Phytochemistry, 1976, Vol. 15, pp. 1353–1359. Pergamon Press. Printed in England.

ENZYMES OF THE PHENYLPROPANOID PATHWAY AND THE NECROTIC REACTION OF HYPERSENSITIVE TOBACCO TO TOBACCO MOSAIC VIRUS

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(*Received* 15 *December* 1975)

Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco mosaic virus; hypersensitivity; necrotic reaction; phenylalanine ammonia-lyase; cinnamic acid-4-hydroxylase; caffeic acid-*O*-methyltransferase; peroxidase.

Abstract—Pronounced increases in activity of phenylalanine ammonia-lyase (PAL), cinnamic acid-4-hydroxylase (CAH) and caffeic acid-*O*-methyltransferase (OMT) occur following infection by TMV of hypersensitive tobacco cv. Samsun NN. The maxima of PAL and CAH activities occur at exactly the same time, whereas those of OMT and peroxidase activities are delayed. The cells showing increases in these enzyme activities are not synchronally affected by the stimulus. There is a radial spread of the stimulated PAL, CAH and OMT activities from the center of the local lesions. Stimulation of enzyme activities in cells peripheral to the center of the local lesion precedes infection by the virus. Shortly after the initial appearance of the local lesions the stimulus spreads much faster than necrosis, but later in the infection the spread of the stimulus slows down while that of necrosis continues almost unabated. Similar results concerning the changes in the activities of these enzymes were obtained after inoculation of the same host with other strains of TMV inducing smaller lesions. All these data show that enzymes of the phenylpropanoid pathway are good biochemical markers of the necrotic reaction. Thus symptoms of necrosis and changes in enzyme activity occur at exactly the same time, while increases in enzyme activities are proportional to the number of developing necrotic lesions. Also, the greater the increase in enzyme activities, the larger the final size of the necrotic lesion. Finally, almost all the cells which show a stimulation of PAL, CAH and OMT activities are destined to be destroyed by necrobiosis.

INTRODUCTION

It has been known for some time that an increased production of phenolic compounds occurs in many plants infected by various phytopathogens [1–3]. Infection of plants by viruses leads either to a generalized invasion or to the hypersensitive reaction. This latter reaction is characterized by the appearance of necrotic local lesions on the inoculated leaves and by a limitation of virus spread in the vicinity of these lesions. It has been shown that the virus is not localized by the barrier of necrotized cells, but is also present in an active state in the cells around the lesion [4]. There is, however, no movement of the virus to uninoculated leaves as in systemic viral infections. The mechanism of localization of the virus is still unknown and represents probably the most interesting feature of hypersensitivity. It seems to be associated with necrosis which is the important visible event of the hypersensitive reaction. Since the production of phenolics is stimulated in necrotic reacting hosts rather than in systemic hosts, these compounds have been suspected of involvement in necrogenesis according to this hypothesis: a dramatic increase in the levels of phenols and a concomitant stimulation of polyphenoloxidases [5–8] and peroxidases [7–9] would be responsible for a rapid accumulation of toxic concentrations of quinones which in turn would lead to disruption of the cells.

In the case of infections of hypersensitive tobacco plants with tobacco mosaic virus (TMV), the most striking quantitative changes are found in phenolic compounds derived from phenylalanine, such as chlorogenic acids, coumarins and flavonoids [10–12]. Since the maxima of their levels are reached late after appearance of symptoms it has been suggested that these compounds cannot be involved in necrogenesis [11]. Since, however, the enzymes which catalyse the catabolism of these phenolics are also activated, a conclusion based upon the time of maximum accumulation of such compounds might be misleading. To determine the exact time of stimulation of the phenylpropanoid pathway we have investigated both the rate of incorporation of radioisotopes and changes in enzyme activities. We have already reported that increases in the rate of incorporation of labeled phenylalanine into phenylpropanoids occur rather early: at the time of local lesion appearance [12]. Moreover, preliminary experiments have also shown an early increase in the levels of phenylalanine ammonia-lyase (PAL) around the local lesions [13].

The present paper summarizes the results of investigating the relationship between the activation of the phenylpropanoid pathway, the necrotic process and the localization of the virus. Experiments were conducted using hypersensitive tobacco plants, *Nicotiana tabacum*, cv. Samsun NN, infected by one of three strains of TMV. Four enzymes were assayed and tentatively used as biochemical markers. These were PAL, cinnamic acid-4-hydroxylase (CAH), caffeic acid *O*-methyltransferase (OMT) and peroxidase. In recent years, considerable attention has been devoted to the response of PAL, the first enzyme of the pathway, to various stimuli applied

to plant tissue [14]. However, rather little is known about changes in PAL in relation to plant disease. CAH, the second enzyme, catalyses the conversion of cinnamic acid to *p*-coumaric acid and is concentrated in the membrane fraction [15,16]. Caffeic acid-*O*-methyltransferase (OMT) of tobacco appears to be similar (Legrand, M., unpublished) to the enzyme extracted from pampa grass [17,18]. We also assayed the level of peroxidase which catalyses the catabolism of many phenylpropanoid compounds and has been linked by several authors to symptom severity and resistance [19].

RESULTS

Changes in enzyme activities in TMV-infected Samsun NN leaves

Figure 1 shows that the 4 enzymes tested are sensitive to TMV infection but that they are not stimulated at the same time. PAL and CAH activities are already increasing when the local lesions become detectable, that is 36 hr after inoculation. The maximum activity of both enzymes occurs 50–60 hr after inoculation and then enzymatic activity decreases. The methylation activity increases at a lower rate and its maximum is reached at about the 4th day following inoculation. The curve of peroxidase activity parallels that of OMT, but with a longer time lag. Thus, there appears to be a sequence in the changes of these enzymes with time of infection. PAL and CAH activities increase first, but the PAL curve is sharper and the maximum of CAH activity occurs slightly later than that of PAL. OMT activity starts to increase at about the same time as PAL and CAH but at a lower rate and is still increasing when PAL and CAH are already decreasing.

Since the changes in enzymatic activities depend on the number of lesions [13], it is advantageous to use heavily infected leaves in order to increase the sensitivity

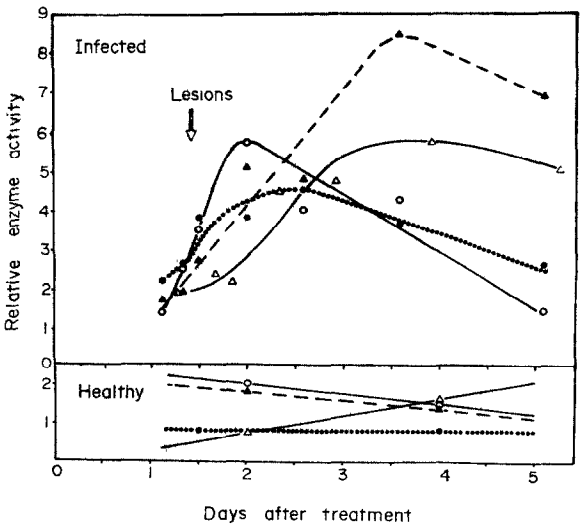

Fig. 1. Changes in enzyme activity during viral infection. Leaves have been inoculated with TMV at a concentration of 0.2 μg/ml in order to develop 250 local lesions. The vertical arrow indicates the time of appearance of the lesions. Symbols used for enzymic activities: O——O PAL; ●····● CAH; ▲---▲ OMT; △——△ peroxidase.

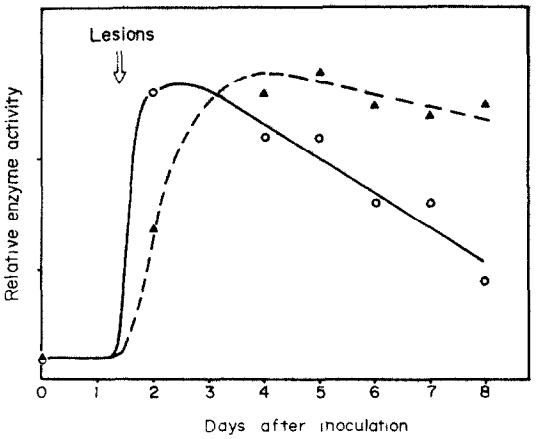

Fig. 2. Time course curves of PAL and OMT activities in discs (diameter of 5 mm) centered on the necrotic lesions. Leaves have been inoculated with TMV at a concentration of 0.04 μg/ml in order to develop 20–50 local lesions. Enzymic activity indicated for the first day after inoculation represents the average enzyme activity over the period studied in 5 mm discs punched on water-inoculated leaves. Symbols used for enzyme activities: O——O PAL; ▲---▲ OMT.

of measurement in the time course curves. Under these circumstances, however, it must be pointed out that after several days of infection the leaves show some drying out and the lesions, when present in large number, become coalescent. This reduces the number of healthy cells around the lesions and hence all enzymatic activities. Therefore, to measure enzyme activities after long infection periods, we assayed discs of 5 mm diameter containing the local lesion in their center. We have already reported that these samples contained the stimulated PAL activity [13]. Figure 2 summarizes the results obtained for PAL and OMT with such discs punched from leaves carrying only a few lesions. It is clear that the sharp rise in PAL activity is not followed by an equally sharp fall as has been mentioned by Simons and Ross [20]; instead the enzymatic activity decreases progressively over a period of several days. The stimulated OMT activity, on the other hand, is stable; only a slight decrease occurs between the 4th and 8th day following inoculation. In the experiment of Fig. 1, 300 local lesions were induced per leaf, resulting in some coalescence of the enlarging lesions and in turn to an over-pronounced decrease in PAL and OMT activities.

The changes in enzyme activities in response to TMV infection show some similarities to the behaviour of the enzymes of flavone glycoside biosynthesis in illuminated cell suspension cultures of parsley [21]. Activities of all enzymes involved in this pathway increased, but differences were observed in the point of maximum enzyme activity. As in our case, PAL and CAH were found to be rapidly responding enzymes whereas the *O*-methylation enzyme (*S*-adenosylmethionine: luteolin 3′-*O*-methyltransferase) fell into a second group of enzymes whose activity was still rising at a time when activities of the first group had already decreased. Concerning the relationship between CAH and PAL, we find in the case of infection a close parallel between the activities of the two enzymes, as has been found in other plant materials during ageing [22] or after light treatments [21,23].

How can these changes in enzyme activities be related to changes in the levels of the individual phenolics of the phenylpropanoid pathway? The sharp increase in PAL and CAH activities at the time of appearance of the lesions is in good agreement with a concomitant rise in the levels of all the phenylpropanoids and with an increased rate of incorporation of labelled phenylalanine into these compounds [12]. Between the second and third day following inoculation, PAL and CAH activities reach their maximum level and then start to decline. The rate of incorporation of labelled phenylalanine into all phenolics is immediately reduced as a result of the decrease in activity of these two key enzymes. At the same time, the levels of *o*-dihydroxyphenols such as chlorogenic acids and rutin fall abruptly whereas, surprisingly, those of methoxylated compounds such as scopoletin, scopolin and conjugates of ferulic acid increase. This enrichment in *m*-methoxy, *p*-hydroxy phenyl compounds appears to be closely correlated with the high OMT activity which lasts for several days. A similar correlation between OMT activity and production of ferulic acid has been observed in wheat during lignification [24]. The aforesaid methoxylated compounds are substrates for peroxidases whose activity is also enhanced at this time. Both methylation of *o*-diphenols and enhanced catabolism of the resulting methoxylated compounds are in good agreement with the decrease in the levels of total phenols which is observed after 3 days of infection [11,20].

All these data show that, even though the maximum concentration of some compounds may occur rather late, the onset of stimulation of the phenylpropanoid pathway appears to be an early event in the course of the disease and not, as previously proposed [11], a secondary effect of viral infection.

Localization of the stimulated PAL and OMT activities and development of necrosis

Figure 3 shows that the growth of the necrotic lesions is relatively fast during the first week of infection but slows down thereafter. There is no significant enlargement of these lesions between 2 and 3 weeks after inoculation. To see how PAL activity, OMT activity and virus titers are localized in relation to necrosis, we assayed discs and rings punched on the leaves with cork borers. The sizes of the different samples were the following: (D) disc with a diameter of 2.5 mm; (R_1) ring with internal and external diameters of 2.5 and 5.0 mm respectively; (R_2) ring with internal and external diameters of 5.0 and 8.0 mm respectively; (R_3) ring with internal and external diameters of 8.0 and 13.0 mm respectively. Leaves were inoculated with low titers of TMV in order

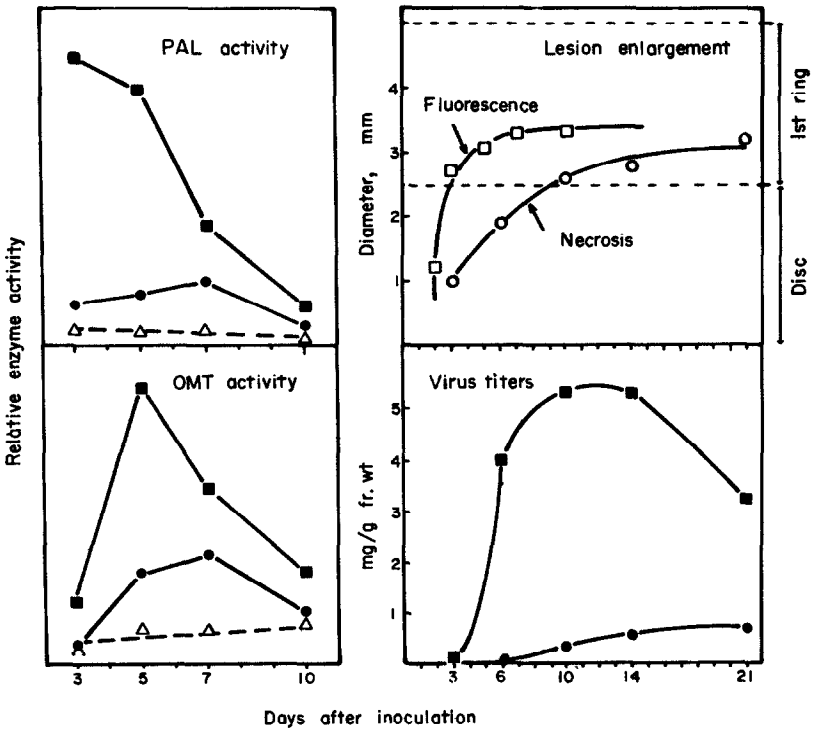

Fig. 3. Localization of the stimulated PAL and OMT activities and of the virus particles during lesion enlargement. Legend: Leaves have been inoculated with 0.01 μg/ml TMV suspension in order to induce 5–20 local lesions per leaf. The samples assayed for PAL and OMT activities and for virus titers are as follows: ■——■ disc D with a diameter of 2.5 mm and centered on the center of the lesion. ●——● ring R_1 with diameters of 2.5 and 5.0 mm and centered on the center of the lesion. △——△ ring R_2 with diameters of 5.0 and 8.0 mm and centered like disc D and ring R_1. Enzymic activities in ring R_2 of infected leaves were the same as those in disc D and rings R_1 and R_2 of healthy leaves. Therefore R_2 may be considered as the control sample. The average diameter ○——○ of the necrotic lesions has been measured using a magnifying glass. The fluorescent rings around the necrotic areas have been viewed in UV light at 360 nm and their external diameter □——□ measured.

to induce 5–20 lesions per leaf. Each ring or disc was centered upon a local lesion. Similar samples were punched from water-inoculated leaves and served as controls. We observed that stimulated PAL and OMT activities were detectable only in disc D and ring R_1. At any time, enzymatic activities in rings R_2 and R_3 of infected leaves were the same as those in disc D and rings R_1, R_2 and R_3 of healthy leaves. Therefore, ring R_2 of infected leaves could also be considered as a control sample. Figure 3 shows the changes with time of infection of PAL and OMT activities in disc D and rings R_1 and R_2. These curves indicate how the stimulated enzymatic activities contained in a disc of 5 mm in diameter (see Fig. 2) are shared between the smaller disc D and ring R_1 during development of necrosis. In these graphs enzymatic activities are expressed relative to the same amount of soluble proteins of the crude extract. Total activities in disc D and first ring R_1 are not comparable since necrosis develops in the first but not in the second sample at the time of these measurements. The curves of PAL and OMT activities in disc D and ring R_1 indicate that the reaction of all the cells to the stimulus is not synchronous. For both enzymes the maxima of activity are not coincident in the two samples. Furthermore, in disc D there is a decrease in PAL activity between 3 and 5 days whereas OMT activity increases very sharply in the same sample during the same period. Such concomitant increase in one enzyme and decrease in another is a matter of enzyme regulation and not of reduction in the number of living cells as a result of necrosis. Figure 3 shows that there is a radial spread of the stimulation of PAL and OMT activities in a manner similar to the spread of the virus and to the progression of necrosis. Moreover, each cell affected by the stimulus shows first an enhancement of PAL and later a stimulation of OMT.

Electron microscopy has shown that, at any time of infection, clumps of virus particles are present in the necrotic areas and in a layer of a few cells surrounding the local lesions [4]. Five days following inoculation, virus was detected even further in advance of necrosis, up to 1.4 mm from the center of the lesions (Roussel, G., unpublished). These results are in good agreement with the curves of virus titers (Fig. 3) which show that one can detect significant amounts of virus in first ring R_1 only after necrosis has approached the edge of this sample. However, stimulation of enzymes are detectable in ring R_1 much earlier.

The curve of fluorescence indicates the minimum size of the zone containing stimulated cells. This is because fluorescence is brought about by an accumulation of scopoletin and its glycoside, scopolin [12], both of which require PAL and OMT for their synthesis [25]. At the time of appearance of the local lesions the fluorescent rings are already clearly visible (diameter of approximatively 1 mm); they extend much faster than necrosis during the following 4 days. Later in the infection the rate of growth of the fluorescent rings slows down while the spread of necrosis continues almost unabated. Two weeks after inoculation, fluorescence is restricted to a zone with a width of 0.2 mm surrounding the necrotic lesion. It is clear from Fig. 3 that at the time of large increases in PAL and OMT activities the stimulated cells are situated in advance of the zones showing high virus titers. Although the presence in these cells of a few virus particles cannot be excluded, it is apparent that extensive multiplication of virus in non-necrotic cells occurs later and in a small layer of cells in advance of necrosis.

Since the fluorescent ring extends to a maximum diameter of 3.5 mm, it follows that only about 1/4 of the cells of ring R_1 are affected by the stimulus (Fig. 3). Thus the degree of stimulation of PAL and OMT activities in this sample is diluted by 75% of unstimulated cells. The conclusion is that PAL and OMT activities increase almost to the same extent in the stimulated cells of disc D and ring R_1 and that all these stimulated cells are progressively necrotized thereafter.

Changes in activity of enzymes in relation to the size of the local lesions induced by 3 strains of TMV

In the experiments described above, Samsun NN leaves were inoculated with the common strain of TMV. To confirm these results, we assayed the same enzymes in Samsun NN leaves inoculated with one of three strains of TMV: the common strain, strain 2 and strain 3. Strain 2 is the GTAMV strain [26]. Strain 3 has been isolated from the common strain after several transfers on tobacco tissue cultures and cloning on leaves of *Nicotiana tabacum*, cv. Xanthi n.c. [27]. Strains 2 and 3 induce much smaller lesions on Samsun NN leaves than the common strain (see Fig. 4), indicating that the plant is more resistant to these other two strains.

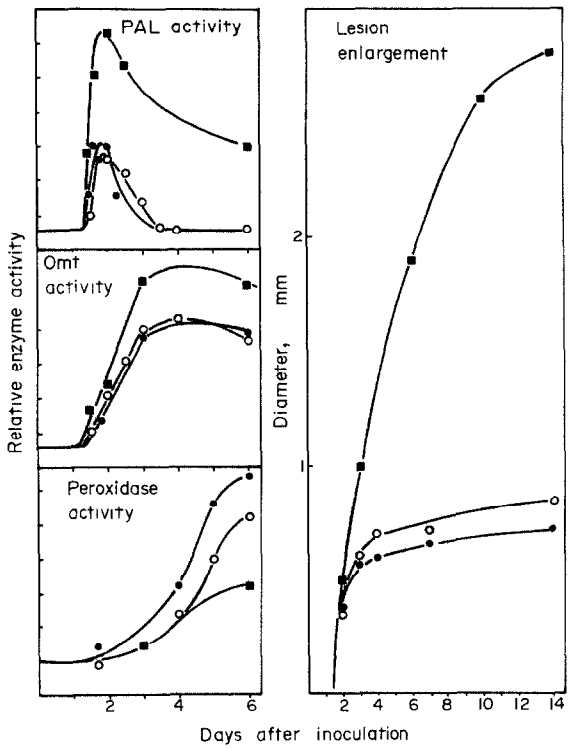

Fig. 4. Changes in activity of PAL, OMT and peroxidases and growth of local lesions induced by three strains of TMV. Enzyme activities were assayed on whole leaves. Virus titers have been adjusted in order to induce approximately 300 local lesions per leaf for each strain. Enzymic activity drawn on each graph for the first day of infection represents that of water-inoculated leaves. Diameters of local lesions have been measured using a graduated magnifying glass. Each determination represents the average of 500 measurements. Symbols used for virus strains: ■—■ common strain; ●—● strain 2; ○—○ strain 3.

Figure 4 summarizes the results obtained for the stimulation of PAL, OMT and peroxidase activities after inoculation of Samsun NN leaves with the three strains of TMV. The local lesions induced by the different strains appear exactly at the same time, i.e. 36 ± 2 hr after inoculation. This is also exactly the time at which PAL and OMT activities are increasing. It appears in Fig. 4 that the time course curves of all enzymic activities are paralleling each other during the increase in activity. After the maxima of enzymic activities is obtained, the decrease in activity is sharper when leaves have been infected by strains 2 and 3. This is apparent in Fig. 4 for PAL and has also been shown for OMT for times of infection longer than 6 days. As will be discussed below, this is the result of a more restricted spread of the stimulus.

The localizations of the stimulated PAL and OMT activities are compared in Table 1. The same samples as those described in Fig. 3 have been punched on Samsun NN leaves infected by one of the three strains. For none of these strains does the stimulus reach ring R_2, i.e. spread further than 2.5 mm from the center of the lesion. Around the local lesions produced by the common strain, increased PAL and OMT activities are found in disc D and ring R_1 as expected from Fig. 3. However, it is clear from Table 1 that, on leaves infected by strains 2 and 3, the stimulated PAL and OMT activities are restricted to the disc D with a diameter of 2.5 mm. These data on the localization of stimulated enzyme activities are in good agreement with the results described in Table 2. The fluorescent rings delimit the zone of enrichment in methoxylated phenylpropanoids. They extend into the ring R_1 when leaves are infected by the common strain but are contained in disc D in the case of infection by strains 2 and 3. Thus, there is a close relationship between the spread of the stimulus and that of necrosis: the larger the size of the necrotic reaction, the larger the zone with a stimulated metabolism of the phenylpropanoids. Moreover, the relative rate of spread of the stimulus and necrosis are similar for the three strains. At the time of appearance of the lesions the fluorescent zone spreads faster than necrosis but later the reverse situation occurs. During advanced stages of infection with all three strains of TMV there remains a fluorescent ring with a width of 0.2 mm in which diffusion or multiplication of the virus (or both) are almost suppressed. It is noteworthy that all these events (appearance and spread of the fluorescent zone and of necrosis) are approximatively the same for the three strains till 2 days after inoculation. At that time, the movements of fluorescence and of necrosis are slowing down on leaves infected by strains 2 and 3, and 1 day later, i.e. 3 days after inoculation, growth of the lesions is almost undetectable. A similar situation occurs only 10 days after inoculation with the common strain.

Table 2. External diameters (in mm) of the fluorescent rings around the necrotic lesions induced by three strains of TMV

Time of infection (days)	Virus strains Common	Strain 2	Strain 3
1.5	1.20	0.90	0.95
3	2.71	1.22	1.31
5	3.07	1.27	1.31
10	3.33	1.31	—

The fluorescent rings containing high levels of methoxylated phenols have been viewed and measured in uv light at 360 nm.

In the experiments described in Fig. 4, virus titers were adjusted in order to induce the same number of lesions for the three strains. It appears that the degree of stimulation of PAL and OMT activities is the highest for the necrotic reaction against the common strain. This is confirmed by the data of Table 1. Therefore one can conclude that the larger the size of the lesions, the higher the total stimulated PAL and OMT activities. However, if one takes into account the number of stimulated cells, it appears that the amplitude of the stimulations is higher for a cell surrounding a smaller local lesion. A calculation using the sizes of the fluorescent zones shows that the number of cells affected by the stimulus is about 7–8 fold lower after inoculation with strains 2 and 3. The stimulation of PAL activity, however, is reduced by only 2 fold and that of OMT activity by even less. With peroxidase activity, the total stimulation as well as the average stimulation per cell is higher in the case of smaller local lesions, suggesting a strong oxidative metabolism.

DISCUSSION

In the case of the host–virus combination tobacco Samsun NN-TMV, there is no drastic change in the total

Table 1. Localization of the stimulated PAL and OMT activities on Samsun NN leaves infected for 3 or 6 days by one of 3 strains of TMV

		3 days			6 days		
	Virus strain	Disc	Ring 1	Ring 2	Disc	Ring 1	Ring 2
PAL activity relative to control	Common	20	2.8	1.0	15	4.2	1.1
	Strain 2	10	1.0	1.1	2.8	0.9	1.0
	Strain 3	12	1.2	1.0	2.0	1.1	0.9
OMT activity relative to control	Common	4.8	1.5	0.9	7.0	3.5	1.1
	Strain 2	5.1	1.1	0.9	5.3	1.1	0.9
	Strain 3	4.0	1.1	1.0	4.2	1.2	0.9

Leaves have been inoculated with low virus titers in order to induce 5–20 local lesions per leaf. The samples assayed for PAL and OMT activities are exactly the same as those of Fig. 3: disc D with a diameter of 2.5 mm, ring R_1 with internal and external diameters of 2.5 and 5.0 mm, ring R_2 with internal and external diameters of 5.0 and 8.0 mm. Enzymic activities of all samples punched from infected leaves are relative to enzyme activities in identical samples punched from water-inoculated leaves.

amount of phenols, the concentration of total phenols being increased only by 50–100% [11]. However, not all the phenols are affected to the same extent. The most striking quantitative changes concern methoxylated compounds (conjugates of ferulic acid, scopoletin and scopolin) whose concentrations are increased by over 100 fold. Such changes and the sequence of stimulation of enzyme activities described here are in every way compatible with an involvement of phenolics in necrosis. It is noteworthy that the time course curves obtained by assaying whole leaves do not reflect exactly the regulation of enzymic activity at the level of the individual cell. Experiments in which the zones of stimulation was visualized by fluorescence in UV light and in which small and concentric samples were assayed allowed us to show that not all cells are affected synchronously by the stimulus. There is a radial spread of the stimulus with an almost constant amplitude from cell to cell. In each cell the stimulation of PAL and CAH activities occurs first and is followed by a stimulation of OMT activity. For each cell these stimulated enzyme activities last for a much shorter time than is apparent when whole leaves are assayed. Moreover we have shown that the stimulus moves in advance of high viral multiplication. Its movement is much faster than necrosis just after appearance of the symptoms and slower than the necrotic process a few days later. Similar results concerning the relative spreads of the stimulus and of necrosis were obtained after inoculation of the same host by two other strains of TMV producing smaller local lesions. These experiments have also indicated that the level of stimulation of enzyme activity of a given cell affected by the stimulus does not depend only on the host. It depends rather on the virus strain responsible for the stimulus and is higher for cells surrounding smaller lesions.

All these data show that there is a close parallel in time and amplitude between necrosis and the stimulation of the phenylpropanoid pathway. Moreover, the total stimulation of enzymes increases with the size of the necrotic area. Therefore, enzymes of this pathway appear to be good biochemical markers of the necrotic reaction. Finally, if one takes into account the number of stimulated cells, activities of these enzymes may also be considered as markers of the resistance which will be developed by the host against viral infection.

EXPERIMENTAL

Plant material. Experiments were performed using 3-month-old tobacco plants *Nicotiana tabacum*, cv. Samsun NN, with 6 or 7 leaves which were grown in an air conditioned greenhouse at 22° ± 2°. We used the 2 or 3 first fully expanded leaves from the top of each plant.

Inoculation of leaves with tobacco mosaic virus. The leaves were inoculated with an aqueous suspension of highly purified virus (wild strain) by rubbing in the presence of Celite. Inoculations with TMV strains 2 and 3 were performed with crude extracts of systemically infected leaves of cv. Samsun. Infections were allowed to develop in a growth chamber at 22° ± 1°. At this temperature infection of Samsun NN leaves by any of the 3 strains of TMV leads to the production of local lesions that appear 34–37 hr after inoculation.

Extraction of enzymes. PAL, CAH and OMT were extracted by grinding 30 g of leaves in a Virtis homogenizer in the presence of 300 mg activated charcoal and of 75 ml ice-cooled 0.1 M Pi buffer pH 7.4 containing 15 mM 2-mercaptoethanol. For PAL and OMT of discs and rings, extraction was carried out with 20 ml buffer per g fr. wt and activated charcoal was omitted. The mixture was filtered through a double layer of cheesecloth and the filtrate was centrifuged at 20000 *g* for 30 min. This supernatant was used to assay PAL and OMT. For the determination of CAH activity a second centrifugation was performed at 100000 *g* for 90 min to yield a pellet containing the membrane fraction. Extraction of peroxidases was carried out by grinding 10 g of leaves at 4° in a mortar in the presence of quartz sand and 30 ml of 0.1 M Pi buffer pH 6.0 free of mercaptoethanol. After centrifugation at 20000 *g* for 20 min, we used the supernatant to assay peroxidase activity.

Determination of enzyme activities. Phenylalanine ammonia-lyase. The reaction mixture consisted of 0.5 ml of 20000 *g* supernatant, 1.5 ml 0,1 M borate buffer pH 8.8 and 0.1 ml of the same buffer containing 0.05 μCi of L-phenylalanine U-^{14}C and 2 mM L-phenylalanine. After 2 hr incubation at 37° the reaction was stopped with 0.1 ml 10 N H_2SO_4. After addition of 8 ml H_2O and 15 ml of a ether–cyclohexane (1:1) mixture, each tube was shaken for 0.5 min. 10 ml of the organic phase were removed and mixed with 10 ml Bray's [28] scintillation solution for counting. The background (contamination of cinnamic acid by traces of labelled phenylalanine) was determined by inactivating the enzymic soln with 0.1 ml 10 N H_2SO_4, then incubating it with the same amount of radioactive substrate. The reaction mixture was treated as described above and the radioactivity measured taken as the background. All assays were run in triplicate. Caffeic acid *O*-methyltransferase. The reaction mixture contained 0.5 ml of the 20000 *g* supernatant, 2.5 mM caffeic acid, 150 μM *S*-adenosyl-L-methionine and 0.1 μCi *S*-adenosyl-L-methionine [methyl-^{3}H] in a final volume of 0.6 ml. After 1 hr of incubation at 37° the reaction was stopped with 0.1 ml 10 N H_2SO_4. The reaction mixture was then treated as described above for measurement of PAL. All assays were run in triplicate. Cinnamic acid 4-hydroxylase. The pellet of 100000 *g* centrifugation was resuspended and homogenized in a vol of 0.5 ml of Pi buffer. The reaction mixture contained 100 μl of microsomes, 100 μl of cinnamate-[3-^{14}C] (0.5 mM, 5 μCi/ml) and 50 μl of a regeneration mixture (6 mM NADP, 10 mM glucose-6-phosphate, 8 units/ml of glucose-6-phosphate dehydrogenase). This mixture was incubated for 30 min at 30° with continuous shaking. The reaction was stopped with 0.1 ml 4 N HCl and 200 μg of carrier *p*-coumaric acid and 10 ml H_2O were added. The mixture was then extracted with 3 portions Et_2O and the organic phase was then washed 2× with H_2O and dried. After evaporation of Et_2O, TLC was performed on pre-coated Si gel plates with the upper phase of C_6H_6–HOAc–H_2O (6:7:3). *p*-Coumaric acid (R_f 0.26) was eluted with EtOH, recovery was determined by UV spectroscopy and the radioactivity measured by scintillation counting of an aliquot of the ethanolic fraction. The background (contamination of *p*-coumaric acid by some tailing of the cinnamic acid peak) was determined by inactivating the enzymatic solution with 0.1 ml 4 N HCl. After incubation as described just above, the radioactivity of *p*-coumaric acid was taken as the background. Four parallel samples were run for each assay. Peroxidases. The 20000 *g* supernatant was diluted 10–500 fold, depending on activity, with 0.1 M phosphate buffer pH 6.0. We used 20 mM guaïacol and 0.1% (w/v) H_2O_2 as substrates and measured changes in A. vs time at 420 nm. The slopes at zero time of the A. curves were determined for several concentrations of enzyme and averaged to calculate enzyme activity.

Measurement of virus titers. These measurements are based on the fact that the number of local lesions induced on leaves of hypersensitive tobacco depend on the concentration of the viral soln used for inoculation. The curve of number of lesions vs virus concentration shows an almost linear relation in the range of 30–150 local lesions per leaf. Discs and rings to be assayed for virus titers were ground at 4° in a mortar in the presence of quartz sand and 0.01 M Pi buffer pH 7.0. Supernatant of the centrifugation at 20000 *g* for 20 min was used for inoculation. To obtain the standard curve several dilutions of a purified TMV suspension of known concentration were inoculated on the 3 first fully expanded leaves of 3 plants

of a lot of Samsun NN plants. Several dilutions of the supernatants to be assayed were similarly inoculated the same day on leaves of the same lot of plants. After 4 days of infection local lesions were counted and the average number per leaf calculated for the 9 leaves inoculated with the same extract. The dilutions leading to a number of local lesions situated on the linear portion of the standard curve were used to calculate the virus titers. This method is very sensitive and allows the determination of virus concentrations as low as 0.01 μg/ml. Proteins were measured according to Lowry *et al.* [29].

Acknowledgements—The skilled technical assistance of Mrs. P. Geoffroy is gratefully acknowledged. This work was supported by a grant of the Commissariat à l'Energie Atomique.

REFERENCES

1. Bollard, E. G. and Matthews, R. E. F. (1966) *Plant Physiology* (Stewart, F. C. ed.), Vol. IV B. Academic Press, New York.
2. Goodman, R. N., Kiraly, Z. and Zaitlin, M. (1967) *The Biochemistry and Physiology of Infectious Plant Disease.* Van Nostrand, New Jersey.
3. Hughes, J. C. and Swain, T. (1960) *Phytopathology* **50,** 398.
4. Hayashi, T. and Matsui, C. (1965) *Phytopathology* **55,** 387.
5. Solymosy, F. and Farkas, G. L. (1963) *Virology* **21,** 210.
6. Cabanne, F., Scalla, R. and Martin, C. (1968) *Ann. Physiol. Vég.* **10,** 199.
7. Suseno, H. and Hampton, R. E. (1966) *Phytochemistry* **5,** 819.
8. Van Loon, L. C. and Geelen, J. L. M. C. (1971) *Acta Phytopathol. Acad. Sci. Hung.* **6,** 9.
9. Birecka, H. and Catalfamo, J. L. (1975) *Plant Physiol.* **55,** 611.
10. Fritig, B. and Hirth, L. (1971) *Acta Phytopathol. Acad. Sci. Hung.* **6,** 21.
11. Tanguy, J. and Martin, C. (1972) *Phytochemistry* **11,** 19.
12. Fritig, B., Legrand, M. and Hirth, L. (1972) *Virology* **47,** 845.
13. Fritig, B., Gosse, J., Legrand, M. and Hirth, L. (1973) *Virology* **55,** 371.
14. Camm, E. L. and Towers, G. H. N. (1973) *Phytochemistry* **12,** 961.
15. Russel, D. W. and Conn, E. E. (1967) *Arch. Biochem. Biophys.* **122,** 256.
16. Potts, J. R. M., Weklych, R. and Conn, E. E. (1974) *J. Biol. Chem.* **249,** 5019.
17. Finkle, B. J. and Masri, M. S. (1964) *Biochim. Biophys. Acta* **85,** 167.
18. Finkle, B. J. and Kelly, S. H. (1974) *Phytochemistry* **13,** 1719.
19. Simons, T. J. and Ross, A. F. (1970) *Phytopathology* **60,** 383.
20. Simons, T. J. and Ross, A. F. (1971) *Phytopathology* **61,** 1261.
21. Hahlbrock, K., Ebel, J., Ortmann, R., Sutter, A., Wellmann, E. and Grisebach, H. (1971) *Biochim. Biophys. Acta* **244,** 7.
22. Camm, E. L. and Towers, G. H. N. (1973) *Can. J. Botany* **51,** 824.
23. Amrhein, N. and Zenk, M. H. (1970) *Naturwissenschaften* **6,** 312.
24. Glass, A. D. M. and Bohm, B. A. (1972) *Phytochemistry* **11,** 2195.
25. Fritig, B., Hirth, L. and Ourisson, G. (1970) *Phytochemistry* **9,** 1963.
26. Knight, C. A., Silva, D. M., Dahl, D. and Tsugita, A. (1962) *Virology* **16,** 236.
27. Hirth, L. and Durr, A. (1970) Colloques Internationaux (CNRS ed.), *Les Cultures de Tissus de Plantes.*
28. Bray, G. A. (1960) *Anal. Biochem.* **1,** 279.
29. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265.