THE COLORIMETRIC DETERMINATION OF PLANT SUCCINIC DEHYDROGENASE

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Demonstration of the presence of succinic dehydrogenase in certain plant tissues has been difficult and often unsuccessful. Previous attempts have failed to reveal succinic dehydrogenase activity by the Thunberg technique (18), by use of reversibly oxidized dyes like methylene blue or thionine, in many plant materials. These materials include oat (Avena sativa) and rice (Oryza sativa) seedlings (1, 4, 7), spinach (Spinacia oleracea) leaves (6), and mungo bean (Phaseolus mungo) leaves and shoots (7). Thunberg (19) reported that he could not detect succinic dehydrogenase activity in seeds from 100 species of plants. However, the enzyme has been found in certain seeds (8), seedlings (7, 8), roots (10), embryos (9, 15), legume pods (7), and pollen grains (14). Malonate inhibition in vivo has been used as indirect prima facie evidence for the participation of succinic dehydrogenase in the respiration of several plants, but malonate studies with intact plant tissues have appeared more complex than mere competitive inhibition (2, 5, 6, 17, 20).

Failure to demonstrate succinic dehydrogenase in some plants has produced a skeptical attitude regarding the universal operation of a tricarboxylic acid cycle in higher plants. This paper describes a method of determining the succinic dehydrogenase activity of plant tissues by means of anaerobic reduction of the tetrazolium salt, p,p'-diphenylenebis-2-(3,5-diphenyltetrazolium chloride) (DBDTC). The oat (Avena sativa) was selected as a source of the enzyme because of previous difficulties in demonstrating the presence of succinic dehydrogenase in preparations from the embryos, coleoptiles, or whole coleoptiles with enclosed foliage leaves (1, 4).

EXPERIMENTAL

Preparation of Cell-Free Enzyme Extracts from Avena Seeds and Seedlings

Oats were soaked 2 hours in distilled water and then spread on moist

1 The p,p'-diphenylenebis-2-(3,5-diphenyltetrazolium chloride) is commercially available under the trade name "neotetrazolium" from the following: Amend Drug and Chemical Company, Inc., New York; Dajac Laboratories Division of Monomer-Polymer, Inc., Leominster, Massachusetts; General Biochemicals, Inc., Chagrin Falls, Ohio; Montclair Research Corporation, Montclair, New Jersey.

2 Clinton variety oats were supplied by the Pennsylvania Agricultural Experiment Station Foundation Seed Stocks.
Cheese-cloth in glass-covered trays. Seeds were germinated in the dark at 20°. Length of culture period depended upon the phase of the investigation under consideration.

The tissues were minced in vacuo in a Waring blender for 3 minutes with twice their weight of cold 0.1 M potassium phosphate buffer at pH 7.4. The system was evacuated for 3 minutes prior to blending and the evacuation was continued throughout the blending period. Preliminary experiments indicated that blends made in vacuo exhibited greater reducing activity than those prepared in air. Cellular debris was removed by squeezing the blended material through cheese-cloth. The resulting filtrate was centrifuged for 5 minutes at about 1800 × g. The white to sienna-colored opalescent supernatant liquid was decanted and used immediately in tests for enzyme activity. The preparation time did not exceed 30 minutes.

Properties of DBDTC and Its Reduction Product—Reduction of DBDTC with an excess of sodium hydrosulfite or with about one-half the amount of sodium hydrosulfite required for complete reduction yielded only one colored product as determined spectrophotometrically. The same colored reduction product was formed by enzymatic action. The reduction presumably goes to completion, with the formation of a diformazan, and there is no interference in photocolorimetric measurements from an intermediate monoformazan. Unlike some other tetrazolium salts which are sensitive to light, DBDTC in solution does not undergo any measurable reduction after 4 hours exposure to direct sunlight.

The reduction product of DBDTC was only slightly soluble in dilute aqueous solutions of pyridine, acetone, ethanol, or propanol. However, reduced DBDTC could be extracted from aqueous systems with ethyl acetate, diethyl ether, and n-butanol. n-Butanol, being the least volatile of these solvents, was selected as the extraction solvent for making colorimetric determinations of reduced DBDTC. The formazan is stable in n-butanol and extracts could be kept as long as 12 hours without any measurable change in light transmittance. The water-insoluble diformazan of DBDTC produces a magenta-colored solution with organic solvents. The diformazan showed maximal light absorption at a wave-length of 520 mp in n-butanol and this wave-length was accordingly used for colorimetric determinations. In the presence of colloidal cellular material, the diformazan often appears purple in color and is difficult to extract. Because of the presence of different shades of color, some of which can be extracted preferentially by solvents, Antopol et al. (3) raised the question whether more than one colored compound is formed. We have found that the purple color is a result of the association of diformazan with cellular components and that it is not caused by the formation of other colored compounds.
**DBDTC**

*p,p'-Diphenylenebis-2-(3,5-diphenyltetrazolium chloride)*

(Pale yellow in aqueous solution)

\[ \text{DBDTC} \xrightarrow{+4e+4H^+} \]

**Diformazan of DBDTC**

*p,p'-Diphenylenebis-2-(3,5-diphenylformazan)*

(Insoluble in water; magenta in n-butanol)

*DBDTC Colorimetric Procedure*—DBDTC in aqueous solution was reduced with sodium hydrosulfite to produce a diformazan suspensoid, which was used to make a series of dilutions. The reduced DBDTC of each dilution was extracted with twice the volume of water-saturated (26°) n-butanol. Extracts of reduced DBDTC, read at a wave-length of 520 m\(\mu\) in a Leitz Rouy photometer, followed Beer’s law in the range between 2.5 and 25 \(\gamma\) (20 to 85 per cent \(T\)). The slight yellow color of unreduced DBDTC did not interfere with the spectrophotometric readings.

Tests for enzyme activity with DBDTC were conducted anaerobically in Thunberg tubes, because oxygen interfered with the reduction of DBDTC. Presumably at least a part of the oxygen effect is a result of the competitive action of the cytochrome-cytochrome oxidase system for electrons. Under anaerobic conditions, the addition of cyanide to inactivate the cytochrome system was not required. Enzyme systems in Thunberg tubes were made up in the following manner: To the main tubes were...
added 1 ml. of freshly prepared *Avena* cell-free enzyme extracts, 0.5 ml. of either 0.2 M substrate or distilled water, 1 ml. of 0.1 M potassium phosphate buffer at pH 7.4, and 2 ml. of distilled water; 0.5 ml. of a 0.2 per cent DBDTC solution was placed in the bulbed side arm stopper. The total volume of the system was always 5 ml. DBDTC was not reduced by heat-inactivated enzyme preparations. Blanks were run to correct for the color found in tissue extracts.

The Thunberg tubes were evacuated for 3 minutes at 10-15° and then placed in a 37° bath for 10 minutes before the DBDTC solutions in the side arms were tipped into the main tubes. The reactions were stopped by mixing 10 ml. of water-saturated (26°) *n*-butanol with the contents of the tubes. After several minutes of centrifuging, a clear butanol extract of reduced DBDTC was obtained. The proteinaceous material from the enzyme preparation was concentrated at the butanol-water interface. The transmittance of the supernatant butanol extract was determined and dehydrogenase activity was reported as the number of micrograms of tetrazolium salt reduced per gm. of fresh tissue.

**Effect of Enzyme Concentration on Reduction of DBDTC**—Various increments of a cell-free enzyme extract prepared from oats germinated 31 hours were added to the Thunberg tubes and the reaction systems were brought to a final volume of 5 ml. with buffer. Determinations were made with and without added succinate. The reaction time was 4 hours. There is a linear correlation between the amount of enzyme preparation and the reduction of DBDTC (Fig. 1). A slight dilution effect occurred at the lower enzyme concentrations.

**Effect of Reaction Time on Dehydrogenase Activity As Determined by DBDTC Reduction**—To study the rate of enzymatic reduction of DBDTC, a cell-free extract was prepared from oats germinated 20 hours. In one set of Thunberg tubes the extract was tested with succinate and in another set of tubes no substrate was added. At 30 minute intervals, the reactions in one tube from each set were stopped by the addition of water-saturated *n*-butanol. Dehydrogenase activity, as determined by DBDTC reduction with added succinate as substrate, was a linear function of the reaction time for the 4 hour period investigated (Fig. 2).

**Effectiveness of DBDTC As Electron Acceptor**—Investigations were conducted to compare the effectiveness of DBDTC and 2,3,5-triphenyltetrazolium chloride (TTC) as indicators of dehydrogenase activity. The butanol extract of reduced TTC was read at a wave-length of 490 m\(\mu\). When DBDTC was used as an acceptor, large differences were obtained between determinations with and without added succinate (Table I). In contrast, tubes with TTC produced insignificant differences.
Preliminary studies indicated that the apparent oxidation-reduction potential of DBDTC was higher than that of TTC and in the proper range for DBDTC to act as an electron acceptor in the succinate oxidation. Since the reported oxidation-reduction potential of the succinic-fumaric

![Graph 1](http://www.jbc.org/)

**Fig. 1.** The effect of enzyme concentration on the reduction of DBDTC. The cell-free enzyme extract was prepared from oats germinated 31 hours. The reaction time was 4 hours at 37°. O, succinate added as substrate; Δ, no substrate added.

![Graph 2](http://www.jbc.org/)

**Fig. 2.** The effect of reaction time on the dehydrogenase activity of preparations from oats germinated 20 hours as determined by DBDTC reduction at 37°. O, succinate added as substrate; Δ, no substrate added.
SUCCINIC DEHYDROGENASE

system is 0.00 volt at pH 7.0 (13), TTC, for which the potential is −0.08 volt at pH 7.0 (12), would not be expected to accept electrons directly at this step. Failure to obtain reduction of TTC by the addition of succinate without the addition of diphosphopyridine nucleotide to preparations of corn embryos (Zea mays) has been reported by Jensen et al. (11). Smith (15) found similar results with a corn embryo preparation which displayed succinic dehydrogenase activity by the Thunberg methylene blue technique.

TABLE I
Comparison of DBDTC and TTC As Electron Acceptors in Determination of Succinic Dehydrogenase

<table>
<thead>
<tr>
<th>Electron acceptor</th>
<th>Dehydrogenase activity, γ indicator reduced per gm. tissue*</th>
<th>No substrate added</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBDTC</td>
<td>434</td>
<td>336</td>
</tr>
<tr>
<td>TTC</td>
<td>38</td>
<td>36</td>
</tr>
</tbody>
</table>

Germinating Avena seeds (23 hrs.); reaction time, 4 hrs. at 37°

Avena coleoptiles with foliage leaves (100 hrs.); reaction time, 1 hr. at 37°

<table>
<thead>
<tr>
<th>Electron acceptor</th>
<th>Dehydrogenase activity, γ indicator reduced per gm. tissue*</th>
<th>No substrate added</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBDTC</td>
<td>283</td>
<td>182</td>
</tr>
<tr>
<td>TTC</td>
<td>9</td>
<td>8</td>
</tr>
</tbody>
</table>

* Average of two determinations.

DISCUSSION

Determination of dehydrogenase activity by the quantitative measurement of the amount of colored stable formazan produced, calculated as DBDTC reduced, employs the precision of photoelectric instrumentation. Previous methods with reversible dyes, such as methylene blue, depend on the visual estimation of the decolorization time of the dye. Photometric estimation of decolorization can only be performed in Thunberg tubes of special dimensions when the reaction systems are not turbid. These estimations must be conducted anaerobically to prevent the dye from returning to the oxidized state. Tam and Wilson (16) found that the reduction rate of methylene blue with most substrates is not linear, but logarithmic. For accurate results they recommend that the rate of reduction should be determined by taking individual readings at definite time intervals. The anaerobic reduction rate of DBDTC with succinate was found to be linear (Fig. 2).
Experiments with enzyme preparations from oats have repeatedly shown remarkably high differences in the amounts of DBDTC reduced between control tubes and tubes with succinate added as substrate (Figs. 1 and 2; Table I). Although the endogenous dehydrogenase activity of Avena preparations was high, the addition of succinate in each instance increased the amount of DBDTC reduced. DBDTC reduction can be used as an index of dehydrogenase activity because the quantity of DBDTC reduced is linearly related to the concentration of the enzyme preparation (Fig. 1).

Malonate (0.02 M), a specific inhibitor of succinic dehydrogenase, inhibited DBDTC reduction with succinate as much as 70 per cent. Inhibition by malonate shows that the succinic dehydrogenase reaction was a limiting step. However, the increased DBDTC reduction with added succinate cannot be taken as a direct measure of succinic dehydrogenase activity alone, but must be considered as a measure of the increased general dehydrogenase activity. Although the DBDTC colorimetric procedure is not specific, it can be applied advantageously to study multiple enzyme systems, as have the Thunberg methylene blue and Warburg manometric techniques.

**SUMMARY**

1. A relatively sensitive colorimetric method was developed to determine the dehydrogenase activity of plant tissues in vitro by using *p*,*p*-diphenylenebis-2-(3,5-diphenyltetrazolium chloride) (DBDTC) as an electron acceptor.

2. Application of the DBDTC colorimetric procedure demonstrated succinic dehydrogenase activity in cell-free enzyme extracts prepared from germinating Avena seeds and seedlings. This fact is offered in support of the view that plants follow a metabolic pathway similar to the Krebs tricarboxylic acid cycle.

3. The quantity of DBDTC reduced was linearly related to the amount of dehydrogenase preparation present.

4. Dehydrogenase activity of an extract from germinating Avena seeds, as measured by the reduction of DBDTC with added succinate, was a linear function of the anaerobic reaction time for the 4 hour period investigated.

5. DBDTC was much more sensitive as an electron acceptor than 2,3,5-triphenyltetrazolium chloride.

**BIBLIOGRAPHY**

SUCCINIC DEHYDROGENASE

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