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ANOMALOUS EFFECTS OF CYCLOHEXIMIDE ON PHENYLALANINE AMMONIA-LYASE: ROLE OF SYNTHESIS AND INACTIVATION IN LEAF DISKS OF *HELIANTHUS ANNUUS**

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Key Word Index—*Helianthus annus*; Compositae; Sunflower Phenylalanine ammonia-lyase; Cycloheximide; Inactivation.

Abstract—The activity of phenylalanine ammonia-lyase (PAL) increases dramatically in leaf disks of sunflower (*Helianthus annuus*) cultured on 0·1 M sucrose in the dark. If disks are subsequently transferred to water, PAL activity decays rapidly. After inactivation the level of PAL can be increased again by transferring the tissue back to sucrose. The initial increase in PAL activity appears to involve an increase in the rate of PAL formation and the appearance is inhibited by cycloheximide. Inactivation of the enzyme is also inhibited by cycloheximide. A comparison of cycloheximide inhibition at different concentrations showed that inactivation was much more sensitive to the inhibitor than PAL formation. The rate of PAL inactivation was very low in fresh disks placed directly on water ($t_{1/2}$ = > 1 day) but increased greatly after culture on sucrose ($t_{1/2}$ = 2 to 4 hr). Therefore, culture appears to increase PAL inactivation as well as PAL formation. Reappearance of PAL activity after inactivation is stimulated rather than inhibited by cycloheximide. The change in effect of cycloheximide from inhibition to apparent stimulation can best be explained by the observation that (1) the turnover of PAL, both formation and inactivation, increases greatly as a result of culture on sucrose and (2) inactivation is more sensitive to cycloheximide than formation. Thus, even where an anomalous cycloheximide insensitive appearance of PAL activity occurs, a mechanism other than reactivation of the enzyme may be involved.

INTRODUCTION

PHENYLALANINE ammonia-lyase PAL (E.C. 4.3.1.5) is the first enzyme in a biosynthetic pathway leading to a large number of diverse phenolic constituents in plants. The level of PAL activity can be increased dramatically in many plant tissues by exposing the material to a variety of stimuli, both chemical and physical.[1,2] In a number of tissues, the initial increase in PAL is followed by decay of enzyme activity either in response to the removal of the stimulus or as part of the chain of events initiated by the original stimulus. In several systems the increase in enzyme activity has been linked directly to *de novo* synthesis of PAL. In others this relationship has been inferred from inhibitor studies. It has been suggested that active PAL arises from an inactive PAL protein[3] although attempts to demonstrate an inactive pool of PAL by density labelling techniques have thus far been unsuccessful.[4] It is not clear at present whether decay involves a specific inactivating system or

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[1] CAMM, E. L. and TOWERS, H. N. (1973) *Phytochemistry* **12**, 961.
[2] ZUCKER, M. (1972) *Ann. Rev. Plant Physiol* **23**, 133.
[3] ATTRIDGE, T. H. and SMITH, H. (1973) *Phytochemistry* **12**, 1569.
[4] IREDALE, S. E. and SMITH, H. (1973) *Phytochemistry* **12**, 2145.

is part of the general mechanism of protein turnover in plant and animal tissues. The data do indicate though that changes in the rate of inactivation as well as in the rate of synthesis of PAL can occur in response to external stimuli. Furthermore, protein components of inactivating systems appear to undergo rapid turnover as evidenced by the sensitivity of inactivation to cycloheximide. Thus, mechanisms controlling the level of PAL in plant tissues involve regulation of both synthesis and turnover as has been demonstrated for enzyme induction in a number of higher animal systems.[5]

Engelsma has proposed[6] that inactivation of PAL involves the formation of a reversible, inactive complex with a protein inhibitor rather than proteolytic or enzyme inactivation. Under certain conditions following inactivation of PAL in gherkin seedlings he has observed a cycloheximide insensitive reappearance of PAL activity. He concluded that the reappearance of PAL could not involve *de novo* synthesis but must represent reactivation of an inactive complex. The data presented here indicate that cycloheximide insensitivity is not a sound criterion for lack of *de novo* synthesis of PAL in a system where changes in the rate of both formation and inactivation determine the level of PAL activity attained. Our data, and we believe those of Engelsma, are consistent with a specific or general mechanism of turnover involving proteolysis.

RESULTS

The level of PAL activity increases rapidly in sunflower leaf disks floated on sucrose solution. The change in enzyme activity does not require light in contrast to other green leaf systems.[7-9] The rapid increase in PAL activity is inhibited by cycloheximide. If disks which have attained a relatively high level of PAL activity are transferred from sucrose to water, they lose activity rapidly. The decay proceeds logarithmically suggestive of the first order kinetics of systems involving turnover. Cycloheximide inhibits loss of enzyme activity under these conditions. The effect of cycloheximide concentration on the appearance and decay of PAL is compared in Table 1. The data indicate that inactivation of PAL is more sensitive to cycloheximide than is the appearance of PAL. Inactivation of PAL was inhibited almost completely by 10 μg/ml of cycloheximide while the initial rate of increase in level of PAL in the disks was inhibited only 50% by this concentration of cycloheximide. The inhibitor concentration had to be increased to 100 μg/ml before PAL appearance was inhibited completely.

This differential sensitivity to cycloheximide provides a basis for understanding anomalous effects of the inhibitor which are shown in Fig. 1. Added initially to disks placed on sucrose solution, 10 μg/ml of cycloheximide produced an expected 50% inhibition of PAL appearance. However, if addition of cycloheximide were delayed 12 hr, then little inhibition of PAL appearance occurred. In fact, as shown in Fig. 1, the net rate of increase in PAL activity was stimulated slightly by cycloheximide. If addition were delayed 24 hr, then cycloheximide produced a sizeable increase in the accumulation of PAL compared with disks cultured on sucrose alone. Disks transferred from sucrose to water after 24 hr rapidly lost PAL activity. However, enzyme activity could be increased again to a high level by

[5] SCHIMKE, R. T. and DOYLE, D. (1970) *Ann. Rev. Biochem.* **39**, 929.
[6] ENGELSMA, G. (1969) *Naturwissenschaften* **56**, 563.
[7] AMRHEIN, N. and ZENK, M. H. (1971) *Z. Pflanzenphysiol.* **63**, 145.
[8] CREASY, L. L. (1968) *Phytochemistry* **7**, 1743.
[9] ZUCKER, M. (1969) *Plant Physiol.* **44**, 912.

transferring disks back to sucrose solution even after PAL inactivation was virtually complete. If at that point disks were transferred to sucrose solution containing 10 μg/ml cycloheximide, the inhibitor stimulated the reappearance of PAL activity in contrast to its initial inhibitory effect in fresh disks. The stimulatory effect of cycloheximide on reappearance persisted throughout the period of decay even after PAL activity had disappeared from the tissue. Cycloheximide at 100 μg/ml was able to inhibit the reappearance of PAL in sucrose even after the period of decay (Fig. 2). The reappearance of PAL must therefore be due to renewed synthesis or activation since it is still cycloheximide sensitive and not due to reactivation of inactivated PAL protein.

TABLE 1. DIFFERENTIAL EFFECT OF CYCLOHEXIMIDE ON PHENYLALANINE AMMONIA-LYASE APPEARANCE AND DECAY

Cycloheximide concn. (μg/ml)	% Inhibition of PAL decay	% Inhibition of PAL synthesis
0	0	0
2·5	24	—
5·0	41	—
7·5	67	45
10·0	95	52
15·0	—	74
25·0	100	86
50·0	100	—
100·0	93	100

PAL appearance was measured by determining the change in PAL activity after sunflower leaf disks were cultured 16 hr at 23° in the dark on 0·1 M sucrose. Increase in activity was linear for 24 hr under these conditions. During the period of culture PAL activity increased from an endogenous level of 0·01 mU/cm^2 of leaf area to 1·44 mU/cm^2 in control disks on sucrose alone. Decay of PAL activity was followed in disks transferred to water (or cycloheximide) after a 20-hr period of culture on 0·1 M sucrose. PAL activity at the time of transfer was 1·32 mU/cm^2. The half-life of enzyme in control disks was 6·5 hr.

The change in response to cycloheximide during culture was not due to a general decrease in effectiveness of cycloheximide as an inhibitor of protein synthesis. Incorporation of [^{14}C]-isoleucine into protein was similarly inhibited by cycloheximide at all stages of culture (Table 2). A concentration of 10 μg/ml which inhibited the increase of PAL activity in fresh disks placed on sucrose solution by 70% in this experiment inhibited incorporation of [^{14}C]-isoleucine into soluble protein 74%. If disks were cultured for 24 hr on sucrose and then labeled with radioactive isoleucine in the presence and absence of cycloheximide, a 74% inhibition of incorporation was still observed even though addition of cycloheximide to the sucrose culture solution at that point stimulated the apparent net increase in PAL activity. Note that incubation on sucrose not only increased the level of PAL activity in the tissue, but increased the rate of isoleucine uptake as well compared to fresh disks. No signs of gross microbial contamination were evident in the cultures. Incorporation into insoluble protein (precipitated from borate extracts of the tissue by centrifugation at 20 000 *g* for 15 min) was also inhibited by cycloheximide at all stages of culture of the sunflower disks.

The anomalous stimulatory effect of cycloheximide on the accumulation of PAL in the tissue can be explained by taking into account the differential sensitivity of PAL formation and PAL inactivation to cycloheximide. If we assume that formation and inactivation of PAL occur simultaneously in sunflower disks cultured on sucrose solution for 24 hr, then the increase in PAL activity shown in Fig. 1 only represents the net accumulation of PAL.

The true rate of PAL synthesis would be the sum of the rate of PAL accumulation observed during culture of disks on sucrose and the maximal rate of inactivation observed after transfer of disks to water. Average rates calculated from a number of experiments similar to that shown in Fig. 1 are 0·037 mU/hr for accumulation and 0·150 mU/hr for PAL inactivation in disks transferred to water after 24 hr of culture on sucrose. The true rate of PAL formation calculated as the sum of these values would be 0·187 mU/hr, five to six times the actual rate of increase in PAL activity observed. Since the true rate of PAL formation and the rate of inactivation are much greater than the observed rate of accumulation, a differential inhibition of inactivation by cycloheximide could lead to an increase in the rate of accumulation even though an actual inhibition of PAL formation occurred. In other words, stimulation of the rate of PAL accumulation by low concentrations of cycloheximide can occur in a tissue where there is a rapid turnover of the enzyme and the inhibition of PAL inactivation by cycloheximide is more than that of PAL formation.

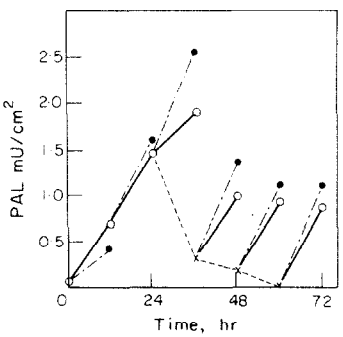

FIG. 1. EFFECT OF CYCLOHEXIMIDE ON THE ACCUMULATION OF PHENYLALANINE AMMONIA-LYASE IN LEAF DISKS. Disks were transferred to 10 μg/ml cycloheximide in 0·1 M sucrose (●) or to sucrose solutions (○) every 12 hr from either sucrose or from water (×).

If the above assumptions are correct, then the net inhibition of PAL appearance by cycloheximide during the first 12 hr of culture suggests that turnover of the enzyme does not occur or occurs at a very low rate during this initial period of culture. A suggestion of a low rate of turnover in freshly cultured tissue has been obtained in disks from leaves of flowering plants. The endogenous level of PAL activity in such tissue is usually higher than in leaf tissue from younger plants although other aspects of induction are similar. Thus, by placing disks from flowering plants on water, the endogenous rate of PAL inactivation in fresh tissue can be measured directly without an intervening period of culture on sucrose necessary to increase the activity of PAL to a measurable level. Data in Table 3 indicate that the rate of PAL inactivation in such fresh disks is indeed very much less than in the same disks after they have been cultured on sucrose. Thus, culture on sucrose appears to increase not only the rate of PAL formation, but the level of a PAL inactivating system as well.

The persistence of the stimulatory effect of cycloheximide throughout the period of decay, as noted above, suggests that once the PAL inactivation system if formed it is maintained even though PAL activity itself is lost from the tissue after transferred to water. However, the formation of the inactivating system will not occur initially on water alone but appears to require sucrose. Disks cultured on water for 24 hr do not show a stimulatory effect of cycloheximide when transferred to sucrose. Rather, cycloheximide inhibits appearance of PAL activity as in freshly cut disks.

TABLE 2. INHIBITION OF PROTEIN SYNTHESIS BY CYCLOHEXIMIDE AT DIFFERENT STAGES DURING THE FORMATION AND DECAY OF PAL IN CULTURED SUNFLOWER LEAF DISKS

Pretreatment	PAL activity after pretreatment (mU/cm²)	Change in PAL activity after 8 hr (mU/cm²)		Uptake of [¹⁴C]-isoleucine in 4 hr (nC/cm²)		Incorporation of [¹⁴C]-isoleucine into soluble protein % of uptake		% Inhibition of soluble protein synthesis by cycloheximide
		Sucrose	Sucrose + cycloheximide	Sucrose	Sucrose + cycloheximide	Sucrose	Sucrose + cycloheximide	
None (fresh disks)	0·53	+0·30	+0·09	103	60	25·0	6·6	74
24 hr Sucrose	1·15	−0·23	+0·57	235	160	11·5	3·0	74
24 hr Sucrose +24 hr water	0·09	+0·52	+1·20	261	245	8·6	3·0	65

PAL activity was measured in extracts of fresh disks, in the disks after 24 hr of culture on 0·1 M sucrose and in the disks cultured 24 hr on sucrose followed by 24 hr on water. Replicate sets of each kind of disk were then transferred to fresh solutions of either 0·1 M sucrose of 0·1 M sucrose + 10 μg/ml of cycloheximide, and the change in PAL activity was measured after an additional 8 hr of culture. Replicate sets of disks were supplied with [¹⁴C]-isoleucine (460 nC: 1·3 nmol per cm² of leaf area) for 4 hr beginning 2 hr after transfer and followed by 2 hr in the appropriate isoleucine-free solution. PAL activity and radioactivity were determined as described in the text. Each replicate consisted of 5 leaf disks which were incubated at 23° in the dark.

Calculation of the true rate of PAL synthesis described above is based on the assumption that formation but not inactivation of the enzyme stops when disks are transferred from sucrose to water. The true rate of formation can be calculated more directly from the observed rate of increase in PAL activity in disks exposed to sucrose containing 10 μg/ml of cycloheximide and thus provide a check for the assumption. This concentration of cycloheximide virtually eliminates inactivation but decreases PAL formation only 50%. Therefore, the true rate of formation would be double that observed in the presence of cycloheximide. The data in Fig. 1 indicate that at 24 hr the true rate calculated on such a basis would be 0·183 mU/hr, a value close to that mentioned above (0·187 mU/hr). Thus, the assumption that decay of PAL activity on water occurs in the absence of PAL formation appears to be valid. Another test of this assumption can be made by adding cycloheximide to disks transferred from sucrose to water, i.e. during the period of decay. If no enzyme formation occurred during the period of decay, addition of 10 μg/ml of cycloheximide should stop inactivation, halting further change in the level of enzyme activity. However, if formation of PAL as well as inactivation occurred during the period of decay, then addition of cycloheximide would inhibit inactivation but allow enzyme formation at half maximal rates resulting in an increase in PAL activity on water. Figure 3 shows that this is not the case. Addition of cycloheximide during the period of decay produces only a temporal increase in PAL activity possibly representing a residual effect of sucrose followed by a slow decay in enzyme activity at a rate approximately 10% of that observed in the absence of cycloheximide. Thus, PAL formation appears to stop in the absence of sucrose.

DISCUSSION

The observation reported here (Table 3) that the rate of inactivation of PAL in freshly cut sunflower leaf disks is relatively low ($t_{1/2} = >1$ day) provides information about the regulation of endogenous levels of PAL. Since endogenous PAL activity is usually very low, the rate of PAL formation, like the rate of turnover, must also be low. The increase in PAL activity observed when fresh disks are floated on sucrose solution can be ascribed to an increase in the rate of PAL formation because 100 μg/ml of cycloheximide virtually abolishes any increase in the initial endogenous level of PAL activity (Table 1).

The rate constant for inactivation of PAL increases in disks cultured 24 hr on sucrose (Table 3) suggesting an augmentation of the endogenous level of a PAL inactivating system as a result of culture. Cycloheximide will prevent inactivation of PAL in sunflower disks as in other tissues.[1,2] This cycloheximide inhibition of inactivation has been interpreted to indicate that synthesis of a labile protein component(s) as the inactivating system is blocked by the inhibitor. Such a protein(s) would be a logical candidate for the component of the inactivating system that is augmented during culture of the disks.

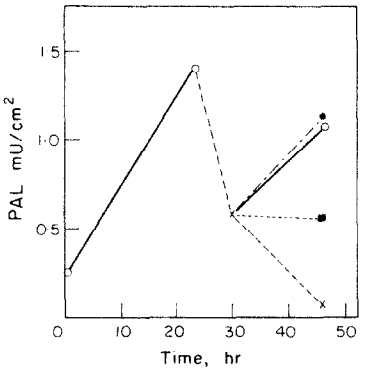

FIG. 2. (Left) THE EFFECT OF CYCLOHEXIMIDE ON THE RESYNTHESIS OF PHENYLALANINE AMMONIA-LYASE (PAL) FOLLOWING INACTIVATION. Leaf disks were transferred from 0·1 M sucrose (○) to water (×) and then to water, sucrose, sucrose with 10 μg/ml cycloheximide (●) or sucrose with 100 μg/ml cycloheximide (■).

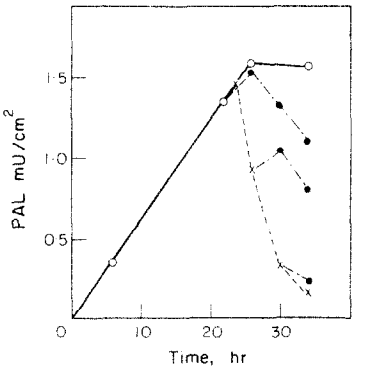

FIG. 3. (Right) THE EFFECT OF CYCLOHEXIMIDE IN WATER SUPPLIED AT VARIOUS TIMES DURING THE INACTIVATION OF PHENYLALANINE AMMONIA-LYASE. Leaf disks after pretreatment with 0·1 M sucrose (○) were transferred to water (×) and then at intervals during inactivation to 10 μg/ml cycloheximide in water (●).

Cycloheximide has been found to inhibit appearance of PAL initially in almost every system examined. The subsequent non-inhibitory or even stimulatory effects of delayed addition of cycloheximide reported here have also been found in other tissues. Hyodo and Yang[10] have observed that the increase in PAL activity induced by exposing pea seedlings to ethylene becomes progressively less sensitive to cycloheximide and is eventually stimulated by cycloheximide if the inhibitor is added late during induction. They reasoned that the anomalous effects of cycloheximide during the later stages of induction resulted from the ability of the inhibitor to shift the balance of turnover of PAL from inactivation to synthesis. Our finding that cycloheximide inhibits inactivation of PAL more than formation (Table 1) provides experimental evidence for this hypothesis. Addition of cycloheximide to a tissue in which rapid turnover of PAL occurred would shift the balance to net accumulation of PAL by inhibiting inactivation more than formation. Cycloheximide "insensitive" formation would only be apparent, not real.

Engelsma[6] proposed a different explanation for the cycloheximide insensitive accumulation of PAL in gherkin seedlings. A rapid appearance of PAL and a subsequent decay of PAL activity can be induced by irradiating etiolated gherkin seedlings. This initial PAL induction is cycloheximide sensitive.[11] Once PAL activity has disappeared from the tissue, it will reappear again if the seedlings are given a cold treatment. This reappearance was found by Engelsma[6] to be insensitive to a concentration of cycloheximide that completely

[10] HYODO, H. and YANG, S. F. (1971) *Plant Physiol.* **47**, 765.
[11] ENGELSMA, G. (1967) *Naturwissenschaften* **54**, 319.

blocked the initial radiation induced appearance of PAL. He reasoned that the reappearance of PAL activity does not therefore require *de novo* synthesis of enzyme but rather may involve the reactivation of an inactive PAL-protein inhibitor complex.[12] Attridge and Smith[3] also using the gherkin seedling system showed that PAL activity could be increased by treating seedlings in the dark with cycloheximide. This was interpreted as evidence of a pool of protein formed some time after inhibition[4] which was converted to PAL by the application of cycloheximide. An inactive PAL-complex pool resulting from light stimulated PAL inactivation was similarly thought to be reactivated by cycloheximide application.

TABLE 3. HALF-LIFE OF PAL IN FRESH AND CULTURED SUNFLOWER LEAF DISKS

Pretreatment	First-order rate constant of inactivation (k_d)	Action of cycloheximide (10 μg/ml)
None (fresh disks)	0·0–0·038	Inhibition
24 hr Sucrose culture	0·14–0·35	Stimulation

Freshly cut disks and disks cultured for 24 hr on 0·1 M sucrose were placed on water and the decay of PAL activity in both sets was measured at intervals over a period of 24 hr. The first-order rate constant of inactivation, k_d, was calculated according to Schimke.[5] The range of values observed is listed. Action of cycloheximide refers to its effect on accumulation of PAL in disks placed on sucrose (see Fig. 1).

Our sunflower system appears to be very similar to the pea seedlings system of Hyodo and Yang. It is also similar to the gherkin system in that PAL activity can be made to reappear in the tissue once it has disappeared as a result of inactivation. The initial stimulus for induction of PAL synthesis in gherkin seedlings was irradiation. A change to cold treatment was necessary to obtain reappearance of activity, suggesting two different mechanisms, i.e. PAL synthesis and PAL reactivation. In sunflower, however, it was only necessary to reapply sucrose, the initial inducing stimulus, to obtain reappearance of PAL once activity had decayed after removal of exogenous sucrose from the tissue. We do not think that the change from an initial cycloheximide sensitive to a subsequent cycloheximide insensitive increase in PAL activity represents a change in the mechanism of action of sucrose, i.e. stimulation of the rate of PAL formation. Rather, the change in sensitivity to cycloheximide reflects a major increase in the rate of inactivation of PAL as well as in the rate of PAL formation during induction. Under these conditions, the greater sensitivity of inactivation to cycloheximide provides an adequate explanation of the anomalous effects of cycloheximide. Engelsma's data can also be explained on the same basis if we assume that cold treatment inhibits inactivation more than formation of PAL in gherkin seedlings. In fact, Engelsma has demonstrated that there is indeed a differential sensitivity of inactivation and formation to low temperature in his seedlings.[11]

The present work is based on the assumption that cycloheximide inhibition of PAL inactivation results from the direct inhibition of production of some labile protein inactivator of PAL. The effect of cycloheximide could very well be more indirect. An observation made recently on the sunflower system suggests a type of indirect action. We have found

[12] ENGELSMA, G. (1970) *Planta (Berlin)* **91**, 246.

that the anomalous effects of cycloheximide can be mimicked to some degree by L-phenylalanine,[13] the substrate of the PAL reaction. It is possible that phenylalanine inhibits inactivation of PAL by stabilizing the enzyme. This stabilization would be analogous to that provided by tryptophan for tryptophan oxygenase (see 9). Cycloheximide need not inhibit the production of a specific protein inactivator system but may only block protein synthesis in general raising the level of phenylalanine and thereby stabilizing PAL.

Whatever the mechanism of the anomalous cycloheximide effect, it appears to involve turnover of the enzyme as a vital factor. Regulation of the level of a number of enzymes of amino acid metabolism in animal tissues involves control of the rate of inactivation as well as the rate of synthesis.[5] It should not be surprising, therefore, that the regulation of an enzyme of amino acid metabolism such as PAL in plant tissues also involves control of the rate of turnover as a mechanism of prime importance.

EXPERIMENTAL

Sunflower plants (*Helianthus annuus*) were grown from seed in pots in the greenhouse. At the start of an experiment 1·0 cm dia. disks were cut from the lamina of the youngest fully expanded leaves and placed in petri dishes on filter paper floating on appropriate solns. All incubations were carried out in darkness at 23°. At the time of sampling or transfer to other solns the disks were gently blotted and if being sampled were frozen in liquid nitrogen and stored overnight at −20°. PAL was extracted by grinding disks at liquid nitrogen temp. and then mixing the frozen powder well with 0·1 M borate pH 8·8 containing 0·5 mM mercaptoethanol and centrifuging at 20000 *g* for 20 min. The supernatant was used as enzyme and was assayed as previously described.[9] Each sample consisted of 5 disks extracted in 5 ml of borate buffer. A unit (U) is defined as the production of 1 μmol cinnamic acid/min at 30°. The uptake and incorporation of [^{14}C]-isoleucine was followed by moistening disks with a soln containing 4·6 μC; (13 nmol) of [^{14}C]-isoleucine per ml at a rate of 0·1 ml/cm^2 of leaf area. After a 4 hr period of uptake followed by 2 hr on a soln with no isoleucine, the disks were rinsed well, blotted and frozen in liquid nitrogen. An aliquot of the total brei prepared as above and suspended in borate was counted for total [^{14}C]-isoleucine uptake and the remainder centrifuged. The supernatant was assayed for PAL activity. The soluble protein in the enzyme extract was precipitated with 5% TCA in the cold and resuspended for counting. Radioactivity was measured by liquid scintillation counting with quenching standards prepared by parallel methods from non-labeled disks.

[13] CREASY, L. L., ZUCKER, M. and WONG, P. Abstract, 13*th Annual Meeting Phytochemical Society of North America.*