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PHENYLALANINE AMMONIA-LYASE IN TOBACCO MOSAIC VIRUS-INFECTED HYPERSENSITIVE TOBACCO

DENSITY-LABELLING EVIDENCE OF DE NOVO SYNTHESIS

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Summary

A strong increase in phenylalanine ammonia-lyase (EC 4.3.1.5) activity occurs in tobacco mosaic virus-infected tobacco leaves developing necrotic local lesions. Comparison of physicochemical properties of the partially purified enzymes extracted from healthy and infected leaves showed that the hypersensitive reaction leads to an increase in the pool size of the same active enzyme molecules as those present in non-infected material. The molecular mechanism of enzyme formation was investigated by radiolabelling with [^{3}H]leucine and by density labelling with ^2H_2O. Abnormal patterns of incorporation of radioactivity into all soluble proteins were found in infected leaves carrying local lesions. In contrast, uptake of deuterium into the amino acid pool was the same in healthy and infected leaves. Unstimulated phenylalanine ammonia-lyase was shown to be a long-lived enzyme (half-life: 25—35 h). Results of comparative density labelling experiments unequivocally demonstrated that the increased enzyme pool size arose from an increased rate of synthesis mediated by the hypersensitive reaction.

Introduction

Infection of plants by viruses sometimes leads to a hypersensitive reaction, in which the spread of the virus is limited to the vicinity of local necrotic lesions. The localization of the virus is accompanied by a strong reaction of the host [1,2] with highly increased production of phenolic compounds derived from phenylalanine [3—7]. It is assumed that phenolic compounds are involved in

Abbreviation: TMV, tobacco mosaic virus.

the formation and development of the local lesions: a strong increase in the levels of these compounds followed by a stimulation of polyphenoloxidases [8] and peroxidases [9,10] would be responsible for a rapid accumulation of toxic concentrations of quinones that would disrupt the cells.

Enzymes of the phenylpropanoid pathway, such as phenylalanine ammonia-lyase, cinnamic acid-4-hydroxylase and catechol *O*-methyltransferases, are strongly stimulated in tobacco mosaic virus (TMV) infected leaves of hypersensitive tobacco [11—13]. Considerable attention has been devoted in recent years to the mechanisms which might explain the pronounced increase in extractable activity of phenylalanine ammonia-lyase (EC 4.3.1.5) following various stimuli applied to plant tissue, such as blue light, red light, wounding, aging and chemicals [14—16]. However, very little is known about the regulation of phenylalanine ammonia-lyase in plants infected by bacteria, fungi or viruses, although stimulation of the phenylpropanoid pathway is a common feature of many host-parasite combinations. It is mostly found when the host develops resistance to a general invasion by the phytopathogen [2].

The factors which lead to an increased amount of catalytically active enzyme in the diseased tissue may operate by one or more of the following processes: increased rate of synthesis of the polypeptide chains of the enzyme, activation of pre-existing but catalytically inactive enzyme, reduced inactivation of catalytically active enzyme, reduced degradation of the polypeptide chains of active enzyme molecules. Several techniques offer themselves for the demonstration of one or more of these processes: immunology, radioactive labelling and density labelling. The first two methods require the purification of the enzyme to a single homogeneous protein. A method of purification of phenylalanine ammonia-lyase extracted from tobacco leaves (*Nicotiana tabacum* L., var. Burley 2) has already been described [17]. We show here that the purification of the enzyme by this procedure was insufficient and that the transport of radioactive amino acids was strongly disturbed when local lesions developed on the leaves, so that radiolabelling could not be used. In contrast, ^2H_2O is taken up to the same extent by healthy and necrotic-reacting mesophyll tissue. This allowed us to use the comparative density labelling technique and to show that the increased phenylalanine ammonia-lyase activity occurring in infected leaves arose from an increased rate of synthesis of catalytically active enzyme molecules.

Materials and Methods

Plants

Three-month-old tobacco plants *Nicotiana tabacum*, variety Samsun NN, were grown in an air-conditioned greenhouse at 22°C ± 2°C. We used the two first fully expanded leaves from the top of each plant, which had 6 to 7 leaves altogether.

Inoculation

The leaves were rubbed with a 5 μg/ml aqueous suspension of purified virus in the presence of Celite. This treatment induced the formation of 500—700 local lesions per leaf. The corresponding leaves of control plants were inoculated with plain water.

Radioactive labelling

After the desired period of infection, leaves were detached and fed with tritiated leucine by absorption through the cut petioles. 10 ml of a 30-fold diluted Knop mineral medium containing 0.5 mCi of L-[4-^{3}H]leucine (5 Ci · mmol^{-1}) were absorbed by 30 g of leaves within 4 h. Another procedure of labelling consisted in stripping off the lower epidermis of the leaves and floating them on the radioactive solution in Petri dishes.

Density labelling

Leaves were detached and fed with ^2H_2O also by absorption through the cut petioles. In our first experiments we used 95% ^2H_2O, but this high concentration led to some isotopic stress of the leaves and was subsequently reduced to 70% and even 60%. The plants were incubated at 24° C for 8—94 h in small growth chambers producing an air current, to activate transpiration of the leaves and speed up the replacement of H_2O by ^2H_2O. In our conditions, 0.1 ml of 60% ^2H_2O was absorbed per h and per g fresh weight in the case of both healthy and TMV-infected leaves.

Extraction of phenylalanine ammonia-lyase

30 g of freshly harvested leaf tissue (midvein removed) was ground at 4° C in a Virtis homogenizer in the presence of 0.3 g activated charcoal and of 80 ml 0.025 M phosphate buffer, pH 7.4, containing 30 mM 2-mercaptoethanol. The mixture was filtered through a double layer of cheesecloth. The filtrate was centrifuged at 20 000 × *g* for 20 min.

The enzyme was then purified by the method of O'Neal and Keller [17] that consists of 6 steps: $(NH_4)_2SO_4$ precipitation, low pH treatment, heat treatment, acetone precipitation, chromatography on hydroxyapatite, polyacrylamide gel electrophoresis.

For isopycnic centrifugation we used less purified fractions. These were obtained as follows: to the supernatant of the 20 000 × *g* centrifugation was added $(NH_4)_2SO_4$ to 55% saturation. After centrifugation at 20 000 × *g* for 20 min, the pellet was dissolved in 10 ml of 0.025 M phosphate buffer, pH 7.4, and cold acetone (—30° C) was added to a final concentration of 47% v/v. The mixture was centrifuged at 20 000 × *g* for 5 min. The pellet was blotted dry on filter paper and resuspended in 2 ml of 0.1 M borate buffer, pH 8.8, containing 30 mM 2-mercaptoethanol and 0.4 g/ml CsCl. This mixture was again centrifuged at 20 000 × *g* for 30 min and the supernatant containing the partially purified phenylalanine ammonia-lyase and acid phosphatase was called "enzyme fraction I". For isopycnic centrifugation we used also "enzyme fraction II" that was obtained by the same procedure, except that the acetone treatment was replaced by gel filtration over Sephadex G-25.

Isopycnic equilibrium centrifugation

We used the type 50 Ti fixed-angle rotor in a Beckman L2-65 ultracentrifuge. This allows better radial separations than swinging-bucket rotors [18].

2 ml of enzyme fraction I or II were mixed with 7 ml of 0.1 M borate buffer, pH 8.8, containing 10 mM 2-mercaptoethanol, 0.4 g/ml CsCl and 0.4 units of β-galactosidase. The density of the mixture was adjusted to 1.310 for all tubes

with a saturated CsCl solution. In some experiments RbCl was used instead of CsCl, and the density was then adjusted to 1.318. The tubes were then centrifuged at 45 000 rev./min at 2° C for 60 h.

After centrifugation a 20 μl micropipette was lowered to the bottom of the tube and 1-drop fractions (25 μl) were collected by means of a peristaltic pump. Every fifth fraction was used to measure refractive index in an Abbe 60 refractometer. All other fractions were used to assay phenylalanine ammonia-lyase, β-galactosidase and acid phosphatase. To improve the accuracy of buoyant density determination for each of these enzymes, the portion of its activity profile situated above half-maximum activity was sampled more often as follows: among the fractions of density in the range 1.325—1.380, two out of every three were used to assay acid phosphatase, one out of every six to assay β-galactosidase and one out of every six to assay phenylalanine ammonia-lyase. In the density ranges 1.308—1.325 and 1.250—1.308, β-galactosidase and phenylalanine ammonia-lyase were the most assayed enzymes, respectively.

The values of refractive index were converted to density units using the equations of Ifft et al. [19,20].

Assays of phenylalanine ammonia-lyase

To each fraction to be assayed was added 0.7 ml of 0.1 M borate buffer, pH 8.8, containing 10 mM 2-mercaptoethanol and 20 mM L-phenylalanine. The reaction mixture was incubated for 12—48 h at 35° C. At the end of the reaction we measured the absorbance at 290 nm using a Jobin-Yvon model Monospac 103 spectrophotometer.

To assay phenylalanine ammonia-lyase of crude extracts and of polyacrylamide gel slices we used a radiochemical procedure that has already been described [13].

Assays of β-galactosidase (EC 3.2.1.23)

The enzyme (source: *Escherichia coli)* was purchased from Sigma Chemical Company (St. Louis, U.S.A.). To each fraction to be assayed was added 0.4 ml 0.1 M sodium phosphate buffer, pH 7.2, containing 10 mM 2-mercaptoethanol and 2 mM *O*-nitrophenyl β-D-galactopyranoside. The reaction mixture was incubated for 1 h at 35° C. The reaction was stopped by the addition of 0.8 ml of 10% sodium carbonate solution and the absorbance at 410 nm was measured in a Jobin-Yvon model Monospac 103 spectrophotometer.

Assays of acid phosphatase (orthophosphoric-monoester phosphohydrolase, EC 3.1.3.2)

To each fraction 0.5 mg *p*-nitrophenyl phosphate dissolved in 0.7 ml of 0.02 M sodium acetate buffer, pH 5.2, was added. The reaction mixture was incubated at 4° C for 15 min. The reaction was stopped by the addition of 1.4 ml of 10% sodium carbonate. The concentration of the reaction product, *p*-nitrophenol, was determined by measuring the absorbance at 410 nm.

Acid phosphatase activity of crude extracts was assayed by incubation of 100 μl of enzyme solution in 2.9 ml acetate buffer, pH 5.2, containing 1.5 mg of *p*-nitrophenyl phosphate. The formation of *p*-nitrophenol versus time was observed at 410 nm and at room temperature in the Jobin-Yvon model Monospac 103 spectrophotometer equipped with the Logispac 1200 unit.

Polyacrylamide gel electrophoresis

Samples (centrifuged crude extracts or enzyme fraction I) contained about 100 μg of protein. Cylindrical tubes (8 × 1 cm) containing 7.5% polyacrylamide gels were electrophoresed for 8 h at a current of 5 mA per gel and in the presence of 0.025 M Tris, 0.2 M glycine buffer, pH 8.4. After electrophoresis, the gels were stained [21] with either amidoblack or Coomassie Brilliant Blue. Alternatively, 1.5 or 2 mm slices were cut and either used for phenylalanine ammonia-lyase activity determination according to a radiochemical procedure [13], or were homogenized with 30% H_2O_2 and the resulting fluid was mixed with Bray's scintillation liquid [22] for radioactive counting.

Results

Time course of phenylalanine ammonia-lyase activity in TMV-infected leaves

Fig. 1 shows that a large increase in extractable phenylalanine ammonia-lyase activity occurred when the necrotic local lesions appear, but not in water-inoculated leaves. This increased activity lasted for about two days and then decreased slowly. In the present work, we considered periods of infection ranging from 25 h to about 60 h after inoculation in order to elucidate the mechanism(s) by which enzyme activity was increased. As shown in Fig. 1, acid phosphatase activity was not significantly affected by viral infection during this period and could, therefore, be considered as an internal standard in all labelling experiments.

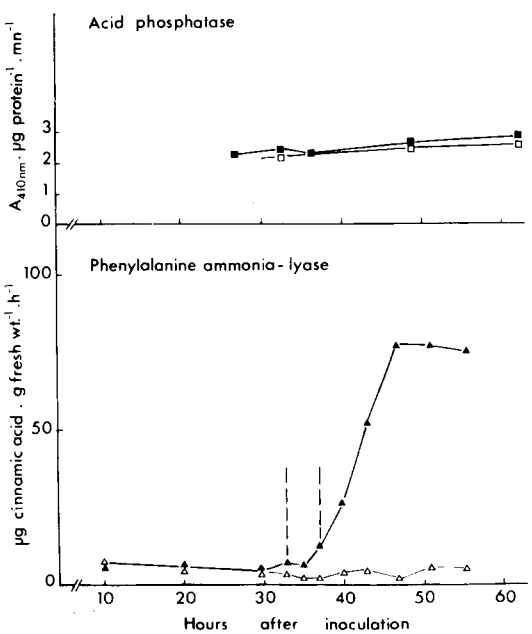

Fig. 1. Activities of phenylalanine ammonia-lyase and acid phosphatase in leaves of Samsun NN tobacco plants as a function of time after inoculation with a 2 μg/ml aqueous suspension of TMV (which induced 300 local lesions per leaf), or with water. Lesions appeared at times between the two vertical dotted lines. Symbols used: phenylalanine ammonia-lyase activity of healthy (△———△) and infected (▲———▲) leaves; acid phosphatase activity of healthy (□———□) and infected (■———■) leaves.

The increase in phenylalanine ammonia-lyase activity in infected leaves depends on the number of local necrotic lesions [13]. The higher the titers of virus inocula, the higher the number of induced local lesions, and in turn the higher the increase in levels of phenylalanine ammonia-lyase. It follows that in our labelling experiments we had to consider 5 main factors. These were: (1) the number of local lesions produced, (2) the duration of incubation with radioactive or density marker, (3) the time of feeding the precursor relative to the time at which the stimulus appeared, (4) the turnover rate of the enzyme and (5) the rate of equilibration of the pool of amino acids with radioactive or density marker. Taking these factors in order: (1) we induced 200—500 local lesions per leaf in order to observe a 5—20-fold increase over the control in phenylalanine ammonia-lyase activity; (2) the duration of the feeding depended on the turnover rate of the enzyme and should not be longer than the half-life of the enzyme [23—25]; (3) it is generally assumed that the feeding of precursors should be performed at the time at which the stimulus is applied [23,25]; (4) and (5) these factors are characteristic of the plant material and cannot be easily changed. Moreover, when results obtained in the presence and in the absence of a stimulus are to be compared, it must be checked that factor no. 5 is not affected by the stimulus itself. This could occur if the stimulus led to changes in the size of the pool of amino acids or in the transport of the radioactive or density marker.

Comparison of the stimulated and unstimulated phenylalanine ammonia-lyase

Did the increased extractable enzymatic activity of infected material arise from an identical pool of activated enzyme or from an increased pool of the same enzyme as in healthy material? Or were new isoenzymes produced in response to viral infection?

We used the procedure described for tobacco [17] to purify the enzyme(s) extracted from either healthy or TMV-infected leaves of *N. tabacum*, var. Samsun NN. We found that acetone precipitation was the most efficient but also the most critical step in the purification procedure, as already mentioned by O'Neal and Keller [17]. When the active fraction obtained after the hydroxylapatite chromatography step was gel electrophoresed, a major protein band was observed. O'Neal and Keller [17] concluded that this band corresponded to phenylalanine ammonia-lyase and represented 0.7% of the total amount of soluble proteins of leaf tissue.

During the purification, we found that the quantitative data (degree of purification, recovery) were very similar for the enzymes extracted from healthy and infected leaves, all steps being carried out exactly in parallel. After disc gel electrophoresis and staining we observed that, surprisingly, the amounts of the major protein band were exactly the same whereas enzymatic activity was, as expected, much higher for the sample extracted from infected leaves. This suggested that the increased level of enzyme activity resulted from an increased specific activity of the enzyme and, hence, that the regulatory mechanism was activation. However, enzymatic activity was determined on slices of a gel whereas the positions of the protein bands were revealed on a parallel gel by staining that leads to some swelling of the gel. Thus, an exact correspondence was difficult to establish. Furthermore, when electrophoresis was carried out

for long times (15 h), we noticed that the migration of the peak of enzyme activity was slightly faster than that of the major protein band revealed by staining. This was observed for the samples obtained from both healthy and infected leaves. It was unlikely, therefore, that phenylalanine ammonia-lyase corrsponded to this major protein band, as confirmed by the radiolabelling experiments that are described below.

After the acetone precipitation step, we compared a number of physico-chemical properties of the partially purified stimulated and unstimulated enzymes. Behaviours upon chromatography on Sephadex G200 and on DEAE-cellulose were exactly the same and a single peak of activity was observed in each case. After isopycnic centrifugation on CsCl or RbCl, we observed a true Gaussian distribution of enzymatic activity for the two enzymes and the same buoyant densities 1.301 ± 0.001 kg $\cdot$ l^{-1} and 1.3010 ± 0.0005 kg $\cdot$ l^{-1} on CsCl and RbCl gradients, respectively. Electrophoretic migrations of the two enzymes were the same: (25 ± 1) mm on 7.5% polyacrylamide gels subjected to electrophoresis for 15 h at 5 mA/gel. Identical K_m values of 85 ± 5 μM were measured for both enzymes. These results suggested that viral infection led to an increase in the pool size of the same active enzyme as in healthy material.

Labelling of healthy and TMV-infected leaves with radioactive amino acids

To see if this increased pool of active enzyme molecules arose from an increased rate of de novo synthesis or from an activation of a pool of inactive molecules already present in healthy leaves, we performed radiolabelling experiments. Leaves were fed with L-[4-^{3}H]leucine or L-[*U*-^{14}C]leucine or L-[U-^{14}C]-phenylalanine by absorption through the cut petioles. Similar patterns of distribution of radioactivity in all studied fractions were obtained with these three precursors, but the best rate of incorporation of radioactivity into the proteins was observed with [^{3}H]leucine, because of its high specific radioactivity available. Fig. 2 summarizes the results obtained when healthy and infected leaves were fed with L-[4-^{3}H]leucine. Figs. 2a and 2b correspond to "enzyme fraction I" obtained after the acetone precipitation step, i.e. to a 30—50 fold purified enzyme. Each gel slice was used to measure both enzyme activity and incorporation of radioactivity into the proteins. For the samples obtained from healthy leaves (Fig. 2a) there was a major peak of radioactivity that is not perfectly superimposable on the peak of enzyme activity and that apparently corresponds to the major protein band revealed by staining. Enzyme fraction I was also centrifuged on a CsCl gradient and each fraction was again used for both enzyme activity determination and scintillation counting of ^{3}H. We found that the buoyant density of the peak of radioactivity was significantly lower than that of the peak of enzyme activity. Therefore, we do not confirm the results of O'Neal and Keller [17] and our conclusion is that phenylalanine ammonia-lyase does not correspond to the major protein band revealed by staining of the gels. Comparison of the samples obtained from healthy and infected leaves (Figs. 2a and b) shows that, as expected, the enzymatic activity of the latter was much higher. But the radioactivity incorporated into all the proteins of enzyme fraction I was much lower, even though staining of the two parallel gels revealed equivalent intensities of the corresponding protein bands. It has already been shown by direct autoradiography of the leaves [26] that radioac-

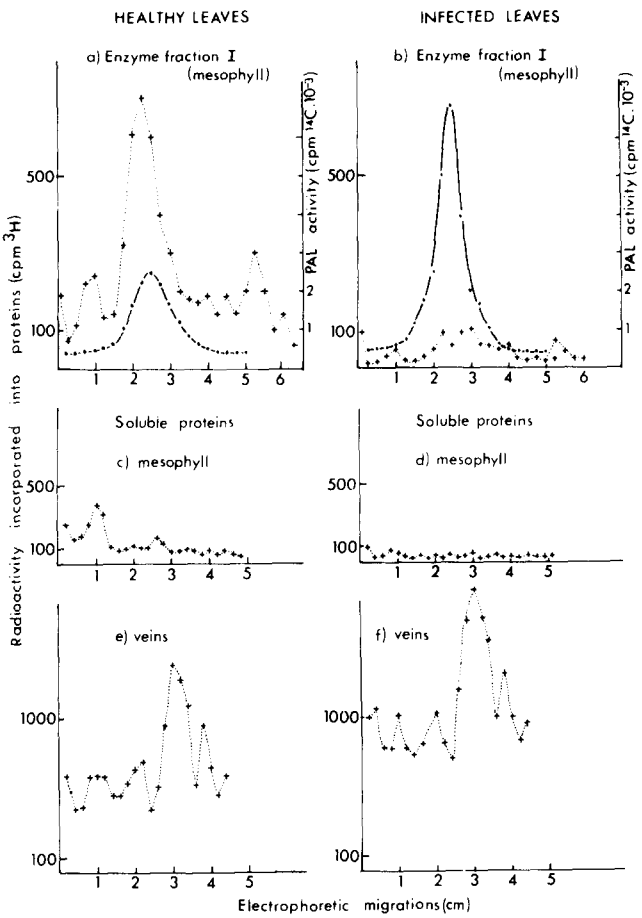

Fig. 2. Electrophoretic patterns of incorporation of L-[4-^{3}H]leucine into the soluble proteins of healthy and TMV-infected leaves. After a period of 44 h following inoculation with TMV or water, leaves were detached and fed for 4 h with the radioactive precursor. The 7.5% polyacrylamide gels were loaded in all cases with 50 µl of protein fraction containing approximately 100 µg of protein. These fractions were: (a, b), the 30 to 50-fold purified enzymatic fraction obtained after the acetone precipitation step (enzyme fraction I); (c,d), centrifuged crude extracts of mesophyll; (e,f), centrifuged extracts of midveins and secondary veins. Electrophoresis was conducted for 15 h at 5 mA/gel. Gels were cut into 2 mm slices that were either (c, d, e, f) homogenized with 30% H_2O_2 for scintillation counting of ^{3}H, or (a, b) again cut diametrically into 2 fragments, one of these being also homogenized with H_2O_2 for ^{3}H counting, the other being incubated with L-[U-^{14}C]phenylalanine in order to measure phenylalanine ammonia-lyase activity [13]. Symbols used: •———•, phenylalanine ammonia-lyase activity (expressed as number of cpm ^{14}C incorporated into the reaction product, cinnamic acid) and +- - - - -+, radioactivity incorporated into proteins.

tivity accumulates in the veins of local-lesion-carrying leaves of *N. tabacum*, var. Xanthi nc, fed with [^{14}C]leucine. This result suggested that the overall metabolism of the proteins was disturbed by the presence of enlarging local lesions on the leaves. Figs. 2c, 2d, 2e and 2f show the disc gel electrophoresis patterns of radioactivity incorporated into the proteins of centrifuged crude homogenates. Mesophyll tissue and veins (midvein + secondary veins) of healthy and infected leaves fed with [^{3}H]leucine by the petioles were extracted separately. The stained gels again revealed similar patterns of protein distribu-

tion and intensity. However, rates of incorporation of radioactivity into the proteins were very different. In healthy leaves, soluble proteins of the veins showed higher specific radioactivity than thos of the mesophyll tissue. In infected leaves, the label of the mesophyll soluble proteins was almost undetectable whereas that of the soluble proteins of the veins was increased. These results show that the stimulus itself affected the endogenous specific radioactivity of the amino acid supplied to the leaves, probably because of changes in the transport and in the metabolism of the radioactive precursor absorbed through the petioles.

We tentatively used another radiolabelling procedure that involves stripping off the lower epidermis of the leaves and floating the resulting material on water or various plasmolytica containing the radioactive amino acid. We found that, under these conditions, the labelling of the proteins of the mesophyll was very efficient and of the same order of magnitude for healthy and infected leaves. Unfortunately, the levels of phenylalanine ammonia-lyase activity were drastically increased even in healthy tissue, probably as a result of osmotic stress (Kopp, M. et al., unpublished). Therefore, this labelling procedure also could not be used.

There are two prerequisites for meaningful results when the mechanism of modulation of extractable enzyme activity is investigated by radiolabelling. These are (1) purification of the enzyme to a single homogeneous protein, and (2) demonstration that the stimulus itself does not affect the labelling of the pool of amino acids. The results above indicate that none of these conditions have been fulfilled here and that it is difficult to use radiolabelling in this type of experiments. Even if phenylalanine ammonia-lyase could be purified after addition of several tedious steps of purification, the problem of feeding the radioactive precursor would still remain.

Labelling of the pool of amino acids of healthy and infected leaves with ^2H_2O

The labelling of enzymes with ^2H_2O is increasingly being used to investigate the mechanisms underlying changes in extractable enzyme activity in intact organisms in response to various stimuli [23—25,27—37]. Density labelling has the major advantage over radiolabelling that a high degree of enzyme purification is not necessary. Contaminating proteins do not affect the two parameters that are classically [23—25] used to describe the activity profiles of density labelled enzymes on density gradients: (1) the density shift from the buoyant density of native, non-deuterated enzyme and (2) the broadening of the activity profile. However, it is still necessary that the pool of amino acids be the same in the presence and in the absence of the stimulus in all types of labelling experiments.

The degree of deuteration of the pool of amino acids from externally supplied ^2H_2O after increasing periods of infection was determined from the buoyant density of an enzyme that is not sensitive to the stimulus. We used the classical internal marker acid phosphatase [23,32,33,36,37], since it is not changed after viral infection (Fig. 1). A high acid phosphatase activity was present in enzyme fraction II (Sephadex G25 filtrate) and even in enzyme fraction I containing the 30- to 50-fold purified phenylalanine ammonia-lyase. Table I shows some of the data obtained for the density labelling of acid phosphatase.

TABLE I

DENSITY LABELLING OF ACID PHOSPHATASE IN HEALTHY AND TMV-INFECTED LEAVES

After inoculation with TMV or with water, leaves were fed with 60% ^2H_2O (Expt. 1) or 70% ^2H_2O (Expts. 2, 3 and 4). The samples were centrifuged to equilibrium on RbCl (Expts. 1, 2 and 3) or on CsCl gradients (Expt. 4) and the measured buoyant densities were corrected using β-galactosidase as external marker.

Experiment	Time of infection (h)		Duration of labelling (h)	Density shifts from native acid phosphatase ($kg \cdot l^{-1}$)	
	before leaf detachment	end of labelling		Healthy leaves	Infected leaves
1	22	→ 41	19	0.0015 ± 0.0005	0.0015 ± 0.0005
	22	→ 50	28	0.0025 ± 0.0005	0.0025 ± 0.0005
	22	→ 60	38	0.0045 ± 0.0005	0.0050 ± 0.0005
2	25	→ 51	26	0.0015 ± 0.0005	0.0020 ± 0.0005
	25	→ 66	41	0.0050 ± 0.0005	0.0045 ± 0.0005
3	21	→ 57	36	0.0055 ± 0.0005	0.0060 ± 0.0005
4	43	→ 64	21	0.0020 ± 0.0008	0.0040 ± 0.0008
	29	→ 64	35	0.0040 ± 0.0008	0.0060 ± 0.0008

The high amounts of enzyme made the shifts in buoyant density low but progressively increasing with time. When ^2H_2O was fed for periods shorter than 15 h, there was no significant shift in buoyant density compared with the native enzyme. After more than 15 h, the samples extracted from healthy and infected leaves showed similar shifts in density, except in a few experiments in which infection was allowed to develop for more than 60 h and in which many local lesions have been induced on the leaves (example: experiment 4 of Table I). Such heavily infected leaves dried off and absorbed significantly more ^2H_2O than the controls. Thus, some increased density shifts observed for the infected sample probably resulted from faster replacement of H_2O by ^2H_2O than from changes in amino acid pool size. Furthermore, we were interested mainly in the 30—55 h period following inoculation during which the levels of phenylalanine ammonia-lyase activity increased strongly. Table I shows that during this period the rate of uptake of density label into the pool of amino acids is the same for healthy and TMV-infected leaves. Thus, in contrast to the data presented above and concerning radiolabelling with amino acids, feeding of ^2H_2O by absorption through the cut petioles appeared to be a reliable procedure for comparative labelling studies.

Turnover rate of phenylalanine ammonia-lyase in healthy leaves

To identify the biochemical site of action of a stimulus leading to increased extractable enzyme activity requires that one must first establish a definitive mechanism for the turnover of the enzyme [25]. We did this by observing the density labelling of phenylalanine ammonia-lyase after increasing periods of incubation of healthy leaves with ^2H_2O. At the same time we searched for the optimum concentration of ^2H_2O.

The effects of increasing concentrations of ^2H_2O on the growth, development, morphology and transpiration patterns in *N. tabacum* L. have been reported recently [39]. Very high (90—100%) concentrations of deuterium caused extensive tissue necrosis. Since such isotopically induced stress is not

specific for tobacco and since it is well known that phenylalanine ammonia-lyase activity is sensitive to wounding [15,16], it is very surprising that most of the experimental data reported so far for the density labelling of this enzyme have been obtained with externally supplied 99—100% ^2H_2O. When we detached healthy tobacco leaves and supplied them with 95% ^2H_2O, large translucent zones appeared within a few hours along the midvein and veins. These leaves showed several-fold higher phenylalanine ammonia-lyase activity than the corresponding controls fed with H_2O. Detailed experimental data concerning this isotopic stress will be published elsewhere and we shall only indicate briefly here how this difficulty was overcome. We first reduced the concentration of ^2H_2O to 60—70%, and this efficiently decreased the effects of isotopic stress on enzyme levels. In addition, we assayed various strips of such leaves and found that the isotopic stress was the most pronounced in 8 mm strips containing the midveins and 6 mm strips containing the secondary veins. Therefore, in all experiments described hereafter, such strips were removed from the leaves before grinding and the remaining leaf tissue showed only a slightly increased level of enzyme activity over corresponding leaf tissue supplied with light water.

To investigate the rate of turnover of phenylalanine ammonia-lyase, we have taken account of general considerations that have been reviewed by several authors [23—25,33]. For example, the rate of deuteration of an enzyme is absolutely limited by the degree of deuteration of the amino acid pool from which it is being synthesised. Also, the bandwith of activity profile of deuterated enzyme is maximal when the population is half native and half deuterated, i.e. when half of the initial population of molecules has been renewed. But the bandwith test can be used only when the labelling of amino acids is significantly faster than enzyme turnover.

Phenylalanine ammonia-lyase of tobacco leaves was not significantly labelled (less than 0.1% increase in buoyant density) when the density marker was supplied for less than 10 h. For labelling periods of 12 h with 60—70% ^2H_2O we observed a 0.2—0.3% increase in density for this enzyme, whereas no label was detectable in acid phosphatase and in the mixture of the three catechol *O*-methyltransferases used as internal markers. When ^2H_2O was supplied for 20 h, all the enzymes cited above were significantly density labelled. Table II indicates the degree and heterogeneity of deuteration of phenylalanine ammonia-lyase for various periods of uptake of ^2H_2O: the buoyant density of the enzyme increased progressively between 20 and 90 h of labelling. Furthermore, the shifts in density from the native enzyme were accompanied by very significant band-broadening, indicating heterogeneity of the population of labelled molecules. The largest bandwiths were observed for periods of labelling in the range 35—45 h. These results indicate that phenylalanine ammonia-lyase was continuously synthesised and degraded in healthy leaves, that it had a rather low rate of turnover and that this turnover was slower than the rate of uptake of label into amino acids. According to the bandwith measurements, half of the population of enzyme molecules seemed to be renewed after 35—45 h of labelling. This was also demonstrated by a direct estimation of the proportion of native enzyme molecules still present at that time. The resolution of the density gradients could be increased by using RbCl or, even better, KBr [40] instead of

TABLE II

DENSITY LABELLING OF PHENYLALANINE AMMONIA-LYASE IN HEALTHY TOBACCO LEAVES

The bandwith was calculated from the density increment at half peak height. Increase in bandwith (%) represents the relative difference between bandwiths of labelled and native enzyme and this difference was calculated from gradients of the same run.

Experiment	Concentration of ^2H_2O (%)	Period of labelling (h)	Salt used for centrifugation	Shift in density from native enzyme ($kg \cdot l^{-1}$)	Increase in bandwith (%)	Proportion of native enzyme molecules still present (%)
1	70	21	CsCl	0.0082 ± 0.0012	38 ± 8	—
		35	CsCl	0.0120 ± 0.0012	44 ± 8	—
2	70	26	CsCl	0.0063 ± 0.0012	40 ± 8	—
		41	CsCl	0.0105 ± 0.0012	60 ± 8	
			RbCl	—	—	40 ± 10
3	70	28	RbCl	0.0075 ± 0.0008	50 ± 10	>50
		38	RbCl	0.0083 ± 0.0008	60 ± 10	40 ± 10
4	60	48	CsCl	0.0103 ± 0.0012	40 ± 8	46 ± 10
5	55	94	CsCl	0.0140 ± 0.0012	30 ± 8	—
			KBr	0.0145 ± 0.0008	—	10 ± 5

CsCl. Under these conditions, a peak corresponding to native enzyme molecules was clearly visible and its area was estimated relative to the total area of all the peaks. These results are also shown in Table II and agree well with the bandwith tests: after 35—40 h of labelling with 60—70% ^2H_2O, the population of enzyme molecules still contained 40—50% of native phenylalanine ammonia-lyase. Therefore, when density shifts in the presence or absence of the stimulus are to be compared, periods of incubation with ^2H_2O should not exceed 35—40 h.

Because of the 10 h delay to observe a significant density labelling of the enzyme (due to several factors: absorption of the marker by the petioles, transportation to all parts of the mesophyll, replacement of H_2O by ^2H_2O and entry of ^2H_2O into the pool of amino acids), we estimated the half-life to be in the range 25—35 h. When the density marker was supplied for 94 h, there remained a few native enzyme molecules and a significant band-broadening. This does not contraduct the estimated half-life, but indicates that the amount of label in the pool of amino acids had still increased between 40 and 90 h, so that not all the renewed phenylalanine ammonia-lyase molecules were deuterated to the same extent.

Comparative density labelling of phenylalanine ammonia-lyase in healthy and TMV-infected leaves

We first investigated the effect of ^2H_2O on the appearance and enlargment of the local lesions and on the time course curve of the stimulated phenylalanine ammonia-lyase activity. When ^2H_2O was supplied to leaves already carrying local lesions, the growth curve of these lesions and the time course curve of enzyme levels were not significantly disturbed. When supplied before 33 h of infection, i.e. before appearance of the local lesions, the density marker did not

delay the formation of the lesions but it reduced by 3—5 fold the number of these lesions and, hence, the stimulated enzyme levels. This difficulty was overcome by inoculating the leaves to be supplied with ^2H_2O with more virus in order to induce approximately the same number of lesions as leaves supplied with H_2O.

For comparative density labelling studies, periods of feeding did not exceed the half-life of the unstimulated enzyme, as estimated in the previous para-

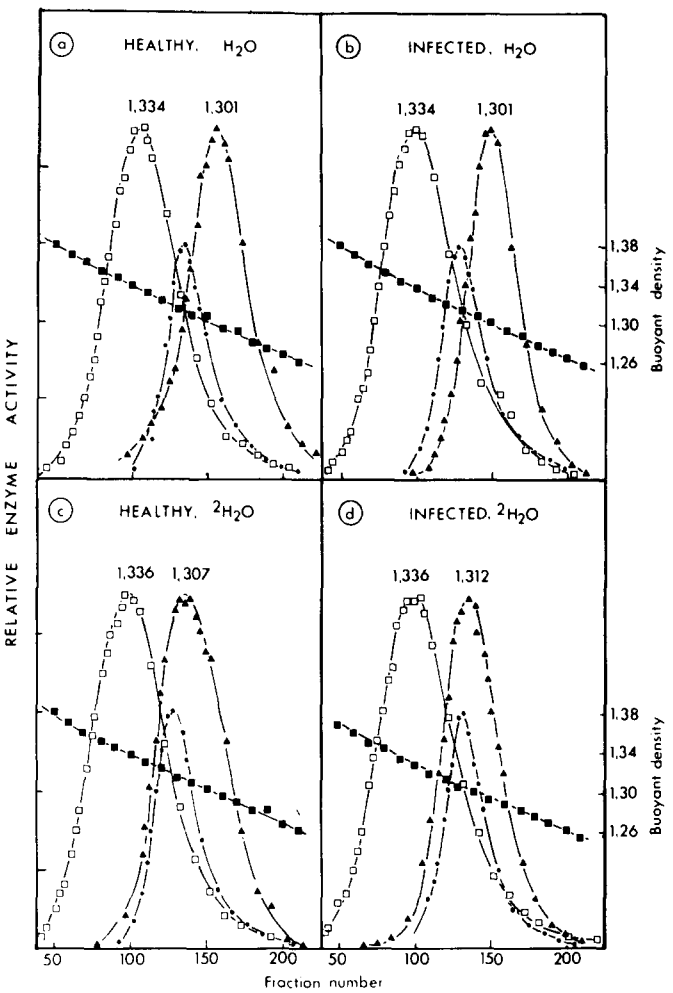

Fig. 3. Comparative density labelling of phenylalanine ammonia-lyase in healthy and TMV-infected leaves. Isopycnic centrifugation in CsCl of β-galactosidase (●———●) used as external marker, of acid phosphatase (□———□) and of phenylalanine ammonia-lyase (▲———▲) extracted from tobacco leaves treated in the following ways: leaves detached 25 h after inoculation with water (a) or with virus (b) and fed with ^2H_2O for 26 h, or detached 25 h after inoculation with water (c) or with virus (d) and fed with 70% for 26 h. Density profiles of the gradients (■———■) were obtained from refractive index measurements using the equation of Ifft et al. [19]. In this particular experiment, sample (d) contained 7.5-fold higher phenylalanine ammonia-lyase activity than the corresponding control sample (c) at the end of the treatment. Thus, the total amount of deuterium incorporated into phenylalanine ammonia-lyase of sample (d) equalled approximatively (1.312—1.301/1.307—1.301 × 7.5 = 15)-fold that incorporated into the enzyme of the control sample (c).

TABLE III

COMPARATIVE DENSITY LABELLING OF PHENYLALANINE AMMONIA-LYASE IN HEALTHY AND TMV-INFECTED LEAVES

Experiment	Concentration of ^2H_2O (%)	Time of infection (h)		Duration of labelling (h)	Density shifts from native enzyme ($kg \cdot l^{-1}$)	
		before leaf detachment	end of labelling		Healthy leaves	Infected leaves
1	60	22	→ 41	19	0.0075 ± 0.0008	0.0120 ± 0.0008
2	60	22	→ 50	28	0.0090 ± 0.0008	0.0135 ± 0.0008
3	70	25	→ 51	26	0.0063 ± 0.0012	0.0110 ± 0.0012
4	60	22	→ 60	38	0.0083 ± 0.0008	0.0130 ± 0.0008
5	70	29	→ 64	35	0.0080 ± 0.0012	0.0120 ± 0.0012
		43	→ 64	21	0.0060 ± 0.0012	0.0080 ± 0.0012
6	70	43	→ 64	21	0.0060 ± 0.0015	0.0065 ± 0.0015
7	70	36	→ 48	12	—	0.0050 ± 0.0015
		43	→ 55	12	—	0.0010 ± 0.0015
8	70	32	→ 46	14	0.0070 ± 0.0015	0.0050 ± 0.0015
		43	→ 57	14	0.0070 ± 0.0015	0.0005 ± 0.0015

graph. Typical activity profiles of phenylalanine ammonia-lyase from healthy and infected leaves supplied with H_2O or 70% ^2H_2O are shown in Fig. 3. In all experiments we compared the profiles from a complete set of 4 samples centrifuged together in a single rotor. Table III summarizes the results obtained when the density marker was introduced at various stages of the infection process. If it was introduced before appearance of the local lesions, i.e. before increase in enzyme levels, the stimulated enzyme had a significantly higher buoyant density than the unstimulated enzyme whatever the period of labelling (Expts. 1—5). The reverse situation was observed when ^2H_2O was supplied to leaves already carrying local lesions and, therefore, already containing increased levels of enzyme activity. The later the introduction of ^2H_2O, the lower the shift in buoyant density from the native enzyme (Expts. 7 and 8).

Discussion

The data presented clearly show that the stimulus of a hypersensitive reaction increases the pool size of catalytically active phenylalanine ammonia-lyase. The presence on the leaves of necrotic local lesions very strongly disturbed the rate of uptake of externally supplied amino acids into the amino acid pool. Therefore, radiolabelling with amino acids could not be used. In contrast, when ^2H_2O was supplied through the petioles, the rates of uptake of deuterium into the pool of amino acids were very similar in healthy and infected leaves. Furthermore, fully expanded leaves appeared very suitable for density labelling with ^2H_2O, since replacement of H_2O by ^2H_2O could be speeded up by increasing the transpiration rate in a stream of air.

Several authors [23,25,33] have discussed the interpretation of activity profiles of density-labelled enzymes with and without a stimulus. They do not think that density labelling can demonstrate an increased rate of enzyme synthesis if the half-life of the enzyme is short compared with the period of incubation with ^2H_2O. This is because both the stimulated and the short-lived unsti-

mulated enzyme will be of predominantly high buoyant density and their density difference would not be significant. We have demonstrated that phenylalanine ammonia-lyase of healthy tobacco leaves has a rather long half-life, which should make it easier to observe any increase in the rate of synthesis. But the stimulus could increase the extractable enzyme activity by activating existing inactive enzyme or by reducing the rate of enzyme degradation. In both cases the stimulated enzyme would be less dense than the unstimulated one by an amount that would increase with the rate of turnover of the unstimulated enzyme. Therefore, in the case of tobacco leaves, it will be difficult to demonstrate activation of existing inactive enzyme or reduction in the rate of degradation, should they occur.

Table III shows that when ^2H_2O was introduced before appearance of the lesions, the buoyant density of the stimulated phenylalanine ammonia-lyase was significantly higher than that of the unstimulated control. This unambiguously demonstrates an increased rate of synthesis of catalytically active enzyme [23,25,33]. Lower differences, or even no difference, in density were found when the stimulus and the density marker were applied together. This result does not contradict the previous conclusion and is caused by the slow entry of the label into the amino acid pool of fully expanded leaves. As mentioned above, no significant label in unstimulated phenylalanine ammonia-lyase appears in less than 10 h. Therefore, if incubation with ^2H_2O and increased synthesis of enzyme begin at exactly the same time, enzyme molecules synthesised during the first 10 h are predominantly unlabelled. The later ^2H_2O is introduced, the more pronounced are the isotopic dilution effects, which may lead to a much lower measured buoyant density and even to a total suppression of the density shift for the stimulated enzyme (Expts. 7 and 8 of Table III). Such situations were easily simulated by centrifuging mixtures containing various proportions of labelled and unlabelled enzymes (Table IV).

Our results show that the models already proposed to interpretate density

TABLE IV

ISOTOPIC DILUTION OF LABELLED UNSTIMULATED PHENYLALANINE AMMONIA-LYASE BY UNLABELLED STIMULATED ENZYME

Healthy leaves were supplied with ^2H_2O, infected leaves with H_2O. In Expt. 1, the mixture of enzymes was extracted from a mixture of healthy and infected leaves. In Expts. 2 and 3, the mixture of enzymes was made from a mixture of crude homogenates of healthy and infected leaves ground separately. The proportions of native and labelled enzymes indicated represent the relative enzyme activities that have been mixed. All buoyant densities in a given experiment were measured from CsCl gradients of the same run and were corrected using β-galactosidase as external marker.

Experiment	Buoyant densities of enzymes centrifuged separately ($kg \cdot l^{-1}$)		Proportions (%) of enzymes in the mixture native/labelled	Buoyant density of mixture of enzymes ($kg \cdot l^{-1}$)	
	Native	Labelled		Measured	Calculated
1	1.300 ± 0.001	1.310 ± 0.001	40/60	1.306 ± 0.001	1.306 ± 0.001
2	1.300 ± 0.001	1.312 ± 0.001	50/50	1.306 ± 0.001	1.306 ± 0.001
			75/25	1.302 ± 0.001	1.303 ± 0.001
3	1.3010 ± 0.0005	1.3113 ± 0.0008	25/75	1.3093 ± 0.0008	1.3087 ± 0.0007
			50/50	1.3068 ± 0.0008	1.3065 ± 0.0007
			75/25	1.3035 ± 0.0008	1.3036 ± 0.0007

labelling data [23,25,33] should be applied with caution. Though a higher buoyant density of a stimulated enzyme is unequivocal evidence of an increased rate of synthesis, a lower buoyant density does not necessarily imply slower degradation or activation of enzyme from an enzyme-inactivator complex. We observed that the stimulated enzyme might be less dense even with faster synthesis if deuterium was not applied before the stimulus. Furthermore, we found that, in strips containing the midvein and cut from both healthy and infected leaves, phenylalanine ammonia-lyase became highly labelled as a result of isotopic stress (Duchesne, M. et al., unpublished). The disturbances brought about by isotopically induced enzyme were easy to detect because of the low turnover rate of the basal levels of unstimulated enzyme. In plant materials containing short-lived enzyme, the probable difficulty of detecting isotopic stress helps to explain discrepancies between results [23,41] obtained from density labelling studies on the same stimulus applied to the same plant material.

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