Phytochemistry, 1972, Vol 11, pp. 19 to 28 Pergamon Press Printed in England

PHENOLIC COMPOUNDS AND THE HYPERSENSITIVITY REACTION IN *NICOTIANA TABACUM* INFECTED WITH TOBACCO MOSAIC VIRUS

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(*Received* 27 *November* 1970, *in revised form* 26 *March* 1971)

Abstract—The hypersensitivity of *Nicotiana tabacum* cv. Xanthi to tobacco mosaic virus infection leads to the production and accumulation of a great number of phenolics (flavonol glycosides, caffeoylquinic, feruloylquinic and *p*-coumaroylquinic acids, glucose esters and glucosides of cinnamic and benzoic acids) An increase in temperature inhibits the hypersensitive reaction, resulting in the disappearance of these substances. The differences between the healthy and infected leaves become important when the synthesis of the virus is practically brought to completion and the hypersensitivity taken hold The phenolic compounds do not appear to be responsible for the necrotic hypersensitivity and their production is one of the secondary effects of the virus infection

INTRODUCTION

TOBACCO mosaic virus (TMV) when inoculated into a plant, especially into *Nicotiana*, can either produce a systemic infection (*Nicotiana tabacum* cv. Samsun) or necrotic local lesions (*Nicotiana tabacum* cv. Xanthi n.c.). In inoculated Xanthi the virus remains confined to the necrotic tissues and to its immediate environs. This hypersensitive reaction serves to protect the plant from the effects of systemic virus infection. It has been shown[1] that TMV-induced local necrotic lesions prevent the development of new lesions on subsequent inoculation.

The behaviour of TMV in Xanthi also depends on the temperature at which infected plants are growing. At temperatures below 29°, the strain produces only necrotic local lesions,[2] but the speed with which these lesions develop and the size they reach is increased by raising the temperature. At about 30°, the type of reaction changes, and the plants develop systemic symptoms.[2]

The experiments conducted so far with regard to the biochemistry of the hypersensitive reaction are almost unanimous in emphasizing the role of the phenolic metabolism in plants.[3] In the case of several host-parasite complexes, phenol accumulation or the very intensive oxidation of polyphenols are processes leading to the hypersensitive reaction.[4]

Best[5,6] reported that 6-methoxy-7-hydroxycoumarin (scopoletin), a normal constituent of many plants, accumulates in *Nicotiana tabacum* L. around primary lesions of tomato spotted wilt virus (TSWV) infection and around TMV lesions in *N. glutinosa* L. A slight

[1] A. F. ROSS, *Virology* **14**, 329 (1961).
[2] C. MARTIN and M GALLET, *Compt. Rend* **262**, 646 (1966).
[3] G. L. FARKAS and Z. KIRALY, *Phytopathol. Z.* **44**, 105 (1962).
[4] G L. FARKAS and F. SOLYMOSY, *Phytopathol. Z.* **53**, 85 (1965).
[5] R J BEST, *Austral J Exptl Biol Med* **14**, 199 (1936)
[6] R J BEST, *Austral J Exptl Biol Med* **22**, 251 (1943)

accumulation was noted in plants systemically infected with these viruses. This same compound had been reported in other plants infected by various pathogens

Hampton *et al.*[7] reported that two unidentified fluorescent compounds accumulate in TMV-infected hypersensitive tobacco, but not in systemically infected tobacco Both compounds appeared at the time of lesion formation and were localized around the necrotic lesions Neither of the compounds was scopoletin In previous investigations[8,9] we have reported a significant accumulation of several phenolic compounds in TMV-inoculated leaves of Xanthi at 20° The present work concerns the identification and quantitative differences of certain aromatic compounds found in leaves of Xanthi at 20° and 30° before and after TMV inoculation

RESULTS AND DISCUSSION

I *Phenolic Compounds in TMV-inoculated Leaves of Xanthi at* 20°

Phenolic compounds accumulated in Xanthi leaves showing TMV-induced lesions are listed in Table 1. Analyses of these phenols (chlorogenic acids, rutin, feruloylquinic and *p*-coumaroylquinic acids, feruloylglucose, scopolin) made in relation to time on healthy and TMV-infected leaves at 20° are depicted in Fig 1.

(a) *Total phenols.* The total phenols in the leaves were significantly increased as a consequence of TMV infection Their levels reached a maximum between 60 and 156 hr after inoculation, and then dropped below the level of the control leaves.

TABLE 1. PHENOLS ACCUMULATED IN XANTHI LEAVES SHOWING TMV-INDUCED LESIONS AT 20°

Phenolic acids	Coumarins		Flavonols
Quinic esters	3-Caffeoylquinic acid (chlorogenic acid) 4-Caffeoylquinic acid 5-Caffeoylquinic acid (neochlorogenic acid) 3-Feruloylquinic acid 4-Feruloylquinic acid 5-Feruloylquinic acid 3-*p*-Coumaroylquinic acid		
Glucose esters	1-Caffeoylglucose* 1-Feruloylglucose* 1-*o*-Coumaroylglucose* 1-*o*-Coumaroylgentiobiose*		
Glycosides	Melilotoside* (*o*-coumaric acid glucoside) Melilotic acid glucoside*	Scopolin* (scopoletin 7-glucoside) Cichoriin* (esculetin 7-glucoside)	Rutin (quercetin 3-rutinoside) Nicotiflorin (kaempferol 3-rutinoside)
	Vanillic acid glucoside*	Scopoletin* 7-gentiobioside*	
	p-Hydroxybenzoic acid glucoside* Gentisic acid glucoside*		Isoquercitrin (quercetin 3-glucoside)

* Phenols which were produced in Xanthi leaves showing TMV-induced lesions.

[7] R E HAMPTON, R SUSENO and D M BRUMAGEN, *Phytopathology* **54**, 1062 (1964).
[8] J TANGUY and M GALLET, *Compt Rend* **269**, 589 (1969)
[9] J TANGUY and M GALLET, *Compt Rend* **269**, 773 (1969)

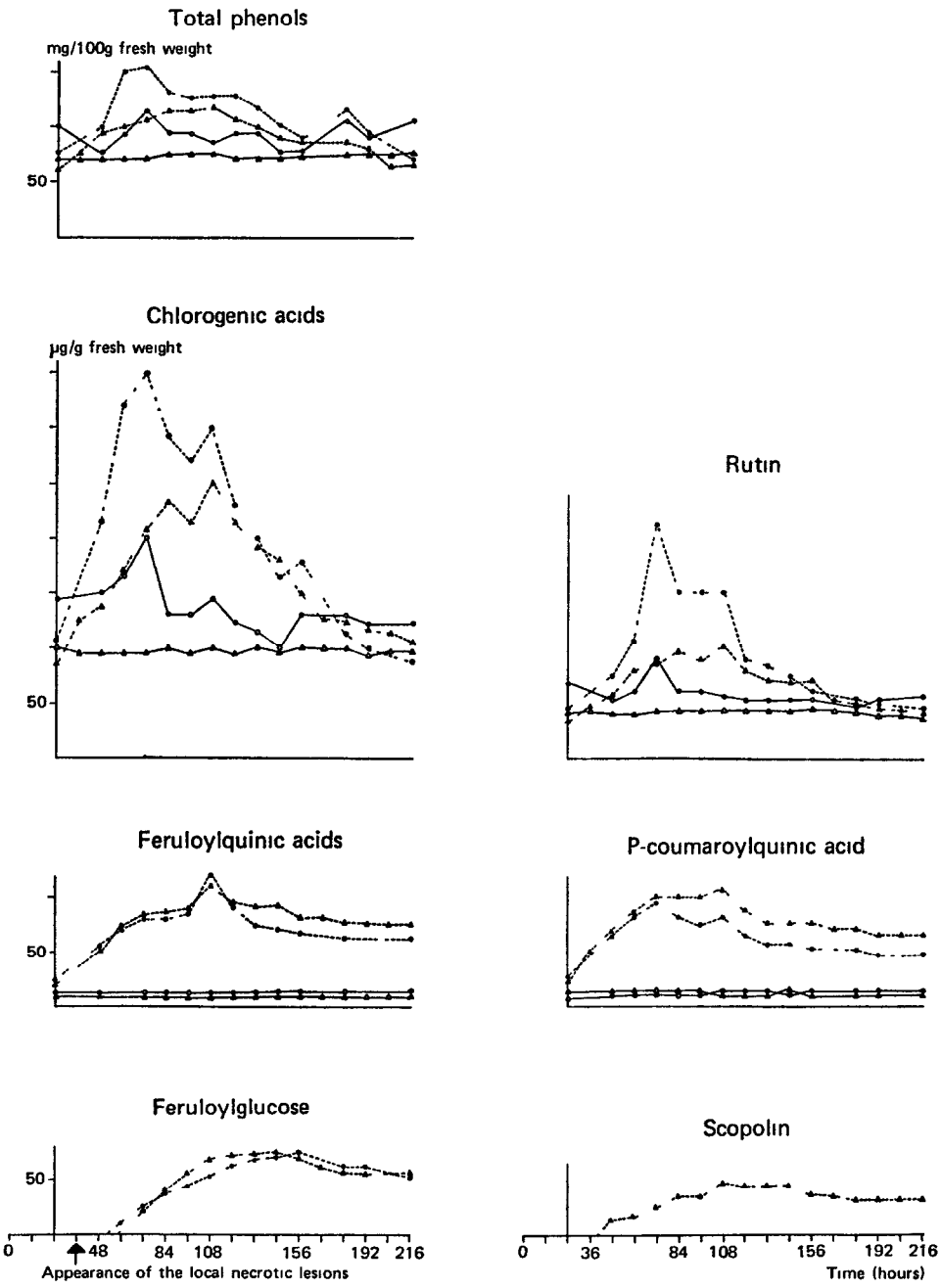

FIG 1 CHANGES IN PHENOLIC CONCENTRATIONS IN TMV-INOCULATED LEAVES OF XANTHI AT 20°. Control plants (January 1968)(○), Control plants (April 1968)(△); TMV-infected plants (January 1968)(●); TMV-infected plants (April 1968)(▲).

(b) *Individual phenols* Virus-infected leaves had considerably larger amounts of caffeoylquinic acids than did the corresponding unaffected leaves These substances increased in concentration rapidly between 60 and 156 hr after inoculation. The chlorogenic acids content in TMV-infected leaves, 72 hr after inoculation, was more than twice that of the controls Thereafter, chlorogenic acids levels decreased and fell to a level comparable with that of the control plants. Rutin increased in similar manner, and reached its maximum concentration 72 hr after inoculation with TMV During this period rutin level was approximatively twice as high in infected leaves than in corresponding healthy leaves The formation of local necrotic lesions was accompanied by an increase in the levels of feruloyl and *p*-coumaroylquinic acids. These compounds were present in small amounts in uninoculated leaves The greatest increase in the production of feruloylquinic acids was observed 108 hr after inoculation (120 μg per g fr. wt) Thereafter, feruloylquinic acids content dropped and remained steady just above the level of the normal plants *p*-Coumaroylquinic acid content reached a maximum between 60 and 108 hr after inoculation with TMV Feruloylglucose and scopolin could not be detected in healthy leaves but both compounds were found in leaves bearing local necrotic lesions They could not be detected until lesions were apparent, i e 36 hr after inoculation About 72 hr after infection the lesion was completely necrotic The greatest increase in the production of scopolin occurred 108 hr after TMV-inoculation The concentration of feruloylglucose showed a remarkable increase between 120 and 156 hr after infection with the virus

In brief, the accumulation and production of phenolic compounds in TMV-inoculated leaves were detectable several hours after the appearance of the local necrotic lesions. The differences between the healthy and infected leaves became important when the synthesis of the virus was practically complete and the hypersensitivity established These variations occurred between 60 and 156 hr after inoculation. Thereafter, they had a tendency to disappear gradually

II *Phenolic Compounds in Xanthi Leaves Systemically Infected with TMV*

At temperatures above 29°, the plant developed systemic symtoms This process was associated with a decrease in total phenolics content.[10] They began to decrease about 36 hr after inoculation, and they dropped quickly below the level of the controls (Fig 2). There was a relative decrease from 10 to 20% in concentration Thereafter, differences became less apparent and 180 hr after inoculation diseased leaves contained relatively more total phenols than the corresponding normal leaves Leaves systemically infected with TMV showed a characteristic rapid decline in amounts of chlorogenic acids and rutin (Fig 2) These decreases took place between 36 and 156 hr after inoculation with TMV. The caffeoylquinic acids content in systemically infected leaves was about half that of the controls Rutin levels decreased in a similar manner. Within 204 hr after inoculation, levels of chlorogenic acids and rutin in diseased leaves were comparatively higher than levels found in the corresponding uninoculated leaves Coumarins, glucosides and esters of phenolic acids which were formed in the leaves showing TMV-induced lesions, could not be detected in leaves systemically infected with the virus

The rapid decline in phenol content in leaves systemically infected with TMV between 36 and 156 hr after inoculation might be directly related to a extensive virus multiplication. Martin[2] reported a rapid synthesis of virus during the first 96 hr following TMV-inoculation

[10] J. TANGUY, *Compt Rend* **271**, 74 (1970)

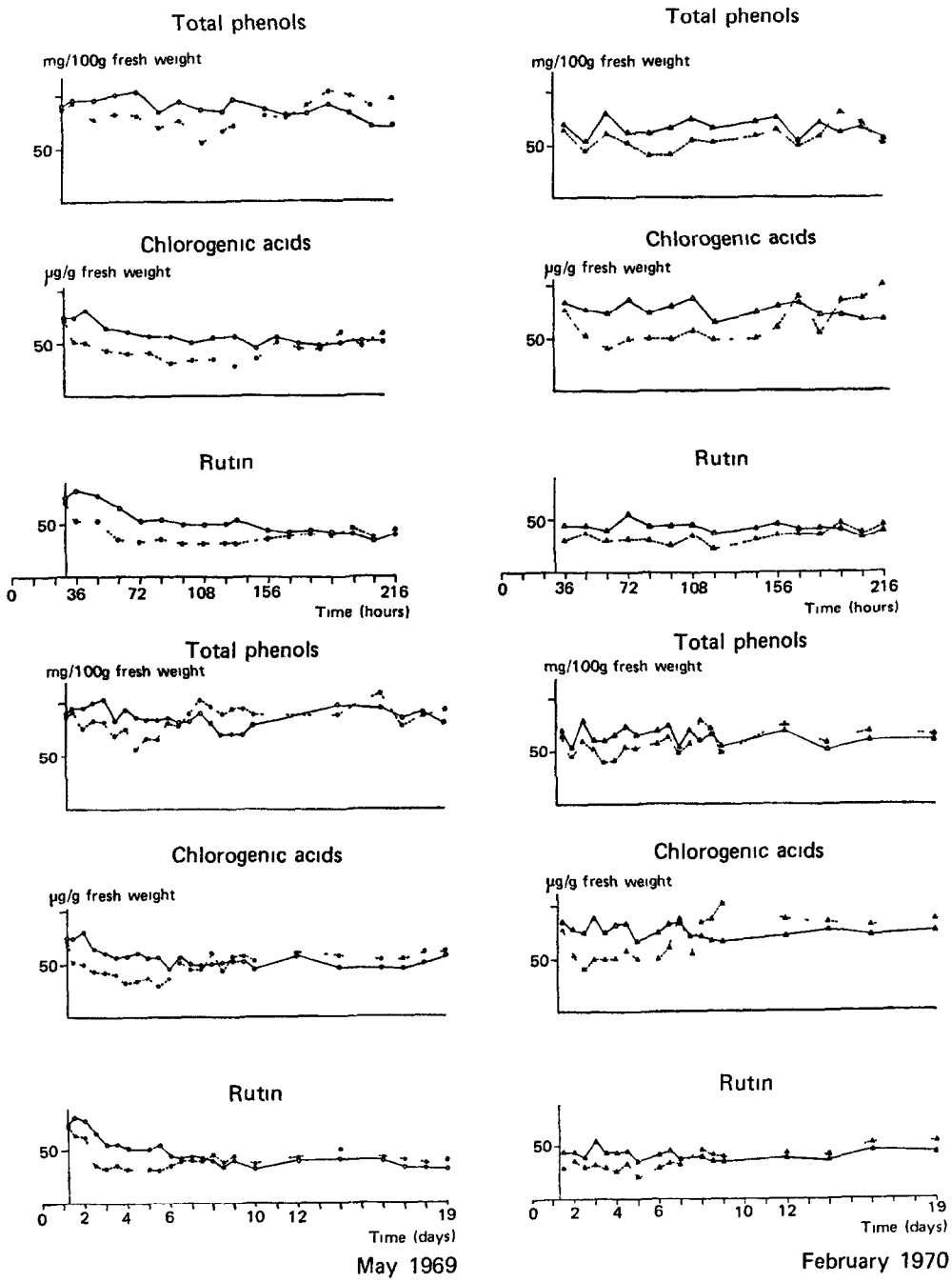

FIG 2. CHANGES IN PHENOLIC CONCENTRATIONS IN TMV-INOCULATED LEAVES OF XANTHI AT 30°
Control plants (May 1969)(○), Control plants (February 1970)(△), TMV-infected plants (May 1969)(●), TMV-infected plants (February 1970)(▲).

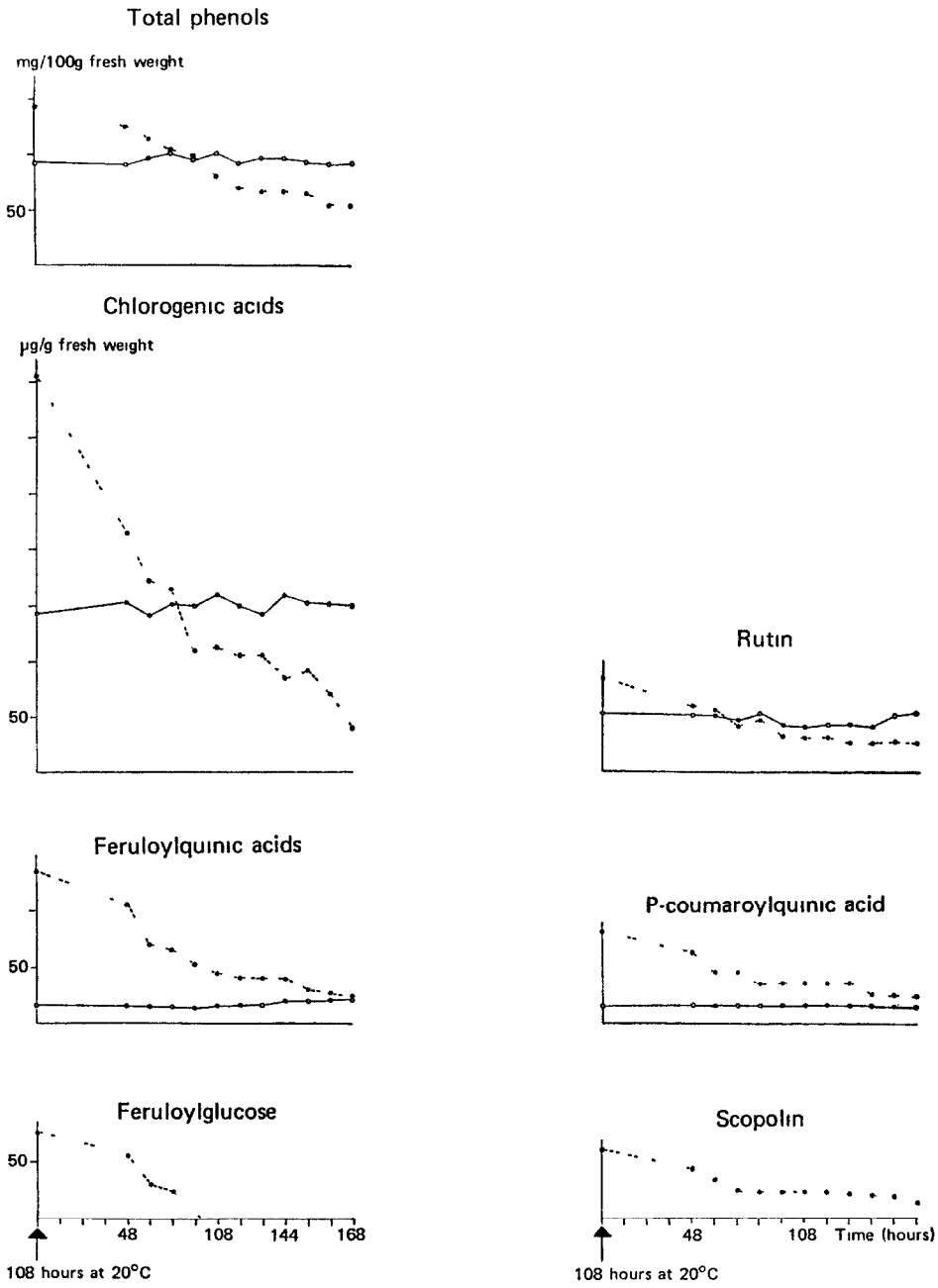

FIG 3 CHANGES IN PHENOLIC CONCENTRATIONS IN XANTHI LEAVES MAINTAINED AT 20° FOR 108 hr AND THEN TRANSFERRED TO 30°.

Control plants maintained at 20° for 108 hr and then transferred to 30° (February 1969)(○), TMV-infected plants maintained at 20° for 108 hr and then transferred to 30° (February 1969) (●)

After this flush of virus synthesis, phenolic compounds such as caffeoylquinic acids and rutin increased in concentration Virus multiplication in Xanthi leaves systemically infected with TMV might lead to an increased utilization of some metabolites which play a part in the phenolic synthesis These metabolites could be used in the host-phenol metabolism when the virus reaches its maximum concentration in inoculated leaves

Marked changes were also observed in the phenolic content of TMV-infected leaves of the cultivar Samsum In this plant, systemic infection was accompanied by an increase in chlorogenic acids,[11] scopolin and rutin levels This process led to an accumulation of *p*-coumaroylquinic acid. However, numerous compounds detected in Xanthi leaves showing local lesions were not found in TMV-inoculated leaves of Samsun

III *Phenolic Compounds in TMV-inoculated Leaves of Xanthi after Changes in Temperature*

(a) *From* 20° *to* 30° (*Fig.* 3). Virus-infected plants kept at 20° for 108 hr and exhibiting 100–150 local lesions per leaf, were transferred to 30° This transfer, leading to a systemic infection in inoculated leaves, was associated with a decrease of all the phenols examined. The decline in phenol content was apparent about 48 hr after a transfer to 30°. Scopolin, feruloylglucose and numerous unidentified compounds which were found in TMV-inoculated leaves bearing necrotic local lesions, disappeared in infected leaves 84 hr after a transfer to 30°. In these conditions chlorogenic acids and rutin levels were reduced to about 40–50% below those in corresponding healthy controls Feruloylquinic acids content declined from an initial value of 137 μg/g fresh wt. (in TMV-inoculated Xanthi leaves maintained at 20° for 108 hr) to nearly 30 μg 84 hr after transfer to 30°. *p*-Coumaroylquinic acid showed a similar decline

(b) *From* 30° *to* 20° (*Fig* 4). Plants inoculated with TMV and kept 36–48 hr at 30° developed systemic infection. The hypersensitive reaction was initiated on transfer to 20°. The hypersensitivity then affected the majority of the infected cells of the leaf In these experiments, necrotic lesions began to appear about 8–10 hr after a transfer to 20°. From the 30–20° change in the thermal environment a rapid rise in phenol content occurred in TMV-inoculated leaves [12] Production of chlorogenic acid in TMV-inoculated leaves, following a 60–70 hr exposure at 20°, increased approximatively two- or three-fold over uninoculated controls. Under the same conditions the rutin level was about twice that of the controls, and the concentration of *p*-coumaroylquinic acid and feruloylquinic acids increased rapidly. Feruloylglucose and scopolin were found several hours later when the lesions were well defined.

With the change from 30° to 20°, no accumulation of phenols was observed prior before necrosis became visible in TMV-inoculated leaves. These results indicate that phenolic substances do not induce the formation of local necrotic lesions and illustrate a situation where production of phenols is a result rather than the cause of the hypersensitive reaction.

Lesion development was associated with a marked increase in polyphenoloxidase (PPO) activity Previous experiments in our laboratory[13] showed that the virus-induced activation of PPO did not precede the appearance of lesions. In TMV-infected leaves kept at 20° the maximum PPO activity was found 3 days after inoculation, when necrotic symptoms were visible. The activation of PPO is not responsible for the development of necrotic lesions and

[11] C MARTIN, *Dissertation*, Paris, France (1958)
[12] J TANGUY and C MARTIN, *Compt Rend* **271**, 497 (1970)
[13] F. CABANNE, R SCALLA and C MARTIN, *Compt Rend*, **268**, 59 (1969)

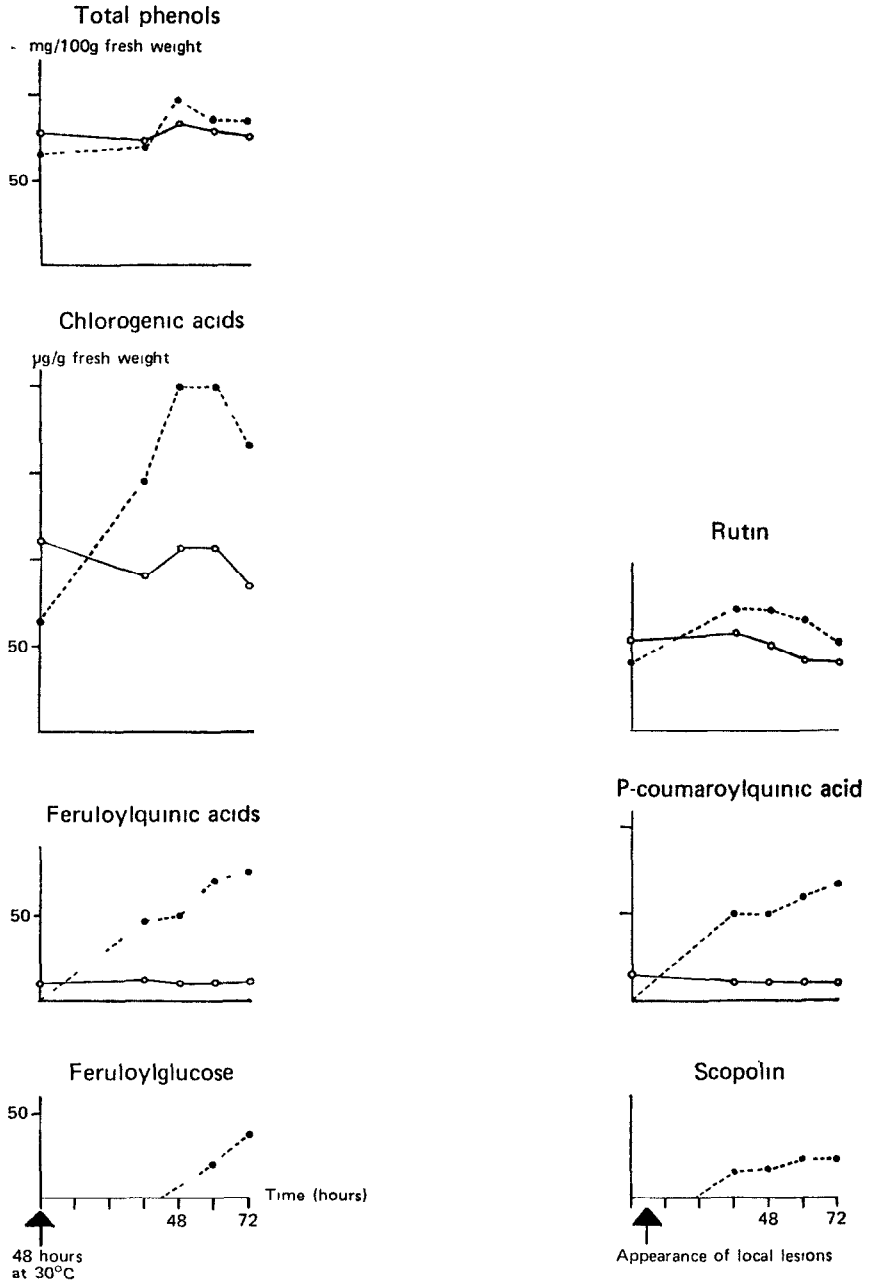

FIG 4 CHANGES IN PHENOLIC CONCENTRATIONS IN XANTHI LEAVES MAINTAINED AT 30° FOR 48 hr AND THEN TRANSFERRED TO 20°

Control plants maintained at 30° for 48 hr and then transferred to 20° (January 1970)(○), TMV-infected plants maintained at 30° for 48 hr and transferred to 20° (January 1970)(●)

the formation of toxic quinones and their polymerized products are unlikely to be responsible for lesion development

EXPERIMENTAL

Plant materials Tobacco plants were grown in a greenhouse to the 4–5 leaf stage (about 2-months-old) and transferred to a controlled environment room 3 days before inoculation in which the temperature was controlled from 20–30°, and light was provided at 8000 lx with a 16 hr photoperiod Inoculation was made with a purified preparation of TMW on the upper leaf surface of a mature leaf, inoculation of Xanthi always resulted in 100–150 lesions per leaf

Authentic compounds 1-Feruloylglucose and 1-caffeoylglucose were isolated by paper chromatography from the petals of *Petunia hybrida* [14] 3-Feruloylquinic acid, 4-caffeoylquinic acid and 5-caffeoylquinic acid were obtained from coffee beans,[15–17] 3-*p*-coumaroylquinic acid was isolated from cider apples [18] Melilotic acid was obtained from the acid or enzymic hydrolysate of an extract of *Dipteryx odorata* [19] The other compounds were obtained from commercial sources

Extraction Leaves were extracted with methanol and conserved in the methanol for 24 hr After centrifuging and re-extraction of the residue, the supernatants were combined and evaporated *in vacuo* to a small volume The concentrate was filtered and the filtrate washed successively with light petroleum The extract was evaporated to dryness and then made up to a known concentration with 50% MeOH

Separation and purification of phenolic compounds by chromatographic procedures These water-soluble and alcohol-soluble components were subjected to paper chromatography on Whatman No 1 and No 3 papers Solvents were BAW (*n*-BuOH–HOAc–H_2O, 4 1 5, upper layer), BEW (*n*-BuOH–EtOH–H_2O, 4 1 2), BAm (*n*-BuOH–2 N NH_4OH, 1 1, upper layer), BPW (*n*-BuOH–pyridine–H_2O, 14 3 3), KFW (*l*-BuCOMe–HCO_2H–H_2O, 14 3 2), and 2% HOAc With the exception of BPW, all systems were descending Separations were usually accomplished with BEW by one-dimensional chromatography on Whatman No 3 The band corresponding to the desired compounds was cut from the chromatogram and then eluted with 70% EtOH. The eluate was concentrated and purified by repeated chromatography as necessary in BAW, BPW or 2% HOAc.

Identification of phenolic compound Phenolic compounds were identified by fluorescence and R_f values, spectral measurements, identification of the aglycone and the non-phenolic compound produced by hydrolysis and co-chromatography in at least six solvents with authentic materials The UV spectra were determined in 95% EtOH In measuring the spectra of compounds eluted from paper chromatograms allowance for the impurities in the paper was made by using eluates of an appropriate paper blank Diagnostic shifts were determined by adding to solutions in the spectrophotometer cells (about 3 ml) 3 drops of 5% $AlCl_3$ in EtOH, (2) 3 drops of 2 N NaOH, (3) excess NaOAc, (4) the preceding plus 0 75 ml of a saturated H_3BO_3 in EtOH

Hydrolyses Alkaline hydrolysis was effected at room temp with 2 N NaOH for 30–60 min The solution was acidified and extracted with ether, the ether washings taken to dryness and the residue taken up in EtOH and transferred to a chromatogram The non-aromatic phase was concentrated to a small volume. This concentrate was examined for the presence of quinic acid and sugars by paper chromatography.

Acid hydrolysis consisted of 1 hr refluxing in 1 N HCl followed by ether extraction of the aglycone The aqueous fraction containing the sugars was neutralized with di-*n*-octylmethylamine,[20] concentrated under reduced pressure at 40° and chromatographed

For β-glucosidase hydrolysis the phenolic glucoside was dissolved in 1 ml H_2O (EtOH absent). Two drops of an acetate buffer pH 5 were added and this incubated with 1 mg β-glucosidase, for 1–4 hr at room temp The solution was heated at 100° to destroy the enzyme and transferred to a paper chromatogram on which reference spots of the suspected acid and sugar had been placed

Separation of phenolic acids and coumarins was accomplished through one-dimensional chromatography on Whatman No 1 in TAW (Toluene–HOAC–H_2O, 4 1 5, upper layer) Two-dimensional descending chromatograms on Whatman No 1 were used to separate these compounds. The first solvent was BzAW (benzene–HOAc–H_2O, 6 7.3, upper layer), and the second was Na F (HCO_2Na–HCO_2H–H_2O, 10 1 200) as described by Ibrahim and Towers [21] Flavonols were chromatographed on Whatman No. 1 with Forestal (HOA–HCl–H_2O, 30 3 10), and BAW

[14] J B Harborne and J J Corner, *Biochem J* **81**, 242 (1961)
[15] C. Lentner and F. E Deatherage, *Food Res* **24**, 483 (1959)
[16] J Corse, E Sondheimer and R Lundin, *Tetrahedron Letters* **18**, 1207 (1962).
[17] E Sondheimer, *Arch Biochem Biophys.* **74**, 131 (1958).
[18] A. M Williams, *Chem & Ind* 120 (1955)
[19] L A. Griffiths, *J Exptl Bot* **13**, 169 (1962)
[20] E. L Smith and J. E. Page, *J Soc Chem Ind.* **67**, 48 (1948).
[21] R K Ibrahim and G H N Towers, *Arch Biochem Biophys* **87**, 125 (1960)

Phenolic acids, coumarins and flavonols were identified by direct comparison with known specimens for colour reactions, absorption spectra and R_f values Phenolic acids and coumarins were visualized under UV light before and after exposure to NH_3 They were also located by their colour reactions when chromatograms were sprayed with diazotized *p*-nitroaniline [22]

The sugars were separated by one-dimensional paper chromatography in BAW and APW (EtOAc–pyridine–H_2O, 2 1 2, upper layer) and located by dipping in aniline hydrogen phthalate,[23] and heating at 100° for 5 min

Quinic acid was separated in BAW in IBW (*iso*-PrOH–*n*-BuOH–H_2O, 7 1 2) and in EAW (EtOH–NH_4OH–H_2O, 20 1 4) and detected by the periodate-nitroprussate-piperazine reagent [24]

Quantitative determination Determination of total phenols was based on oxidation with the Folin Ciocalteu reagent [26] The intensity of the resulting blue complex was estimated with a spectrophotometer (the complex had λ_{max} at 725 nm) Standardization was made with chlorogenic acid The same method was applied on a microscale to individual compounds separated by paper chromatography [26] Paper blanks of comparable size to some of the phenolic spots were always included to determine background colour The concentration of the identified phenolic was then ascertained by comparison with suitable standard Data reported are the average of two replicates (7–10 plants/replicate) with two determinations for each replicate

[22] T SWAIN, *Biochem J* **53**, 200 (1953)
[23] S BAAR *Biochem J* **58**, 175 (1954)
[24] R A CARTWRIGHT and E A H ROBERTS, *Chem & Ind*, 230 (1955)
[25] H G BRAY and W V THORPE, *Methods of Biochemical Analysis* (edited by D. GLICK), Vol 1, p 27, Interscience, New York (1954)
[26] R W KEITH, D LE TOURNEAU and D MAHLUM, *J Chromatog* **1**, 534 (1958).

Key Word Index—Nicotiana tabacum, Solanaceae, tobacco mosaic virus, hypersensitivity, phenolic compounds, flavonoids, cinnamic acids, scopolin