Metabolic Changes Associated with Systemic Induced Resistance to Tobacco Mosaic Virus in Samsun NN Tobacco

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ABSTRACT

The induction of systemic resistance in upper leaves of Samsun NN tobacco by inoculation of lower leaves with tobacco mosaic virus (TMV) was not accompanied by permanent change in the rate of oxygen uptake or in the activities of phosphohexoisomerase, aconitase, cytochrome oxidase, polyphenol oxidase, ascorbic acid oxidase, or glucose-6-phosphate dehydrogenase. Peroxidase and catalase activities increased parallel with the development of resistance and remained at high levels.

Following inoculation with high-titer TMV inocula, oxygen uptake and the activities of all enzymes increased markedly at about the time of lesion formation, reached a maximum shortly afterwards, then declined sharply (except catalase and peroxidase); increases were detected sooner and the maxima reached were earlier and higher in resistant than in nonresistant leaves. Lesions appeared sooner and in greater numbers in resistant than in nonresistant leaves.

We conclude that inoculation with a lesion-inducing virus elicits a nonspecific host response characterized by a general increase in enzyme synthesis and oxidative metabolism in infected and surrounding cells. Resistance is considered a consequence of high peroxidase activity, which leads to early killing of infected cells, which in turn leads to rapid and enhanced changes in advance of infection, including early formation of a barrier to virus spread. Phytopathology 61:293-300.

Additional key words: hypersensitivity, local lesions, virus localization.

Tobacco varieties containing the *N* gene for hypersensitivity (6) respond to infection with tobacco mosaic virus (TMV) by the formation of local lesions, to which the virus stays confined, at the sites of infection. Lesions appear on the 2nd day after inoculation and expand rapidly for the next 3-5 days; subsequent increments of growth are minimal, or no further enlargement occurs. A zone of tissue surrounding a mature lesion displays a highly increased capacity to resist infection by TMV or any of several other local lesion-inducing viruses (14). Presumably, this induction of resistance in advance of the virus may be involved in eventual virus localization. Inoculation of the lower leaves effects induction of a high level of resistance to TMV infection in the noninfected upper leaves; this resistance is expressed as an early cessation of lesion growth and a reduction in lesion size (15, 16). Available data indicate (16) that mechanisms that normally function to limit lesion expansion are stimulated in resistant leaves.

Since the paper of Szent-Gyorgyi & Vietorisz (20), altered patterns of oxidative and phenol metabolism have been associated by many with the functioning of the hypersensitive reaction. In our work, aspects of this approach are brought to bear on a study of the systemic resistance to TMV induced in noninfected upper leaves of Samsun NN tobacco by previous inoculation of the lower leaves with TMV.

Total respiration rates of leaf tissue during and subsequent to resistance induction were compared with those of appropriate controls. The activities of selected enzymes that catalyze specific steps of the glycolytic (EMP), hexose monophosphate (HMP), and tricarboxylic acid (TCA) reaction sequences; of selected terminal oxidases; and of the peroxide-dependent enzymes, peroxidase and catalase, also were examined. These systems were also compared in resistant and nonresistant leaves subsequent to challenge inoculation.

In order to amplify changes possibly occurring only in infected cells, challenge inoculations were made routinely with high-titer TMV inoculum to ensure that all samplings contained large numbers of infected cells. Also, an attempt was made to quantify the amount of necrosis in resistant and nonresistant leaves at intervals after inoculation corresponding to the sampling times for enzyme assays.

MATERIALS AND METHODS.—*Nicotiana tabacum* L. 'Samsun NN', which contains the *N* gene from *N. glutinosa* L. (6), was used. The plants were grown in steam-sterilized, composted soil in 4-inch pots in a greenhouse in which the temp averaged 21 C in winter months but sometimes exceeded 30 C in summer. Plants used in oxygen uptake studies were held after inoculation in a small air-conditioned room of this greenhouse kept at 21 C (±2 C) and fitted with a single bank of Sylvania warm white fluorescent tubes suspended about 2 ft over the plants to provide supplemental light for 12 hr/day. Plants used as sources of enzymes were kept after inoculation in a controlled environment chamber at 21 C and illuminated for a 16-hr day with 1,400 ft-c of light, mostly from cool white VHO fluorescent tubes.

Inocula were prepared from frozen clarified juice of Turkish tobacco plants systemically infected with TMV (common strain). Inocula were applied with artist's brushes to leaf surfaces previously dusted with 400-mesh Carborundum, and leaves were rinsed with tap water immediately after inoculation. In simulated inoculations of leaves of control plants, tap water was substituted for the virus inoculum.

In most experiments, two inoculations were made. The day before the first inoculation, Samsun NN tobacco plants in the six- to eight-leaf stage were topped and trimmed to two or three well-expanded lower leaves and two expanding upper ones. The lower leaves were inoculated with TMV inoculum diluted to induce 300-400 lesions/leaf. Challenge inoculations of upper leaves were normally made 6-8 days later. Where the degree of resistance was being estimated, inocula diluted to induce 50-100 lesions/leaf were used for the challenge inoculation; otherwise, high-titer inocula (juice diluted 10^{-1} or 10^{-2}) were used.

Lesion counts and measurements were made through a binocular stereoscopic microscope at ×10. Early lesions were made more clearly visible for counting by soaking infected tissues in a 5% solution of pyrogallol.

Oxygen uptake.—Samples for oxygen uptake studies consisted of 15 leaf discs, each 7 mm in diam, and were constituted of three 5-disc lots, with discs of each lot punched by means of a sharp cork-borer from leaves of a single plant. The discs were immediately placed in the main compartment of a respirometer flask to which 1 ml of distilled water had previously been added. Prior to measurements, in a Gilson differential respirometer, 0.5 ml of 10% KOH and a filter paper wick were added to the center compartment of each respirometer flask. The loaded flasks were placed in the water bath of the respirometer, which was kept at 25 C and equilibrated for 30 min. Oxygen absorption was measured in the dark at 30-min intervals for 2 hr after equilibration. Results were calculated as μliters of O_2 absorbed/hr per mg dry wt.

Preparation of leaf extracts.—Each homogenate for enzyme assay was prepared from a sample consisting of three equal lots of discs (diam 2.1 cm), each of which had been freshly harvested from the interveinal tissues of leaves of a single plant of the same treatment, or from five such lots in the case of peroxidase assays. Each sample was ground in a small volume of chilled buffer (kind and concn given later for each type of assay) in a Virtis "45" homogenizer at high speed for 90 sec at approx 27 C. The triturated tissues were then filtered rapidly at 5 C through glass wool and subsequently maintained at or below this temp. The filtrate was centrifuged at 10,000 *g* for 30 min prior to phosphohexoisomerase, glucose-6-phosphate (G-6-P) dehydrogenase, aconitase, and cytochrome oxidase assays; prior to all other assays, filtrates were centrifuged at 3000 *g*. The yellowish supernatant fluids were decanted into test tubes and kept in ice baths until assayed. At each sampling, samples duplicating those described above were taken for dry-wt determinations, which were made by drying to constant wt at 135 C.

Enzyme assays.—A Bausch and Lomb Spectronic "20" colorimeter was used for peroxidase and polyphenol oxidase assays and a Beckman DB spectrophotometer for those of phosphohexoisomerase, G-6-P dehydrogenase, and aconitase. In the case of the latter instrument, silica cells with a 1-cm light path were used in all assays, and changes in extinction of reaction mixtures were recorded continuously by a Linear/Log Varicord "43" strip recorder. All manometric measurements were based on techniques described by Krupka (11) and were made as outlined in a previous section. Specific activities of test samples (including those from rubbed controls) were commonly calculated on the basis of dry wt and are presented relative to the activities of noninoculated, nontreated, though otherwise similar leaves.

Phosphohexoisomerase activities in extracts prepared from 6 g of leaf tissue in 15 ml of 0.1 M Tris [tris (hydroxymethyl) amino methane] buffer (pH 7.4) were measured spectrophotometrically (19). Commercial G-6-P dehydrogenase was diluted afresh before each group of assays in such a way that 0.1 ml induced a change of 0.1 optical density (OD) per min in a system containing 1.0 μM of G-6-P and 0.1 μM of nicotinamide adenine dinucleotide phosphate (NADP) in a volume of 3 ml. Specific activities, based on the initial rate of appearance of reduced NADP (in reaction mixtures containing 2.5 μM of fructose-6-phosphate [F-6-P] in 2 ml distilled water, 0.4 ml of 0.1 M Tris buffer at pH 7.4, 1.0 μM of $MgCl_2$ in 0.4 ml of buffer, 0.1 ml of diluted G-6-P dehydrogenase solution, 0.3 μM of NADP in 0.2 ml of distilled water, and 0.1 ml of leaf extract) were calculated as change in OD at 340 nm/hr per mg dry wt. In reference mixtures, the sugar phosphate solutions were replaced by equal volumes of distilled water.

G-6-P dehydrogenase activities in extracts prepared from 6 g of leaf tissue in 15 ml of 0.1 M glycylglycine buffer at pH 7.5 were measured spectrophotometrically by procedures based on those of Kornberg & Horecker (10). Specific activities, based on the rate of appearance of reduced NADP (in reaction mixtures containing 0.5 ml 0.1 M glycylglycine buffer at pH 7.5, 0.4 ml 0.1 M $MgCl_2$, 0.2 ml of 1.5×10^{-3} M NADP, 2 ml of 0.01 M G-6-P, and 0.1 ml of leaf extract), were calculated as change in absorbance at 340 nm/hr per mg dry wt. In reference mixtures, G-6-P and NADP solutions were replaced by equal volumes of distilled water.

Aconitase in extracts prepared from 6 g of leaf tissue in 15 ml of 0.1 M glycylglycine buffer at pH 7.8 was assayed as described by Anfinsen (1). Specific activities, based on the appearance of *cis*-aconitate (in reaction mixtures consisting of 50 μM of citric acid in 3 ml of 0.1 M glycylglycine buffer at pH 7.8 and 0.1 ml of leaf extract) was calculated as change in absorbance at 240 nm/hr per mg dry wt. Reference mixtures were similarly constituted, except that the enzyme had been inactivated by heating the leaf extract in a boiling water bath for 30 min.

Activity of polyphenol oxidase in extracts, prepared from 6 g of leaf tissue in 15 ml of 0.01 M phosphate buffer at pH 5.8 and dialyzed for 6 hr in the cold against the buffer prior to assay, was measured by the procedure of Kenten (9). Reaction mixtures contained 1 ml of freshly prepared catechol solution (0.4 mg catechol/ml distilled water), 2.0 ml of distilled water, and 2.0 ml of leaf extract. Activity was calculated as change in absorbance per mg dry wt between 30 and 180 sec after addition of substrate.

Cytochrome oxidase assays were based on manometric measurements of reduced cytochrome *c* in the

presence of leaf extract prepared from 6 g of leaf tissue in 15 ml of 0.01 M phosphate buffer. Reaction mixtures were equilibrated 15 min and then 0.5 ml 2×10^{-4} M cytochrome *c* and 0.5 ml of freshly prepared 0.12 M *p*-phenylenediamine (neutralized to pH 7.0 with 0.1 M HCl) were added from separate side arms into the main compartment of a respirometer flask containing 2 ml of leaf extract; O_2 uptake was then measured at intervals of 10 min for 30 min. Results, determined on the basis of initial O_2 uptake, were calculated as μliters O_2 absorbed/hr per mg dry wt, correction being made for oxidation of *p*-phenylenediamine in the absence of cytochrome *c*.

In the ascorbic acid oxidase assays, O_2 uptake was measured manometrically. Reaction mixtures consisted of 2 ml leaf extract, prepared from 6 g of leaf tissue in 15 ml of 0.05 M phosphate buffer (pH 5.8), and 0.5 ml 0.25 M ascorbic acid (dissolved in 0.05 M phosphate buffer at pH 5.8). The reactants were held separately 10 min (ascorbic acid in the side arm and leaf extract in the main compartment) for equilibration to temp; they were then mixed, and O_2 uptake was recorded at 5-min intervals for 30 min. Specific activities, calculated on the basis of the initial rate of O_2 uptake, were determined as μliters O_2/hr per mg dry wt.

Peroxidase activity was measured as previously described (18). In catalase assays, oxygen evolution from the decomposition of H_2O_2 in the presence of leaf extract (prepared from 5 g of leaf material in 15 ml 0.01 M phosphate buffer (pH 7.0) and diluted 1:4 with the buffer prior to use) was measured manometrically. After a 10-min equilibration period, 0.2 ml leaf extract was added from the side arm to 3.0 ml 0.01 M phosphate buffer (pH 7.0) and 0.2 ml of 0.2 M H_2O_2 in the main compartment, and the evolution of O_2 was measured at 1-min intervals for 5 min. Specific activities were calculated as μliters O_2 evolved/min per 0.1 ml of leaf extract.

RESULTS.—*Oxidative metabolism of noninfected leaves during and after the induction of systemic resistance.*—In four experiments, in which upper leaves were sampled 4, 5, 6, and 8 days following inoculation of the lower leaves of test plants with TMV, or rubbing those of control plants with water, the rate of O_2 uptake of discs from upper leaves was independent of the treatment of lower leaves and also independent of the time interval. This was true whether results were calculated on the basis of fresh wt, dry wt, or protein content. Each group was challenge-inoculated immediately following the taking of discs; high levels of resistance in the upper leaves of the test plants were detected on the 5th day and subsequently. Thus, resistance induction in the noninfected upper leaves appears not to be accompanied by increased O_2 uptake.

At no time after primary inoculation did the activities of phosphohexoisomerase, aconitase, cytochrome oxidase, or ascorbic acid oxidase in extracts of test leaves differ significantly from those in extracts of control leaves (Table 1). The temporary increase in G-6-P dehydrogenase activity detected on the 5th day in test leaves did not persist beyond the 8th day, whereas resistance persists much longer than this (16). An analysis of our data on polyphenol oxidase led us to conclude that the single significant difference found (Table 1) was due to inadequate sampling, for in separate tests and earlier (2), much plant-to-plant and leaf-to-leaf variation was found.

The activities of catalase and peroxidase were observed to be significantly increased in resistant leaves at 5 days after primary inoculation and subsequently (Table 1). The increases in peroxidase activity, which have been described in greater detail elsewhere (18), were approximately threefold, and those of catalase activity approached twofold (Fig. 1). The increased catalase activity coincided both with increased peroxidase activity and the development of resistance (18). In another experiment, the catalase activity of extracts of highly resistant leaves sampled 14 days after primary inoculation was found to be 1.83 times greater than that of extracts of control leaves; thus, the increased level of catalase activity persists after the max is reached, just as do the increased levels of peroxidase activity (18) and resistance (16).

Oxidative metabolism of resistant and nonresistant leaves subsequent to inoculation with a potent TMV inoculum.—In five experiments, the upper leaves of

TABLE 1. Increases in activity of respiratory enzymes found in extracts of upper leaves of Samsun NN tobacco in association with the induction of systemic resistance following inoculation of lower leaves with tobacco mosaic virus (TMV)[a]

Enzyme	No. tests	Increases found on day indicated[b]									
		1	2	3	4	5	6	7	8	9	10
Phosphohexoisomerase	2			0		0			0		
G-6-P dehydrogenase	4		0		0	+	+	+	+	0	0
Aconitase	2		0		0	0		0		0	
Cytochrome oxidase	2				0		0		0		
Polyphenoloxidase	6		0	+	0	0		0		0	
Ascorbic acid oxidase	2				0		0		0		
Peroxidase	4	0	0	0	0	+	+	+	+		
Catalase	2	0	0	0	0	+	+	+	+		

[a] Extracts were prepared at intervals from upper leaves of test plants, whose lower leaves were inoculated on day 0 with TMV and from comparable control plants, whose lower leaves were rubbed with water. A high level of resistance had developed in upper leaves by the fifth day.

[b] Days are numbered from the day when lower leaves were inoculated. A plus sign denotes a significantly higher activity in the extract from test leaves (inoculated plant) than in the corresponding extract from control plants; a zero indicates no significant difference. Where three or more sets of data were collected, they were analyzed by Student t-test.

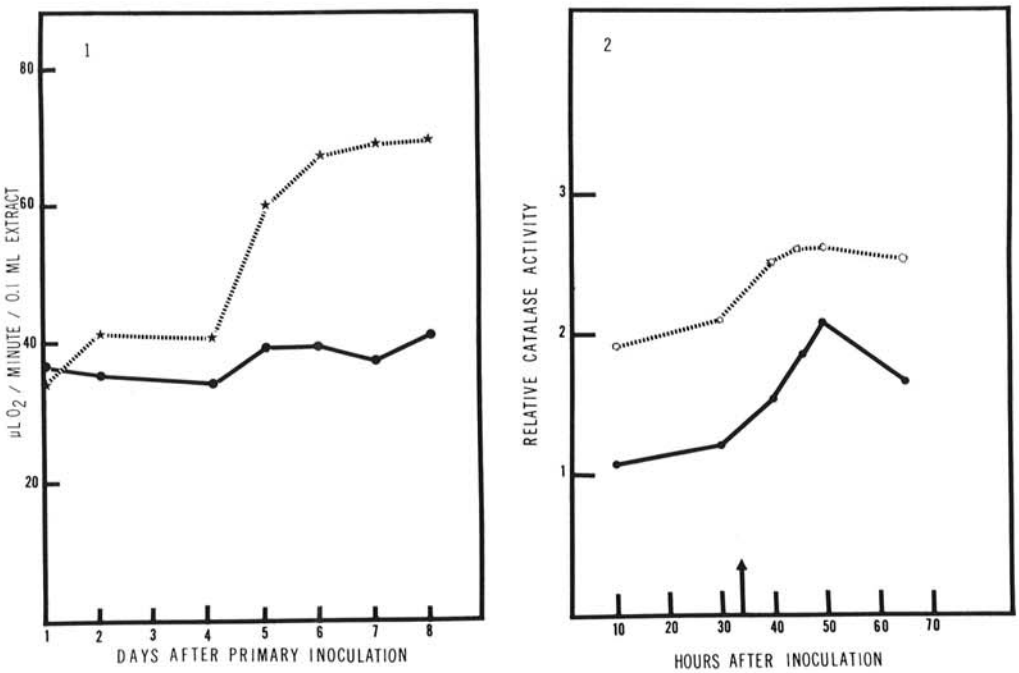

Fig. 1-2. Catalase activity, as indicated by rate of decomposition of H_2O_2 of extracts of upper leaves of Samsun NN tobacco, the lower leaves of which had previously been inoculated (broken line) with tobacco mosaic virus (TMV) or rubbed with water (solid line). **1)** Noninoculated upper leaves sampled at intervals following treatment of lower leaves. Development of resistance in inoculated plants paralleled the upper curve, reaching a max in 5-7 days. **2)** Upper leaves sampled at intervals following challenge inoculation with high-titer TMV inoculum (juice diluted 10^{-2}) 7 days after inoculation and rubbing of lower leaves. Activity is expressed relative to that of extracts from control leaves (activity = 1) which were noninoculated, nontreated leaves (from noninoculated, nontreated plants) that were otherwise comparable. Arrow on horizontal axis indicates approximate average time of lesion appearance. Level of resistance was 71%, as indicated by the formula in legend for Fig. 4-9.

sets of six plants, three resistant and three nonresistant, were inoculated with potent TMV (crude juice diluted 10^{-2}) 75, 65, 60, 50, 46, 30, 20, and 10 hr previous to the removal of discs for O_2 uptake measurements.

The averaged results of five experiments (Fig. 3), in each of which a similar trend was apparent, indicate an increased respiratory rate due to infection in both resistant and nonresistant leaves. This increase became apparent 30 hr after inoculation and remained significant thereafter. After local lesion formation, 45-46 hr after inoculation, O_2 uptake of both types of leaf tissue increased sharply, the magnitude of the increase being much greater in resistant than in nonresistant leaves at 50 hr after inoculation and for 10-15 hr thereafter. Respiration rates attained a max at 60 and 65 hr after inoculation in discs of resistant and nonresistant leaves, respectively; subsequently, respiration decreased in both cases.

As was the case for peroxidase (18), the activity of catalase prior to, during, and subsequent to lesion formation was substantially greater in resistant than in nonresistant leaves (Fig. 2). Although the differences subsequent to lesion formation (Fig. 2) are not so divergent as those reported for peroxidase (18), it is clear that catalase activity remained greater in the former than in the latter, despite the development of more extensive necrosis in the latter.

In general, the activity patterns of all other enzymes (Fig. 4-9) investigated paralleled the patterns of O_2 uptake of discs cut from similar leaves after challenge (Fig. 3). In all cases (Fig. 4-9), a characteristic activity increase occurred just preceding or during the appearance of visible lesions, reached a peak some hr later, and usually decreased subsequently. Each characteristic phase of the activity profile occurred sooner, and this generally by a few hr, in resistant leaves than in nonresistant ones. Furthermore, the max or peak activity in resistant leaves was almost always significantly greater than that in nonresistant leaves.

Stimulation of the major pathways of glucose and two-carbon-unit catabolism in inoculated leaves is indicated by the increased activities of phosphohexoisomerase (Fig. 4), G-6-P dehydrogenase (Fig. 5), and aconitase (Fig. 6). The activity increases of the last two enzymes mentioned are clearly associated with the approx time of lesion formation. The relative activity increase of G-6-P dehydrogenase was more striking than that of phosphohexoisomerase, a sharp rise in the former being evident in both resistant and nonresistant leaves at about the time of lesion formation. A sharp drop in activity of G-6-P dehydrogenase in resistant samples between 40 and 42 hr coincided with a continuing rise in activity in nonresistant ones. This is convincing evidence that max activity of this enzyme

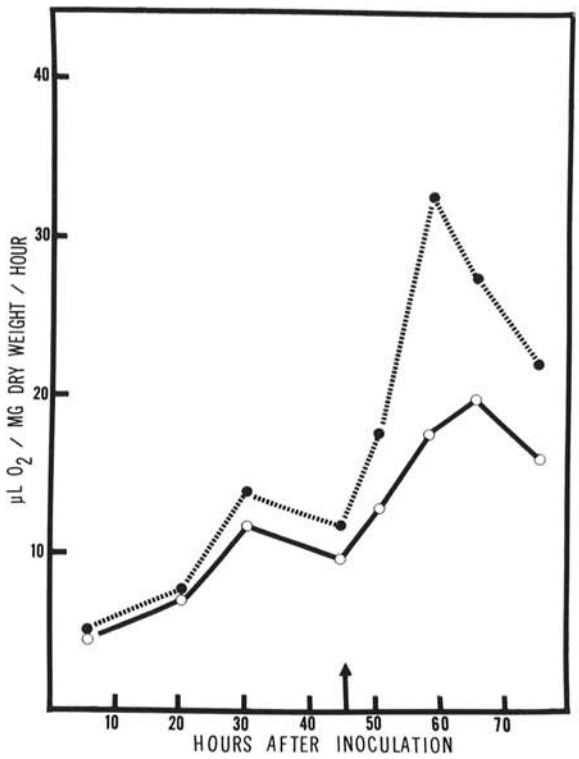

Fig. 3. Oxygen uptake by discs cut from resistant (broken line) and nonresistant (solid line) Samsun NN tobacco leaves at intervals following inoculation with high-titer tobacco mosaic virus (TMV) inocula (juice diluted 10^{-2}). Arrow on horizontal axis indicates approximate average time of lesion appearance. Resistance was induced by prior inoculation of lower leaves with TMV.

is achieved sooner in the former than in the latter. The postinoculation activity profiles of aconitase (Fig. 6) qualitatively resemble those of G-6-P dehydrogenase, although the relative activity increases are not so great in either resistant or nonresistant leaves.

Increased aconitase and, presumably, TCA cycle activity prompted investigation of cytochrome oxidase activity, since "normal" energy-coupled oxidation of reduced NAD is through the cytochrome system. The qualitatively similar patterns of cytochrome oxidase activity (Fig. 7) in extracts of resistant and nonresistant leaves may possibly indicate a coupling of enhanced glucose catabolism to increased oxidative phosphorylation.

Activation of both polyphenol oxidase (Fig. 8) and ascorbic acid oxidase (Fig. 9) occurred early and was greater in challenged resistant than in challenged nonresistant leaves. Although polyphenol oxidase activity appeared to increase significantly prior to lesion formation, a more critical study with a greater number of samplings is needed to establish this point definitively. The profiles of ascorbic acid oxidase activity (Fig. 9) illustrate strikingly the general tendencies indicated above as characterizing the postinoculation activities of most of the enzymes investigated in resistant and nonresistant leaves.

Necrogenesis in resistant and nonresistant leaves subsequent to inoculation with a potent TMV inoculum.—In a typical experiment (Fig. 10) involving challenge with potent inoculum, lesions appeared earlier, and the number of lesions was always significantly greater, in resistant than in nonresistant leaves. The beginning of a rapid phase of lesion appearance occurred 4-6 hr sooner in resistant than in nonresistant leaves, and at the end of this phase the number of lesions in the former still exceeded that in the latter by a factor of two. At the time of their first appearance, lesions in resistant and nonresistant leaves seemed to be of the same size. The expansion of lesions slowed down sooner in resistant than in nonresistant leaves, so that lesions in nonresistant leaves were very soon detectably larger than those in resistant leaves; despite their containing dense populations of pinpoint lesions, resistant leaves remained virescent and erect, whereas nonresistant leaves collapsed about 48 hr after challenge inoculation because of the continued expansion of lesions until they coalesced.

The actual amount of necrosis in leaves could not be determined accurately from lesion measurements, however, because of the irregular shape of lesions in dense populations. A possible approach to the problem was suggested by the results of Owen (13), which indicated that the decreased wt of infected tissues due to water loss was a function of the amount of necrotic tissue in them. In a single preliminary experiment, the wt of nonresistant leaves relative to those of equal areas of resistant leaves at 34, 38, and 42 hr after challenge inoculation were 1.11, 1.04, and 0.77, respectively. From these and additional similar results it is inferred tentatively that the amount of necrotic tissue in nonresistant leaves becomes equal to that in resistant leaves approx 40 hr after inoculation, after which it increases relative to the amount in resistant leaves. Before this time, however, inoculated resistant leaves presumably contain more necrotic tissue than do nonresistant ones.

Discussion.—The present and earlier reports (2, 4, 16, 17) indicate that the induction of systemic resistance does not result in appreciable alteration in cellular activities prior to challenge inoculation, but instead that resistance can be equated with an enhanced capacity of tissue to respond nonspecifically to subsequent inoculation. This general response includes enhancement of the activities of a variety of oxidative enzymes (Fig. 4-9) (19, 22), an increase in oxidative metabolism (Fig. 3) (21, 24), and increased cellular synthesizing activities (7, 19). These changes, or at least some of them (7, 19), occur not only in the cells infected by the virus but also in a zone of tissue peripheral to the infected cells. Presumably, at least some of the changes occurring in advance of infection play a role in the eventual localization of infection; this eventual blocking of cell-to-cell spread of virus could be due to an induced juvenility similar to that of meristematic cells, which often are incapable of supporting virus multiplication; to accumulation in this zone of materials toxic to the virus or capable of

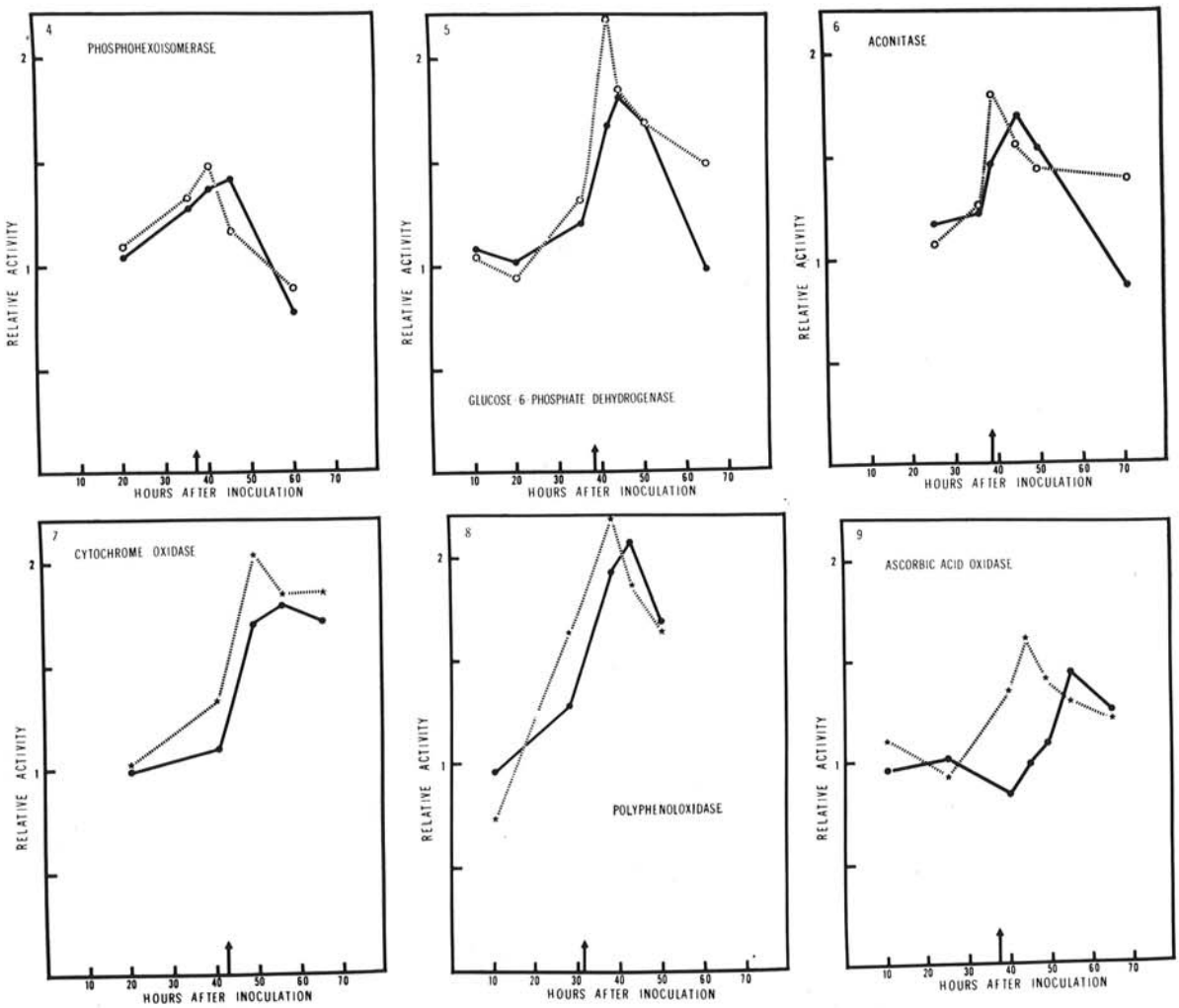

Fig. 4-9. Activities of oxidative enzymes in extracts made at intervals following challenge inoculation, with high-titer tobacco mosaic virus (TMV) inocula (crude juice diluted 10^{-1}), of resistant (broken lines) and nonresistant (solid lines) upper leaves of Samsun NN tobacco. Enzyme activities are expressed relative to those of extracts of control leaves (activity = 1) which were noninoculated, untreated leaves (from noninoculated, untreated plants) that were otherwise comparable. Arrows on horizontal axes indicate approximate average time of lesion appearance. Resistance levels, where stated, were calculated by the formula $100 - (100 \times T/C)$, where T is the average diam of 200 lesions in test (resistant) leaves challenge inoculated with dilute TMV and C the average for a like number of lesions in control leaves. **4)** Phosphohexoisomerase. Level of resistance = 68%. **5)** Glucose-6-phosphate dehydrogenase. Level of resistance = 72%. **6)** Aconitase. Level of resistance = 71%. **7)** Cytochrome oxidase. Level of resistance = 65%. **8)** Polyphenol oxidase. Level of resistance = 68%. **9)** Ascorbic acid oxidase. Level of resistance judged by observation to be greater than 65%.

blocking viral synthesis; or to a series of changes that make the cells in advance of infection progressively more labile until eventually those nearest the lesion collapse either before or very soon after invasion by the virus (17). Whatever the nature of the limiting mechanism, it appears identical in resistant and nonresistant leaves, with the two types of leaves differing only in the speed and extent to which this mechanism acts following inoculation.

The similarity of the effect of inoculation on all of the enzymes studied points to a general stimulation of protein synthesis, and thus of enzyme synthesis, rather than to a more specific type of reaction, such as conversion of inactive forms of enzymes to active forms. Increased protein or enzyme synthesis in the vicinity of lesions in tobacco is also indicated by the finding of an increased amount of protein in leaves with lesions (23) and in cells peripheral to lesions (19), by the report of an increase in the number of mitochondria following inoculation of a local-lesion host (22), and by electron microscopy indicating a general increase in the amount of cytoplasm and in the number of subcellular organelles in cells surrounding local lesions (7). In all cases, except possibly for catalase and peroxidase, enhancement of enzyme synthesis was transient, for a sharp drop in enzyme content began within

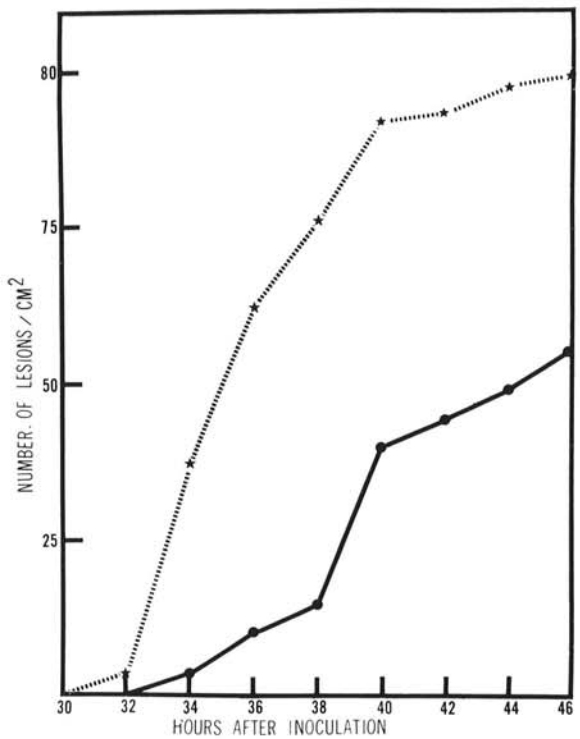

Fig. 10. Lesion density, measured at intervals after challenge inoculation with high-titer tobacco mosaic virus (TMV) inoculum (crude juice diluted 10^{-1}), in resistant (broken line) and nonresistant (solid line) upper leaves of Samsun NN tobacco. Resistance was induced by prior (7 days) inoculation of lower leaves with TMV. For each count, two 2.1-cm discs were punched at random from each of three leaves of each type. From each disc, four smaller ones (diam = 7 mm) were removed, and all lesions in these were counted.

a few hr after the onset of necrosis. This decrease in enzyme content may have been due in part to the killing and drying out of cells; this could not be the only cause, however, because in that case the drop would have always been much greater in nonresistant than in resistant leaves because of the greater amount of necrosis in the former in the later stages of infection. Enzyme synthesis also could have stopped because the stimulus was transient or because of rapid depletion of the energy reserves of the tissues. Subsequently, enzyme loss could be due to a rapid protein turnover.

Although the activity patterns of all enzymes (and oxygen uptake) were similar regardless of the type of leaf used, some consistent differences between resistant and nonresistant tissues point to an accelerated and unusually extensive response of resistant leaves to inoculation. In each instance, the characteristic phases of the activity profile occurred sooner (by a few hr) in resistant than in nonresistant leaves. Not only were max activities achieved sooner in resistant than in nonresistant leaves, but they were usually significantly higher in the former. These patterns of metabolic activity appear directly related to the amount of necrosis developing in the early stages of infection, for lesions appeared sooner and in greater number in resistant than in nonresistant leaves. This correlation did not hold during the later stages of infection, an indication that the enhanced activities are not entirely the consequences of necrosis.

Of the reported differences between resistant and nonresistant leaves, the enhanced peroxidase activity (18) of the former appears to be the one most likely to contribute towards the speedy and enhanced responses to inoculation characteristic of resistant leaves. Loebenstein & Ross (12) reported the presence of an inhibitor(s) of infection in resistant leaves, but there is no evidence that this material affects lesion size. New unidentified proteins have been found in resistant leaves (23), but their appearance seems to follow the development of resistance rather than being concomitant with it. The transient nature of the increased G-6-P dehydrogenase activity detected on day 5 through day 8 (Table 1) discounts the likelihood of the involvement of this enzyme in resistance. It is conceivable that the enhanced capacity of resistant leaves to metabolize H_2O_2, as evidenced by their increased peroxidase and catalase activities, might be a response to stimulation of flavin-terminated electron carrier systems. Preliminary tests, however, gave no indication of an association between resistance and enhanced activity of glycolic acid oxidase or "NADPH" oxidase. Also, the similarity of the amino acid content of resistant and nonresistant leaves (2) minimizes the likelihood of activation of amino acid oxidases, which likewise have terminal flavin cofactors. The activation of catalase, which occurs simultaneously with that of peroxidase and with induction of high levels of resistance, is puzzling; the degree of activation is not striking, and no likely connection between catalase and the hypersensitive reaction is apparent. The facts that both of these heme-containing enzymes have been shown to catalyze the coupled oxidation of hydrogen peroxide (8), that peroxidatic activity with respect to phenols has been demonstrated for catalase (8), and that a catalase subunit has been shown to possess powerful in vivo peroxidatic properties (3) suggest the possibility that we may be dealing with enhanced levels of a single hemoprotein—a cross-functioning subunit of either enzyme; i.e., a catalase subunit with peroxidatic activities or vice versa. On the other hand, the induced changes in peroxidase activity are striking, and the well-known ability of peroxidase to oxidize phenols (5) provides a basis for an hypothesis relating this enzyme to the enhanced response of resistant leaves to inoculation. Structural disruption, brought about by virus infection, presumably would facilitate the mutual availability of peroxidase and its substrates. The resulting quinones may be the actual killers of infected cells. If this were true, then the high peroxidase content of resistant leaves (and the greatly enhanced synthesis of peroxidase following inoculation) would lead to early appearance of necrosis in resistant leaves. Since the changes brought about in cells surrounding the infected cells are most probably caused by the lateral movement of some product(s) of necrosis or of reactions

preceding necrosis, then those reactions induced in advance of infection would also be initiated earlier, proceed faster, and/or result in more extensive changes in resistant leaves than would the same reactions in nonresistant leaves. If, as is assumed, resistance results from one or more of the changes induced in advance of infection, an early limitation of lesion size would result in resistant leaves.

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