OXIDATION OF REDUCED DIPHOSPHOPYRIDINE NUCLEOTIDE BY MITOCHONDRIA FROM NORMAL & CROWN-GALL TISSUE CULTURES OF TOMATO

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Respiration, both in higher plants and animals, is generally assumed to be mediated by a series of electron carriers, including the pyridine nucleotides, flavoproteins, and cytochromes. Many of the electron carriers and catalytic proteins for electron transport from reduced diphosphopyridine nucleotide (DPNH), reduced triphosphopyridine nucleotide (TPNH) or succinate to oxygen have been demonstrated in particulate preparations from potato tubers (7), wheat roots (14), pea seedlings (6, 19), avocado fruits (3), silver beet petiole (15), lupine seedlings (12), Aroid spadix (4, 5, 8, 17), and tobacco roots (18).

With mammalian mitochondria the ability of the enzymes of the particles to react with cytochrome c and DPNH in solution is dependent upon the toxicity of the suspending medium since there is a permeability barrier to these substances (13, 21). Humphreys and Conn (12) have found with lupine mitochondria that the rate of oxidation of DPNH and the effect of cytochrome c on the DPNH oxidation differ in media with and without added sucrose. Although there have been several indirect observations of this nature (10, 17) the toxicity problem has not been considered fully in many other experiments on DPNH oxidation by plant mitochondria.

The purpose of the present study was to examine spectrophotometrically the following electron transfers catalyzed by mitochondria isolated from normal and crown-gall tissue cultures of tomato: from DPNH to cytochrome c (DPNH-cytochrome c reductase activity), to 2,6-dichlorophenol indophenol (diaphorase activity) and to oxygen (DPNH oxidase activity), and from reduced cytochrome c to oxygen (cytochrome c oxidase activity). Special emphasis was put on the effect of toxicity of the reaction medium on these systems.

MATERIALS & METHODS

PREPARING MITOCHONDRIA: The tomato tissue cultures of normal and crown-gall origin were like those used in the previous experiments (23, 24). Tissue cultures originally derived from normal tissue are called normal, and those from crown-gall tissue are called crown gall. Mitochondria were prepared from 3-week old tissues as described before (23, 24). Dihydrogen potassium phosphate (KH₂PO₄) in the grinding medium was replaced by 0.02 M potassium phosphate buffer, pH 7.4, and the adjustment of pH with tris was omitted.

ENZYMATIC ASSAYS: All assays were performed with a Beckman DU spectrophotometer in cuvettes of 1 cm path at room temperature (24°C). The final volume of each reaction mixture was 3.0 ml. Each enzymatic activity was assayed at two or more mitochondrial concentrations. For convenience, the components of these mixtures are presented with the results of experiments. The initial reading was taken 30 seconds after a reaction was started, and subsequent readings were recorded at 30 second intervals for several minutes. Appropriate blanks were used to adjust the instrument for each reading.

Molar extinction coefficients used were 6.22 × 10⁶ cm²/mole for DPNH (11), and 1.91 × 10⁶ cm²/mole for 2,6-dichlorophenol indophenol (1). 1.91 × 10⁶ cm²/mole was used as the difference between the coefficients of reduced and oxidized cytochrome c for the determination of reduced cytochrome c concentration (25).

RESULTS

The mitochondria from normal and crown-gall tissue cultures behaved similarly in the following enzymatic experiments. Only the results with mitochondria from crown-gall tissue cultures are presented as examples of the reactions. The comparison of activities between normal and crown-gall preparations is discussed in the latter part of this section.

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TABLE I

<table>
<thead>
<tr>
<th>CONDITION*</th>
<th>% ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>100**</td>
</tr>
<tr>
<td>Without EDTA</td>
<td>93</td>
</tr>
<tr>
<td>Without MgCl₂</td>
<td>100</td>
</tr>
<tr>
<td>Without mannitol</td>
<td>22</td>
</tr>
<tr>
<td>Phosphate buffer only</td>
<td>24</td>
</tr>
<tr>
<td>With KCN</td>
<td>5</td>
</tr>
<tr>
<td>With Antimycin A</td>
<td>7</td>
</tr>
<tr>
<td>With ascorbic acid</td>
<td>102</td>
</tr>
</tbody>
</table>

* Complete reaction mixture contained $5 \times 10^{-5} \text{ M}$ DPNH, $5 \times 10^{-5} \text{ M} \text{ MgCl}_2$, $5 \times 10^{-4} \text{ M}$ EDTA, $2 \times 10^{-2} \text{ M}$ phosphate buffer, pH 7.4, 0.4 m mannitol, and 0.05 ml (6 $\mu$g N) mitochondrial suspension in a total volume of 3.0 ml. KCN, $2 \times 10^{-4} \text{ M}$; Antimycin A, 0.2 $\mu$g/ml; ascorbic acid, $5 \times 10^{-4} \text{ M}$.

** This corresponds to 1.1 mmoles DPNH oxidized/ min/mg N.

DPNH OXIDASE: The oxidation of DPNH by mitochondria was measured by the rate of decrease in optical density at 340 mμ under the conditions as described in table I. The reaction proceeded at a uniform rate for several minutes. The effect of each component of the reaction medium and inhibitors on DPNH oxidase activity is shown in table I. Omitting MgCl₂ or ethylenediamine tetraacetic acid (EDTA) from the reaction mixture had no appreciable effect on the rate of oxidation, whereas omitting mannitol reduced the oxidation rate to 22%. The addition of ascorbate ($5 \times 10^{-4} \text{ M}$) had no effect on the oxidation. Cyanide ($2 \times 10^{-4} \text{ M}$) or Antimycin A (0.2 $\mu$g/ml) inhibited the DPNH oxidation by 95% and 93%, respectively.

Effect of Sucrose, Mannitol, & Cytochrome c on DPNH Oxidation: The rates of DPNH oxidation at various concentrations of sucrose and mannitol with and without cytochrome c are shown in figures 1 and 2. Without added cytochrome c, the reaction rate increased with increasing amounts of sucrose or mannitol up to 0.2 m. Beyond this, the rate decreased in the sucrose mixture, whereas the maximum rate was maintained up to 0.8 m in the mannitol mixture. In the presence of cytochrome c, the oxidation rate was practically constant at the highest level at all concentrations of sucrose or mannitol.

Effect of pH on DPNH Oxidation: The effect of pH on DPNH oxidation at the optimum concentration of sucrose (0.2 m) or mannitol (0.4 m) is shown in figure 3. Sucrose and mannitol reaction mixtures gave virtually identical curves: the rate of

![Figure 1](image1.png)

**Fig. 1.** DPNH oxidase activity of mitochondria from crown-gall tomato tissue cultures. Sucrose reaction mixture. Complete reaction mixtures were the same as in table I.

![Figure 2](image2.png)

**Fig. 2.** Effect of cytochrome c ($2 \times 10^{-3} \text{ M}$) on DPNH oxidation by mitochondria from crown-gall tomato tissue cultures. Mannitol reaction mixture. All other components were as in figure 1. O.D./min. = change in optical density per minute.
oxidation was practically unchanged at the highest level between pH 6.8 and 7.7.

DPNH-Cytochrome c Reductase: The reduction of cytochrome c was measured by following the increase in optical density at 550 mμ. Under the conditions of the test, as given in table II, the reaction showed apparent first order kinetics with respect to DPN. The reaction rate, expressed as the first order velocity constant, decreased to one-third when mannitol (0.4 m) was added to the reaction mixture (table II). Antimycin A (0.2 μg/ml) partially inhibited the reductase activity.

Cytochrome c Oxidase: The oxidation of reduced cytochrome c was measured by following the decrease in optical density at 550 mμ. The reaction mixture contained 3 × 10⁻⁵ M cytochrome c (approx. 80% in reduced form), 0.2 μg/ml Antimycin A, 2 × 10⁻² M phosphate buffer, pH 7.4, and 0.05 ml (6 μg N) mitochondrial suspension in a total volume of 3.0 ml. Cytochrome c was reduced in aqueous solutions (pH about 8 with tris buffer) by adding a small excess of solid sodium hyposulfite (Na₂S₃O₇). A fine stream of air was passed through the solution for about five minutes to oxidize excess hyposulfite.

The first order velocity constant (min⁻¹) for the disappearance of reduced cytochrome c was calculated according to Smith (20), and a value of 0.11 was obtained. The reaction rate was virtually unchanged in the presence of mannitol (0.4 m), KCN (2 × 10⁻⁴ M) blocked the oxidase activity completely.

Diaphorase: Diaphorase activity was measured by following the decrease in optical density at 600 mμ due to reduction of 2,6-dichlorophenol indophenol. The reaction mixture contained 5 × 10⁻⁷ M DPNH, 2 × 10⁻⁵ M cytochrome c, 2 × 10⁻² M phosphate buffer, pH 7.4, and 0.05 ml (6 μg N) mitochondrial suspension in a total volume of 3.0 ml. Under these conditions, the reaction proceeded linearly with time for the first 2 to 3 minutes, and the rate of reaction was 2.2 mmoles DPNH oxidized/min/mg N.

Comparison of Rates of Electron Transfer Reactions Between Normal & Crown-gall Mitochondria: Similar experiments as described above were carried out using mitochondria isolated from crown-gall tissue cultures of tomato. Mitochondria from crown-gall tissue cultures showed all enzymatic activities observed in mitochondria from normal tissue cultures. Their responses to the inhibitors tested and change of tonicity were also similar. However, the results of three separate, comparative experiments indicated that the rates of reactions catalyzed by crown-gall mitochondria were about 20% lower than those by normal tissue mitochondria.

**Discussion**

In mitochondrial oxidation of DPNH the structural integrity of the particles is important. Humphreys and Conn (12) compared the rates of DPNH oxidation by lupine mitochondria in the reaction mixtures with and without 0.5 m sucrose. In the absence of added cytochrome c, the oxidation rate was higher in the presence of sucrose than in the absence of sucrose, but in the presence of added cytochrome c the reverse was true. Adding cytochrome c enhanced the oxidation threefold in the absence of sucrose, but less than twice in the presence of sucrose.

The present experiments showed that the presence of sucrose or mannitol in the reaction mixture was essential to support the highest rate of DPNH
oxidation (figs 1 & 2). Although the maximum rates of oxidation which were attained by sucrose or mannitol were similar, the range of concentration was different. Sucrose supported the maximum rate only at 0.2 M, and above this concentration the reaction rate decreased. On the other hand, mannitol maintained a maximum rate at a wider range of concentrations, 0.2 to 0.8 M, and hence was preferable to sucrose. When mannitol (or sucrose) was omitted from the reaction mixture, or when the mitochondria were in phosphate buffer only, the rate of DPNH oxidation decreased to one-third normal. Apparently, hypotonic treatment affects the availability of cytochrome c, because an external supply of cytochrome c almost completely restored activity to the highest levels which were obtained in the presence of the optimal concentration of sucrose or mannitol.

In contrast to DPNH oxidase activity, DPNH-cytochrome c reductase activity was reduced to one-third when mannitol was added to the reaction mixture (table II), whereas cytochrome c oxidase activity was unchanged with or without mannitol. These results suggested the importance of toxicity of the reaction medium in determination of catalytic activities of mitochondria.

Oxidation of DPNH by the present mitochondrial preparations was unaffected by the addition of ascorbic acid. This response is distinct from that of the DPNH oxidation systems in soluble fractions of certain plants (2,9,16) and particulate preparations of wheat embryo (22).

Antimycin A inhibited DPNH-cytochrome c reductase activity of the present tissue culture mitochondria about 75%. A partial inhibition (30%) of the reductase activity has also been reported with mitochondrial particles from potato (7) and wheat roots (14). Apparently, added cytochrome c provides an alternate pathway of DPNH oxidation which can bypass the Antimycin A-sensitive factor (7,13). Whether the Antimycin A-insensitive pathway operates within the particles under physiological conditions is not clear.

The previous experiments (24), in which oxygen uptake was measured with an oxygen electrode apparatus, established that crown-gall mitochondria have lower oxidative activities for DPNH and Krebs cycle substrates than do mitochondria from normal tissue cells. The present experiments indicated that the ability to catalyze the electron transport reactions tested was also lower in mitochondria from crown-gall tissue cultures than in preparations from normal tissue cultures. However, there was no qualitative difference in their catalytic properties. It seems that mitochondria from normal and crown-gall tissue cultures have identical pathways for the transport of electrons from DPNH to oxygen. A possible sequence of the electron carriers is like the one suggested by Hackett (7) for the DPNH oxidation system in potato particles, and thus the plant tissue culture mitochondria show a resemblance to mitochondria of higher plants and animals.

Mitochondrial particles isolated from normal and crown-gall tomato tissue cultures were assayed spectrophotometrically for their DPNH oxidase, DPNH-cytochrome c reductase, cytochrome c oxidase, and diaphorase activities. Normal tissue particles always showed higher activities than crown-gall particles. There was no qualitative difference in their catalytic properties, and it was concluded that mitochondria from normal and crown-gall tissue cultures have virtually identical pathways for the transport of electrons from DPNH to oxygen.

The effects of the osmotic properties of reaction mixtures on these enzymatic activities, especially the DPNH oxidase system, were studied. Without added cytochrome c, the maximum rate of DPNH oxidation was obtained in 0.2 M sucrose, and in 0.2 to 0.8 M mannitol. In the presence of cytochrome c, the oxidation rate was maintained at a maximum level at all concentrations used of sucrose or mannitol. DPNH-cytochrome c reductase activity, on the other hand, was accelerated in the absence of mannitol. The activity of cytochrome c oxidase was not affected appreciably in the hypotonic solution. Ascorbic acid had no effect on the rate of DPNH oxidation by the tissue culture mitochondria.

**Acknowledgments**

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**Literature Cited**

Dormant seeds can perform many complex metabolic processes (6, 9) and, in certain instances, mitosis and cytokinesis (5). Very little attention has been given, however, to the possibility that tissue differentiation may occur within dormant seeds. The purpose of this communication is to report an instance of tissue differentiation that we have observed in certain dormant seeds.

**Experimental**

In the course of other studies we noticed that seeds of lettuce (Lactuca sativa L., var. New York) became green even though germination was prevented either by preirradiation of the dry seeds with 300 to 1,000 kr Co⁶⁰ gamma rays or by imbibition of otherwise untreated seeds in solutions of maleic hydrazide (MH). When the seeds are sown on filter paper in petri dishes containing 0.03 M MH at pH 5.7 under 30 to 100 ft-c white light at 23° C, about 60 to 70% remain dormant. After 7 days, however, about 25 to 30% of the dormant seeds are visibly green. Corresponding dark-imbibed controls in MH do not become green. The color is localized in an internal sector near the outer epidermis of the cotyledon which faces the light. Within this sector are chloroplasts of 4 to 5 µ diameter, indistinguishable under the microscope from the chloroplasts in the expanded cotyledons of growing seedlings.

Eighty per cent acetone extracts were prepared from green dormant seeds that had been in 0.03 M MH and exposed to 100 ft-c continuous white light for 7 days. These extracts showed the chlorophyll a peak at 663 mµ (Curve a in fig 1). This peak is absent in the extracts of dark-imbibed controls in MH.

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PIGMENT FORMATION & PHOTOSYNTHESIS IN DORMANT LETTUCE SEEDS

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