sulfinate on succinate oxidation in the arsenate-Dicumarol-preincubated mitochondria. Experimental conditions as in Fig. 1. Cysteine sulfinate (CSA), 3 mM, was added at the point indicated.

Data Bearing on ATP Effect

Concentration of ATP—The amount of added ATP was found to influence the extent of stimulation of succinate oxidation in the arsenate-Dicumarol-pretreated mitochondria. The apparent lowest concentration to give a maximal effect was 1 mM. It should be remembered, however, that under the prevailing conditions, i.e. in the presence of arsenate (2) and Dicumarol (12) (both of which are known to elicit a high ATPase activity), the added ATP was presumably subject to rapid hydrolysis.

Other Nucleotide Triphosphates—in Table II, a comparison is made of the capacity of ATP to stimulate succinate oxidation with those of other nucleotide triphosphates, viz. CTP, UTP, ITP, and GTP, all added in concentrations of mM. All four compounds were markedly less efficient than ATP, with their individual potencies decreasing in the above mentioned order. It may also be noticed that the effects of these compounds had

<table>
<thead>
<tr>
<th>Nucleotide added</th>
<th>Stimulation</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>First minute</td>
</tr>
<tr>
<td>ATP</td>
<td>213</td>
</tr>
<tr>
<td>CTP</td>
<td>117</td>
</tr>
<tr>
<td>UTP</td>
<td>30</td>
</tr>
<tr>
<td>ITP</td>
<td>12</td>
</tr>
<tr>
<td>GTP</td>
<td>0</td>
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Fig. 5. Stimulation of succinate oxidation by Pi and ATP in liver mitochondria pretreated with dinitrophenol and AMP. Experimental conditions as in Fig. 1. The mitochondria used in this experiment were pretreated for 5 minutes with dinitrophenol and AMP as described in "Experimental Procedure."

A well marked lag period perhaps due to transphosphorylation with mitochondrial ADP, thus giving rise to ATP.

Other Agents—No stimulation of succinate oxidation was obtained with AMP (1 to 2 mM) or with ethylenediaminetetraacetate (2 mM). Finally, the inhibited state of succinate oxidation was not altered when the succinate concentration was raised from 13 to 65 mM.

Experiments with Dinitrophenol-AMP-pretreated Mitochondria

It was shown previously (1) that a rapid depletion of the mitochondrial endogenous high energy phosphate could be obtained if mitochondria were incubated for a short period of time at 30° in the presence of dinitrophenol plus AMP. In the experiments summarized in Fig. 5 and Table III, suspensions of mitochondria in 0.25 M sucrose had been incubated in the presence of 0.1 mM dinitrophenol plus 1 mM AMP at 30° for 5 minutes, after which the suspensions were recentrifuged in the cold and washed with 0.25 M sucrose. When these mitochondria were incubated with succinate in the presence of Dicumarol (Fig. 5A), the respiration, as measured with the oxygen electrode, was relatively low and could be stimulated more than 2-fold by the addition of ATP. Similar to the case of the arsenate-pretreated mitochondria, the presence of Pi resulted in a high respiration provided that the phosphate was added before the addition of Dicumarol (Fig. 5A) but not when it was added after the addition of Dicumarol (Fig. 5B).

Data reported in Table III indicate that addition of a DPN-linked substrate, such as β-hydroxybutyrate (or glutamate), to the dinitrophenol-AMP-pretreated mitochondria considerably increased the rate of succinate oxidation beyond that obtained either by adding Pi before or ATP after the addition of Dicumarol. The presence of Pi was essential for observing this effect, which required, furthermore, that both Pi and succinate were present, together with the DPN-linked substrate, before the addition of Dicumarol. This effect was not due merely to a co-oxidation of the DPN-linked substrate with succinate, because it was not abolished to any larger extent by a concentration of Amytal which blocks the oxidation of DPN-linked substrates. These results indicate that in the dinitrophenol-AMP-pretreated system, intramitochondrially generated high energy phosphate may stimulate succinate oxidation more efficiently than does externally added ATP.

**Table III**

<table>
<thead>
<tr>
<th>Order of additions</th>
<th>Respiration</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
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<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td>Succinate, mitochondria, Dicumarol</td>
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<td>48</td>
<td></td>
</tr>
<tr>
<td>Succinate, mitochondria, Dicumarol, ATP</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Succinate, Pi, mitochondria, Dicumarol, β-Hydroxybutyrate</td>
<td>110</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>Succinate, P; mitochondria, Dicumarol, β-Hydroxybutyrate</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinate, β-Hydroxybutyrate, mitochondria, Dicumarol</td>
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<td>400</td>
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</tr>
<tr>
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<td>163</td>
<td></td>
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</tr>
<tr>
<td>Succinate, Pi, mitochondria, Amytal, Dicumarol</td>
<td></td>
<td>140</td>
<td></td>
</tr>
<tr>
<td>Succinate, β-Hydroxybutyrate, mitochondria, Dicumarol</td>
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<td>310</td>
<td></td>
</tr>
<tr>
<td>β-Hydroxybutyrate, mitochondria, Dicumarol, succinate</td>
<td>139</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Hydroxybutyrate, P; mitochondria, Dicumarol, Amytal, succinate</td>
<td>90</td>
<td></td>
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</table>

**DISCUSSION**

Concept of High Energy Phosphate Requirement for Mitochondrial Oxidation of Succinate—The findings of the present paper are consistent with the conclusion that the aerobic oxidation of succinate in liver mitochondria requires a supply of high energy

3 (Added in proof) A similar conclusion has recently been reached by Chance and Hagihara (13) in experiments with aged mitochondria from pigeon heart. Results of their experiments also confirmed some of our earlier data on the ATP-induced activation of succinate oxidation (3) and on the simultaneous reduction of mitochondrial DPN (8).
phosphate. The evidence supporting this conclusion may be summarized as follows:

1. Pretreatment of mitochondria with agents which were previously (1, 2) found to facilitate the depletion of endogenous high energy phosphate leads to a serious depression of the capacity of mitochondria to carry out the aerobic oxidation of succinate. This effect was independent of the nature of the agent used; pretreatment with arsenate, or with dinitrophenol plus AMP, gave similar results.

2. When the arsenate-induced depletion of mitochondrial high energy phosphate was prevented by the addition of Amytal or cysteine sulfinate (2), the capacity for succinate oxidation was preserved.

3. No depression of the succinate-oxidizing capacity was observed if the depleted mitochondria were allowed to regenerate their endogenous high energy phosphate.

4. Addition of ATP to the depleted system resulted in a substantial increase of capacity for succinate oxidation. This effect of ATP was not duplicated (a) by P₄, unless this was added under conditions which allowed a generation of high energy phosphate; (b) by AMP, this ruling out the possibility that adenine nucleotide rather than high energy phosphate was required for the restoration of the capacity for succinate oxidation; and (c) by ethylenediaminetetraacetate, this eliminating a conceivable chelating type of action, rather than that of supplying energy, as the basis for the observed ATP effect.

Lanes of Evidence Eliminating Oxaloacetate as Inhibitory Agent

In order to further fortify the above concept, it was necessary to exclude the possibility that the phenomenon studied here was due to the accumulation of an inhibitor during the preincubation of the mitochondria, removed subsequently by ATP. Of special importance in this connection was to eliminate oxaloacetate as responsible for the observed inhibition. This compound, which is known to be a competitive inhibitor of succinic dehydrogenase (14), has been implicated in several instances in the past when inhibitory effects on succinoxidase were encountered (15-18). Furthermore, it has been shown by Purdie and Potter (10) and by Tyler (20) that added ATP potently counteracts the inhibition of succinoxidase by oxaloacetate. In spite of these indications, however, it has been possible to establish, within the limits of available means, that the depression of the succinoxidase capacity, as studied in the present connections (cf. also (8)), was not due to an inhibition of succinic dehydrogenase by oxaloacetate. The arguments underlying this statement may be summarized as follows:

1. As concluded in “Results,” cysteine sulfinate was not able to remove the inhibition of succinate oxidation after preincubation with arsenate and Dicumarol.

2. It was reported elsewhere (8) that no oxaloacetate could be demonstrated in the arsenate-Dicumarol-preincubated system. The method used for this assay allowed the detection of as little as $5 \times 10^{-8}$ m oxaloacetate in the test system. It has been shown by Greengard et al. (21) that succinic dehydrogenase is inhibited no more than 29% by oxaloacetate if approximately 1000-fold excess of succinate is present. In the present case, an excess of succinate of 250,000- to 1.3 million-fold above the maximal possible concentration of oxaloacetate, $5 \times 10^{-8}$ m, was used. This should have relieved virtually all the inhibition even if allowance were made for the possibility that part of the oxaloacetate may be bound to the mitochondria and its concentration increased by compartmentation. There is no reason to believe that added succinate might not reach the mitochondrial compartment where the succinic dehydrogenase is located.

3. The possibility was also considered that ATP may stimulate succinate oxidation in the present system because of the removal of a trace amount of oxaloacetate by way of the oxaloacetate carboxylase reaction. However, it is known that this enzyme reacts faster with GTP and ITP than with ATP (22, 23). Therefore, if this mechanism were responsible for the stimulation of succinate oxidation by ATP, it should be expected that GTP or ITP should be more efficient in promoting this stimulation than ATP. This was clearly not the case. Moreover, direct analysis reported elsewhere (8) revealed the presence of no measurable amounts of phosphoenolpyruvate in the ATP-restored system; the lower limit of the assay was $5 \times 10^{-8}$ m.

Oxaloacetic Carboxylase as Potential Competitor with Succinooxidase—It was concluded in a previous paper (1) that the $\alpha$-keto glutarate-linked substrate level phosphorylation takes place in a special compartment within the mitochondrion, yielding ATP, which does not freely communicate with ATP originating from respiratory chain phosphorylations. Evidence was presented that a large part of the endogenous ATP found in isolated liver mitochondria may be located in this compartment. The removal of high energy phosphate from this compartment was proposed to proceed via a number of alternative mechanisms. One of these involved the oxaloacetic carboxylase reaction, whereas another mechanism was visualized to act via the activation of succinate oxidation, according to the concepts developed in the present paper. It was pointed out that both of these reactions may preferentially utilize high energy phosphate originating from the $\alpha$-keto glutarate-linked substrate level phosphorylation, the former because of its nucleoside specificity, and the latter in view of its close metabolic relation to the oxidation of $\alpha$-keto glutarate. These considerations thus raise the possibility that an actively operating oxaloacetic carboxylase reaction may constitute a potential competitor with the mitochondrial oxidation of succinate. This possibility is also stressed by the finding (Fig. 4A and 1) that the inhibition of succinate oxidation, after preincubation of mitochondria with arsenate and Dicumarol, could be prevented by cysteine sulfinate, that is, by preventing an active operation of the oxaloacetic carboxylase reaction. Further indication of such a competition is found in the data of Tyler (20), who concluded that ATP could remove the inhibition of succinate oxidation by added oxaloacetate in a kidney homogenate, without essentially lowering the concentration of oxaloacetate. Tyler (20) concluded that this effect of ATP could be due to a transformation of oxaloacetate into a chelated form which does not inhibit succinic dehydrogenase.

On the basis of the present hypothesis, it appears more probable that the added oxaloacetate in Tyler's system caused an efficient removal of ATP from the mitochondria by way of the oxaloacetic carboxylase reaction, thus suppressing the activation of succinate oxidation, and that the role of added ATP was to compensate for this loss. In general, it would appear that caution is
warranted in interpreting effects on succinate oxidation in mitochondria, observed in connection with addition, formation, or removal of oxaloacetate, exclusively on the basis of the classical type of competitive inhibition of succinic dehydrogenase by oxaloacetate.

Mechanism of Activation of Succinate Oxidation—Because succinate is known to be oxidized aerobically by different types of submitochondrial preparations without a requirement for high energy phosphate, it must be assumed that the activation phenomenon here observed is a reflection of an organised state of succinoxidase, present only above a certain level of structural integrity. This assumption is in line with the often stressed opinion (24–27) that the pathways, kinetics, or both, of the aerobic oxidation of succinate may be different in “phosphorylating” and “nonphosphorylating” succoxidase preparations. A transitional decrease of the succinoxidase capacity during aging of isolated liver mitochondria was reported earlier from this laboratory (9). This decrease coincided with the loss of the phosphorylating capacity and could be eliminated by the addition of ATP.

The concept developed in the present work would seem to indicate that one of the differences between “phosphorylating” and “nonphosphorylating” succoxidase systems may lie in the fact that whereas in the “nonphosphorylating” system, the aerobic oxidation of succinate proceeds over a number of electron carriers each of which possesses a higher potential than the preceding one, in the “phosphorylating” system, i.e. in the intact mitochondrion, the reaction is forced to proceed via a thermodynamically unfavorable stage involving an electron carrier (A), which for becoming reduced by electrons derived from succinate, requires an intervention of ATP. When this activated, reduced carrier then is reoxidized by the subsequent carrier (B) in the respiratory chain, the invested ATP may be regenerated. A simple way of visualizing such a mechanism may be:

\[
\text{Succinate} + A + \text{ATP} \rightarrow \text{fumarate} + \text{AH} \sim P + \text{ADP} \quad (1)
\]

\[
\text{AH} \sim P + B + \text{ADP} \rightarrow A + BH_2 + \text{ATP} \quad (2)
\]

That the “nonphosphorylating” succinoxidase system does not involve this mechanism may be explained by assuming that either in this system the electrons from succinate can reach B directly (due to the removal of a structural barrier which must be circumvented by way of A in the intact system), or that A is present in a chemically modified form with an increased oxidation-reduction potential.

The net result proceeding from Reactions 1 and 2 implies neither a loss nor a gain of high energy phosphate. The proposed activation mechanism is thus without implication regarding the generally held concept that the aerobic oxidation of succinate gives rise to two net phosphorylations. The fact that no similar activation mechanism appears to be involved in the oxidation of DPN-linked substrates indicates that the carrier A ought to be located before or at, but not beyond, the point where the electrons from succinate enter the terminal respiratory chain. Possible candidates for A would seem to be the diaphorase flavoprotein, a quinone, or a complex form of succinic dehydrogenase; and for B, cytochrome b.

Studies conducted in collaboration with Klingenberg (8) revealed that the activation of succinate oxidation by ATP in the high energy phosphate-depleted mitochondria was paralleled by a reduction of the mitochondrial pyridine nucleotides. This finding suggested that the high energy intermediate formed in the activation reaction was partly utilized for reducing DPN. With use of the present terminology, this reaction may be written as

\[
\text{AH} \sim P + \text{DPN} = A + \text{DPNH} + P_i
\]

which would thus represent an alternative reaction pathway for \(\text{AH} \sim I\) in relation to Reaction 2. It is visualized, in other words, that the reduced, phosphorylated electron carrier, generated at the expense of ATP in the activation of succinate oxidation, may be reoxidized, either by way of the terminal respiratory chain with the regeneration of ATP, or, alternatively, by DPN with the liberation of P_i, by way of a reversal of the DPN-flavin-linked oxidative phosphorylation.

It was assumed in the above discussion that the high energy intermediate, \(\text{AH} \sim P,\) is a phosphorylated compound, and not, as, also might be conceivable, a compound of the type \(\text{AH} \sim I\) or \(X \sim I\) (cf. (5)). The basis for this assumption was the observation that the activation of succinate oxidation by ATP, as well as the accompanying reduction of the mitochondrial pyridine nucleotides (8), could clearly take place in the presence of dinitrophenol or Dicumarol, at concentrations of these agents which are known to fully uncouple respiratory chain phosphorylation. A mechanism involving intermediates of the \(\text{AH} \sim P\) type, which allows energy from ATP to reach the level of the respiratory chain in the presence of uncoupling agents, has been proposed from this laboratory (28, 29); no similar mechanism involving intermediates of the \(\text{AH} \sim I\) or \(X \sim I\) type has yet been suggested.

SUMMARY

Preincubation of rat liver mitochondria in a sucrose-KCl medium in the presence of 2 to 3 mM arsenate and 0.06 mM Dicumarol or 0.1 mM 2,4-dinitrophenol for 3 to 4 minutes results in a marked depression of the succinoxidase capacity. No depression is found when the preincubation is made in the presence of Amytal, cysteine sulfinate, or inorganic phosphate. Addition of adenosine triphosphate after the preincubation stimulates succinate oxidation several-fold. The effect of adenosine triphosphate is not duplicated by cysteine sulfinate, inorganic phosphate, ethylenediaminetetraacetate, adenosine 5'-phosphate, cytidine triphosphate, uridine triphosphate, inosine triphosphate, or guanidine triphosphate. Similar results are obtained with mitochondria pretreated with dinitrophenol and adenosine 5'-phosphate.

The data are consistent with the conclusion that conditions leading to a depletion of the endogenous content of mitochondrial high energy phosphate result in a reversible depression of the succinoxidase capacity.

The concept is developed that the aerobic oxidation of succinate in intact liver mitochondria requires an activation by high energy phosphate. Some implications of this concept regarding the enzymic organization of mitochondrial electron transport and oxidative phosphorylation are discussed.

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