decreasing pH. From measurements of K absorption as a function of time and other data, it was concluded that the effect of hydrogen ion on K absorption could be interpreted as a direct hydrogen ion effect on the K absorption mechanism. The observed hydrogen ion effects on K absorption were consistent with the ion absorption hypothesis of Jacobson and Overstreet.

The presence of Ca ion in solution was found to decrease considerably the depressing effect of hydrogen ion on K absorption. An explanation of this phenomenon in terms of the Jacobson-Overstreet hypothesis was presented.

Increasing hydrogen ion concentrations depressed Na absorption from NaBr solution in a similar manner as K absorption from KBr solution.

**LITERATURE CITED**


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**CYTOCHROME OXIDASE CONTENT AND RESPIRATORY RATES OF ETIOLATED WHEAT AND BARLEY SEEDLINGS**

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Hill and Hartree (10) have reviewed the data which have accumulated during the last several years regarding cytochrome oxidase of plants, and the bulk of the evidence favors the view that this terminal oxidase plays an important role in respiratory oxygen consumption. Also Webster’s recent survey (21) stressed the widespread distribution of this enzyme in dicotyledonous plants. Even so, there are several reports that the enzyme is missing from older cereal tissues. For example, Waygood (20) concluded that the oxidase is not present in extracts of wheat seedlings after the third day of development. Butler (4) reported inability to detect cytochrome oxidase in extracts from 5-day wheat roots, although Lundegârdh (16), using a specially designed spectrophotometer, was able to observe characteristic cytochrome absorption bands in intact roots of older wheat. Albam and Eichel (1) demonstrated that the oxidase was present in extracts from oat seedlings one or two days old, but did not detect the enzyme in extracts from older seedlings (up to 6 days), and concluded that cytochrome oxidase operated only during the embryonic stages. James (12) detected cytochrome oxidase in barley embryos, but found no evidence for its exist-

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ence in the tips of older barley roots (10 to 11 days). In a recent review article, James (13) summarized the information relating to terminal oxidases in barley roots and supported the hypothesis that a transition in respiratory agents takes place during maturation.

Although the finding that cytochrome oxidase is present in any particular tissue would not prove that it operates in respiratory oxygen absorption, nevertheless a demonstration of its presence in a tissue previously thought to be without the enzyme removes the most potent argument against the possibility of its participation. Thus it is of considerable interest that we were able, in a re-investigation of this problem, to detect the enzyme at all stages of development of etiolated wheat, barley, rice and oat seedlings. Having established that the enzyme was present, experiments were instituted in which the enzyme content of seedlings of different ages were evaluated. For comparison, parallel experiments were conducted with pea, in which Stafford (19) has shown that the cytochrome oxidase persists at least to the 6-day stage, the oldest seedling investigated. Enzyme assay values, when compared to the respiratory oxygen absorption of the living tissue, allowed a decision to be made as to whether sufficient cytochrome oxidase was present in the extracts to account for the oxygen absorption of the tissue from which they were derived. Experiments of this nature cannot by themselves exclude the possibility of participation of other terminal oxidases. However, any suggestion from experiments in vivo that cytochrome oxidase is an important terminal oxidase in a given tissue would seem to require for its support a demonstration that a sufficient amount of the enzyme is present to mediate a major portion of the respiratory oxygen absorption.

**MATERIALS AND METHODS**

**Preparation of Enzyme Extracts:** Etiolated seedlings of Vigo wheat (Triticum vulgare), Kentucky No. 1 barley (Hordeum vulgare), Alaska pea (Pisum sativum), Victory oat (Avena sativa) and rice (Oryza sativa) were used as sources of cytochrome oxidase. The seeds were disinfected by soaking for 8 hours in a 1% solution of Arasan SF (E. I. DuPont de Nemours and Company) to which a sprinkling of “Tide” had been added as a wetting agent. Pea seeds were planted in vermiculite (heat-expanded mica), and were watered daily with tap water; the cereal seeds were treated similarly, except that the supporting medium was sand. For a few preliminary experiments, plants were grown as described in (7).

The soaking and subsequent germination and growth took place at 20°C in a dark room with occasional red light. At harvest time, sand or vermiculite was washed away with running water, and a sample of seedlings (usually 50 to 100) was selected. The pea cotyledons and the cereal endosperms were removed and discarded, and the plants were separated into root and shoot as required. After drying with paper toweling and weighing, the tissue was cut into small pieces with scissors, and stored at about 10°C for one to two hours. The enzyme extract was prepared by grinding the tissue with half its weight of 0.1 M phosphate buffer (pH 7.7) in a previously chilled mortar and pestle; a small quantity of white sand was sprinkled on the tissue to facilitate grinding. The mash was recovered and strained through a piece of broadcloth, hand pressure being exerted to assist the extraction of plant juice, and the volume of the extract was recorded. The plant extract, containing the cytochrome oxidase, was used without delay and in the spectrophotometric experiments it was maintained at 0°C until it was added to the cuvettes.

The pH of the extract was 6.9 to 7.3, depending upon the kind and age of the tissue. For spectrophotometric experiments, no adjustment of pH was made, since the volume of plant juice added to a cuvette was very small in comparison to the volume of reduced cytochrome c solution which was buffered at pH 7.2. For manometric experiments, the extract was adjusted to pH 7.4 except for certain experiments with pea juice, as noted, when the pH was adjusted to 7.0.

**Rate of Respiration:** The rates of oxygen absorption of intact seedlings, or of the separated roots and shoots, were measured manometrically at 25°C. The tissue was cut into small pieces with a razor blade and placed into Warburg flasks containing water; KOH-saturated paper was placed in the center well. Seedlings selected for measurement of respiratory rates were of average size, and the recorded rates of oxygen absorption represent the means of several determinations.

**Manometric Technique:** The manometric method of measuring cytochrome oxidase activity involves the addition of cytochrome c and a reducing agent to the enzyme preparation, the function of the reducing agent being to reduce the cytochrome c non-enzymatically, after it is oxidized by the oxidase (18). The reducing agent used in most of the experiments was p-phenylenediamine (PPD) and for each experiment a solution (0.33 M adjusted to pH 7.4) was prepared a few minutes before use.

Measurement of oxygen consumption was made in standard Warburg respirometers at 25°C; the gas phase was air. The flasks contained enzyme extract (1.0 ml for wheat and barley and 0.5 ml for pea), cytochrome c solution (made by dissolving 2.0 mg “Sigma” cytochrome c per ml water), reducing agent (0.75 ml in the side-arm) and deionized water to a volume of 2.5 ml. Each flask contained KOH saturated paper in the center well. After tipping, readings were taken at 5-minute intervals for 15 minutes. The initial rate of oxygen uptake was estimated graphically, and converted to the rate per hour after subtracting the value for autoxidation of the reducing agent; these rates were a measure of activity at any given concentration of cytochrome c.

The rate of autoxidation of the reducing agent was determined by measuring its rate of oxygen uptake in the presence of a constant concentration of cytochrome c, at several concentrations of enzyme, and
extrapolating to zero enzyme concentration, according to the method outlined by Schneider and Potter (17) and Slater (18). The rate of autoxidation of PPD per Warburg flask was small (20 μl O₂/hour at the highest cytochrome c level employed) in comparison with the rate of enzymatic oxidation (from 150 to 600 μl O₂/hour).

Spectrophotometric Technique: The spectrophotometric method of determining cytochrome oxidase activity, introduced by Hogness (11) is based upon the direct enzymatic oxidation of reduced cytochrome c by oxygen, and the rate of oxidation is measured by the rate of decrease of optical density at 550 mμ, where the alpha-band of reduced cytochrome c exhibits a peak. It should be stressed that the spectrophotometric method of measuring cytochrome oxidase is much more sensitive than the manometric method. Thus, a readily measurable decrease in optical density of 0.02 divisions per minute during the oxidation of reduced cytochrome c corresponds to the absorption of approximately one μl oxygen per hour, an uptake which is scarcely detectable manometrically.

The procedure was based upon that described by Cooperstein and Lazarow (5). Cytochrome c was dissolved in 0.03 M phosphate buffer (pH 7.7) so that there was 0.66 mg cytochrome c per ml, and reduction was accomplished by adding sufficient solid sodium hyposulfite (Na₂S₂O₄) to give a concentration of 0.004 M. A fine air stream was passed through the solution for 3 to 5 minutes to oxidize excess hyposulfite. (After aeration, the pH of the solution was 7.2). Reduced cytochrome solutions were prepared in small quantities as needed, and reduction was effected immediately before use.

Measurements of absorbance at 550 mμ were made at room temperature (21 to 25°C) with a Beckman Model B spectrophotometer. The absorbance was recorded 15 seconds after adding the plant extract (usually 0.02 ml) to 3.0 ml of a cytochrome c solution and readings were taken at half-minute intervals. Linear curves were obtained for 1.5 to 2 minutes and optical density change per minute was the measure of activity at any given concentration of cytochrome c. The change in optical density was converted to μl oxygen absorbed, by the procedure outlined below. Sufficient oxygen is present in solution so that it does not limit the rate of the reaction and autoxidation of the reduced cytochrome c during the 2-minute reaction period was negligible.

No change was ever detected upon the addition of catalase (Bios Laboratories, Inc., New York) in the ability of each enzyme extract—prepared from either root or shoot systems of wheat, pea or barley seedlings—to catalyze the oxidation of reduced cytochrome c, when tested spectrophotometrically. Consequently, the possibility of peroxidase participation in cytochrome c oxidation was rendered unlikely.

Expression of Results: The procedure developed by Slater (18) was followed in assaying for cytochrome oxidase content and results are expressed in terms of catalytic ability of the enzyme at infinite cytochrome c concentration. To obtain these assay values, enzyme activities were determined at several levels of cytochrome c, and the activity at infinite concentration of cytochrome c was estimated by the extrapolation procedure of Lineweaver and Burk (15).

For the manometric method, the reciprocal of the rate of oxygen absorption plotted against the reciprocal of the concentration of added cytochrome c; the rate of oxygen absorption in μl per hour at infinite concentration of cytochrome c was estimated by extrapolation, and was expressed as Vₘₐₓ per ml enzyme extract. Since the number of seedlings used and the volume of the extract were known the cytochrome oxidase content of a single seedling could then be derived from this figure.

In the spectrophotometric method (used when the seedlings were separated into root and shoot), the optical density change (ΔD) per minute per 0.02 ml enzyme extract was determined for several levels of cytochrome c. As above, the reciprocals of these functions were plotted, and the optical density change (ΔD) per minute at infinite concentration of cytochrome c was estimated by extrapolation. This value was then converted into Vₘₐₓ per ml extract by application of equation (1):

(1) \[ \Delta D/\text{min for 0.02 ml extract} \times 2500 = \mu l \text{ O}_2 \text{ absorbed/hour per ml extract} = V_{\text{max}} \text{ per ml enzyme extract} \]

Then, as in the manometric method, the value for the cytochrome oxidase content of the root or shoot system (Vₘₐₓ per root or shoot system) was readily computed.

Equation (1) was derived from the fundamental relationship described by previous workers (2, 5, 9):

(2) \[ \text{concentration of oxidized cytochrome c} = \frac{D_r - D}{1.96 \times 10^4} \text{ moles per liter} \]

where Dᵣ is the optical density reading after complete reduction with sodium hyposulfite, and D is the reading at any other time. The difference between the extinction coefficients for reduced and oxidized cytochrome c, at 550 μμ is 1.96 x 10⁴.

From equation (2) the following expression can be obtained.

(3) \[ \text{Oxygen utilized in μl/hr × ml enzyme extract} \cdot \Delta \text{D/min for 0.02 ml extract} = \frac{50 \times 3 \times 10^{-3} \times 60 \times 22.4 \times 10^6 \times 0.25}{1.96 \times 10^4} \]

where 50 = conversion of 0.02 ml enzyme extract to 1.0 ml extract

3 x 10⁻³ = volume of reaction solution in liters

60 = conversion of minutes to hours

22.4 x 10⁶ = conversion of moles of O₂ to μl of O₂

0.25 = equivalence in moles between cytochrome c and oxygen.

Equation (3) can be simplified to equation (1).
RESULTS

FACTORS AFFECTING ACTIVITY: A series of manometric studies was performed to ascertain the effect of various factors upon the activity of cytochrome oxidase, and to clarify the best conditions for extraction and the requirements for measurement of maximal activity. For this purpose, extracts from 4-day wheat seedlings served as the source of enzyme.

The addition of PPD to the wheat extract produced a marked increase in the rate of oxygen absorption (fig 2, curve 3). Reasons are given in the discussion for believing that this catalysis of PPD oxidation is ascribable to cytochrome oxidase and the endogenous cytochrome c of the extracts. The addition of cytochrome c to the extract caused no increase in the rate of oxygen absorption; however, when cytochrome c was added to a mixture of the extract and PPD, a sharp stimulation in the rate of oxygen absorption was brought about (fig 2).

The effect of increasing the concentration of PPD upon the rate of its oxidation by the wheat extract in the presence of a fixed amount of added cytochrome c is shown in figure 1. It is evident that low concentrations of reducing agent limit the rate of the reaction, and to obtain maximal rates of oxygen uptake, it is necessary to employ a saturating level of the reductant. Accordingly, a final concentration of 0.1 M PPD in the Warburg flask was used in all later experiments.

Figure 2 shows that the rate of oxygen uptake (which was shown to be thermolabile) also depended upon the amount of cytochrome c which was added. The endogenous oxygen absorption (curve 1) was virtually unaffected by the addition of cytochrome c alone (curve 2). Curve 3 shows the oxygen absorption in the presence of a saturating amount of PPD. The rate of oxidation was increased (curves 4 to 6) by the addition of cytochrome c to the mixture of PPD and extract, and this stimulation demonstrates the existence of cytochrome oxidase in the extract. It is clear from figures 3 and 4 that higher rates of O₂ uptake can be induced as the concentration of cytochrome c is raised above 2.5 × 10⁻⁵ M and that, in fact, cytochrome c ceases to be a limiting factor only when its concentration is infinitely high. As a result of this, measurements of cytochrome oxidase activity at a cytochrome c level of 10⁻⁵ M give values which are only slightly greater than half of those possible at an infinitely high concentration of substrate, and at 2.5 × 10⁻⁵ M cytochrome c, the highest level employed, the observed rates are still less than 85% of the maximum ones.

The effect of several other variables was also investigated. Increasing the phosphate concentration in the reaction vessel retarded the enzymatic oxidation of PPD. However, absence of phosphate, a condition obtained by using water as a grinding medium, resulted in extracts in which PPD was oxidized at a lower rate than when the enzyme was extracted in 0.1 M phosphate buffer. Also it was found that within rather wide limits on the alkaline side, the pH of the grinding medium had little effect upon the subsequent activity of the cytochrome oxidase; thus, equally active cytochrome oxidase extracts could be prepared from grinding media between pH 7 and pH 10. But if the pH of the extracting medium was more acid than pH 7, the activity of the oxidase was markedly reduced. It appears that if certain limits of acidity are exceeded during the extraction process, maximum values of oxidase activity will not be obtained. The general procedure adopted was to grind the plant tissue in 0.1 M phosphate buffer (pH 7.7), the volume of buffer being equal to half the fresh weight of the tissue. The extracted juice (the pH of which was approximately 7.0) was adjusted to pH 7.4, since this was found to be the optimum pH for the enzymatic oxidation of PPD.

CHOICE OF REDUCING AGENT: Slater (18), in his manometric study of the cytochrome oxidase of heart-muscle preparations, showed that the activity of cytochrome oxidase, measured by extrapolation to infinite concentration of cytochrome c, was the same when ascorbic acid was used as the reducing agent, as when PPD was used. Comparable results have now been obtained when extracts from 3-day pea seedlings were used as the source of oxidase, and when

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Fig. 1. Effect of p-phenylenediamine concentration on the rate of its oxidation by 1.0 ml extract from 4-day wheat seedlings in the presence of 1.21 × 10⁻⁴ M added cytochrome c.

Fig. 2. Oxidation of p-phenylenediamine in the presence of 1.0 ml extract from 4-day wheat seedlings and increasing amounts of added cytochrome c. Concentrations of added cytochrome c and PPD: Curve 1—no cyt c, no PPD. Curve 2—2.42 × 10⁻⁴ M cyt c, no PPD. Curve 3—no cyt c, 0.1 M PPD. Curve 4—0.97 × 10⁻³ M cyt c, 0.1 M PPD. Curve 5—1.45 × 10⁻⁴ M cyt c, 0.1 M PPD. Curve 6—2.42 × 10⁻⁴ M cyt c, 0.1 M PPD.

Fig. 3. Effect of concentration of added cytochrome c on the rates of oxidation of p-phenylenediamine and hydroquinone by 0.5 ml extracts from 3-day pea seedlings. Curve 1—0.1 M p-phenylenediamine; curve 2—0.12 M hydroquinone.

Fig. 4. Data of fig 3, plotted according to the procedure of Lineweaver and Burk (15).

Fig. 5. The oxidation of reduced cytochrome c by extracts from 11-day barley roots and shoots. The enzyme extract was added at zero time and the optical density was recorded at 550 mλ. Curve 1—0.03 ml root extract. Curve 2—0.12 ml root extract. Curve 3—0.12 ml boiled root extract. Curve 4—0.03 ml shoot extract. Curve 5—0.12 ml shoot extract. Curve 6—0.12 ml boiled shoot extract.

Fig. 6. The oxidation of reduced cytochrome c by cytochrome oxidase from 10-day barley roots. Root extract (0.3 ml) was added to reduced cytochrome c solution at 5 min, and a few crystals of sodium hyposulphite were added at the times indicated.
PPD and HQ were the reductants. Ascorbic acid was not used in the present studies, since the rate of oxygen absorption in the presence of pea, wheat and barley extracts which contain ascorbic oxidase, was excessively rapid, and subsequent addition of cytochrome c resulted in only a small stimulation of oxygen uptake.

The rates of oxidation of saturating concentrations of PPD and HQ, in the presence of 0.5 ml of extract from 3-day pea seedlings and increasing quantities of added cytochrome c, are shown in figure 3. The concentration of HQ required to produce maximal rates of oxygen absorption was 0.12 M. Since it was shown (6, 14) that autoxidation of HQ is very small at pH 7.0, but increases rapidly at higher pH values, the data for this experiment were obtained at pH 7.0, whether PPD or HQ was the reductant. Autoxidation of HQ was estimated by the method used with PPD, and found to be small (25 μl O₂ per hour per Warburg flask).

The rate of oxygen absorption, at the concentrations of cytochrome c used, was dependent upon the reducing agent employed, higher rates being obtained with PPD than with HQ. However, extrapolation of the data of figure 3 by the procedure of Line- weaver and Burk (15) showed that the activity becomes independent of the reducing agent employed at infinite cytochrome c concentration (fig 4). These results demonstrate that if enzyme activity is measured at low concentrations of cytochrome c, values obtained will be dependent upon the particular reducing agent employed. But at infinite cytochrome c concentration, this dependence upon reducing agent vanishes. Therefore, to evaluate the full activity of the enzyme, it is necessary to extrapolate to infinite cytochrome c concentration, a thesis already advanced by Slater (18); under these conditions, the assay values are the same when PPD or HQ is used.

**Cytochrome Oxidase from 11-Day Wheat and Barley:** In view of the reports regarding the disappearance of cytochrome oxidase from the tissues of cereal seedlings in post-embryonic stages (1, 12, 13, 20), it is of interest that we were able to demonstrate the presence of the enzyme in extracts from the roots, as well as from the shoots, of etiolated wheat and barley seedlings as old as 12 days, by both the manometric and the spectrophotometric methods. (In less exhaustive studies with extracts prepared from the whole seedling of etiolated oat and rice, cytochrome oxidase was readily demonstrated by the manometric method at all ages up to two weeks.)

The results of manometric tests for cytochrome oxidase in root and shoot extracts from 11-day barley and wheat seedlings are shown in table I. When cytochrome c was added to the mixture of PPD and root extract, a small but definite increase in the rate of oxygen uptake was obtained, and demonstrated the presence of cytochrome oxidase in these extracts; on the other hand, when the shoot extracts were employed, the corresponding rates were almost doubled. Further additions of cytochrome c caused no further increases in oxygen absorption when PPD was added to the root extract. Since the manometric assay for cytochrome oxidase depends upon the ability of added cytochrome c to cause progressive increases in the rate of oxygen uptake, and the extrapolation of this data to infinite cytochrome c concentration, it is apparent that the small increases obtained with the root extracts do not lend themselves to this treatment and would in fact involve considerable error. Thus the manometric method is not suited for the assay of the enzyme in the root extracts.

On the other hand, the ability of extracts prepared from roots and shoots of 11-day barley and wheat seedlings to catalyze the oxidation of reduced cytochrome c solutions could be readily measured with the spectrophotometer (the results for barley are shown in figure 5). On a volume basis, the extracts prepared from shoots were found to be more active than those from the roots, as was found in the manometric method. The successive oxidation of reduced cytochrome c by the enzyme and its reduction by added crystals of sodium hyposulphite is shown in figure 6; if an excess of crystals is avoided, the cycle of oxidation and reduction can be repeated several times. Since the rates of oxidation of several concentrations of reduced cytochrome c could be readily determined by this method, and the data could be extrapolated to infinite concentration of cytochrome c, the spectrophotometric method was used in later work for the determination of cytochrome oxidase values of various tissue extracts, and served to confirm earlier results obtained by the manometric method.

**Cytochrome Oxidase Assay Results:** The determination of the cytochrome oxidase values of etiolated wheat, barley and pea seedlings, in terms of catalytic ability at infinite concentration of cytochrome c (Vₘₐₓ), was made by manometric technique for seedlings at several ages of development, and these results are tabulated in table II, together with the respiratory rates of the living seedlings. In table III are shown the corresponding spectrophotometric

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**TABLE I**

**Manometric Demonstration of Cytochrome Oxidase in Extracts from the Roots and Shoots of 11-Day Barley and Wheat Seedlings**

<table>
<thead>
<tr>
<th></th>
<th>Barley</th>
<th>Wheat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root extract</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>&quot; + cytochrome c</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>&quot; + PPD</td>
<td>164</td>
<td>144</td>
</tr>
<tr>
<td>&quot; + cytochrome c + PPD</td>
<td>192</td>
<td>174</td>
</tr>
<tr>
<td>Shoot extract</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>&quot; + cytochrome c</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>&quot; + PPD</td>
<td>132</td>
<td>308</td>
</tr>
<tr>
<td>&quot; + cytochrome c + PPD</td>
<td>239</td>
<td>592</td>
</tr>
</tbody>
</table>

The reaction mixture was at pH 7.4; 1.0 ml of extract was used; PPD concentration was 0.1 M and the added cytochrome c was 2.42 x 10⁻⁵ M.

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data, but in this case, the root and shoot tissue was analyzed separately. The studies were terminated at the 12-day stage, when the etiolated seedlings had reached or passed their period of most active growth, as measured by size and weight and rate of respiration.

An obvious feature of the data of table II is that the assay values for pea, whether expressed as $V_{\text{max}}$ per seudding or otherwise, are consistently higher than the corresponding values for wheat and barley. In addition, columns 2 and 3 show that even though the amount of cytochrome oxidase in a unit volume of extract and in a gram fresh weight of tissue decreases as the seedlings mature, the absolute amount of enzyme per seedling (column 4) actually increases during the first week of growth. Thereafter, the amount of enzyme per seedling is more or less maintained (wheat and barley) or is still further increased (pea). In this connection, it is interesting to note that the respiratory rate of a wheat or barley seedling does not increase significantly after the fourth day (column 5). In the case of pea, where the respiratory rate continued to rise throughout the 11-day period, it may be that production of protoplasmic material is continuing during this time, and that this is reflected in the parallel increase in cytochrome oxidase.

It is apparent from comparing values in columns 4 and 5 (table II) that the assay values for the cytochrome oxidase content of a single seedling, in all these plants, are higher than the rates of respiration of seedlings of the same age; in the case of pea, these values are several times higher. (To the extent that our methods are comparable with those of Stafford (19), the results may be regarded as extending to at least 12 days the period over which cytochrome oxidase in excess of respiratory needs can be obtained from etiolated pea seedlings). Thus it would appear that there is more than sufficient cytochrome oxidase present in a single seedling of these plants to meet the respiratory oxygen requirements. However, the further investigation of the separated root and shoot systems (by the spectrophotometric method) revealed the important fact that the relationship between cytochrome oxidase value and respiratory rate is not the same in the two tissues (table III). For the shoot system, the values for $V_{\text{max}}$ (column 2) are in all cases considerably higher than the corresponding values for respiratory rates (column 3). But for the root systems of wheat and barley, the values for cytochrome oxidase (column 4) are consistently lower than the corresponding respiratory rates (column 5); on the other hand, the reverse is true for pea roots. It is therefore clear that whereas the shoot systems of all three kinds of plants, and the root system of pea, contain amounts of cytochrome oxidase which are more than adequate to account for the $O_2$ absorption in respiration, the root systems of wheat and barley are apparently deficient in this regard. Also, it is evident that the cytochrome oxidase values for wheat and barley shoots were sufficiently in excess of the values for respiratory rates, that they masked the deficiency of the enzyme in the roots when the entire

| Table II |
| Manometric Assay Values of Cytochrome Oxidase Content ($V_{\text{max}}$) and the Rates of Respiration of Single Seedlings of Various Ages |

<table>
<thead>
<tr>
<th>AGE IN DAYS</th>
<th>$V_{\text{max}}$**/ML ENZYME EXTRACT</th>
<th>$V_{\text{max}}$/GM FRESH WT</th>
<th>$V_{\text{max}}$/SEEDLING</th>
<th>RESP RATE OF SEEDLING*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.2</td>
<td>632</td>
<td>712</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>3.0</td>
<td>555</td>
<td>625</td>
<td>35</td>
<td>24</td>
</tr>
<tr>
<td>4.2</td>
<td>513</td>
<td>478</td>
<td>43</td>
<td>30</td>
</tr>
<tr>
<td>5.4</td>
<td>490</td>
<td>440</td>
<td>54</td>
<td>31</td>
</tr>
<tr>
<td>6.1</td>
<td>466</td>
<td>415</td>
<td>58</td>
<td>35</td>
</tr>
<tr>
<td>7.4</td>
<td>456</td>
<td>445</td>
<td>54</td>
<td>32</td>
</tr>
<tr>
<td>9.1</td>
<td>414</td>
<td>390</td>
<td>50</td>
<td>29</td>
</tr>
<tr>
<td>11.0</td>
<td>384</td>
<td>328</td>
<td>42</td>
<td>31</td>
</tr>
<tr>
<td>Barley</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.1</td>
<td>390</td>
<td>424</td>
<td>39</td>
<td>32</td>
</tr>
<tr>
<td>6.2</td>
<td>329</td>
<td>346</td>
<td>48</td>
<td>36</td>
</tr>
<tr>
<td>8.1</td>
<td>363</td>
<td>327</td>
<td>53</td>
<td>39</td>
</tr>
<tr>
<td>10.5</td>
<td>266</td>
<td>280</td>
<td>47</td>
<td>33</td>
</tr>
<tr>
<td>12.1</td>
<td>259</td>
<td>270</td>
<td>43</td>
<td>31</td>
</tr>
<tr>
<td>Pea</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.8</td>
<td>1000</td>
<td>1015</td>
<td>260</td>
<td>51</td>
</tr>
<tr>
<td>5.0</td>
<td>870</td>
<td>850</td>
<td>261</td>
<td>72</td>
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<tr>
<td>6.9</td>
<td>770</td>
<td>852</td>
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<td>97</td>
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<td>8.3</td>
<td>714</td>
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</tr>
<tr>
<td>9.3</td>
<td>690</td>
<td>786</td>
<td>587</td>
<td>180</td>
</tr>
<tr>
<td>11.1</td>
<td>523</td>
<td>616</td>
<td>1104</td>
<td>190</td>
</tr>
</tbody>
</table>

* Respiratory rate is expressed in $\mu$l O$_2$/hr.
** $V_{\text{max}}$ is a measure of the cytochrome oxidase activity of the tissue in terms of $\mu$l O$_2$/hr at infinite cytochrome c conc (see text for details).

| Table III |
| Spectrophotometric Assay Values of Cytochrome Oxidase Content ($V_{\text{max}}$) and the Rates of Respiration of the Root and Shoot Systems of Single Seedlings of Various Ages |

<table>
<thead>
<tr>
<th>AGE IN DAYS</th>
<th>SHOOT SYSTEM</th>
<th>ROOT SYSTEM</th>
<th>SUM OF $V_{\text{max}}$ PER SHOOT + ROOT SYSTEMS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{\text{max}}$**</td>
<td>RESP RATE</td>
<td>$V_{\text{max}}$</td>
</tr>
<tr>
<td>Wheat</td>
<td>4</td>
<td>49</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>72</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>53</td>
<td>15</td>
</tr>
<tr>
<td>Barley</td>
<td>4</td>
<td>36</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>31</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>40</td>
<td>18</td>
</tr>
<tr>
<td>Pea</td>
<td>4</td>
<td>110</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>400</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>820</td>
<td>127</td>
</tr>
</tbody>
</table>
seedling (including root and shoot systems) was analyzed.

By summing the separate contributions of the root and shoot systems, the cytochrome oxidase values for the entire seedling were obtained (table III, column 6). These spectrophotometric values can be compared with those achieved by the manometric method for the same seedlings at the same age (table II, column 4). The closeness of agreement between these two sets of data indicates that the two independent methods for assaying for cytochrome oxidase yield similar results.

**DISCUSSION**

In efforts to arrive at values for the enzyme content of a tissue, the criterion of measurement must necessarily be the catalytic ability of the enzyme. For the purpose of such an assay, precautions must be taken to extract as much of the enzyme as possible, and to measure its activity under conditions shown to lead to maximal rates; for the special case of cytochrome oxidase, the activity must be extrapolated to infinite concentration of cytochrome c. Since the physiological function of cytochrome oxidase in the tissue is the catalysis of oxygen absorption, the measure of its catalytic ability in vitro can be compared to the rate of respiratory oxygen absorption. It is assumed, in a comparison of this type, that the cytochrome c in the tissue, probably by virtue of its favorable spatial distribution, does not limit the rate of respiration.

No direct attempt has been made here to answer the question regarding the relative contributions of cytochrome oxidase and other terminal oxidases to the respiration. Indeed, there is nothing in this report which serves as direct proof of the participation of cytochrome oxidase in the respiration of any of the tissues examined. Rather, we have addressed the question: Can sufficient cytochrome oxidase be extracted from a tissue to account for the measured rate of oxygen uptake of the tissue? From the results shown in table III, and to the extent to which the assay values are comparable to the actual respiratory rates, we have concluded that etiolated pea tissues, and barley and wheat shoots, at all stages of development (up to 12 days) contain amounts of cytochrome oxidase adequate to account for the respiratory rate. By contrast, the root tissues of wheat and barley do not yield the required amounts of the enzyme under the conditions of the assay. If it were shown that quantitative extraction from the tissues had been achieved, and the conditions of activity measurement were no less favorable than those under which the enzyme operates in respiration, this apparent inadequacy of cytochrome oxidase could be used as evidence that other terminal oxidases participate in oxygen absorption of these root tissues. However, it should be stressed that since even the best assay procedure is most unlikely to be 100% effective in extracting all of the enzyme from the tissue, and transferring it without loss to the measuring vessel, the assay values reported here are to be regarded as minimal. One obvious correction which would, no doubt, lead to an upward revision of the recorded values if that for the enzyme discarded with the press cake after squeezing the mash through muslin. In this connection also, it may be mentioned that Goodwin and Waygood (8) have recently shown that the presence of hydrolytic enzymes in barley seedlings may profoundly reduce the yields of succinoxidase. This qualification must be kept in mind in those cases where the measured content of cytochrome oxidase was found to be insufficient to cope with the respiratory requirements of the tissue. And, in those cases where the assay results were in excess of respiratory needs, the conclusions are strengthened because the actual enzyme content must almost certainly be greater than that which we were able to measure.

The enzyme extracts used in this study catalyzed oxygen absorption by PPD to some degree even in the absence of added cytochrome c (figs 2 and 3). There are strong reasons for believing that the bulk of this oxygen absorption is catalyzed by cytochrome oxidase and the endogenous cytochrome c. The evidence in favor of this view is: (a) Phenoloxidase, which might be considered to be a possible catalyst for PPD oxidation, is apparently absent from these seedlings (12, 20); the feeble oxygen absorption induced by the addition of catechol to the extracts is probably ascribable to the cytochrome system, with catechol acting as a reducing agent (18). (b) For the pea extract, the activity of cytochrome oxidase at infinite cytochrome c concentration was the same whether PPD or HQ was used as the reductant. (c) The possible participation of peroxidase is unlikely, since addition of catalase did not affect the enzymatic oxidation of PPD. (d) Measurement of the cytochrome oxidase content by the manometric method provided assay values which agreed closely with those obtained by the more direct and independent spectrophotometric method; if the PPD oxidation in the absence of added cytochrome c was not mediated by the cytochrome system, consistently higher values should have been obtained by the manometric method. For example, the assay value (V_max per seedling) for 4-day barley seedlings is 39 by the manometric method (table II, column 4) and the corresponding value obtained spectrophotometrically is 40 (table III, column 6). Now, if the PPD oxidation observed without added cytochrome c had not in fact been due to cytochrome oxidase, then it would have been necessary to subtract this amount (which is calculated to be 16) from the manometric assay value, a correction which would have reduced the manometric value to almost one-half that of the spectrophotometric. The fact that the assay values obtained by the two methods were almost the same when such a correction was not applied, seems to justify the conclusion that endogenous cytochrome c is largely responsible for the oxygen uptake when PPD alone is added to the extract. (e) The oxida-
tion of PPD by the extracts is sensitive to cyanide and azide, which when applied at concentrations of 0.005 M to 7-day wheat root extracts, inhibited the oxygen uptake by 55% and 50%, respectively.

Slater (18) has already shown that endogenous cytochrome c (which may be bound, in contrast to added cytochrome c, which is soluble (3)) is much more active catalytically than added cytochrome c, due probably to its more favorable spatial relationship with respect to the oxidase. Thus, for those enzyme extracts containing relatively large amounts of endogenous cytochrome c, the subsequent addition of soluble cytochrome c to a mixture of extract and PPD would be expected to result in only small stimulations of oxygen absorption. Such as apparently the case in the barley and wheat root extracts we have examined (table 1), and this consideration, no doubt, goes far toward explaining the difficulty experienced by several previous workers (1, 12, 13, 20) in attempts to detect cytochrome oxidase manometrically. Other factors contributing to the difficulty may be the failure to take precautions essential to success, such as rapid extraction of the enzyme in the cold, and provision of adequately high amounts of cytochrome c and reductant. (See also Goodwin and Waygood (8).) However, in these cases, and indeed wherever it is feasible, it seems that the spectrophotometric method of measuring cytochrome oxidase is greatly to be preferred, since not only is it more sensitive and direct, but it is independent of the endogenous cytochrome c.

**SUMMARY**

Extracts prepared from etiolated seedlings of pea, wheat and barley seedlings were shown to contain cytochrome oxidase at all stages of development (up to 12 days) by both the manometric and spectrophotometric methods. When the amount of the enzyme in the tissue was evaluated in terms of activity at infinite concentration of cytochrome c, it was found that the cytochrome oxidase content of pea shoots and roots, and wheat and barley shoots, was more than adequate to account for the observed respiratory activity of the tissue from which it was derived. However, for the roots of wheat and barley, although the enzyme is present at all stages of development, the amounts extracted were not capable of catalyzing all of the respiratory oxygen absorption.

**LITERATURE CITED**