Studies on the Physiology of Crop Plants in Response to the Effect of High Temperature

II. Inhibition by high temperature of cytochrome c oxidase activity and its restoration by phospholipid.*

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In the previous paper, it was reported that respiratory rate of several species of crop plants was inhibited by the treatment of temperature lower than 40°C, under which it is unlikely that cellular protein undergoes heat coagulation or denaturation.

With the intention of deducing these changes in respiratory activity, the present paper deals with the behavior of cytochrome c oxidase which is generally known as a terminal oxidizing enzyme of respiratory system.

Materials and Methods

Preparation of plant materials: Rice plant and orchard grass were grown in Wagner pots of 1/5,000 are in size by conventional water culture method in green house. Prior to the preparation of enzyme extracts, temperature pretreatments of plant materials were carried out. The detached roots were submerged for 3 hours in the water of various temperatures. In another case, the intact roots were similarly treated for a night, about 16 hours. The temperature treatments of leaves were carried out by the same method as described in the first paper.

Preparation of enzymes: Immediately after the temperature pretreatments, 1-5 gm. of the plant materials were homogenized with 30 ml of phosphate buffer of 0.1 M and pH 7.0, containing succrose of 0.25 M. Following the centrifugation of the homogenate at 600 G for 10 minutes, the precipitates were discarded and the supernatants were further centrifuged at 14,000 G for 20 minutes. The precipitates were suspended in 1 ml of the abovementioned buffer solution and offered as enzyme preparations.

Addition of phospholipid to enzyme preparations: Commercially furnished soybean lecithin was employed as phospholipid. Lecithin was added in advance to the buffer solution with which the plant materials were homogenized. Twenty milligrams of lecithin solved in 0.2 ml of ethanol were mixed with 100 ml of the buffer solution and stirred into emulsion.

Determination of cytochrome c oxidase activity: Determination of the enzyme activity was carried out by conventional Warburg’s manometry. Constituents of reaction mixture are shown in table 1. Endogenous oxygen consumption and autooxidation of cytochrome c were simultaneously determined and subtracted from the oxygen uptake of complete system with the result which is referred to cytochrome c oxidase activity.

Examination of possible oxidation of phospholipid by cytochrome c: In order to examine whether soybean lecithin is oxidized or not by cytochrome c, being subjected to be as a substrate for cytochrome c oxidase, oxygen consumption of the mixture shown in table 2 was determined.

Results

Oxidation of lecithin by cytochrome c: Oxygen consumption of the mixture shown in table 2 was determined with the result that there was scarcely any enhancement of oxygen consumption due to the presence of cytochrome c. (table 3)

Table 1. Reaction mixtures for determination of cytochrome c oxidase activity.

<table>
<thead>
<tr>
<th>Component</th>
<th>Complete system</th>
<th>Endogenous O₂ consumption</th>
<th>Autooxidation of cytochrome c</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 M phosphate buffer, pH 7.0</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>1% hydroquinone</td>
<td>-0.2</td>
<td>0.2</td>
<td>...</td>
</tr>
<tr>
<td>1% cytochrome c</td>
<td>-0.1</td>
<td>...</td>
<td>0.1</td>
</tr>
<tr>
<td>enzyme solution</td>
<td>-0.5</td>
<td>0.5</td>
<td>...</td>
</tr>
<tr>
<td>water</td>
<td>-1.2</td>
<td>1.3</td>
<td>1.9</td>
</tr>
<tr>
<td>total</td>
<td>-3.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

* Received for publication, Jan. 11, 1965.
ingly there is no need to pay consideration for
the oxidation of lecithin by cytochrome c in the
determination of the enzyme activity.

It is generally recognized that haemine catalyzes
oxidation of unsaturated fatty acid. However,
Lewis and Wills observed that the oxidation of
unsaturated fatty acid was inhibited in the pre-
sence of cytochrome c at the concentration more
than 10^{-4} M, in contrast with the enhancement of
the oxidation in the presence of less amount of
cytochrome c.

Cytochrome c oxidase activity as affected by
high temperature: Cytochrome c oxidase activity
in orchard grass roots and leaves was inhibited by
the temperature pretreatments of 32°C for 3
hours and 28°C for 16 hours respectively. (table
4 and 5) In the rice roots, the enzyme was in-
hibited by the temperature pretreatments of 38°C
for 3 hours, though the treatment of 32°C for 3
hours had no influence on it. (table 4)

These results are in consistence quite well with
the results reported in the first paper on the in-
hibitive effect of high temperature on respiratory
rate of leaves and roots of crop plants.

It is now clear that the retardation of cyto-
chrome c oxidase activity induced by high tem-
perature is responsible for the reduced respiratory
rate caused by high temperature treatment.

Effect of phospholipid on the cytochrome c ox-
idase activity: It was observed that the cytochrome c
oxidase activity of orchard grass roots which
was inhibited by the pretreatment of 32°C for 3
hours was completely restored by the addition of
lecithin. The same was true for the rice roots
pretreated at 36°C for 16 hours. (table 6) Sur-
prisingly enough, it was apparently shown that the
thermal inactivation of cytochrome c oxidase
activity, i.e. the inhibition of cytochrome c ox-
idase activity due to high temperature, could be
completely reversed by the addition of a sort of
phospholipid, lecithin.

This fact furnishes an interesting evidence that
the respiratory activity of higher plants is under
the regulation of cellular lipid components and
that high temperature exerts some adverse influ-
ence on the role of cellular lipid components, but
not on the cytochrome c oxidase itself.

**DISCUSSION**

In the light of the results shown in the first
paper and the present one, it is presumed that the
inhibition of respiratory enzyme activity would
responsible for the decline of respiratory activity
of crop plants caused by high temperature even
lower than 40°C, under which the enzyme protein
could hardly undergo heat denaturation.
The results presented here is suggesting an im-
portant aspect of physiological role of cellular lipid
components and also a promising new avenue for the reseach
on the physiological signifi-
cance of temperature on plant
growth and metabolism.

Recent evidence has indicat-
ed that lipids or lipid-soluble
substances may be important
for the normal activity of res-
piratory enzymes. Nason and
Lehman observed the res-
toration by α-tocopherol (vitamin E) of DPNH-
and succinate-cytochrome c re-
ductase activity of rat skeletal
muscle following isocyanate ex-
traction to reduce the activi-

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Table 2. Reaction mixtures for determina-
tion of lecithin oxidation by cytochrome c.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 M phosphate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>buffer, pH 7.0</td>
<td>1.0 ml</td>
<td></td>
</tr>
<tr>
<td>1% hydroquinone</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>1% cytochrome c</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>lecithin emulsion*</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>water</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>3.0</td>
<td></td>
</tr>
</tbody>
</table>

* 0.2 mg/ml

Table 3. Lecithin oxidation by cytochrome c.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>O₂ consumption, mm³/vessel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 min.</td>
</tr>
<tr>
<td>with cytochrome c</td>
<td>0.17</td>
</tr>
<tr>
<td>without cytochrome c</td>
<td>0</td>
</tr>
<tr>
<td>difference</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Table 4. Effect of temperature pretreatment on the cytochrome c oxidase activity of rice and orchard grass roots.

<table>
<thead>
<tr>
<th>Grass Type</th>
<th>Temperature of Pretreatment</th>
<th>Cyt. c Oxidase Activity O₂ Uptake, mm³/f. w./hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>orchard</td>
<td>32°C</td>
<td>31.2</td>
</tr>
<tr>
<td></td>
<td>cont.*</td>
<td>51.2</td>
</tr>
<tr>
<td>rice</td>
<td>32°C</td>
<td>48.1</td>
</tr>
<tr>
<td></td>
<td>cont.**</td>
<td>45.4</td>
</tr>
<tr>
<td>rice</td>
<td>38°C</td>
<td>35.1</td>
</tr>
<tr>
<td></td>
<td>cont.**</td>
<td>40.7</td>
</tr>
</tbody>
</table>

* Left stand at room temp. (about 16°C)
** Ditto (about 22°C)
Table 5. Effect of temperature pretreatment on the cytochrome c oxidase activity of orchard grass leaves.

<table>
<thead>
<tr>
<th>Temperature of pretreatment</th>
<th>Cytochrome c oxidase activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>28° cont. **</td>
<td>43.0</td>
</tr>
<tr>
<td></td>
<td>71.7</td>
</tr>
</tbody>
</table>

* O₂ uptake, mm³/f. w./hr.
** Left stand at room temperature (about 16°C)

Martius³ proposed phylloquinone (vitamin K₁) is a necessary component of the DPNH-cytochrome c reductase system. Crane et al.⁵ reported the isolation from beef heart mitochondria of a quinone with an absorption maximum at 275 mμ (designated as Q275). This compound reactivated the succinoxidase system of heptan-extracted mitochondria.

More recently, Green and his collaborators⁸ made investigations to the wide extent on the electron transport system in beef heart mitochondria and its lipid component. They observed that the mitochondria contained about 26% by weight of lipid and in excess of 90% of it is phospholipid, i.e. 37% of phosphatidylcholine (lecithin), 31% of phosphatidylethanolamine (cephalin), 16% of cardiolipin, and 10% of phosphatidylinositol. The balance is the neutral lipid fraction, the principal constituents of which are coenzyme Q, cholesterol, carotinoid, and glycereid esters. A mixture of 90% aceton and 10% water could extracted the bulk of the phospholipid from mitochondria or submitochondrial particles. Such extracted particles lost the capacity to carry out electron transfer and this capacity could in large measure be restored by adding back mitochondrial phospholipid and coenzyme Q to the particles. An unambiguous requirement for lipid was demonstrated in the following reactions; succinate−O₂; succinate−coenzyme Q; reduced coenzyme Q→cytochrome c; reduced cytochrome c→O₂. They further reported that purified β-hydroxybutylic dehydrogenase showed an absolute requirement for lecithin.⁶,¹⁵

As regards enzyme other than mitochondrial electron transport system, too, Colman and Hübscher⁹ reported the lipid requirement for phosphatidic acid phosphatase activity.

Concerning cytochrome c oxidase, Marinetti et al.¹ⁱ found that a purified cytochrome c oxidase preparation contained 33% lipid which consisted of neutral fat, cholesteral, and phospholipids. Wainio and Greenlees,¹⁰ attempting to purify the cytochrome c oxidase of beef heart mitochondrial fragments by extracting with surface active agents such as deoxicholate found relatively inactive cytochrome c oxidase and its reactivation by adding back deoxicholate extract. They further reported that reactivation of the inactive cytochrome c oxidase was accomplished by adding crude phospholipid from beef brain or heart, purified lecithin, lysolecithin, phosphatidylserine, phosphatidyl ethanolamine, or inositolphosphatide.¹⁴,¹⁵

In view of those reports, it may be concluded that the inhibition by high temperature of cytochrome c oxidase activity in crop plants could possibly be resulted from some degenerations of cellular lipids and/or some disturbances of interactions between the enzyme and lipids. In this connection, Green et al.¹⁴ has generally classified the nature of bonds between lipid and protein into two types, one of which—ionic in character is exemplified by the interaction of basic proteins with acidic phospholipids, and the other—non ionic and largely hydrophobic is exemplified by the interaction between the structural protein of mitochondria with phospholipids whether acidic or

Table 6. Restoration by phospholipid of rice and orchard root cytochrome c oxidase activity affected by high temperature pretreatment.

<table>
<thead>
<tr>
<th>Temperature of pretreatment</th>
<th>Phospholipid</th>
<th>Cytochrome c oxidase activity*</th>
<th>Percentag of no phospholipid addn.</th>
</tr>
</thead>
<tbody>
<tr>
<td>orchard grass</td>
<td>addition</td>
<td>51.5</td>
<td>183</td>
</tr>
<tr>
<td></td>
<td>none</td>
<td>28.1</td>
<td>100</td>
</tr>
<tr>
<td>control**</td>
<td>addition</td>
<td>47.7</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>none</td>
<td>49.4</td>
<td>100</td>
</tr>
<tr>
<td>rice plant</td>
<td>addition</td>
<td>44.0</td>
<td>133</td>
</tr>
<tr>
<td></td>
<td>none</td>
<td>33.0</td>
<td>100</td>
</tr>
<tr>
<td>control***</td>
<td>addition</td>
<td>43.1</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>none</td>
<td>41.8</td>
<td>100</td>
</tr>
</tbody>
</table>

* O₂ uptake, mm³/f. w./hr.
** Left stand at room temperature (about 16°C)
*** Ditto (about 22°C)
zwitterionic.

**Summary**

With the intention of making clear the physiological mode of respiratory inhibition by high temperature of crop plants, cytochrome c oxidase activity of orchard grass and rice plant which were pretreated with various temperatures was determined.

The enzyme activities of orchard grass and rice roots were inhibited by the temperature treatments of 32°C and 38°C for 3 hours respectively. In orchard grass leaves, it was inhibited by the treatment of 28°C for 16 hours.

The inhibited enzyme activities of orchard grass and rice roots were restored by the addition of soybean lecithin.

A possible mode of the enzyme inhibition by high temperature was discussed.

**Literature Cited**


