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THE MAJOR PROTEINS IN EXTRACTS OF TOBACCO LEAVES THAT ARE RESPONDING HYPERSENSITIVELY TO VIRUS-INFECTION

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco mosaic virus; hypersensitive reaction; PR-proteins; glycoproteins; chitinase.

Abstract—Extracts from the leaves of tobacco plants which are reacting hypersensitively to TMV-infection, contain the four 'novel' pathogenesis-related (PR-) proteins, and seven other major protease-resistant proteins which are absent or present in small amounts in uninfected leaves, and which can be separated and characterized by electrophoresis and chromatofocusing. None of the seven contain the protein subunit which characterised PR-Ia, b and c. Three of them may be lectins; they stain as if they contained carbohydrate and are absorbed onto chitin. If leaves of infected plants are exposed to $^{14}CO_2$ as lesions develop, PR's-Ia,Ib,Ic,II and one of the other proteins accumulate radioactivity, although it is not clear if they are actively 'turning over'.

INTRODUCTION

Some cultivars of tobacco contain a gene for resistance which confers on them the ability to respond hypersensitively to tobacco mosaic virus (TMV). Infection does not then spread systemically, but is restricted to necrotic lesions around infection sites. Extracts of such hypersensitively responding leaves contain appreciable amounts of four 'novel' proteins which are characteristic of the cultivars [1, 2]. Three of them have been purified and partially characterised as charge isomers [3]. These pathogenesis-related (PR-) proteins may occur in uninfected tissue following chemical and hormonal stimulation [4, 5] or flowering [6], but interest in them is sustained by the possibility that they are part of the hypersensitive process which restricts virus spread: thus there are a number of situations when their presence coincides with resistance to further infection [see 7]. Messenger RNA, which directs their synthesis, has been identified in similar amounts in both uninfected and infected tobacco plants [8], and it is presumed that its translation into protein is initiated by infection. There is evidence that the newly synthesized PR-proteins accumulate in intracellular spaces [van Loon, personal communication], but it is unclear if they are stored there undegraded or are metabolically 'turning over'.

The four proteins, called PR-Ia, Ib, Ic and PR-II [3], are preferentially extracted from tobacco leaves at low pHs [9], and are resistant to proteolytic enzymes [1, 10]. However, when extracts of infected leaves are clarified by autolysis [11] or added enzymes [1, 10] and analysed electrophoretically, they are shown to contain an additional six to eight proteins that are either not present in extracts of uninfected leaves, or are present in smaller amounts. Although these additional proteins may be modified by the digestion procedure, it seems unlikely that they are complete artefacts of the process. It is not known if they have any chemical or physiological relationships to the other four proteins, or whether they are always induced by the same stimuli.

Here we describe experiments on the electrophoresis of the proteins of infected leaves, and also on their labelling with $^{14}CO_2$, which were designed to clarify their chemical relationships and explore their metabolic states.

RESULTS

Major proteins of leaf extracts

Extracts made at pH 2.8 from TMV-infected leaves of Xanthi-nc tobacco, contain at least nine major bands of protein visible after polyacrylamide gel electrophoresis (PAGE). These are not appreciably affected in size or mobility when the extracts were digested overnight with trypsin and chymotrypsin (Fig. 1A). This treatment removed some minor bands and also much unresolved 'background' staining. The four most mobile proteins (R_f 0.86, 0.71, 0.63 and 0.57) are PR-Ia, b, c and PR-II, respectively, and the slower five peaks (R_f 0.53, 0.43, 0.37, 0.3 and 0.16) are, following van Loon [1], referred to as N–R in order of decreasing mobility (Fig. 1A). It is not implied that N–R are single proteins: of the five similar protein peaks present in trypsin-treated extracts of infected Samsun NN tobacco leaves [1], that corresponding to R is more clearly resolved into two components, R and S.

Components P and Q were consistently made visible after gels were treated with periodate–Schiff reagent in conditions which stained ovalbumin but not serum albumin; they may therefore contain carbohydrates. R was also stained initially but became obscured by extensive staining in the region R_f 0.1–0.2. When gels were fixed with TCA before periodate treatment, other protein components including PR-Ia became faintly coloured. Purified PR-Ia however did not react in this way.

Extracts from untouched or sham-inoculated leaves were more variable; and the profile in Fig. 1B is from an extract containing about a sixth as much protein as a

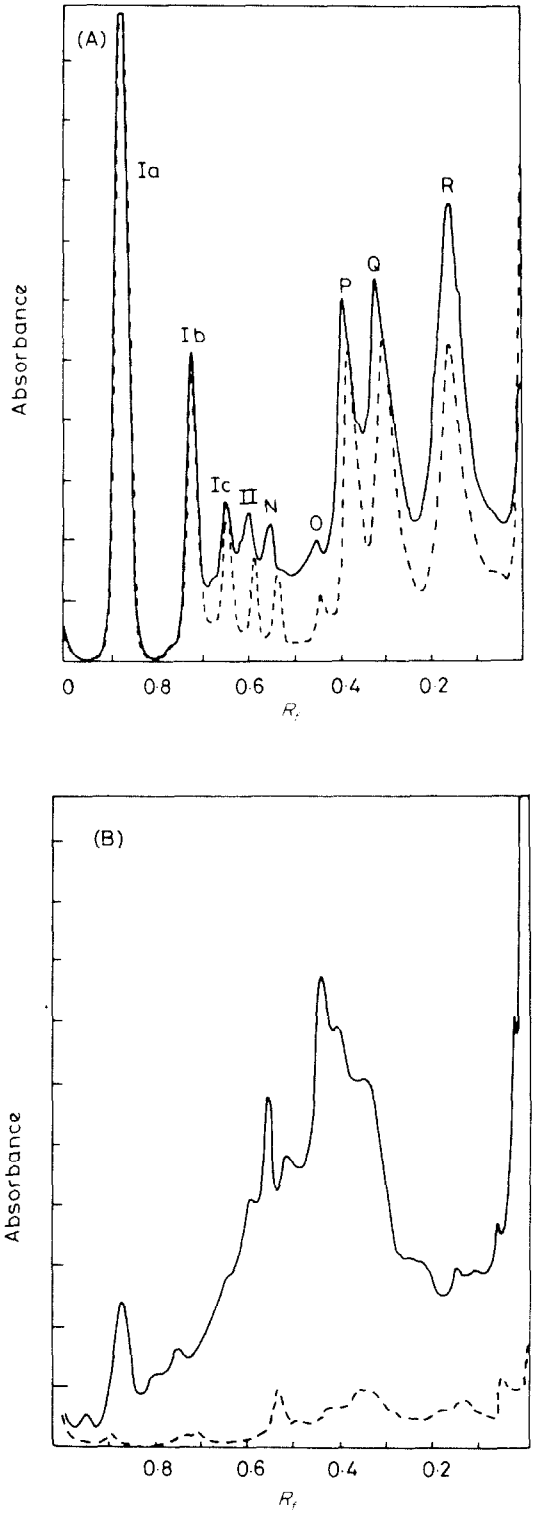

Fig. 1. Electrophoresis of major proteins of leaf extracts. Extracts from TMV-infected (A) and uninfected (B) leaves of tobacco which contained 100 μg protein (corresponding to *ca* 0.27 (A) and 1.6 (B) g of leaf material) were electrophoresed in 10% polyacrylamide gels before (————) and after (– – –) they had been incubated with trypsin and chymotrypsin. Gels were stained and scanned.

similar extract from infected leaves. Most proteins were diminished or removed by proteolytic digestion, including that one (R_f 0.85) which may have been previously mistaken for PR-Ia [see 10]. There was little evidence in these experiments for the new component (R_f 0.7; 10% gels) that was previously produced by digestion [10]. The amounts of proteolytic enzymes used were too small to give visible bands.

Separation of proteins by chromatofocusing

Eleven major protein components were detected by electrophoretic analysis of the fractions produced by passing an extract of infected leaves down a column of ion-exchanger under chromatofocusing [12] conditions (Fig. 2). They were identified by their electrophoretic mobility as PR's-I, II, N, O and Q, the major components of R and P plus two components, *R'* and *P'*, which co-migrated with R and P during electrophoresis. The proteins were eluted from the column in order of decreasing isoelectric points, and the pH at which each emerged gave an indication of its isoelectric point. With the pH gradient used in the experiment described in Fig. 2, only R', R, Q, P and PR-Ia were recovered free from other recognizable major proteins.

Subunit composition of proteins from infected leaves

Proteins from infected leaves were separated in non-denaturing acrylamide gels, the gels sliced, and the proteins extracted into an SDS-containing buffer and subject to SDS-PAGE. The results, summarized in Fig. 3, produce no suggestion that the SDS-peptide which characterizes PR-Ia, b and c, can be produced from the other major components. The fast migrating peptide derived from R, was readily distinguished from SDS-PR-Ia by co-electrophoresis. PR-II was usually associated with two SDS-components, present, judging by staining

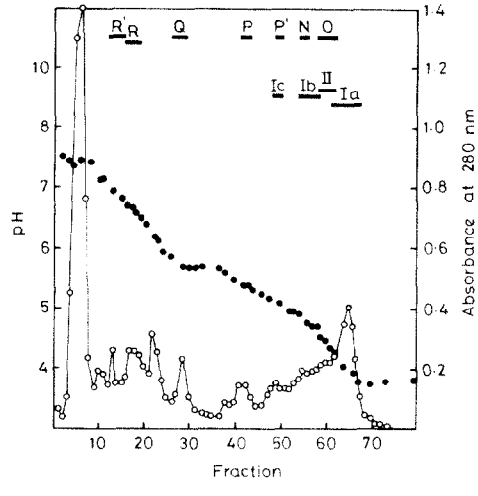

Fig. 2. Separation of leaf proteins by chromatofocusing. An extract containing the proteins from 33 g fr. wt of TMV-infected leaves was passed down a column of ion-exchanging material under chromatofocusing conditions, and fractions of the eluate examined for pH (●), absorbance at 280 nm (○) and presence of proteins (—).

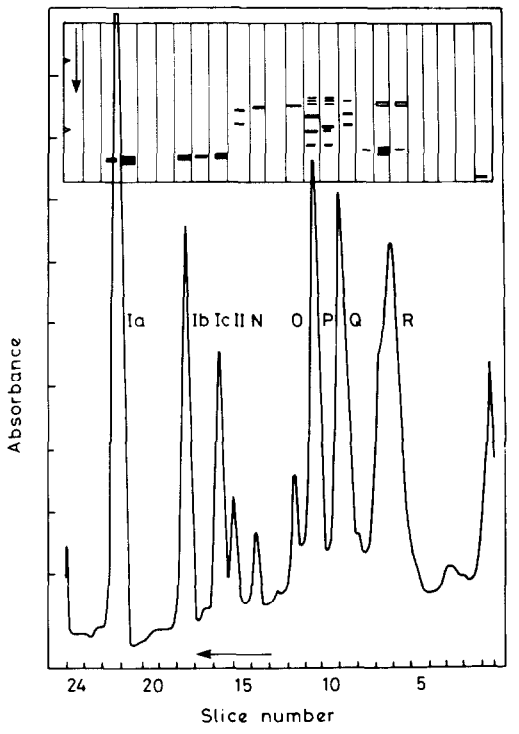

Fig. 3. Subunit composition of proteins from infected leaves. An extract of TMV-infected leaves was divided into two and subject to PAGE in two rod gels. One rod was stained and the proteins bands scanned (lower trace). The other was sliced, proteins extracted from the slices in SDS-buffer, and subject to SDS–PAGE, in a slab gel (upper diagram). The diagram is arranged so that sub units are directly above the protein peaks from which they are believed to have been derived. The notches in the left hand side of the SDS–PAGE gel represent the downward migration of phosphorylase b (60 000 d) and carbonic anhydrase (29 000 d).

intensities, in unequal amounts: this association has also been observed by others [8]. The SDS-peptides of N and O migrated similarly. P and R gave rise to two or more SDS-peptides,. as would be expected because of the presence of P′ and R′.

Chitinases and protease-inhibitors

Protease-inhibitors are produced during the mechanical damage of the leaves of solanaceous plants [13, 14], and in some plants, chitinases are induced by ethylene [15] as are PR-proteins in tobacco [16]; it seemed possible therefore that either of these activities might be associated with one of the observed virus-induced proteins. An exochitinase, active against synthetic substrates, was present in extracts of TMV-infected leaves; but although it migrated electrophoretically close to R (R_f 0.15), it was also present in extracts of uninfected leaves and did not increase on a protein N basis as a result of infection. An endochitinase was also present in the extracts and increased 2–3 fold on infection, but it could not be detected in slices of gels after electrophoresis, and probably has too high a pI [> 9, Boller, personal communication] to allow it to enter these gels. However, three protein components of extracts of infected leaves; Q and probably P (rather than P′) and R′ (rather than R) did interact with chitin; when extracts were passed down columns of either colloidal or non-colloidal chitin, these components were specifically retained and eluted only on subsequent washing with buffer or dilute base (Fig. 4). This behaviour would be expected of either chitinases or of *N*-acetylglucosamine-specific lectins.

A sample of tobacco chymotrypsin-inhibitor 1, purified by Dr. C. A. Ryan [17], migrated as a wide band of R_f 0.1 during electrophoresis. Although it is likely to be present in extracts made from infected leaves at low pH's, it migrates too slowly to be identified with any of the major virus-induced components.

Incorporation of $^{14}CO_2$ into leaf proteins

Leaves of plants which had been inoculated with TMV or sham-inoculated, were exposed to $^{14}CO_2$ four or five days later when lesions were well established. After three further days, radioactivity had been incorporated into proteins which could be extracted at low pH's. Radioactivity was not unequivocally associated with any of the protein bands that could be resolved by PAGE from extracts of uninfected leaves, and this held whether the extracts had been treated with proteolytic enzymes (Fig. 5) or not. Conversely protein bands Ia, Ib, Ic, II and R + R′ resolved from extracts of infected leaves were consistently associated with radioactivity (Fig. 6). Incorporation into proteins N–Q was questionable and inconsistent. This pattern of incorporation was sharpened but not materially altered by proteolytic digestion and subsequent dialysis.

There are three reasons for believing that the radio-

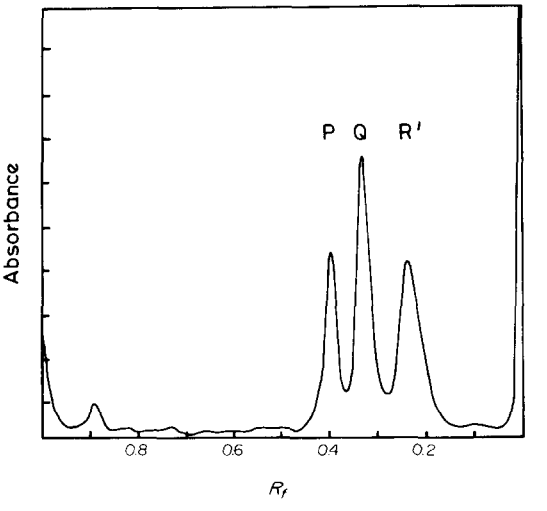

Fig. 4. Absorption of proteins on chitin. An extract of TMV-infected leaves (1.2 ml; 2 mg protein; pH 5.3) trickled through a small (0.5 ml) column of chitin which was washed free of unabsorbed protein with *ca* 4 ml of phosphate–citrate buffer. Retained protein was eluted with either more buffer or 10 mM NaOH, and detected by electrophoresis. The trace is of a stained electrophoretic gel on which a fraction eluted by alkali was examined.

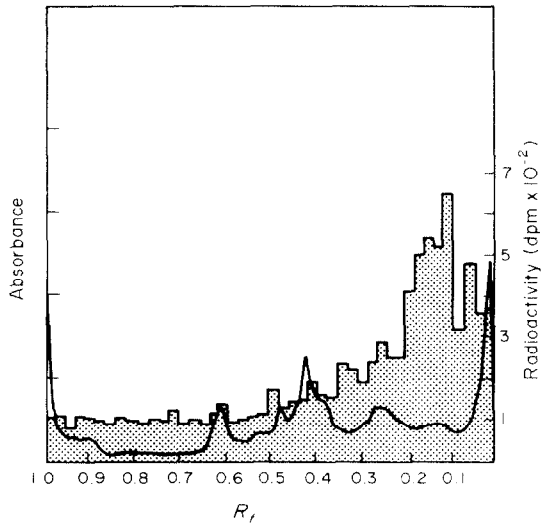

Fig. 5. Radioactivity in proteins extracted from uninfected leaves. The extract (58 μg protein; equivalent to 0.75 g leaf) was digested with proteolytic enzymes and then subject to PAGE. The gel was stained, scanned (continuous trace), sliced and radioactivity (hatched histogram) measured in the slices.

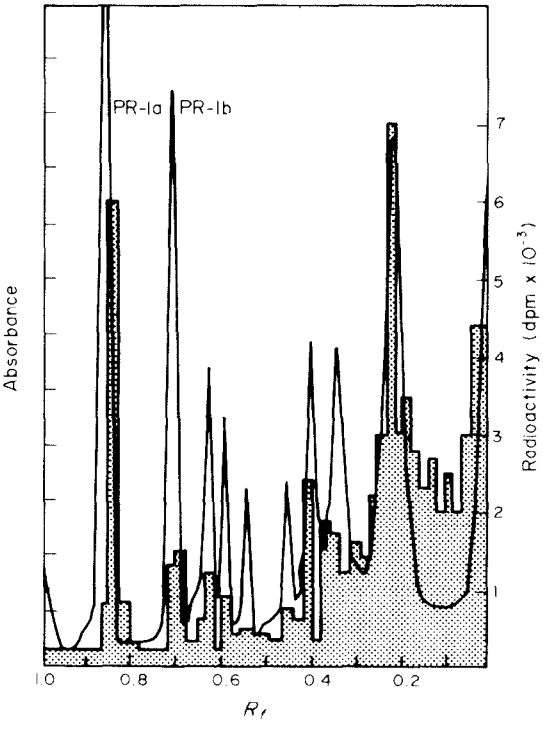

Fig. 6. Radioactivity in proteins extracted from infected leaves. The extract (270 μg protein; equivalent to 0.66 g leaf) was digested with proteolytic enzymes and then subject to PAGE. The gel was stained, scanned (continuous trace), sliced and radioactivity (hatched histogram) measured in the slices.

activity that migrates with PR-Ia belongs to the protein and is not present fortuitously. Firstly, the radioactivity migrated with the same R_f as PR-Ia in polyacrylamide gels of three different compositions (7.5%, 10% and 15%). Secondly, when PR-Ia was extracted from PAGE gels into SDS-buffer and subject to SDS–PAGE, about 80% of the extracted radioactivity migrated as did SDS-PR-Ia (R_f 0.89 in 12% polyacrylamide gel) while most of the remainder stayed at the origin. Thirdly, in some experiments a batch of leaves was inoculated with virus but exposed to $^{14}CO_2$ at different times relative to the inoculation. They were allowed to metabolize the radioactive assimilate for four days before being extracted, so that samples were obtained from leaves with lesions in different stages of development. Within the limits of the procedure (Fig. 7), the amount of radioactivity associated with PR-Ia was proportional to the amount of PR-Ia.

When leaves were extracted six days after inoculation, the radioactive labelling of PR-Ia and -Ib altered comparatively little with changes in the time at which $^{14}CO_2$ had been administered. In the experiment summarized in Table 1, the profile of radioactivity along PAGE-gels was similar whether $^{14}CO_2$ was given before, along with, or after TMV-inoculation. Extracts made from leaves fed after inoculation contained more radioactivity, but a larger proportion of it migrated more slowly (R_f 0.05–0.2) than the protein bands. The specific activity of PR-Ia, estimated as described in Table 1, is not appreciably less following the earlier applications of $^{14}CO_2$ than following applications six days later, suggesting that the ^{14}C assimilated by leaves has a long 'wash-through' time.

Incorporation of ^{3}H-leucine into PR-proteins in detached leaves

Leaves which have been detached after inoculation and

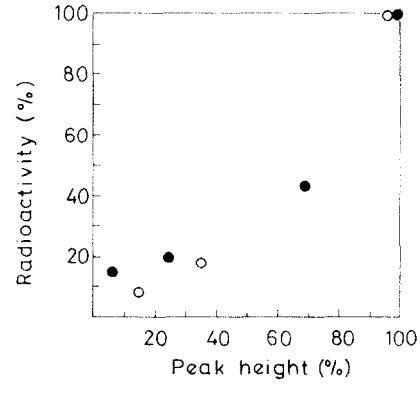

Fig. 7. Relationship between amount of PR-Ia and associated radioactivity in different extracts. The youngest expanded leaves on each of 3 (○) or 4 (●) pairs of tobacco plants were exposed to $^{14}CO_2$ 2 days before, 1 day before, 1 day after or 2 days after inoculation with TMV. Duplicate leaves were harvested 4 days after exposure, so that in different samples lesions were in different stages of development. Protein was extracted and incubated with trypsin plus chymotrypsin. Samples corresponding to approximately half (43–55%) the extract of a leaf were analysed by PAGE and the peak height of PR-Ia as well as its associated radioactivity were measured. Results of the two experiments have been normalized.

Table 1. Incorporation into PR-proteins of $^{14}CO_2$ administered at different times relative to infection

	Time of exposure to $^{14}CO_2$ relative to inoculations		
	3 days previous	Same day	3 days later
Radioactivity in gel (dpm)	7000; 7000	18000; 20000	17000; 26000
Radioactivity in PR-Ia (dpm)	580; 480	550; 1360	620; 1200
Amount PR-Ia (mm height of peak)	13; 9	16.5; 16	12.5; 15
'Specific activity' of PR-Ia	45; 50	34; 85	50; 80

The youngest expanded leaves on six tobacco plants were exposed, in pairs, to $^{14}CO_2$ either 3 days before, the same day, or 3 days after inoculation with TMV. They were harvested 6 days post-infection, duplicates pooled and protein extracted. Samples containing approximately 110 μg protein, were incubated with trypsin and chymotrypsin, analysed in duplicate by PAGE, and the stained gels scanned, sliced and examined for radioactivity. An estimate of the specific activity of PR-Ia was obtained from its radioactive content and the height of its peak in the gel scans. Values from two experiments are given separately.

kept in the laboratory, produced the same proteolysis-resistant proteins as do attached leaves; extracts made from sham-inoculated leaves that have been detached are usually more resistant to proteolysis than those from attached leaves [see also 10]. Detached leaves, both infected and uninfected, incorporate ^{3}H-leucine into extractable proteins. Although the radioactivity is not so clearly associated with protein peaks in PAGE gels as is that incorporated from $^{14}CO_2$, it is associated with PR-Ia, and probably with R. Attempts to 'chase' the radioactivity out of PR-Ia have not been successful. Leaves were fed amounts of ^{3}H-leucine (50 μCi; 0.34 mμ mol) sufficient to produce a small but detectable labelling of PR-Ia, and after 70 hr, were either extracted, or fed a larger amount of unlabelled leucine (7.6 μmol in 5 mM KCl) and kept for a further 60 hr before extracting. Extracts from the latter contained 2–3 times as much PR-Ia as the former, and it was associated with 3–4 times as much radioactivity.

DISCUSSION

There must be many proteins whose presence in a leaf changes quantitatively or in a major qualitative way during the hypersensitive reaction to a virus infection. New enzymes concerned with the replication of viral-RNA may appear, and large changes in such leaf enzymes as peroxidases, polyphenoloxidases and ribonucleases are well known [e.g. 18]: partly degraded or quinone-modified proteins may be produced in necrotic areas: proteins analogous to 'heat shock' proteins may be formed [19], as may, it is widely believed [7], 'phyto-interferon(s)' which help limit the spread of virus. The seven proteins (N–R′) described here could belong to any of these classes. So far there is no evidence that they have a chemical resemblance to the four better known PR-proteins for which a role in resistance has been argued [2]. Thus their subunit constitution makes it unlikely that they are a polymeric family based on PR-Ia. Moreover they do not, apart from R + R′, appear to accumulate ^{14}C to the same extent as the PR-proteins, although the evidence on this point (Fig. 6) is not thoroughly discriminating. Any physiological relationship to PR-proteins will be clearer when it is known if N–R′ are, like PR-proteins, produced systemically and induced by the chemicals [4] which confer resistance to further infections.

The production of protease-inhibitors in leaves following mechanical or insect damage [13, 14] bears some resemblance to the production of PR-proteins following the virus-induced hypersensitive reaction. Thus both types of proteins are formed in leaves that are some distance from the damaged one. Moreover the 'wound-hormones' that act as messengers to initiate the synthesis of the protease-inhibitors, have been characterized as degradation products of plant or fungal cell walls [20], and a chitosan that is highly effective [21], will induce the synthesis of small but definite amounts of PR-Ia in detached tobacco leaves [Pierpoint, W. S., unpublished experiments]. However, the formation of the inhibitors, unlike that of the PR-proteins [8], is normally controlled at the transcription stage [22], and we have previously shown [10] that the trypsin-inhibitory activity of TMV-infected leaves does not belong to PRs-Ia,b,c or II. We now report that the chymotrypsin-inhibitor I from tobacco leaves, cannot be identified electrophoretically with either PR-I or -II, or proteins N–R′. Nor can any of these proteins be identified with either an endochitinase, or the exochitinase that increases following either TMV-infection [Boller, personal communication] or ethylene treatment [15] of leaves. However, P (or P′), Q, and R′ (or R) are absorbed by chitin, and from their staining with Schiff reagent, may contain carbohydrate moieties. They may therefore be acetylglucosamine-specific lectins. Tobacco leaves are known to contain hydroxyproline-rich lectins of this specificity [23], and these may be rendered more readily extractable during necrosis. Moreover similar lectins are known to accumulate in leaves as a result of fungal or viral infection [24], and have been suggested by Brown and Kimmins [25] to localize viruses by blocking plasmodesmata.

The experiments with $^{14}CO_2$ demonstrate that photosynthetically fixed carbon, is incorporated into PRs-I and II as lesions develop over a few days. This is some evidence against the view that PR-proteins are products of the degradation of pre-existing structural cell components with long half-lives. Convincing evidence against this view of course, is the occurrence of specific messenger-RNA's for PR-proteins [8]. Neither the experiments with $^{14}CO_2$

nor those with ^{3}H-leucine established pulse-labelling conditions and so they produce no evidence for the 'turnover' or degration of these proteins. This may indicate that the proteins are stable end-products, or it may reflect the difficulties of 'pulse-labelling' experiments in heterogeneous, unsynchronized tissue. However, evidence that the PR-proteins of TMV-infected Samsun NN tobacco can be degraded, is their apparent disappearance when the temperature is raised to 30° [1]: moreover, the amounts of PR-proteins in infected Xanthi-nc tobacco also decreases 2–3 weeks after infection [26], although this could be because their extraction becomes more difficult as the leaves senesce.

EXPERIMENTAL

Nicotiana tabacum cv Xanthi-nc was grown in the glass house [3] and when plants were about 30 cm high, expanded leaves were inoculated with a suspension of purified type-strain TMV (3 μg/ml) and carborundum. Control leaves were 'sham-inoculated' with water, or left untouched.

Radioactive labelling. When $^{14}CO_2$ was administered, attached leaves were enclosed in a perspex chamber (500 ml) which could be fitted around the petiole and sealed with plasticine. The chamber contained 0.1 ml of sodium (^{14}C) bicarbonate (usually 0.2 mCi; 3.5–3.8 μmol; CFA 3, Amersham International plc), and $^{14}CO_2$ was generated by injecting 1 ml of lactic acid into its compartment with a hypodermic syringe. The leaf was illuminated for *ca* 30 min by a 1000 W Hg-vapour lamp suspended 45 cm above it. During illumination the perspex chamber was cooled with air, and the rest of the plant shaded with metal foil. Afterwards, the chamber was removed and the plant left in the fume cupboard overnight before being returned to a compartment of the glass house.

^{3}H-Labelled leucine was fed to detached leaves which were supported in blocked-off filter funnels [10], contained in a plastic box in a fume cupboard. The leaves were allowed to absorb *ca* 0.5 ml of 5 mM KCl soln containing L[4,5-^{3}H] leucine (50 μCi; 0.34 m μmol; TRK 636 Amersham International plc), followed by further lots (0.5 ml) of 5 mM KCl, without letting the petiole tips dry out. They were then kept for 4–6 days in 5 ml water which was changed daily. They were illuminated for 12 hr each day by 3 × 20 W fluorescent lamps giving about 50 micro-einsteins/m^2/sec. Temperature oscillated between 24° (day) and 17° (night).

Extraction of leaf proteins. Leaves were extracted in a citrate-phosphate buffer, pH 2.8, and the extracts centrifuged and chromatographed on G-50 Sephadex [10]. Protein-containing fractions were pooled, freeze-dried, dissolved in water (0.5–1.0 ml) and dialysed against Tris (5 mM)–glycine (38.5 mM) buffer pH 8.3 for 24 hr. The solns were clarified by centrifugation and protein estimated spectrophotometrically at 260 and 280 nm using the data for enolase [see 10] as standard. The procedure extracted about five times more protein from infected as from uninfected leaves: only a little more (< 10%) PR-Ia was obtained from the residues of infected leaves by re-extraction at pH 8.

Extracts for chromatofocusing and treatment with chitin, were made in pH 2.8 buffer, centrifuged and dialysed for 1 day against half-strength buffer and a further 2 days against water. They were freeze-dried, and the protein re-dissolved and re-dialysed against appropriate buffer as required.

Proteolytic digestion of protein samples. To an extract containing a known weight of protein was added 1/50 of this weight of trypsin, and also of chymotrypsin, in 0.05 vols of 50 mM Tris, pH 8, containing 1 mM $CaCl_2$. The mixture was incubated at 25° for 16 hr and dialysed for a further 24 hr against Tris–glycine buffer at pH 8.3.

Polyacrylamide gel electrophoresis (PAGE). Proteins were separated in rod gels containing 10% (w/v) polyacrylamide, cross-linked with 0.2% *NN'*-methylenebisacrylamide (MEB), which were overlaid with a stacking gel (2.5% w/v polyacrylamide; 0.23% w/v MEB) as described previously [10]. They were stained with Coomassie Brilliant blue G250 in $HClO_4$ for 1 hr, destained in 5% (v/v) HOAc overnight and scanned spectrophotometrically at 610 nm. Electrophoresis in rod gels containing SDS (SDS–PAGE) has been described previously [27]. Gels were stained for carbohydrates with HIO_4 and Schiff reagent [28] after fixation in 10% (v/v) HOAc or 12.5% (w/v) TCA.

Proteins were extracted from non-SDS gels for further electrophoresis by two methods. (1) Unstained gels were washed with water, sliced into 1 mm segments, and the slices suspended in pairs in 0.15–0.2 ml of soln containing Tris buffer (0.07 M), 10% (w/v) glycerol, 3.3% (w/v) SDS and 1% (v/v) 2-mercaptoethanol, pH 6.8. The slices were minced with a scalpel, heated at 80° for 3 min, allowed to stand at room temp. for a few hours and left overnight at 5°. Extracts, adjusted to pH 6.8 if necessary, were mixed with 5 μl of a bromophenol blue solution, and subject to discontinuous SDS–PAGE in slab gels (usually 12% w/v polyacrylamide; 7.5 × 7.5 cm) essentially as described by Gianinazzi *et al.* [9]. Gels were fixed and stained with Coomassie brilliant blue R250 (0.1% w/v) in 50% MeOH–10% HOAc for 1 hr and destained in 5% MeOH 10% HOAc. (2) Alternatively the rod gels were stained in the Coomassie brilliant blue R-250 solution for 30 min and destained for 1 hr to locate the proteins. The stained bands were excised, extracted into Tris buffer (0.21 M, pH 8.5) containing 1% (w/v) SDS, and dialysed for 24 hr against a more dilute Tris buffer (0.07 M, pH 6.9) containing 0.1% (w/v) SDS. The soln was made up to 1% (w/v) with SDS and 1% (v/v) with 2-mercaptoethanol and heated at 80° for 3 min. After adding 1 drop of glycerol and marker dye, they were subjected to SDS–PAGE as above.

Measurement of radioactivity in gels. Stained gels were washed × 3 with water, dried and sliced. Slices or pairs of slices were put into plastic vials containing 0.5 ml of NCS tissue-solubilizer (Amersham/Searle Corporation), tightly stoppered and heated at 60° for 2 hr [29]. They were stored in the dark overnight, 3 ml Tritosol [30] added, and radioactivity counted in a Beckman LS 250 scintillation counter. Observed values were corrected for quenching by the standard two-channel ratio method.

Chromatofocusing. Extracts of proteins from infected leaves were dialysed against imidazole (0.025 M)-HCl buffer, pH 7.4 and 2–3 ml samples applied to a column (1 × 22 cm) of Polybuffer exchanger (Pharmacia) equilibrated with the same buffer. The column was developed with Polybuffer 74, diluted eightfold, as described in the technical literature supplied by Pharmacia Fine Chemicals. Fractions (1.4 ml) of eluate were examined for pH and absorbance at 280 nm, and then freeze-dried. They were dissolved in 0.3 ml water and dialysed against Tris–glycine buffer pH 8.3 and samples (70 μl) taken for electrophoretic examination. Proteins were recognized by their mobility and by migration during co-electrophoresis with small amounts (10 μl) of original extract.

Absorption of protein onto chitin. Purified chitin (Sigma Chemical Company), was packed into the barrel of a syringe to make a small (0.5 ml) column. Colloidal chitin [31] was packed into a larger (5 ml) column. Both were equilibrated with citrate (0.075 M)–phosphate (0.15 M) buffer at pH 5.3 and extracts of infected leaves, adjusted to pH 5.3, were allowed to trickle through them. The columns were washed with more buffer and then 10 mM NaOH: fractions of the eluate were dialysed against

water, freeze-dried, re-dissolved in Tris–glycine buffer (0.3 ml) and examined by electrophoresis.

Extraction and estimation of chitinases. Infected and uninfected leaves were extracted in citrate buffer pH 6, and precipitated with ammonium sulphate as described by Agrawal and Bahl [32]. The precipitates were dissolved and dialysed and the protein in them judged spectrophotometrically as above. Exochitinase was assayed in extracts and also in slices of gels with 4-nitro-*N*-acetyl-β-D-glucosaminide as substrate [32]. Endochitinase was estimated essentially as described by Jeuniaux [33] except that after incubation, samples (0.3 ml) were diluted with 0.2 ml water, and incubated with 25 μl of snail extract (Sigma; crude β-glucuronidase) for 2 hr at 30° to degrade chitin fragments to acetylglucosamine [34]. Solns were heated on a boiling water bath for 1 min, centrifuged and acetylglucosamine estimated in samples of supernatants.

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