Planta 150, 279–285 (1980)

Planta

Modulation of L-Phenylalanine Ammonia-Lyase by Pathway Intermediates in Cell Suspension Cultures of Dwarf French Bean (*Phaseolus vulgaris* L.)

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Abstract. The increase in extractable phenylalanine ammonia-lyase (PAL; EC 4.3.1.5.) activity induced in French bean cell suspension cultures in response to treatment with autoclaved ribonuclease A was inhibited by addition of the phenylpropanoid pathway intermediates cinnamic acid, 4-coumaric acid or ferulic acid. The effectiveness of inhibition was in the order cinnamic acid > 4-coumaric acid > ferulic acid. Cinnamic acid also inhibited the PAL activity increase induced by dilution of the suspensions into an excess of fresh culture medium. Addition of low concentrations ($< 10^{-5}$ M) of the pathway intermediates to cultures at the time of application of ribonuclease gave variable responses ranging from inhibition to 30–40% stimulation of the PAL activity measured at 8 h. Following addition of pathway intermediates to cultures 4–5 h after ribonuclease treatment, rapid increases followed by equally rapid declines in PAL activity were observed. The cinnamic acid-stimulated increase in enzyme activity was unaffected by treatment with cycloheximide at a concentration which gave complete inhibition of the ribonuclease-induced response. However, cycloheximide completely abolished the subsequent decline in enzyme activity. Treatment of induced cultures with α-aminooxy-β-phenylpropionic acid (AOPPA) resulted in increased but delayed rates of enzyme appearance when compared to controls not treated with the phenylalanine analogue. The results are discussed in relation to current views on the regulation of enzyme levels in higher plants.

Key words: Cell suspension culture – *Phaseolus* – Phenylalanine ammonia-lyase – Phenylpropanoid biosynthesis.

Abbreviations: AOPPA = α-aminooxy-β-phenylpropionic acid; PAL = L-phenylalanine ammonia-lyase (EC 4.3.1.5); AOA = α-aminooxyacetic acid

Introduction

L-Phenylalanine ammonia-lyase (EC 4.3.1.5.) catalyses the deamination of L-phenylalanine to yield trans-cinnamic acid, the first reaction in the biosynthesis of a wide variety of phenylpropanoid compounds in higher plants. Transient increases in the activity of this enzyme precede or parallel the accumulation of lignin, coumarins, esters of hydroxycinnamic acids, flavonoids, isoflavonoids and pterocarpans (Stafford 1974; Dixon and Bendall 1978b), and much attention has been given to the molecular mechanisms underlying such changes in PAL activity, in particular in illuminated parsley cell suspension cultures accumulating flavone glycosides (Hahlbrock and Ragg 1975; Betz et al. 1978) and in potato tuber discs accumulating chlorogenic acid (Lamb et al. 1979).

PAL activity levels may be modulated through effects on the rates of both enzyme synthesis and loss of active enzyme (Lamb et al. 1979), while in some systems activation and inactivation have been proposed as the major factors underlying PAL activity changes (Attridge and Smith 1973, Attridge et al. 1974) although this is still a matter of some debate with respect to the phytochrome-mediated induction of PAL in mustard seedlings (Tong and Schopfer 1976). It is now also appreciated that in vivo concentrations of pathway intermediates may act as indicators of, and thereby regulate, the flux through the phenylpropanoid pathway; trans-cinnamic and 4-coumaric acids have been implicated as feed-back regulators of PAL activity levels, causing inhibition of the induced enzyme increase in gherkin hypocotyls (Engelsma 1968; Johnson et al. 1975) and Jerusalem artichoke and potato tuber slices (Durst 1976; Lamb and Rubery 1976). In potato, 4-coumaric acid prevents the appearance of cinnamic acid 4-hydroxylase (EC 1.14.13.11) activity (Lamb and Rubery 1976) and

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cinnamic acid, in addition to its effects on PAL, also feed-forward stimulates the appearance of hydroxycinnamoyl CoA: quinic acid hydroxycinnamoyl transferase (EC 2.3.1-), the first enzyme in the chlorogenic acid branch pathway (Lamb 1977). The effects of cinnamic acid on PAL activity levels in vivo are independent of the product inhibition of the enzyme activity in vitro (Havir and Hanson 1968; Durst 1976).

Evidence of a physiological role for pathway intermediate modulation of PAL comes from experiments in which endogenous cinnamic acid concentrations are either increased as a result of inhibition of cinnamic acid 4-hydroxylase activity (Durst 1976) or lowered via in vivo inhibition of PAL activity (Amrhein 1979). Such treatments result in either inhibition or stimulation of extractable PAL activity respectively. The stimulation of PAL observed following excision and floating on H_2O of hypocotyl segments (Engelsma 1968, 1979) or dilution of cell suspension cultures into excess medium (Hahlbrock and Wellmann 1973) may also be the result of lowered intracellular concentrations of pathway intermediates. In the present paper we report the effects of pathway intermediates on PAL activity in French bean cell suspension cultures, following induction of the enzyme by dilution of the cultures or treatment with denatured ribonuclease A, an inducer of isoflavone and pterocarpan accumulation (Dixon and Bendall 1978a) preceded by de novo synthesis of PAL (Lamb and Dixon 1978). It is shown that inhibition of extractable enzyme activity by exogenously supplied pathway intermediates is a very rapid process which may, however, be preceded by equally rapid, transient increases in enzyme activity.

Materials and Methods

Cell suspension cultures of French bean cultivar Canadian Wonder were initiated and maintained by regular subculture at 14-d intervals in a modified Schenk and Hildebrandt medium as previously described (Dixon and Fuller 1976). All cultures used in the following experiments were in exponential growth phase (6-7 days after subculture). PAL induction in response to 0.5 mg ml^{-1} autoclaved bovine pancreatic ribonuclease A (Sigma Chemical Co.) was measured in 10 ml batches of culture in sterile 25 ml conical flasks incubated as described elsewhere (Dixon and Bendall 1978a).

Cinnamic, 4-coumaric and ferulic acids (Sigma Chemical Co.) were each re-crystallised three times from aqueous ethanol prior to use.

Cells for enzyme assay were harvested by suction filtration on sintered glass filters, transferred to small stoppered vials and stored frozen at −70° C until required. Extracts were prepared in 50 mM Tris-HCl, pH 8.5, containing 7.1 mM 2-mercaptoethanol, with the inclusion of 1/10th the wet weight of cells of insoluble polyvinylpolypyrrolidone (Av. M. wt. 360.000). Cell debris was removed by centrifugation at 20.000*g* for 30 min. Supernatants were assayed for PAL by a spectrophotometric procedure (Lamb et al. 1979). In all the experiments described, extracts from cells treated with AOPPA or from cells treated with concentrations of cinnamic, 4-coumaric or ferulic acids greater than $4 \cdot 10^{-5}$ M were passed through a column of Sephadex G-15 (4.5·1.5 cm) prior to assay. Protein was determined by a modification of the method of Lowry et al. (Leggett-Bailey 1962).

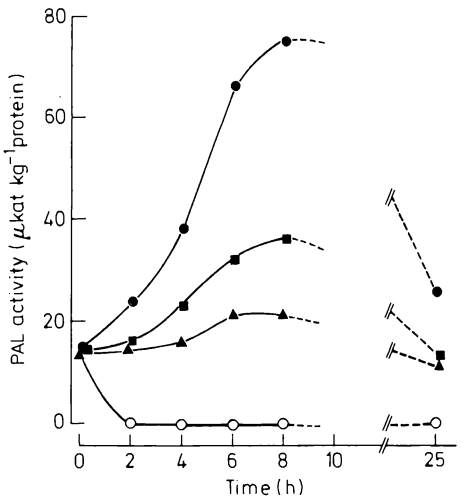

Fig. 1. Time courses for changes in PAL activity following dilution of French bean cell suspension cultures into excess fresh medium. Dilutions were 10-fold (▲–▲). 20-fold (■–■) and 50-fold (●–●). Cells were also diluted 50-fold into fresh medium containing 10^{-3} M trans-cinnamic acid (○–○)

Results

Effects of Cinnamic Acid on PAL Activity Induced by Dilution. PAL activity increased as a result of transfer of French bean cell suspension cultures to fresh culture medium (Fig. 1). Initial increases in enzyme activity were measured at intervals during an 8 h period subsequent to transfer; previous work had indicated that PAL activity in the bean cultures reached a maximum at around 8 h when induced by a fungal elicitor preparation (Dixon and Lamb 1979) and at 8–12 h when induced by denatured ribonuclease (Dixon and Bendall 1978a and below). In the present work the rate and extent of PAL appearance increased with increasing dilution of the cultures. However, dilution into fresh medium containing trans-cinnamic acid (10^{-3} M) completely prevented PAL appearance; extractable enzyme activity decreased to virtually zero by 2 h after transfer. No increases in activity were observed if cells were diluted 50-fold into media in which cultures had previously been growing (conditioned media).

Effects of Pathway Intermediates on Ribonuclease-induced PAL Activity. Six-fold increases in PAL activity were observed in the bean suspensions 8 h after treatment with 0.5 mg ml^{-1} autoclaved ribonuclease

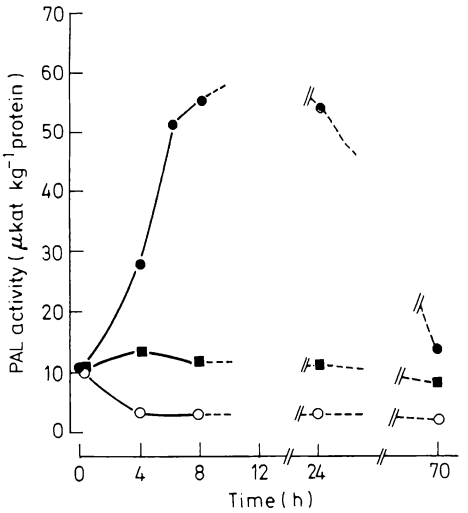

Fig. 2. Time courses for changes in PAL activity in French bean cell suspension cultures in the presence (●–●) and absence (■ ■) of 0.5 mg ml^{-1} autoclaved ribonuclease A, and in ribonuclease-treated cultures in the presence of 10^{-3} M trans-cinnamic acid (○–○)

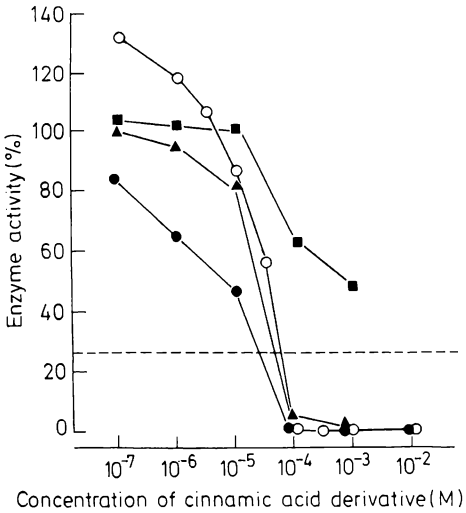

Fig. 3. Dose response curves for the effects of cinnamic acid derivatives on ribonuclease-induced PAL activity in French bean cell suspension cultures. Trans-cinnamic acid (●–●), 4-coumaric acid (▲–▲) or ferulic acid (■–■) were added, at the time of application of inducer, at the final concentrations shown. Cinnamic acid effects were measured in cultures with high (approx. 20 μkat kg^{-1} protein) (●–●) and low (2–3 μkat kg^{-1} protein) (○–○) basal PAL activities. Results are expressed as a % of the PAL activity in ribonuclease-induced controls measured 8 h after application of inducer. The 8 h PAL activity in uninduced controls (for high basal activity cultures) is also shown (– – –)

(Fig. 2). As with the dilution-induced activity, 10^{-3} M cinnamic acid totally prevented the enzyme increase, causing a rapid decline in activity below control (minus ribonuclease) levels. There was no evidence of a later recovery in PAL activity levels.

Dose-response curves for the effects of cinnamic, 4-coumaric and ferulic acids on PAL activity measured 8 h after treatment with ribonuclease indicated that cinnamic acid was the most potent inhibitor (Fig. 3). Ferulic acid (10^{-3} M) added at the time of ribonuclease treatment only gave approximately 50% inhibition of PAL development, while the potency of 4-coumaric acid as an inhibitor lay between that of cinnamic and ferulic acids. Concentrations of cinnamic acid and 4-coumaric acid of 10^{-4} M and above inhibited PAL activity below the level observed at 8 h in non-induced cultures (horizontal dotted line). The filled-in symbols in Fig. 3 are from a single experiment performed with cultures whose basal PAL level was 10 μkat kg^{-1} protein at day 7 of culture. On repeating this experiment several times it was observed that, although the order of effectiveness of the three compounds remained unchanged, the extent of inhibition by low concentrations ($< 10^{-5}$ M) was often quite variable; occasionally increases in PAL activity above ribonuclease-induced levels were noted. This effect was more reproducible in the older cultures, used in later experiments, which had been through more serial subcultures and in which the basal PAL level had fallen to 2–3 μkat kg^{-1} protein at day 7. The stimulatory effect of low concentrations of cinnamic acid in these cultures is shown by the open circles in Fig. 3.

Addition of cinnamic acid to cultures 5 h after ribonuclease treatment did not immediately prevent PAL appearance (Fig. 4a); enzyme activities did not fall below control values until 1.5 h after 10^{-3} M cinnamic acid addition. Prior to this there was a small but reproducible increase in enzyme activity. This phenomenon was more striking in cultures treated with $5 \cdot 10^{-4}$ M cinnamic acid, where a rapid increase in enzyme activity for 1 h after cinnamic acid treatment was followed by an almost immediate decline to activities approaching those in uninduced cultures. These rapid, transient effects resulting from cinnamic acid application were observed in cultures with high (Fig. 4a) and low (Fig. 4b) basal PAL activities. Application of 4-coumaric or ferulic acids to induced cultures also produced transient changes in PAL activity (Fig. 4b), the order of effectiveness of cinnamic, 4-coumaric and ferulic acids ($5 \cdot 10^{-4}$ M) on both enzyme increase and decline being the same as that established from Fig. 3.

The PAL activity increase in response to ribonuclease was rapidly inhibited following addition of cycloheximide 3.75 h after addition of the inducer (Fig. 5). The concentration of cycloheximide used (10 μM) was just sufficient to give 100% inhibition of the activity increase measured 8 h after addition of inducer; 3 μM cycloheximide inhibited this re-

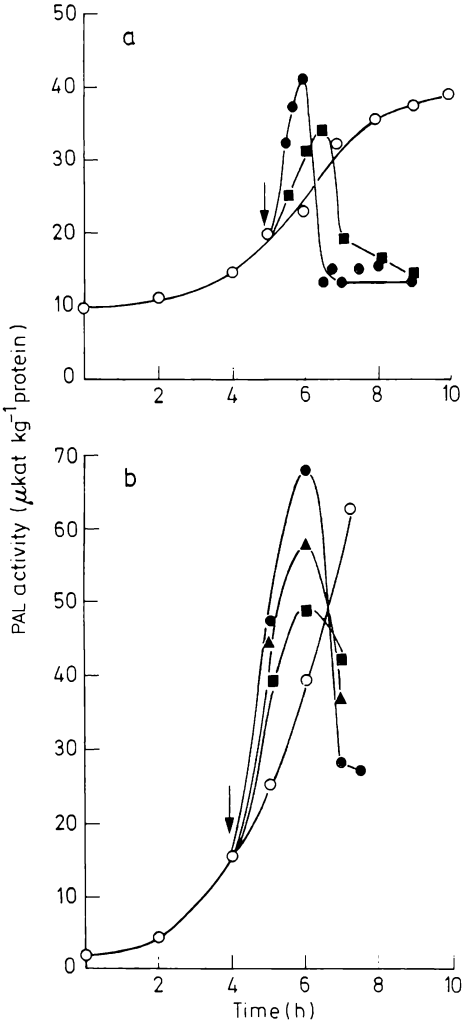

Fig. 4a and b. Transient increases in PAL activity in French bean cell suspension cultures in response to cinnamic acid derivatives added during the ribonuclease-induced response (o–o). Additions were made at the time points indicated by the arrows. **a** Trans-cinnamic acid added to cultures with high basal PAL activity at a final concentration of 10^{-3} M (■–■) and $5 \cdot 10^{-4}$ M (●–●). **b** Trans-cinnamic acid (●–●), 4-coumaric acid (▲–▲) or ferulic acid (■–■) added at a final concentration of $5 \cdot 10^{-4}$ M to cultures with low basal PAL activity

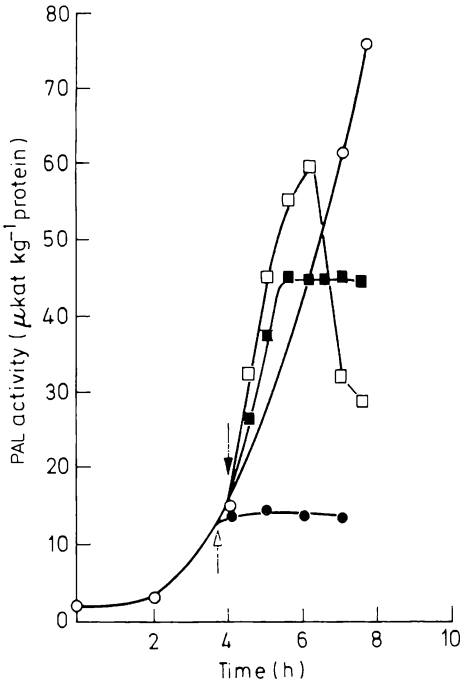

Fig. 5. Effects of cycloheximide on the transient increase in PAL activity observed following addition of cinnamic acid to French bean suspension cultures. PAL activity is shown in response to ribonuclease alone (o–o, ●–●), and in cultures plus $5 \cdot 10^{-4}$ M cinnamic acid (□–□, ■–■). Filled in symbols represent cultures to which cycloheximide (10 μM) was added at 3.75 h after ribonuclease addition (open arrow). Cinnamic acid was added at 4 h (filled-in arrow)

Table 1. PAL activity in normal enzyme assay (a), and in enzyme preparations pre-incubated with $3 \cdot 10^{-6}$ M AOPPA (b and c) or $3 \cdot 10^{-4}$ M AOA (d and e) before and after passage through Sephadex G-15. The enzyme preparation was from cells 6 h after treatment with ribonuclease

	PAL activity (μkat kg^{-1} protein)	PAL activity as % of control
a) Enzyme extract	30.0	100.0
b) Enzyme + AOPPA	1.3	4.3
c) Enzyme + AOPPA + gel filtration	28.9	96.3
d) Enzyme + AOA	0.0	0.0
e) Enzyme + AOA + gel filtration	8.3	27.7

sponse by 80%. The cycloheximide treatment did not, however, eliminate the increase in enzyme activity caused by addition of $5 \cdot 10^{-4}$ M cinnamic acid after 4 h of induction, although the later decline in activity usually observed around 2 h after cinnamic acid addition was completely abolished. The elevated enzyme level attained by 1.5 h after addition of cinnamic acid remained constant for at least the following 2 h.

PAL Induction in the Presence of AOPPA. α-Aminooxyacetic acid (AOA) and α-aminooxy-β-phenylpropionic acid (AOPPA) are potent inhibitors of PAL activity in vitro and in vivo (Amrhein 1979). K_i values for buckwheat PAL are $1.2 \cdot 10^{-4}$ M for AOA and $1.4 \cdot 10^{-9}$ M for AOPPA, while the K_is for phenylalanine transaminase from mungbean are $4.5 \cdot 10^{-7}$ M and $3.0 \cdot 10^{-6}$ M respectively (Amrhein 1979). AOP-PA is therefore the more potent and more specific inhibitor of phenylalanine deamination. To achieve removal of inhibitor from enzyme extracts, homogenates were passed through a column of Sephadex G-15 prior to assay. This method successfully removed

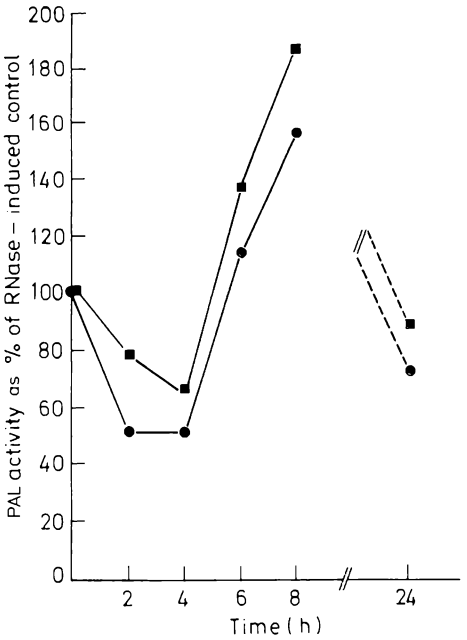

Fig. 6. Ribonuclease-induced PAL appearance in French bean cell suspension cultures in the presence of 10^{-6} M AOPPA. The graph shows 2 separate experiments, and results are expressed as a % of the enzyme activity observed in controls not treated with the phenylalanine analogue

AOPPA from PAL extracts, but did not restore full activity to enzyme preparations treated with AOA (Table 1). PAL induction in ribonuclease-treated cultures in the presence of 10^{-6} M AOPPA was initially (from 0–5 h) lower than in induced controls (Fig. 6); enzyme activities then increased to reach 170% of the control value by 8 h. Figure 6 shows the results from two separate experiments; the PAL activities at 8 h (132–158 μkat kg^{-1} protein) were the highest so far obtained in the bean suspension cultures in response to ribonuclease. AOPPA treatment did not appear to inhibit the subsequent decline in PAL activity, as seen from the enzyme activity at 24 h.

Discussion

Trans-cinnamic acid has been implicated as a feedback regulator of phenylalanine ammonia-lyase appearance in several systems (Johnson et al. 1975; Durst 1976; Lamb and Rubery 1976). In the present paper we demonstrate that cinnamic acid can prevent the appearance of PAL in French bean cell suspension cultures. Our results also suggest, however, that PAL activity in this system may be under positive as well as negative regulation by pathway intermediates.

It is not yet clear why PAL activity increases following dilution of cell suspension cultures or excision and floating on water of hypocotyl segments. An appreciation of the effects of pathway intermediates on in vivo PAL levels has led to proposals that the mechanism may involve (a) release of hydroxycinnamic acids into the surrounding medium, thus relieving their repressive effects on PAL appearance (Engelsma 1968); (b) release of a proteinaceous inhibitor of PAL into the surrounding medium (Engelsma and van Bruggen 1971), or (c) removal of the phenylalanine supply, thus preventing further formation of hydroxycinnamic acids (Engelsma 1979). In the present work our failure to induce PAL by a 50-fold dilution of cells into conditioned medium, coupled with the prevention by cinnamic acid of PAL appearance in response to dilution into fresh medium, is readily compatible with the above proposals without further indicating a specific mechanism.

In potato tuber discs, light-induction of PAL occurs via stimulation of actinomycin-D-sensitive steps (presumably transcription) followed by parallel transmission of the stimuli through cordycepin-sensitive steps (mRNA polyadenylation) and cycloheximide-sensitive steps (translation) (Lamb 1977); however, the inhibitory effects of cinnamic and 4-coumaric acids on PAL activity levels in this system are expressed via a process mechanistically different from the light-mediated effects. This is believed to involve post-transcriptional regulation in the absence of effects on the actinomycin-D-sensitive steps (Lamb 1979). This inhibitor approach has not been applied to the French bean culture system, although comparative density labelling experiments have demonstrated the de novo synthesis of PAL in response to ribonuclease (Lamb and Dixon 1978). As the rate constant for de novo synthesis of an enzyme is a composite function of the rate constants for transcription, translation and activation of newly synthesised polypeptide precursors, it is not yet possible to define fully the site(s) of ribonuclease action on PAL synthesis. Further investigations using a method of residual analysis of enzyme distribution in high resolution KBr density gradients (Lawton et al. unpublished data) have, however, indicated that the PAL activity changes in the bean cultures in response to both ribonuclease and a glucan 'elicitor' from cell walls of the phytopathogenic fungus *Colletotrichum lindemuthianum* are brought about via a programme of changes in the rate constants for both synthesis and loss of active enzyme. Temporal changes in enzyme levels may be described by the equation:

$$\frac{dE}{dt} = k_s - k_d[E]$$

where k_s is the zero-order rate constant for enzyme synthesis, k_d is the first-order rate constant for loss

of active enzyme and dE/dt is the rate of change of enzyme level per unit tissue (E) as a function of time (t). In elicitor-treated bean cultures, the increase in activity occurring 2 h after induction was shown to result from an increase in k_s accompanied by a rapid decrease in k_d. The subsequent decline in activity about 8 h after induction is the result of a decrease in k_s accompanied by an increase in k_d (Lawton et al. unpublished data). This dual programme clearly allows for rapidity and flexibility in the control of induced enzyme activity.

The effects of pathway intermediates reported in this paper may be related to the above phenomena. With one exception ($5 \cdot 10^{-4}$ M cinnamic acid modulation of PAL in high basal activity cultures, Fig. 4a), the decline in PAL activity subsequent to cinnamic acid addition occurs approximately 2 h after application of the compound; this timing would readily allow for the involvement of protein synthesis in the cinnamic acid-mediated decline in enzyme activity (although it is realised that cinnamic acid effects may be limited by the rate of uptake of the compound into the cells). The requirement for protein synthesis is also suggested by the inhibition of the decline by cycloheximide (Fig. 5). However, the use of cycloheximide as an inhibitor of protein synthesis in plant tissues has been questioned in view of the possibility of side effects (McMahon 1975), although it is now generally accepted that the failure of cycloheximide to inhibit a response is evidence for the lack of protein synthesis in the direct determination of that response (McMahon 1975; Schopfer 1977). In view of this, the inhibition of extractable PAL activity by cinnamic acid observed in the present work cannot at present be conclusively ascribed to the stimulation of formation of a proteinaceous inhibitor of the enzyme, although such a mechanism is consistent with earlier proposals (Engelsma and van Bruggen 1971; Durst 1976; Engelsma 1979) and would explain the observed increase in k_d associated with the beginning of the phase of PAL decline in elcitor-treated cultures. In contrast the initial cinnamic acid-mediated increase in PAL activity is very rapid (Fig. 4) and, in view of the ineffectiveness of cycloheximide in inhibiting this response, does not appear to involve protein synthesis (Fig. 5). It is therefore concluded that the cinnamic acid-mediated stimulation of PAL activity in vivo is the result of either enzyme activation or stabilisation (i.e. a lowered k_d value). The half-life for PAL of approximately 2.8 h in non-induced French bean cell cultures corresponds to a k_d value of $0.25\ h^{-1}$ (Lawton et al. unpublished data); 3 h after treatment with high concentrations of fungal elicitor the k_d value decreased to virtually zero. Preliminary data on PAL turnover in ribonuclease-treated cultures likewise suggested reciprocal control by changes in $k_s : k_d$ ratio, although detailed time courses for changes of the two rate constants were not determined (Lawton et al. unpublished data). Clearly, the doubling in the rate of PAL appearance observed in the present work in the 2 h subsequent to exogenous cinnamic acid addition (Fig. 4b) could only occur via cinnamic acid-mediated PAL stabilisation if active enzyme were still turning over during this period. This matter, and the possible alternative of PAL activation, may be resolved by high resolution density gradient analysis of enzyme labelled with ^2H from ^2H_2O (cf. Lawton et al. unpublished data).

The differential quantitative effects of cinnamic, 4-coumaric and ferulic acids on PAL stimulation and subsequent decline need not be associated with the relative abilities of these compounds to stimulate directly a particular regulatory mechanism; different rates of hydroxycinnamic acid uptake and turnover or differential formation of cis-isomers in the illuminated cultures may also contribute to the observed order of effectiveness of these compounds as PAL modulators.

The ability of cinnamic acid derivatives to stimulate as well as inhibit PAL appearance at first seems paradoxical. That such an effect may have physiological significance is suggested by the delay in the "superinduced" PAL increase observed in cultures treated with AOPPA (Fig. 6). The lowered PAL activities observed during the first 5 h following ribonuclease induction in the presence of AOPPA support our proposal that prior to the expression of PAL inhibition, endogenous pools of pathway intermediates may be responsible for stabilising the enzyme against loss by turnover (or, alternatively, activating pre-existing enzyme).

Acceptance of the role of pathway intermediates in regulation of phenylpropanoid synthesis clearly predicts that defined changes in hydroxycinnamic acid pool sizes must occur during the normal course of PAL induction and decline. Detailed analysis of such changes awaits future investigation, although PAL "superinduction" by AOPPA (Fig. 6 and Amrhein 1979), PAL inhibition as a result of anaerobosis (Durst 1976) and, in the present system, the observed differences in the induced catalytic capacities of PAL and cinnamic acid 4-hydroxylase (EC 1.14.13.11) (Dixon and Bendall 1978b) may be taken as indirect evidence of such changes in pool sizes and their importance in the regulation of phenylpropanoid metabolism, although not all plant tissues may respond to such changes in a similar manner (compare Lamb 1977 and Ulrich and Amrhein 1978). Variable concentrations of endogenous hydroxycinnamic acids may account for our initial observations of the different

dose responses for cinnamic acid-mediated PAL increases in cultures with different basal levels of PAL activity.

The phenomenon of bi-directional modulation of in vivo PAL activity by pathway intermediates may provide another level of regulation in the already complex sequence of events associated with maintaining a tightly regulated flow of carbon from general metabolic pools into phenylpropanoid secondary products.

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Received 1 March; accepted 6 May 1980