Oxidase Activities during the Hypersensitive Reaction of *Nicotiana xanthi* to Tobacco Mosaic Virus

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When plant cells die as a result of virus infection, the activity of oxidases, particularly polyphenoloxidases and peroxidases, is altered (Martin, 1958; Farkas, Kiraly & Solymosy, 1960; Farkas et al. 1964). The *in vitro* activity of these two enzyme groups in extracts of infected leaves kept at 20° shows changes that are correlated with the time of appearance and number of local lesions. With most virus/host combinations, the oxidase concentration is merely increased (Van Kammen & Brouwer, 1964; Novacky & Hampton, 1968; Cabanne, Scalla & Martin, 1968), with some there is a change in the relative amount of different isozymes (Bates & Chant, 1970), and with others there is possibly the appearance of new peroxidases (Farkas & Stahmann, 1966) or of a new phenolase (John & Weintraub, 1967). Different workers have interpreted these facts to explain the formation of necrosis and virus localization in different ways (Farkas et al. 1960; Parish, Zaitlin & Siegel, 1965; Suseno & Hampton, 1966). In the experiments reported here we show enzyme alterations by means of a new technique.

Samuel (1931) and Kassanis (1952) showed that the hypersensitive reaction of tobacco to tobacco mosaic virus (TMV) is inhibited by temperatures between 32 and 36°, but when returned to temperatures of 20 to 25° the plants react hypersensitively and necrosis occurs. For instance, when a systemically infected hypersensitive tobacco kept at 32° for 72 hr after infection is put at 20°, necrosis appears within 7 to 11 hr and affects most of the inoculated leaf. This technique enabled Martin & Gallet (1966a, b, c) to show that: (1) The necrosis of tissues is correlated with one of the stages of virus multiplication (Martin & Gallet, 1966a) (2) Necrosis is not the main event responsible for the inhibition of virus multiplication (Martin & Gallet, 1966b) (3) Living cells outside areas of necrosis at 20° contain infective virus (Martin & Gallet, 1966c).

These results suggest that there are at least two distinct mechanisms in the hypersensitive reaction, namely the inhibition of virus multiplication and the death of the cells. We wondered whether oxidases are responsible for the death of cells; to clear up this point we determined the activity of peroxidase and polyphenoloxidase during temperature-shift experiments, as this method allows accurate timing of the appearance of necrosis.

This work was done using the young leaves of 75-day-old plants of *Nicotiana tabacum* L. cr. Xanthi-nc. Two leaves were inoculated on each plant. The whole surface of each leaf was dusted with carborundum (200 mesh), half was rubbed with a TMV solution (4 x 10^{-4} mg./ml.) and the other half with water. The tobacco plants were then put for 72 hr in a controlled chamber to 32° with 80 % humidity and 16 hr photoperiod under 8000 or 18,000 lux. To start the hypersensitive reaction the plants were transferred to 20° (humidity, photoperiod and light intensity as above) and oxidase concentration of extracts was measured at different times. The following method was used for the preparation of the polyphenoloxidase (PPO) solution: 1 g. of green leaf was crushed at 4° in 8 ml. phosphate buffer pH 6.1, 0.1 M, and centrifuged for 1 hr at 8800g. The supernatant contained PPO, and its activity was determined at 20° using the spectrophotometric method of Sisler & Evans (1958) with chlorogenic acid as substrate, and the standards suggested by Van.
Kammen & Brouwer (1964). The peroxidase solution was obtained using the method of Evans & Alldridge (1965): 1 g. fresh leaf was crushed at room temperature in 25 ml. of phosphate buffer, 0.025 M, pH 6.8, filtered and centrifuged at 25,000g for 20 min. The supernatant contained the peroxidase activity, and this was assayed by the method of McCune & Galston (1959); the oxidation of pyrogallol or guaiacol were estimated spectrophotometrically. The peroxidase activity is expressed as the micromolar percentage of the substrate oxidized every 25 sec. for each mg. of dry weight of leaf. The content of the two oxidases in each extract was estimated 4 or 5 times and each result in Fig. 1 and 2 represents the average oxidase content of 2 or 3 leaves picked at a similar level from different plants.

Leaves kept at 20° contained quite different oxidase levels from those kept first at 32° then at 20°. During the comparatively slow hypersensitive reaction in leaves kept at 20°, the PPO and peroxidase activities of cells located outside necrotic lesions, 200/leaf, increased.

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**Fig. 1.** Polyphenoloxidase activity of extracts of the inoculated half-leaves expressed as a percentage of that of corresponding healthy half-leaves. (a) During infection at 20°. (b) After transfer from 32° to 20° 72 hr after infection at 32°. Substrate: chlorogenic acid. The arrow shows time of appearance of local lesions.

**Fig. 2.** Percentage of peroxidase activity determined under conditions of Fig. 1. Substrate: ▲, guaiacol, ■, pyrogallol. The arrow shows time of appearance of local lesions.
when local lesions appeared and doubled 96 and 92 hr respectively after inoculation (Fig. 1a, 2a), results closely similar to those obtained by Martin (1958) and Farkas et al. (1960). By contrast, after a transfer from 32° to 20° there is a fast and violent hypersensitive reaction, yet the activity of these two oxidases does not alter before the first lesions appear (Fig. 1b, 2b). Then, before wilting and generalized necrosis of the leaf, there is an increase of both enzymes, but this increase is small; 12½ hr after a return to 20° the PPO and peroxidase increased by up to 20%. After this the cells of inoculated half leaves were so damaged that no enzyme activity could be determined. Control healthy plants kept at 20° or for 72 hr at 32° contained extractable enzymes in equal amounts (Table 1).

Our results lead to the conclusion that the changes in enzyme activity found during the hypersensitive reaction at 20° are a consequence, not a cause, of the death of the cells. These conclusions are supported by the results of experiments on changes of phenol metabolism induced in tobacco by virus infection; Tanguy showed (1970) that the accumulation of phenols following a transfer to 20° reaches a significant level only after necrosis has appeared. However, though the Michaelis constant is the same at 20° before and after inoculation (Van Kammen & Brouwer, 1964), we cannot tell whether the in vivo increase of oxidase activity represents newly made oxidase co-factors or the breakdown of oxidase inhibitors.

Table 1. Enzyme activity of extracts from leaves of uninfected control plants kept at 20°

<table>
<thead>
<tr>
<th>Time (hr)*</th>
<th>Activity</th>
<th>Time (hr)*</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kept at 20°</td>
<td></td>
<td></td>
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<tr>
<td>24</td>
<td>140</td>
<td>5</td>
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<tr>
<td>32</td>
<td>147</td>
<td>21</td>
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<tr>
<td>36</td>
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<td>48</td>
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<td>72</td>
<td>133</td>
<td>54</td>
<td>0.98</td>
</tr>
<tr>
<td>96</td>
<td>106</td>
<td>68</td>
<td>1.10</td>
</tr>
<tr>
<td>120</td>
<td>160</td>
<td>92</td>
<td>0.98</td>
</tr>
<tr>
<td>72 hr at 32° then transferred to 20°</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>152</td>
<td>0</td>
<td>1.12</td>
</tr>
<tr>
<td>3</td>
<td>133</td>
<td>3</td>
<td>1.12</td>
</tr>
<tr>
<td>6</td>
<td>137</td>
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<td>1.16</td>
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<tr>
<td>9</td>
<td>137</td>
<td>9</td>
<td>1.07</td>
</tr>
<tr>
<td>11½</td>
<td>152</td>
<td>12</td>
<td>1.16</td>
</tr>
<tr>
<td>12½</td>
<td>152</td>
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</table>

* Hr after inoculation of plants. † Hr after transfer from 32° to 20°.

In other words, the importance of oxidases in necrotization is less than had been suggested by Farkas & Kiraly (1962) and Parish et al. (1965), and our results confirm and extend the published results of Van Kammen & Brouwer (1964) and Jockusch (1966).

These results reopen the question of the central cause of local lesion formation and suggest that the temperature shift system we have used will be best for finding this cause. In such experiments it must not be forgotten that there are changes in phosphorus metabolism which can be detected well before the appearance of symptoms and which were mentioned by Scalla & Meignoz (1967).

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REFERENCES


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