Regulation of Succinate Dehydrogenase in Higher Plants

I. SOME GENERAL CHARACTERISTICS OF THE MEMBRANE-BOUND ENZYME

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ABSTRACT

The spectrophotometric phenazine methosulfate assay of succinate dehydrogenase was adapted to use with cauliflower (Brassica oleracea) and mung bean (Phaseolus aureus) mitochondria with suitable modifications to overcome the permeability barrier to the dye. Procedures in the literature for the isolation and sonic disruption of mitochondria from these sources were modified to assure maximal yield and stability of the enzyme. In tightly coupled mung bean mitochondria, as isolated, about half of the succinate dehydrogenase is in the deactivated state, and the enzyme is further extensively deactivated on sonication or freeze-thawing. In cauliflower mitochondria most of the enzyme is in the deactivated form, and little or no further deactivation occurs on sonication or freeze-thawing. Incubation of mitochondria from either source with succinate leads to full activation of the enzyme. The energy of activation for the conversion of the deactivated to the activated form in membrane preparations under the influence of substrate is about 30,000 cal/mole, essentially the same value as in animal tissues. Activation of the enzyme also occurs under the influence of a variety of other agents, among which the action of anions as activators is documented in the present paper. Activation is accompanied by the release of very tightly bound oxaloacetate. As in animal tissues, the enzyme appears to contain covalently bound flavin (histidyl 8a-FAD), and the turnover number is 19,400 moles of succinate oxidized/mole of histidyl flavin at pH 7.5, 38 C.

While the known flavoproteins of the mammalian respiratory chain were isolated and characterized many years ago, characterization of the flavoproteins of mitochondria of higher plants has only recently begun (28). To the authors’ knowledge only one attempt to purify a respiratory chain-linked flavoprotein (succinate dehydrogenase) from higher plants has been reported (6). Because of the recognized importance of succinate dehydrogenase in mitochondrial energy generation and even in the adaptation of organisms to varying conditions of O2 supply (20), it was decided to explore to nature of this enzyme in representative higher plants, particularly in regard to its regulatory properties. The present paper is concerned with the assay of the membrane-bound enzyme in plant material, the demonstration that it contains covalently bound flavin as in animal tissues (8) and yeast (26), and its activation by substrates. The following paper (16) represents a detailed study of the activation of the plant enzyme by a variety of agents. Future reports will be concerned with the isolation and further characterization of succinate and NADH dehydrogenases from higher plant mitochondria.

The properties of succinate dehydrogenase vary with the physiological role of the enzyme (20). In all obligate aerobes, where the enzyme is part of the terminal respiratory apparatus, the kinetic properties of the enzyme are such as to permit the rapid oxidation of succinate to fumarate, it contains covalently bound flavin (histidyl-8a-FAD) as the prosthetic group, and is closely regulated, undergoing rapid and reversible conversion from the deactivated to the activated form under the influence of a variety of modulators (22–24).

Activation of mammalian succinate dehydrogenase by substrates and competitive inhibitors (8) involves the rapid conversion of the enzyme from a form of low (or no) activity to one of high activity and is characterized by a very high activation energy (33,000–35,000 cal/mole) and entropy change, suggesting that a conformational change is involved. The enzyme reverts rapidly to the deactivated state upon removal of the activator (12). This activation has been demonstrated in highly purified, soluble preparations from many tissues, in submitochondrial particles, and in intact mitochondria.

Besides substrates, the enzyme is also activated in membrane or mitochondrial preparations by CoQH2 or by any substrate which reduces internal CoQ0 (such as NADH, NAD-linked substrates, and α-glycerophosphate) (4, 5). The enzyme

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3 Predoctoral Fellow of the Organization of American States, on leave of absence from the University of Chile.

ABBREVIATIONS: CoQ0 and CoQH2: coenzyme Q and its reduced form; DCIP: 2,6-dichlorophenolindophenol; DNP: 2,4-dinitrophenol; DDT: dithiothreitol; ETP: electron transport particle, a nonphosphorylating preparation of the inner mitochondrial membrane; PMS: phenazine methosulfate; NEM: N-ethylmaleimide.
thus activated appears to be identical with the succinate-activated form. It has been observed (4) that in intact mitochondria succinate dehydrogenase activity is high in state 4 but low in state 3, in accord with the fact that in state 4 \(\rightarrow\) 3 transition the oxidation reduction ratio CoQ\(_{10}\)/CoQ\(_{10}\)H\(_2\) rises to a very large extent (13). This explains observations in the literature (18, 29) that in state 3 succinate accumulates and the labeling of malate by \(^{14}C\)-succinate is low, whereas in state 4 the reverse is true. In accord with the fact that uncouplers give rise to rapid oxidation of CoQ\(_{10}\)H\(_2\) (13), they cause extensive deactivation of succinate dehydrogenase in tightly coupled mitochondria (4).

A third means of activation in animal tissues is by ATP (4). The effect has been observed at as low as 6 \(\mu\)M concentration of ATP and is restricted to intact mitochondria. Efforts to demonstrate that the ATP effect involves removal of inhibitory oxaloacetate have given negative results (4). The ATP effect is not mediated by the phosphorylation system, since oligomycin and DNP do not interfere with it.

In addition to these three types of activation, which appear to function in intact mitochondria (4, 24), in submitchondrial particles and in soluble preparations IDP, ITP (but not a series of other nucleotides tested [5, 23]), as well as certain inorganic anions at relatively high concentrations (Cl\(_{-}\), Br\(_{-}\), I\(_{-}\), NO\(_{3}\^-,\)SO\(_4^{2-}\), CI\(_{O}\)\(^ {2-}\)) strongly activate the enzyme (10). Conversion of the inactive to the active enzyme under the influence of anions is much more extensive at pH values below pH 6 than at neutral or alkaline pH. In fact, at any fixed concentration of a given anion, the ratio of deactivated to activated enzyme is strictly a function of pH, with acid pH favoring the active conformation and alkaline pH the deactivated one, in a reversible manner. In submitchondrial particles (e.g. ETP) extensive interconversion of the deactivated and activated enzyme occurs under the influence of pH alone, without added anions, suggesting that the ionization of a critical residue is essential for the process (10). The energy of activation for the pH and anion-catalyzed activation is materially lower than for activation by substrates or CoQ\(_{10}\)H\(_2\), but still high enough to be compatible with a conformation change (10).

The soluble enzyme from animal tissues, as isolated, carries with it a complement of oxaloacetate (9). The oxaloacetate is extremely firmly bound and is not removed by the action of malate dehydrogenase and NADH. It is released, however, on precipitation of the protein with perchloric acid. When the enzyme is activated by incubation with NaBr at pH 6, oxaloacetate is released from the enzyme concurrently, but only at temperatures where activation occurs. The energy of activation for oxaloacetate release is of the same order (18,000 cal/mole) as found for activation of the enzyme under these conditions. The amount of oxaloacetate released from the enzyme preparations is quite consistently in the ratio of about 1 mole/2 moles of covalently bound flavin in soluble preparations and 1 to 1 in membrane preparations.

Although oxaloacetate release has been found (9) to accompany activation by substrates, anions, and CoQ\(_{10}\)H\(_2\) and thus may play a major role in the conversion of the deactivated to the activated form of the enzyme, it is not the only mechanism of activation. The oxaloacetate-free, fully active enzyme is also rapidly deactivated by adjustment to alkaline pH (pH approximately 9) is reactivated at neutral or acid pH (9). There is also evidence that activation involves the appearance of a reactive —SH group in the enzyme (17). Current hypotheses of the physiological purpose of the activation of the enzyme are summarized elsewhere (24).

**MATERIALS AND METHODS**

Cauliflower (Brassica oleracea L.) mitochondria were prepared by the method of Lance (14), except for the following modifications. The upper 3 to 4 mm layer of the flowers was homogenized in a medium (300 ml/200 g fresh weight) containing 0.2 M HEPES buffer, 0.5 M sucrose, 1 mM EDTA, 0.75 mg/ml bovine serum albumin, and 4 mM cysteine, the final solution being adjusted to pH 7.5 at 20 C. Homogenization was for 60 sec in a Waring Blender controlled by an external voltage regulator at 60 v. Adjustment of pH following homogenization was omitted. The second centrifugation was at 20,000 xg for 15 min. The mitochondrial pellet was resuspended for washing in the medium specified above, less cysteine. The final pellet was resuspended in 2 ml of 0.3 M mannitol, containing 1 mM cysteine, at pH 7.5, per 200 g of cauliflower used. The inclusion of cysteine at this stage was essential for preservation of activity.

Cauliflower was obtained from local markets, as fresh as possible, and used within 1 day. Cauliflower collected during summer months was found to have low succinate dehydrogenase activity, and the enzyme from such starting material was difficult to deactivate. Hence, all the work reported here was performed with cauliflower obtained during the cool period of the year.

Mung bean (Phaseolus aureus L. var. Jumbo) seedlings were grown, harvested, and the mitochondria isolated by the procedure of Ikuma and Bonner (7). The quality of the mitochondria was controlled by measurements of the P/O ratios and of respiratory control with succinate, malate, and NADH as substrates; the values were comparable to those reported by Ikuma and Bonner (7). With cauliflower mitochondria these results were more variable. The respiratory control with succinate varied from 1.95 to 3.2 in typical experiments.

Sonication of the mitochondria was performed with a Branson Model S 75 sonifier, using a microprobe, at 5 amps for two 15-sec periods at 3 C, with cooling between. The suspension was externally cooled with an ice-salt bath during the procedure. Prior to sonication, the mitochondria were diluted with 2 volumes of 0.3 M mannitol — 1 mM cysteine, pH 7.5, to give 8 to 10 mg of protein/ml in the case of cauliflower mitochondria. Mung bean mitochondria were diluted with 2 volumes of 0.3 M mannitol — 0.1 M EDTA — 0.1% (w/v) bovine serum albumin (suspension adjusted to pH 7.2 at 0 C), to give about 6 mg of protein/ml. All chemicals used were reagent or analytical reagent grade.

Covalently bound flavin (histidyl flavin) was determined by the procedure of Singer et al. (27), using a Farrand model A-2 fluorometer. Since plant mitochondria tend to give gummy precipitates on treatment with acid-acetone by this method, from which extraction of noncovalent flavin by trichloroacetic acid is difficult, the procedure was modified as follows. The sonicated mitochondria, after dilution with an equal volume of water, were heated for 3 min at 100 C. After cooling, the suspension was repeatedly precipitated with 1% (w/v) trichloroacetic acid and centrifuged, until the supernatant solutions were free from fluorescent material (six to eight centrifugations). The pellet was then washed with acid-acetone (6 ml of acetone + 0.16 ml of 6 N HCl), and the residue was washed twice more by resuspension in 1% (w/v) trichloroacetic acid and centrifugation. The proteolytic digests contained considerable amounts of nonflavin, fluorescent material (i.e. not reduced by dithionite), which interfered with the analysis when the standard riboflavin filters were used. This blank fluorescence was minimized by the substitution of Farrand No. 7-37 filter as the primary and 3-69 as the secondary filter. (Alternatively, the Farrand 450 nm interference filter could be used as the primary and 540 nm filter as the secondary.)

Oxaloacetate was determined fluorometrically with malate dehydrogenase and NADH (30) in perchloric acid extracts of the particles, using the Hitachi-Perkin Elmer MPF-3 spectro.
fluorometer. The determination of succinate dehydrogenase activity is described under "Results." Protein was determined, after precipitation with trichloroacetic acid, by the biuret procedure (15) in cauliflower and by the Lowry method (15) in mung bean preparations.

RESULTS AND DISCUSSION

Determination of Succinate Dehydrogenase Activity. In Hiatt's studies (6) succinate dehydrogenase from higher plants was assayed by the manometric PMS method (11) and by a spectrophotometric adaptation in which the reoxidation of the dye was coupled to the reduction of DCIP, using fixed concentrations of both PMS and DCIP. It is known that in the former method the reoxidation of reduced PMS by O$_2$ may become rate-limiting (1), while the reliability of the spectrophotometric assay at fixed PMS concentration is limited by the fact that the apparent Km for PMS concentration in double reciprocal plots is appreciable for cauliflower mitochondria. It has been ascertained, however, that the apparent Km for PMS does not change on activation or deactivation of the membrane-bound enzyme. Therefore, in the studies reported in the present and following (16) paper the highest PMS concentration shown in Figure 1 (0.3 ml 0.33% (w/v) dye/3 ml reaction mixture) was routinely used, unless otherwise noted.

A special problem in the assay of the dehydrogenase from both plant sources studied is the permeability barrier to PMS in intact mitochondria. While in mammalian mitochondrial pre-treatment of the mitochondria with Ca$^{2+}$ or with snake venom phospholipase A easily overcomes this permeability barrier (25), with cauliflower and mung bean mitochondria neither agent alone nor a combination of the two permitted free penetration of the dye. Thus the apparent succinate dehydrogenase activity did not increase after brief incubation with 0.75 mM Ca$^{2+}$ and 1 mg of Naja naja venom/ml, but freeze-thawing and sonication often increased the apparent activity significantly. The best way to assure that the full activity of the dehydrogenase is measured, is to subject the mitochondria to two cycles of 15-sec sonication, as described under "Materials and Methods." Further sonication or freeze-thawing does not increase the measured activity. That the full activity of the dehydrogenase is assayed after such treatment is indicated by the fact that in sonicated cauliflower mitochondria the turnover number of the dehydrogenase is the same as in mammalian preparations.

Since the enzyme from cauliflower mitochondria is very unstable in the absence of thiols, so that extensive decay is observed even during the 8-min activation period at 30 C unless cysteine or another thiol is present, the preparations were suspended in 1 mM cysteine. The presence of cysteine causes non-enzymatic reduction of both PMS and DCIP and resulting high blanks, however. In order to minimize this interference, 0.01 ml of 0.03 M NEM was added to the assay mixture just prior to the 3-min temperature equilibration preceding the assay. This allowed sufficient time for the combination of cysteine with NEM but not enough time to cause measurable inhibition of succinate dehydrogenase, an SH enzyme (6).

In practice the assays were carried out as follows. A series of spectrophotometer cuvettes were prepared to permit varying the PMS concentration and thus the determination of V$_{max}$ (PMS). Each cuvette contained 0.5 ml of 0.2 M HEPES buffer, pH 7.5, enzyme, 0.3 ml of 10 mM KCN, and 0.01 ml of 0.03 M NEM, and water to give a final volume of 3 ml during assay.

The cuvettes were covered and preincubated for 3 min in a water bath at the assay temperature of 30 C in routine work with the fully activated enzyme, and 15 C in studies of the activation. After the temperature equilibration the cuvettes were transferred to the cell compartment of a recording spectrophotometer, thermostated at the assay temperature. In rapid successions, 0.3 ml of 0.2 M succinate, 0.1 ml of 0.05% (w/v) DCIP, and PMS were added, and the absorbance decline at 600 nm was recorded. Activity was then calculated using the millimolar extinction coefficient for DCIP of 19.1.

Succinate is added just prior to initiation of the assay to prevent activation during temperature equilibration. With fully activated samples it may be added earlier. With each series of experiments a blank was run to correct for spurious dye reduction by performing the assay under identical conditions, except for the omission of succinate. The resulting rate was then subtracted from the experimental value at each PMS concentration.

In accurate work, the amount of PMS used is varied between 0.04 and 0.3 ml of 0.33% (w/v) of PMS per 3 ml reaction mixture, and the results are calculated for double reciprocal plots, as in Figure 1. As noted above, in routine work on activation, 0.3 ml of 0.33% PMS solution gives a satisfactory measure of the activity. The conditions required for full activation are detailed below.

Activation by Substrates. Previous studies by Hiatt (6) on soluble succinate dehydrogenase from Phaseolus vulgaris demonstrated a 2-fold activation of the enzyme on incubation with 0.1 mM succinate at 30 C. The kinetics of the activation and the activation energy for the process were not determined, however. Figure 2 illustrates the kinetics of the activation of succinate dehydrogenase in sonicated cauliflower mitochondria by 0.1 mM succinate at 29 C. The process followed first order kinetics, and the activation energy (Fig. 3) in three experiments ranged from 29,700 to 31,600 cal/mole (average = 30,600 cal/mole), in fair agreement with the figures reported (5, 8) for animal tissues (33,000–35,000 cal/mole).

The time required for full activation by succinate at 30 C depends both on the concentration of the particles and of the substrate present during activation. In batch activation with both cauliflower and mung bean particles in the presence of 0.1 mM succinate 10 to 12 min at 30 C sufficed under the conditions specified in the legend of Figure 3, provided that the protein concentration was below 8 mg/ml. At concentrations in excess of 10 mg/ml a 15-min activation period was necessary. A catalytic concentration of the enzyme, as used in the PMS-DCIP assay, 8 min at 30 C in the presence of approximately 20 mM succinate gave full activation. The technique routinely used in these cases was incubation of the complete reaction mixture, less dyes and NEM, for 8 min at 30 C, transfer of the

![Fig. 1. Succinate dehydrogenase assay with PMS and DCIP in cauliflower mitochondria. Standard assay conditions; temperature, 20 C. Fresh cauliflower mitochondria (9 mg/ml of protein); 50 ml aliquots per assay, preactivated with succinate. Abscissa, reciprocal volume (ml) of 0.33% (w/v) PMS; ordinate, reciprocal absorbance change/min.

5 10 15 20

1/ [PMS]
cuvette to a 15 C bath, addition of NEM, and, after 3-min temperature equilibration, addition of PMS and DCIP to initiate the catalytic reaction. Samples so treated served to determine the maximal level of activation throughout this study as controls for the degree of activation reached with activators other than succinate or in studies of the kinetics of the activation and are referred to as "activated in assay."

**Fig. 2.** Kinetics of activation by 0.1 mM succinate at 29 C. Sonicated cauliflower mitochondria (0.6 ml, containing 4.9 mg of protein) plus 0.11 ml of 0.2 M HEPES, pH 7.5, were brought to 29 C, then 0.01 ml of 0.1 M KCN and 0.08 ml of 1 M Na succinate, pH 7.5, were added. Aliquots of 40 μl were removed at the times shown and placed in cuvettes at 15 C, containing the assay mixture less succinate and dyes. After 30 sec 0.3 ml of 0.2 M succinate and the dyes were added to initiate the enzymatic reaction. Activity is expressed on the abscissa as absorbance change at 600 nm/min/40 μl aliquot at 15 C. The specific activity of the preparation used, measured with 0.3 ml of 0.33% (w/v) PMS per 5 ml, was 0.062 μmole succinate oxidized/min·mg at 15 C.

**Fig. 3.** Arrhenius plot for the activation of succinate dehydrogenase in sonicated cauliflower mitochondria. Experimental conditions were as in Fig. 2. The first order velocity constant for activation by 0.1 mM succinate, shown on the ordinate, was derived from semilog plots of the progress of activation at various temperatures.

### Table I. State of Activation of Succinate Dehydrogenase in Fresh, Sonicated, and Frozen-Thawed Plant Mitochondria

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Specific Activity</th>
<th>Degree of Activation %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mung bean mitochondria, fresh</td>
<td>0.057</td>
<td>63</td>
</tr>
<tr>
<td>Sonicated</td>
<td>0.022</td>
<td>21</td>
</tr>
<tr>
<td>Mung bean mitochondria, fresh</td>
<td>0.054</td>
<td>58</td>
</tr>
<tr>
<td>Sonicated</td>
<td>0.037</td>
<td>34</td>
</tr>
<tr>
<td>Frozen overnight</td>
<td>0.014</td>
<td>15</td>
</tr>
<tr>
<td>Mung bean mitochondria, fresh</td>
<td>0.055</td>
<td>61</td>
</tr>
<tr>
<td>Sonicated</td>
<td>0.050</td>
<td>30</td>
</tr>
<tr>
<td>Frozen overnight</td>
<td>0.020</td>
<td>15</td>
</tr>
<tr>
<td>Cauliflower mitochondria, fresh</td>
<td>0.023</td>
<td>29</td>
</tr>
<tr>
<td>Sonicated</td>
<td>0.024</td>
<td>25</td>
</tr>
<tr>
<td>Frozen overnight</td>
<td>0.026</td>
<td>27</td>
</tr>
<tr>
<td>Cauliflower mitochondria, fresh</td>
<td>0.024</td>
<td>20</td>
</tr>
<tr>
<td>Sonicated</td>
<td>0.017</td>
<td>14</td>
</tr>
<tr>
<td>Frozen overnight</td>
<td>0.029</td>
<td>22</td>
</tr>
<tr>
<td>Cauliflower mitochondria, fresh</td>
<td>0.003</td>
<td>2</td>
</tr>
</tbody>
</table>

1 Cauliflower purchased in Portugal.

**Relation of Activation to Tightly Bound Oxaloacetate.** Table I presents some representative results on the degree of activation of succinate dehydrogenase in freshly isolated mitochondria, in sonicated samples, and preparations kept frozen overnight. In mung bean mitochondria, some 60% of the enzyme is in the activated state immediately after isolation, but sonication increases the extent of deactivation, while in sonicated and frozen-thawed mitochondria only 15 to 20% is in the activated form. In most preparations of freshly isolated cauliflower mitochondria, on the other hand, 20% or less of the enzyme was in activated form but little additional deactivation occurred on sonication or freeze-thawing. In mitochondria isolated from cauliflower available at markets in Portugal, succinate dehydrogenase was even more extensively deactivated: in eight preparations isolated, the degree of activation ranged from 2.5 to 14%, the average less than 10% activated, as shown in the last line of Table I.

Regardless of prior treatment, succinate dehydrogenase was always fully activated by succinate under the conditions given in the previous section. Besides succinate, all the agents known to activate the mammalian enzyme gave complete or nearly complete activation of the enzyme in cauliflower and mung bean particles, as documented in the next paper (16). It was of interest to examine whether that fraction of the plant enzyme which is isolated in the deactivated state contains tightly bound oxaloacetate and whether activation at elevated temperatures involves displacement and dissociation of this oxaloacetate, as has been described recently for the mammalian enzyme (9).

Activation by monovalent anions is particularly useful in following the release of tightly bound oxaloacetate during activation, since the conditions of activation are relatively mild and...
activation comes to completion within a few min at 30 C (Table II). As shown in the table, incubation of cauliflower particles with 0.2 m Br- at pH 6.0 and 30 C gave 90% activation in about 10 min. While the particles contained about 0.06 nmol of oxaloacetate per mg of protein before activation, no oxaloacetate could be detected in the activated and washed preparation. In another similar experiment, conducted with another preparation, the particles (24% activated) were found to contain 0.07 nmol oxaloacetate prior to incubation with Br- and none could be detected after activation and washing by centrifugation.

It is admittedly difficult to conclude from these experiments alone that the oxaloacetate detected in extensively deactivated particles is all associated with succinate dehydrogenase. Ideally, such experiments should be conducted with highly purified or homogeneous preparations of the plant enzyme, which are not yet available, or it should be shown that the tightly bound oxaloacetate is present in one to one proportion to the succinate dehydrogenase content in submitochondrial particles, as is the case with beef heart ETP preparations (9). Although the succinate dehydrogenase content of plant mitochondria may be determined with reasonable accuracy by analysis for histidyl flavin (cf. next section), the oxaloacetate determination in plant particles is too inaccurate because of interfering background fluorescence to permit the calculation of oxaloacetate to succinate dehydrogenase ratios. Nevertheless, from analogy with the mammalian enzyme, in which the relation of oxaloacetate to the activation has been thoroughly explored (9, 24), the observation that plant mitochondria contain tightly bound oxaloacetate which is set free only under conditions of activation suggests that in higher plants also tightly bound oxaloacetate may be associated with the deactivated form of the enzyme. This oxaloacetate may originate either by the action of malate dehydrogenase or from the combined action of succinate dehydrogenase and fumarase on succinate, since succinate dehydrogenase itself oxidizes both L- and d-malate (3).

**Turnover Number.** The availability of a reliable assay method and of a procedure for determining chemically the succinate dehydrogenase content of preparations, which is independent of the state of activation of the enzyme, permits the determination of the turnover number of the enzyme even in crude, particulate preparations. In order to allow comparison with the mammalian enzyme, activity was determined at 38 C. Table III shows that the turnover number of two preparations at pH 7.5, 38 C, at Vmax with respect to PMS, averaged 19,400 ± 2,000, within experimental error the same value (18,000 ± 1,000), as has been reported for intact mitochondria and submitochondrial particles of beef heart from two laboratories (2, 19).

### Table II. Activation of Succinate Dehydrogenase and Release of Tightly Bound Oxaloacetate by Br-

Soniacated cauliflower mitochondria (3.3 ml), repeatedly washed by centrifugation, containing 13.9 mg of protein/ml, were diluted at 0 C with 0.66 ml of 0.4 m 2-(N-morpholino)ethanesulfonic acid buffer, pH 6.0 at 30 C and 0.99 ml of 1 m NaBr and were then incubated at 30 C, 15-µl aliquots were withdrawn and assayed for succinate dehydrogenase activity at 15 C. The specific activity of the fully activated enzyme was 0.068 µmole of succinate oxidized/min/mg at fixed PMS concentration. After 18 min incubation, the sample was chilled to 0, centrifuged for 30 min at 144,000g; the residue, resuspended in 3 ml of 50 mm HEPES buffer, pH 7.5, was centrifuged as above, and the resulting pellet was resuspended in 2.2 ml of 50 mm HEPES buffer, pH 7.5, and precipitated by the addition of 0.14 ml of 70% (w/v) perchloric acid/ml. After centrifuging off the precipitate, the supernatant, containing the oxaloacetate liberated by perchloric acid, was neutralized with 6 µ KOH, clarified by centrifugation, and the oxaloacetate content was determined fluorometrically. The oxaloacetate content of the unactivated enzyme was determined in another sample similarly treated throughout, except that Br- addition was omitted.

<table>
<thead>
<tr>
<th>Time at 30 C</th>
<th>Activity¹</th>
<th>% Maximal Activation</th>
<th>Oxaloacetate Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.025</td>
<td>21</td>
<td>0.06</td>
</tr>
<tr>
<td>5</td>
<td>0.086</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.101</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>0.107</td>
<td>89</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>0.107</td>
<td>89</td>
<td>0</td>
</tr>
</tbody>
</table>

¹ Expressed as ΔA600 nm per min per 15 µl incubation mixture at 15 C. Value for succinate activated enzyme, taken as 100%, was 0.121.

### Table III. Determination of the Turnover Number of Succinate Dehydrogenase in Cauliflower Mitochondria

Soniacated cauliflower mitochondria were batch-activated with 0.1 m succinate for 12 min at 30 C. This preparation was then diluted to 0 C, and aliquots were assayed for succinate dehydrogenase activity, as described in "Materials and Methods," except that the temperature was 38 C. Histidyl flavin content was determined fluorometrically.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Specific Activity¹</th>
<th>Histidyl-Flavin Content</th>
<th>Turnover No.²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.348</td>
<td>0.020</td>
<td>17,400</td>
</tr>
<tr>
<td>2</td>
<td>0.397</td>
<td>0.0186</td>
<td>21,300</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>19,400</td>
</tr>
</tbody>
</table>

¹ Micromoles of succinate oxidized/min/mg of protein at 38 C, pH 7.5 (Vmax).
² Moles of succinate oxidized/min/mole of histidyl flavin, 38 C, pH 7.5, Vmax (PMS).

### Literature Cited