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Virus Resistance Induced in Plants by Polyacrylic Acid

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SUMMARY

Polyacrylic acid (PA) injected into tobacco cv. Xanthi-nc induced complete resistance to infection with TMV or tobacco necrosis virus but only partial resistance to potato virus X. The effect was maximal when the injection was made 2 to 3 days before inoculation. The lesion size was limited when the injection was made after inoculation. Using PA of 3500, 27000, 76000, 230000 and 1×10^6 mol. wt. the resistance decreased with increasing size of the polymer. In plants younger than 7 weeks, only the smallest polymer was active and evidence suggested that cell permeability to the larger polymers might increase with age of plant. The PA-induced resistance disappeared when plants were kept at 32 °C, but the effect of temperature was reversible. Polyacrylamide failed to induce resistance suggesting that the polyanionic structure of the acid polymers is responsible for the phenomenon.

Disc-electrophoresis in 10 % polyacrylamide gels showed that three additional soluble proteins appeared in PA-injected leaves, but only in conditions in which resistance to infection was induced. These new proteins co-electrophoresed with three out of four proteins produced in TMV-infected leaves of cv. Xanthi plants that also are resistant to infection and may be the cause of resistance.

INTRODUCTION

The hypersensitive reaction of plants to virus infection is characterized by localization of the virus in the resulting necrotic lesion. It is now known that the localization of the virus is not caused by the necrosis but takes place in the living cells around the necrotic lesion (Martin & Gallet, 1966*b*). The reason for the inability of virus to move through the host tissue is not understood, but there is evidence that a protective mechanism is developed by the infected plant. This phenomenon was well described by Ross (1961*a*, *b*) in *Nicotiana tabacum* cv. Samsun NN or Xanthi-nc inoculated with tobacco mosaic virus (TMV). Not only healthy tissue around the local lesions, but also the uninoculated opposite half leaves and the leaves above and below the inoculated one became resistant to further infection. Such resistance is not specific, so infection with TMV makes leaves resistant to unrelated viruses, such as tobacco necrosis, turnip mosaic and tobacco and tomato ringspot viruses. The resistance breaks down at temperatures near 30 °C (Ross, 1961*a*, *b*).

Disc-electrophoresis showed that tobacco leaves cv Samsun NN and Xanthi-nc, infected with TMV, contain proteins that are not detectable in healthy plants (Gianinazzi, Vallée &

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Martin, 1969; Gianinazzi, 1970; Gianinazzi, Martin & Vallée, 1970; Van Loon & Van Kammen, 1970). It was been suggested that these proteins might inhibit virus multiplication or its spread in some way analogous to the action of interferon, the antiviral protein produced by cells of many vertebrates in response to virus infection (Gianinazzi, 1970). Various synthetic polyanions stimulate interferon production (De Clercq, Eckstein & Merigan, 1970), so we were interested to see whether they also induce resistance to virus infection in plants. This paper reports that tobacco plants cv Xanthi become totally resistant to infection with TMV and tobacco necrosis virus when injected with polyacrylic acid (PA).

METHODS

Test plants. Nicotiana tabacum cv. Xanthi-nc were grown in 13 cm pots with a sand peat compost at 22 to 25 °C and illuminated in winter for 16 h/day at 4000 lux when young and 2000 lux when old.

Inhibition tests. The inhibitory effect of a chemical on the infectivity of TMV was assayed by inoculating opposite half leaves with virus alone and with virus–chemical mixtures.

Tests for induced resistance. Most Xanthi plants were 9 to 10 weeks old and selected for uniformity. For convenience, plants were trimmed to four fully expanded leaves but similar results were obtained with untrimmed plants, in fact plants younger than 9 weeks were used untrimmed. A solution of the chemical was injected using a fine hypodermic needle into the intercellular spaces of one half of each of the four leaves. Entire half-leaves could be infiltrated with 8 to 10 injections made near the lateral veins in the lower leaf surface (Klement, 1963). The other half was similarly injected with distilled water. The amount of fluid injected was estimated by weighing the leaf before and after injection with water. Fully developed leaves accommodated about 0·52 ml of fluid per g of leaf. The injected fluid was absorbed and the leaves appeared normal within a few hours, except occasionally when a few small light-green patches appeared at the points of injection (Fig. 3). At different times after injection, the entire upper surfaces of the leaf were inoculated with TMV at 2 to 10 μg/ml. The resistance induced by the chemical was calculated as the percentage reduction in the number of lesions produced as compared with their controls. The results are average number of lesions from at least eight half-leaves.

Polymers. Polyacrylic acid (Versicol E) and other polymers, of different mol. wt., were gifts from Allied Colloids, Bradford. We did not modify the chemicals, other than to adjust the solutions in distilled water to pH 6 with 1 N-NaOH. Fresh solutions were prepared for each experiment. Unless otherwise mentioned we used the polyacrylic acid (PA) of mol. wt. 230000.

Soluble plant proteins. Leaf samples (1 g) were ground in a mortar at 4 °C with 1 ml of 0·1 M-tris buffer, pH 8, containing 0·5 M-sucrose and 0·3 % (v/v) mercaptoethanol. The extracts were centrifuged at 8000 ***g*** for 1 h and 50 μl of their supernatant fluids analysed by disc-electrophoresis in 10 % polyacrylamide gel at 4 °C as described by Ornstein (1964) and Davis (1964). After electrophoresis the gels were stained overnight with 0·03 % Coomassie blue in a methanol–acetic acid–water mixture (5:1:5) and destained using 10 % acetic acid. The position of the protein bands in the gel was expressed by the R_F value, taking the distance travelled by the plant phenols as 1·00 (Gianinazzi, 1970).

High temperature. The plants were kept at 32 °C in a glass incubator (Kassanis, 1952).

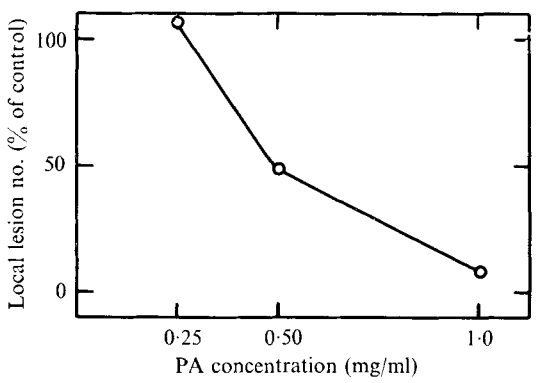

Fig. 1

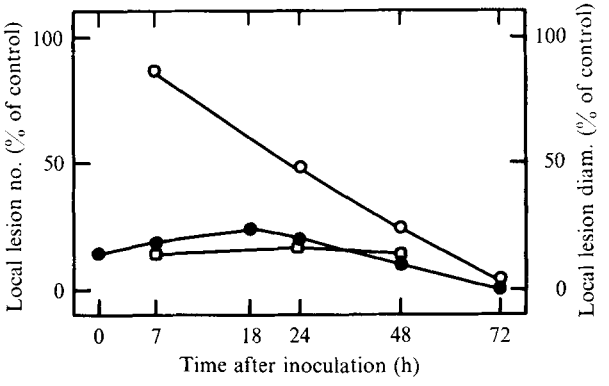

Fig. 2

Fig. 1. The number of lesions as percentage of control in half leaves injected with different concentrations of PA two days before inoculation. 100 % represents 79 lesions per half-leaf.

Fig. 2. The number and diam. of local lesions as percentage of control in half-leaves injected with PA at different times before inoculation. 100 % represents 85 lesions per half-leaf (●—●); 263 lesions per half leaf (○—○) and 3·1 mm lesion diam. (□—□).

RESULTS

Induced resistance to virus infection

Distilled water or different concentrations of polyacrylic acid (PA) of mol. wt. 230000 was injected into opposite half-leaves and at various intervals after injection the entire upper surface of the leaf was inoculated with TMV. PA injected 2 days before inoculation at a concentration of 0·25 mg/ml had no effect on lesion number, but at 1 mg/ml it decreased the number of lesions by 92 % (Fig. 1). When leaves were inoculated with TMV at different intervals after injection with a solution of 1 mg/ml of PA, the percentage decrease in lesion number increased with time, but also depended on the inoculum concentration (Fig. 2). The greater the concentration, the longer it took for the resistance to manifest itself. Using an inoculum which gave an average of 263 lesions per half-leaf in the controls, the lesion number decreased by 15 % when the interval between injection and inoculation was 7 h and by 98 % when the interval was 3 days. By contrast, with an inoculum which gave an average of 85 lesions on the control half-leaves, the lesion number decreased by 80 % after 7 h, 90 % after 2 days and there was complete resistance after 3 days (Fig. 2). On average, the local lesions that appeared on the PA-treated half-leaves were about one-fifth the diam. of the lesions on control half-leaves. The degree of resistance depended on the age of the leaf. When plants were trimmed to four fully expanded leaves the youngest leaf developed resistance quicker than the oldest (Fig. 3).

The results suggest that PA prevents both the infection and later spread of the virus, so that lesions are fewer and smaller. The size difference was more apparent when PA was injected after inoculating the leaves. PA decreased the number of lesions only if injected less than 7 h after inoculation but restricted the size of the lesions even when injected 2 days after inoculation, which is the time when lesions appear (Fig. 4).

PA also induces complete resistance to infection by tobacco necrosis virus. Tobacco cv. Xanthi is hypersensitive to TMV and tobacco necrosis virus, i.e. the two viruses remain localized around the necrotic lesions. To test whether PA would induce resistance to a virus that does not cause a hypersensitive reaction on cv. Xanthi, we used an isolate of potato

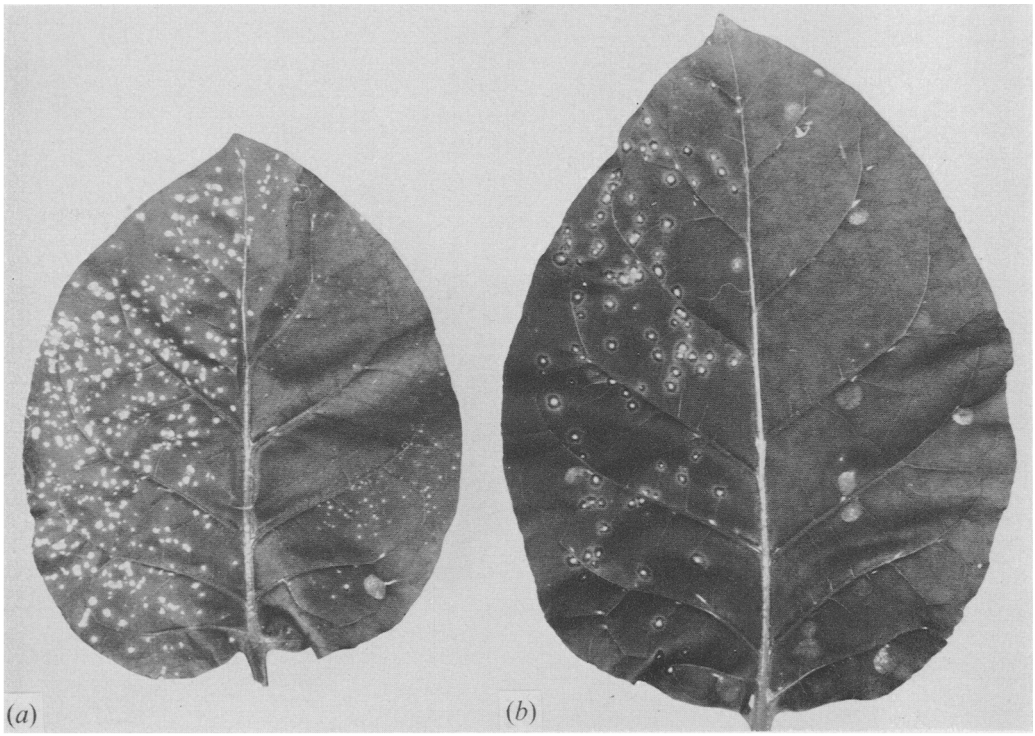

Fig. 3. An old (*a*) and a young (*b*) leaf of cv. Xanthi inoculated with 10 μg/ml of TMV 24 h after PA injection. The right half leaf of each leaf was injected with 1 mg/ml of PA, mol. wt. 230000, and the left half with water. Note the few small lesions in the older right half leaf.

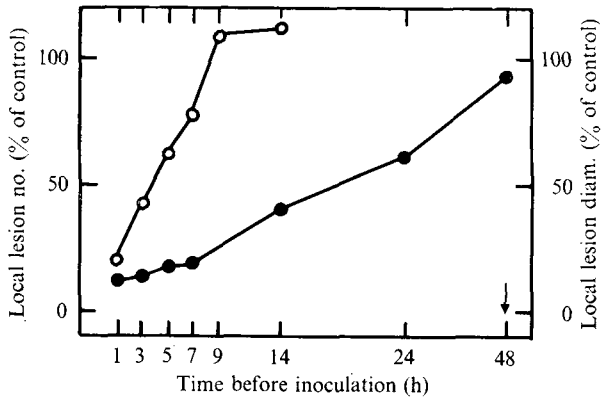

Fig. 4. The number and diam. of lesions as percentage of the control when 1 mg/ml PA was injected at different times after inoculation. One hundred represents 94 lesions/half-leaf (○—○) and 2·9 mm lesion diam. (●—●). Arrow indicates times of appearance of lesions.

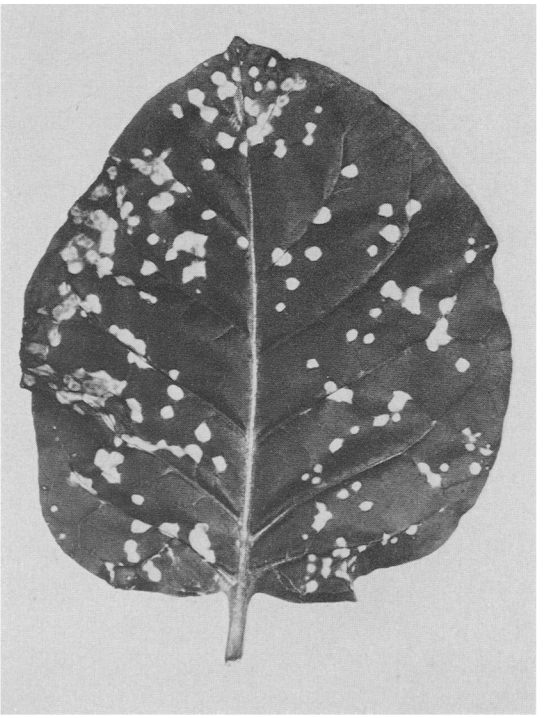

Fig. 5. A leaf of cv. Xanthi injected 14 h after inoculation and kept at 32 °C for 2 days immediately after injection and subsequently at 20 °C. The size of the lesion is the same in the PA-injected right half as in the water-injected left half.

virus X that gives necrotic ring lesions, from which the virus spreads to become systemic. PA failed to induce complete resistance to this isolate. In some tests there was little difference in the number of lesions in the two half-leaves but the lesions appeared 1 day later in the PA injected half. In other tests lesions were not formed in the PA injected half but when the concentration of virus was estimated serologically a considerable amount of virus was found, particularly at high concentrations of inoculum. PA seems to mask potato virus X symptoms but only slightly limits the multiplication of the virus, whereas it completely prevents the initiation or the spread of infection with TMV and tobacco necrosis virus.

The concentration of PA that prevents infection when injected, does not when mixed with the inoculum. When TMV was inoculated with 1 mg/ml of PA, as many lesions were produced as with TMV alone, and the size of the lesions was not affected.

Breakdown of the resistance at 32 °C

Samuel (1931) showed that the hypersensitive reaction breaks down when plants are kept at temperatures over 30 °C after inoculation. TMV does not cause necrotic local lesions on inoculated leaves of Xanthi plants kept at 32 °C but becomes systemic. However, if such plants are then transferred to 20 °C, the tissues where the virus multiplied become necrotic in a few hours (Kassanis, 1952; Martin & Gallet, 1966*a*). Fig. 4 shows that leaves kept at 20 °C and injected with PA 14 h after inoculation produced lesions about half the diam. of the control. However, when kept at 32 °C for 2 days after injection and then at 20 °C, the lesions were the same size in PA-treated and control half-leaves (Fig. 5). When the period

at 32 °C was longer than 2 days, the necrotic lesions in both half leaves became larger, until eventually the entire leaf became necrotic. These results show that at 32 °C PA is not able to stop the multiplication and spread of TMV.

The inability of PA to induce resistance at 32 °C was also shown by moving plants to this temperature after holding them at 20 °C for 3 days after injection with PA. Injected half-leaves of control plants left at 20°C were completely resistant to infection but the others after 1 or 2 days at 32 °C became susceptible. In one experiment PA decreased lesion number by only 24 % after 2 days at 32 °C although the lesions were small. However, when injected plants kept at 20 °C for 3 days, then at 32 °C for 2 days were returned to 20 °C they recovered their resistance, suggesting that PA re-stimulated the mechanism responsible for resistance.

Where no lesions are formed in PA-treated leaves, it is possible that some cells become infected. If so, by putting such plants at 32 °C the infection should spread to the rest of the tissues and become visible when the plants were returned to 20 °C. Plants that had been injected with PA 3 days before inoculation and produced no lesions were put at 32 °C for 3 days. When transferred back to 20 °C the PA injected half-leaves were still free from lesions whereas the lesions in the control halves became larger.

Effect of age of plant on resistance

Plants older than 9 weeks always became resistant when injected with PA of 230000 mol. wt., but plants younger than 7 weeks did not. Between these two ages some injected plants developed resistance, others only partially or not at all. For example, using the smallest and largest plants from a batch of plants 56 days old, only the largest developed resistance, so the change seems very sudden. However, we found that PA of 3500 mol. wt. induced resistance in both old and young plants (6 to 7 weeks), so the most likely explanation for the failures is that cells of young plants are impermeable to PA of high mol. wt. Differential permeability was also suggested by experiments where the leaves were detached and placed with their petioles in a solution of the polymer for 24 h before inoculation. Leaves from young and old plants showed complete resistance when placed in a solution of 50 μg/ml of PA 3500 mol. wt. but there was no resistance at any age with 230000 mol. wt. PA, even when the concentration of the polymer was 1 mg/ml.

Effect of mol. wt. of PA

The ability of polyacrylic acid polymers of mol. wt. 3500, 27000, 76000, 230000 and 1×10^6 to induce resistance increased with decreasing mol. wt. The smallest polymer induced complete resistance 2 to 3 days after injecting a 25 μg/ml solution, whereas the 230000 mol. wt. polymer had this effect only at 1 mg/ml. The largest polymer did not induce resistance at 1 mg/ml and at higher concentrations was too viscous to inject. A sample of PA of 230000 mol. wt. obtained from BDH Chemicals Ltd also induced complete resistance. Most of the work was completed using 230000 mol. wt. polymer before we discovered the increased efficiency of the lower mol. wt. polymers.

Effect of polyacrylamide

Leaves injected with polyacrylamide 103000 mol. wt. induced no resistance to infection with TMV, whereas PA injected into the opposite half-leaf gave complete protection. Polyacrylamide had no effect even when injected at 25 times the concentration of PA 230000 that induced complete resistance.

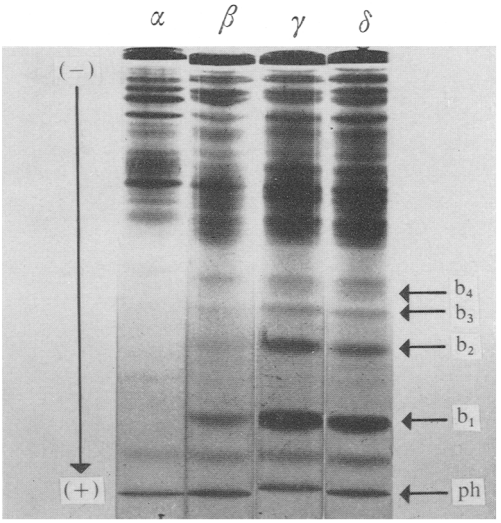

Fig. 6. Electrophoretic gels of centrifuged extracts from plants of cv. Xanthi after injection with water (α), with PA (β) or inoculated with TMV (γ). The last gel (δ) shows co-electrophoresis of PA + TMV extracts. Electrophoresis was conducted at 4 °C for 15 min at 2 mA per tube and subsequently for 90 min at 4 mA per tube in 10 % acrylamide gels: ph, the plant phenol band.

Change in plant protein constitution after PA injection

Fig. 6 shows the protein patterns obtained when 50 μl of centrifuged extracts from water- and PA- injected leaves were separated by electrophoresis in 10 % acrylamide gels. Samples from PA-injected leaves showed additional protein bands at positions R_F = 0·82, 0·67 and 0·59, which are the same as those of three of the four bands that appear when cv. Xanthi is infected with TMV. The additional proteins produced in hypersensitive hosts infected with TMV were designated by Gianinazzi *et al* (1970) as b_1 (0·82), b_2 (0·67), b_3 (0·59) and b_4 (0·55). They were not related to structural TMV protein but were host-specific. Co-electrophoresis of a mixture of equal amounts of centrifuged extracts from PA-injected and TMV-infected leaves gave the same protein bands as each separately (Fig. 6).

We found that the additional proteins appeared in 9-week-old plants injected with a 1 mg/ml solution of PA, 230000 mol. wt. or a 50 μg/ml solution of PA, 3500 mol. wt. When 6 to 7-week-old plants were injected, the proteins appeared only in plants injected with PA, 3500 mol. wt. The b_1 protein appeared at between 2 and 3 days, b_2 at 4 days and b_3 at 7 days after injection. As shown above, the resistance to virus infection disappears when the injected plants are kept for 2 days at 32 °C. Leaf extracts made after this treatment did not contain the additional proteins. There seems, therefore, to be a correlation between the appearance of the additional proteins and the resistance to infection.

DISCUSSION

PA is not an inhibitor of TMV infection because when present in the inoculum it does not decrease the number of lesions. Inhibitors of virus infection have no effect if applied some hours before or after virus inoculation. By contrast, when PA was injected before the virus its effect increased to a maximum when the interval was 2 to 3 days. Injecting PA after inoculation limited the size of lesions even if delayed 2 days – the time when lesions were starting to appear.

Plants of cv. Xanthi injected with PA developed complete resistance to TMV and tobacco necrosis virus, but only partial resistance to potato virus X. In this species, TMV and tobacco necrosis virus cause hypersensitive reactions, but although the strain of potato virus X we used forms local lesions, it is able to spread systemically. These examples suggest that PA induces resistance only to viruses that cause hypersensitive reactions in cv. Xanthi (i.e. when virus is localized around the lesions). Moreover, both the hypersensitive reaction to TMV and the PA-induced resistance disappear above 32 °C. The hypersensitive reaction of cv. Xanthi relies on a single dominant gene derived from *Nicotiana glutinosa* L. (Holmes, 1938). Martin & Gallet (1966*b*) showed that TMV multiplies in tissues of Xanthi beyond those that become necrotic; so further spread of the virus is limited by a mechanism other than necrosis, but which is thermosensitive. Injecting PA may activate this mechanism and so prevent infection.

The smaller the PA polymers used (in the range 3500 to 10^6 mol. wt.) the greater the induced resistance to TMV; smaller polymers may be even more effective. In animal tissues the smallest PA polymer that induced production of interferon was 1000 mol. wt. (De Clercq *et al.* 1970), but in contrast to our results with TMV the effect of PA increased with increasing size of the polymer (De Somer *et al.* 1968). The difference may be attributable to different permeabilities of plant and animal cells, the former probably excluding large polymers. However, plant cells resemble animal cells in their response to polyacrylamide. Polyacrylamide does not induce production of interferon (De Clercq *et al.* 1970) and does not induce resistance in plants. These results suggest that the polyanionic structure of the polymers may be important. PA, like nucleic acid, has a sequence of negative charges whereas polyacrylamide is neutral. Thus it may be relevant that Gicherman & Loebenstein (1968) showed that plants injected with yeast nucleic acid became more resistant to virus infection; it is well known that nucleic acids induce interferon production.

PA of 230000 mol. wt. did not induce resistance in plants younger than 7 weeks whereas PA of 3500 mol. wt. caused resistance in both young and old plants, so it seems probable that cells of young plants are impermeable to the larger polymers. Vallée (1973) showed that in cv. Xanthi plants more than 9 weeks old, floral induction is accompanied by a change in cell permeability. So perhaps Stein & Loebenstein (1972) failed to induce resistance to viruses by injecting PA 60000 to 70000 mol. wt. because they used young plants. The same polymer, kindly provided by Dr Loebenstein, gave complete resistance under our conditions.

In plants 9 weeks old, the young leaves developed resistance more rapidly than the older ones, suggesting that metabolic processes, which are likely to be more intense in young leaves, are involved. This is illustrated by the appearance after PA injection of three additional proteins in the soluble fraction of the leaf extract. These were always present as plants became resistant to infection and disappeared as plants held at 32 °C became susceptible once again. The close correlation between the appearance of the proteins and resistance to infection is also shown by the fact that both occurred after young plants were injected with PA of mol. wt. 3500, but not with PA of mol. wt. 320000. Gianinazzi *et al.* (1970) showed that the same proteins appear in TMV-infected plants when the leaves become resistant to further infection and suggested a causal connexion. Our results with PA also suggest such a connexion. In fact, the action of these proteins in plants may be compared to that of interferon in animals.

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