Phytochemistry, 1974, Vol. 13, pp. 575 to 583. Pergamon Press. Printed in England.

PROPERTIES OF PHENYLALANINE AMMONIA-LYASE EXTRACTED FROM *CUCUMIS SATIVUS* HYPOCOTYLS

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(*Received* 2 *July* 1973. *Accepted* 20 *September* 1973)

Key Word Index—*Cucumis sativus*; Cucurbitaceae; gherkin; phenylalanine ammonia-lyase; negative homotropic cooperativity; end-product inhibition.

Abstract—Some of the *in vitro* properties of PAL from gherkin hypocotyls were investigated. No metal ion requirement for this enzyme could be demonstrated but there were indications that PAL was a sulphydryl enzyme. Kinetic analysis suggested that PAL exhibited negative homotropic cooperativity. Two K_m values were determined, these were K_m^H $2{\cdot}9 \times 10^{-4}$ M and K_m^L $4{\cdot}3 \times 10^{-5}$ M. Strong inhibition of the enzyme was exerted by D-phenylalanine, *trans*-cinnamic acid, *o*-coumaric acid, gallic acid, quercetin and kaempferol. Kinetic studies on the inhibition patterns of these compounds established that D-phenylalanine linearized the curvilinear kinetics, *trans*-cinnamic acid and *o*-coumaric acid acted as competitive inhibitors whilst gallic acid, quercetin and kaempferol acted as mixed inhibitors. Using a number of desensitization techniques PAL was partially desensitized to inhibition by the mixed inhibitors. These results led to the conclusion that PAL may have a regulatory role in phenol, coumarin and flavonoid biosynthesis.

INTRODUCTION

PHENYLALANINE AMMONIA-LYASE (E.C.4.3.1.5) (PAL) catalyses the deamination of phenylalanine to *trans*-cinnamic acid. The cinnamate thus formed can then be metabolized further to form a variety of compounds which include the B ring of the flavonoids, coumarins and lignins.

Since the first *in vitro* demonstration of PAL activity the properties of this enzyme have been studied in a number of plant tissues.[1-7] The results from such studies support the hypothesis that PAL is a regulatory enzyme in secondary metabolism in plants. For example, kinetic analysis of PAL extracted from potato tubers[3] and pea buds[6] suggested that PAL was a regulatory enzyme exhibiting negative homotropic cooperativity. It is possible, however, that these unusual kinetics were due to the presence of two isoenzymes with differing affinities for phenylalanine since isoenzymes of PAL have been detected in potato tubers,[2] sweet potato roots[9] and oak leaves.[8]

Typically, the activity of regulatory enzymes can be modified by substances structurally unrelated to the substrate or product of the enzyme by binding at some site other than the catalytic site. Certain compounds which may be end products of PAL activity, e.g. the

[1] KOUKOL, J. and CONN, E. E. (1961) *J. Biol. Chem.* **236**, 2692.
[2] HAVIR, H. and HANSON, K. R. (1968) *Biochemistry* **7**, 1896.
[3] HAVIR, H. and HANSON, K. R. (1968) *Biochemistry* **7**, 1904.
[4] HAVIR, H. and HANSON, K. R. (1968) *Biochemistry* **7**, 1915.
[5] O'NEAL, D. and KELLER, C. J. (1970) *Phytochemistry* **9**, 1373.
[6] ATTRIDGE, T. H., STEWART, G. R. and SMITH, H. (1971) *FEBS Letters* **17**, 84.
[7] MINAMIKAWA, T. and URITANI, I. (1965) *J. Biochem.* **58**, 53.
[8] BOUDET, A., RANJEVA, R. and GADAL, P. (1971) *Phytochemistry* **10**, 997.
[9] MINAMIKAWA, T. and URITANI, I. (1965) *J. Biochem.* **57**, 678.

flavonoids quercetin and kaempferol, and vanillic and syringic acid (components of lignin), have been found to inhibit PAL activity[5,6,8] whereas gallic acid activated the enzyme.[8] Thus these results suggest that PAL may have a regulatory role in flavonoid,[5,6] lignin[8] and polyphenol biosynthesis.[8] Indeed, PAL has all the attributes of a regulatory enzyme: (a) it catalyses the first unique reaction directing the flow of carbon from primary protein amino acid synthesis to the formation of polyphenols, lignins, flavonoids, coumarins, etc.; (b) it is at a branch-point from the primary pathway of protein synthesis; (c) the reaction is thermodynamically irreversible (at optimum pH).

Experiments were therefore carried out in our laboratory on PAL extracted from light-treated gherkin hypocotyls to determine: (1) how the catalytic properties compared to PAL from other plant tissues; (2) whether PAL is a regulatory enzyme in this plant; (3) whether more than one species of PAL is present in the tissues.

RESULTS

Effect of metal ions on PAL activity

The activity of PAL was determined in the presence of a number of metal ions over a range of concentrations (10^{-2}–10^{-7} M). Table 1 shows that PAL has no obvious metal ion requirement. However, in the presence of the chelating agent EDTA at 10^{-4} M or Mg^{2+} at 10^{-3} M there was a slight stimulation of PAL activity (21%). Two possible explanations for the EDTA effect are: (a) EDTA is removing heavy metal ions which were released from the plant during extraction of the enzyme, or (b) EDTA is removing heavy metal ions present in commercial ammonium sulphate used to prepare the enzyme.

TABLE 1. EFFECT OF METAL IONS ON PAL ACTIVITY

Treatment	PAL activity expressed as a % of the H_2O control (100%) Log molarity −2	−3	−4	−5	−6	−7
NH_3Cl	95	109	107	96	102	102
$CaCl_2$	101	109	109	100	103	96
$CuCl_2$	0	0	0	52	97	101
$CoCl_2$	30	63	97	101	96	95
$FeSO_4$	84	110	108	97	103	101
$FeCl_3$	108	111	109	92	97	102
$MnCl_2$	55	113	114	109	113	111
$MgCl_2$	107	121	108	97	96	100
EDTA	98	116	121	97	103	103

PAL was extracted from the hypocotyls of 3-day-old dark-grown gherkin seedlings, pretreated with 4 hr white light (39·6 W m^{-2}), by $(NH_4)_2SO_4$ precipitation (30–70% saturation). Enzyme activity was estimated in the presence of a range of concentrations of metal ions, and the results expressed as a % of the H_2O control.

Effect of sulphydryl reagents on PAL activity

The effects of various sulphydryl reagents on the activity of PAL were investigated to determine whether PAL was a sulphydryl enzyme, i.e. an enzyme that loses catalytic activity when some or all of its original SH groups undergo chemical modification.[10]

[10] BOYER, P. D. (1959) in *The Enzymes* (BOYER, P. D., LARDY, H. and MYRBÄCH, K., eds.), Vol. I, p. 511, Academic Press, New York.

A preparation of PAL was used which had been frozen in the presence of glutathione. This was 'desalted' to remove all glutathione present, and aliquots of the protein fraction were then incubated in a range of concentrations of sulphydryl reagents. The activity of PAL was measured initially and also after 22 hr. Table 2 shows that mercaptoethanol and glutathione increased the initial activity of PAL at all concentrations tested (122 and 128% of the control for mercaptoethanol and 114 and 118% of the control for glutathione). In addition these compounds maintained the activity of the enzyme over a period of 22 hr whilst the control lost 50% of its activity during this time. Cysteine, meanwhile, consistently inhibited PAL activity, a phenomenon also exhibited in two other plant tissues.[1,5] From the results with glutathione and mercaptoethanol, however, PAL appears to be a sulphydryl enzyme, but reversibility of treatment by iodoacetate or hydroxy mercuribenzoate needs to be examined to confirm this fact.

TABLE 2. EFFECT OF SULPHYDRYL REAGENTS ON PAL ACTIVITY

Treatment	Activity of PAL as a % of the control	
	Initial activity	Activity after 22 hr
Control	100	53
Glutathione 0·5 mM	114	103
Glutathione 5·0 mM	118	102
Mercaptoethanol 0·5 mM	122	112
Mercaptoethanol 5·0 mM	128	113
Cysteine 0·5 mM	109	111
Cysteine 5·0 mM	64	74

PAL was extracted from the hypocotyls of 3-day-old dark-grown seedlings, pretreated with 4 hr white light (39·6 W m^{-2}), by $(NH_4)_2SO_4$ precipitation (30–70% saturation). The enzyme was desalted through a Sephadex G25 column to remove any glutathione present, and aliquots of the protein fraction then incubated in 0·1 M borate buffer pH 8·8 or a range of sulphydryl reagents. Activity of PAL was estimated initially and after 22 hr and the results expressed as a % of the control.

Kinetic analysis of PAL

The Lineweaver–Burk plot of the effect of a range of substrate concentrations on PAL activity shows that there is a departure from simple Michaelis–Menten kinetics since the plot is concave downward at low $1/S$ values and two K_m values could be determined. Using the nomenclature of Datta and Gest[11] K_m^H is $2{\cdot}9 \times 10^{-4}$ M and K_m^L is $4{\cdot}3 \times 10^{-5}$ M. When the data is recast as a Hill plot the interaction coefficient was found to be 0·65. The R_s value (i.e. the ratio of substrate concentrations required for 90 and 10% of V_{max}) was found to be 85·7.

At least two hypotheses can be postulated to account for this kinetic behaviour: (a) there are two species of PAL with differing affinities for substrate, or (b) there is one enzyme which has two substrate binding sites which differ in their affinity for substrate and interact with each other. In an attempt to determine which of these two hypotheses was the more likely possibility three different separation techniques were employed to test for the presence of PAL isoenzymes. These were gel filtration, polyacrylamide gel electrophoresis and sucrose density gradient centrifugation.

[11] DATTA, P. and GEST, H. (1965) *J. Biol. Chem.* **240**, 3023.

Attempts to detect PAL isoenzymes

PAL from light- or dark-treated gherkins was applied to a Sepharose 4B column, previously calibrated with markers of known MW, and run with 0·1 M borate buffer pH 8·8. Two major peaks of PAL activity were observed in either light- or dark-treated PAL preparations suggesting that the enzyme is the same, on a MW basis, in each case. The large peak had a MW of *ca* 316000 whilst the smaller one had a MW of *ca* 619000 (Figs. 1a and b). However, when fractions from the large peak were collected, pooled and run through a Sepharose column again, a similar pattern was observed (Fig. 2). This suggests that aggregation of enzyme molecules with a MW of 316000 was occurring giving rise to what appeared to be a 619000 MW form of PAL.

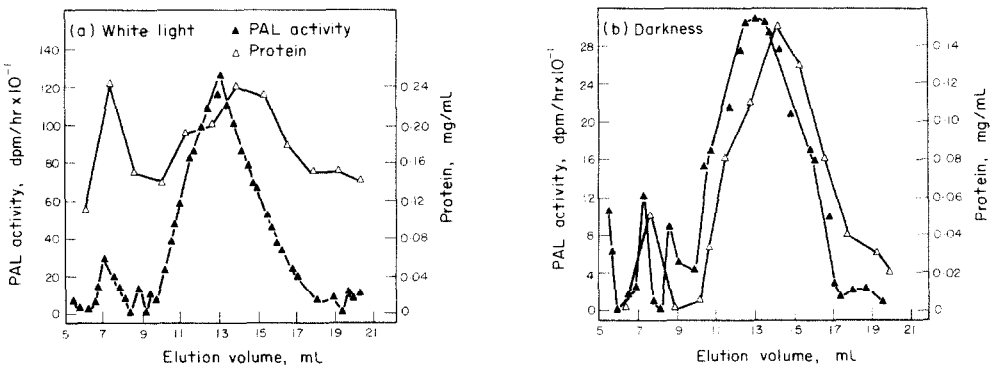

FIG. 1. GEL-FILTRATION OF PAL FROM THE HYPOCOTYLS OF *Cucumis sativus* ON SEPHAROSE 4B. PAL was extracted from the hypocotyls of 3-day-old dark-grown gherkin seedlings given 4 hr white light (39·6 W m^{-2}) or left in darkness. Extraction was by $(NH_4)_2SO_4$ precipitation (30–70% saturation). Enzyme (0·1 ml (a), and 0·25 ml (b)) was run through a 25 × 1·1 cm Sepharose 4B column, equilibrated with 0·1 M borate buffer pH 8·8. After 7·5 ml had been collected 0·25 ml fractions were assayed for PAL activity whilst every fifth fraction was assayed for protein.

The result of running PAL from light-treated gherkins on a polyacrylamide gel is shown in Fig. 3. It can be seen quite clearly that only one species of PAL was detected and the same was true with sucrose density gradient analysis.

Since it was possible to demonstrate by three different separation techniques that only one species of PAL was present in light-treated gherkin hypocotyls, it seems reasonable to assume that the kinetic data shown in Fig. 1 are due to PAL having two substrate binding sites which differ in their affinity for phenylalanine and interact with each other.

Inhibitor studies

The effects of a number of metabolites of the shikimic acid pathway and related compounds are shown in Table 3. Of the compounds tested only D-phenylalanine, *trans*-cinnamic acid, *o*-coumaric acid and gallic acid were significant inhibitors of PAL activity. Kinetic studies on the inhibition patterns of these compounds established that both *trans*-cinnamic acid and *o*-coumaric acid were competitive inhibitors for PAL. This is to be expected for products and related compounds of a reaction. D-Phenylalanine produced a rather unusual effect since, when present, it normalized the curvilinear kinetics for PAL and gave a completely straight $1/V$ vs $1/S$ curve. Similar results to this have been obtained with PAL extracted from pea buds[12] and potato tuber discs.[3] Assuming that PAL has two distinct

[12] ATTRIDGE, T. H. (1969) Ph.D. Thesis, Univ. London.

substrate binding sites the simplest explanation of this phenomenon is that D-phenylalanine preferentially binds to one of the sites and thus simple Michaelis–Menten kinetics result. Gallic acid acted as a mixed type inhibitor for PAL. This type of inhibition will be discussed later.

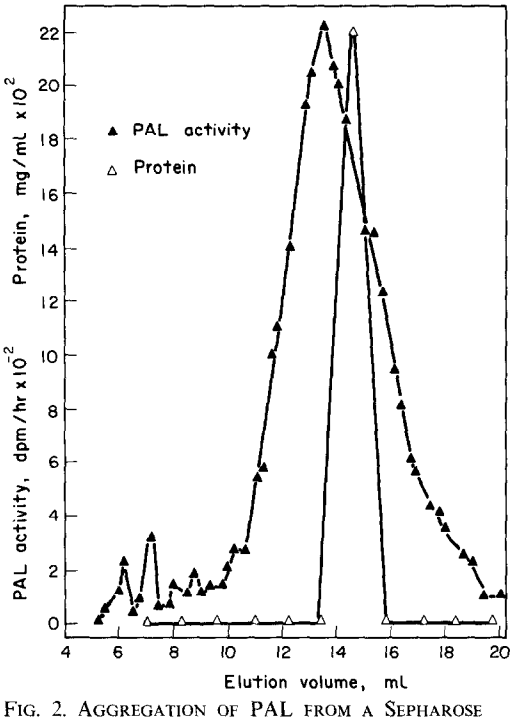

FIG. 2. AGGREGATION OF PAL FROM A SEPHAROSE COLUMN.

Fractions from the major peak in Fig. 1(a) were dialyzed against solid sucrose overnight. The concentrated extract (0·1 ml) was rerun through the same Sepharose 4B column used previously. Every fifth fraction was used for protein determination.

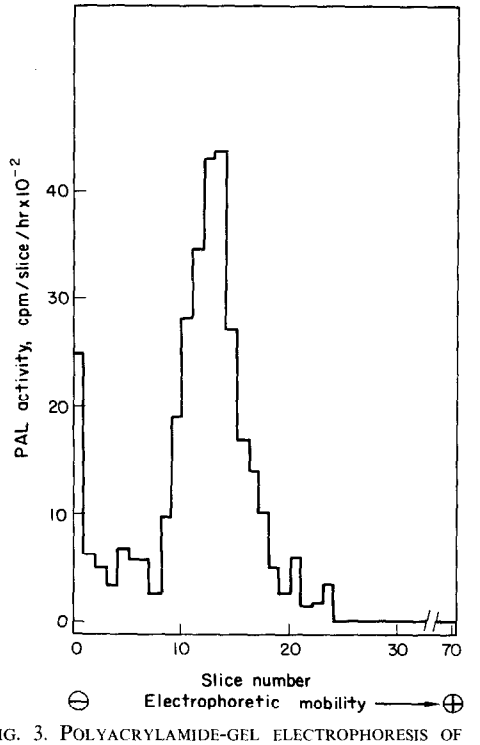

FIG. 3. POLYACRYLAMIDE-GEL ELECTROPHORESIS OF PAL FROM LIGHT-TREATED *Cucumis sativus* HYPOCOTYLS.

PAL was extracted from the hypocotyls of 3-day-old dark-grown gherkin seedlings, pretreated with 4 hr white light (39·6 W m^{-2}), by $(NH_4)_2SO_4$ precipitation (30–70% saturation). The enzyme (0·1 ml) containing 15% sucrose (w/v) was loaded onto a 7% polyacrylamide-gel and run at 3 mA for 3 hr. The gel was then cut horizontally into 8 mm slices, incubated in a reaction mixture, and assayed for PAL activity. The data is expressed as CPM/slice/hr. No enzyme activity at all was detected in slices 30–70 and these have been omitted for clarity.

Investigations into the allosteric properties of PAL

It is characteristic of regulatory enzymes to be susceptible to inhibition by metabolites other than products of the reaction or related compounds. These substances are considered to bind at some site other than the catalytic site, i.e. the regulatory site, and affect the catalytic activity by altering the conformation of the enzyme.[13]

A number of possible end-products of the PAL reaction were tested for their effects on PAL activity (Table 3); only the flavonoids quercetin and kaempferol showed any signifi-

[13] MONOD, J., CHANGEUX, J. P. and JACOB, F. (1963) *J. Mol. Biol.* **6**, 306.

cant inhibition. Lineweaver–Burk plots established that both these compounds were mixed inhibitors, thereby suggesting that they may be acting as allosteric inhibitors for PAL. However, it was possible that it was the B ring of the flavonoids, derived from the shikimic acid pathway, which was the inhibitory portion of the flavonoid due to its structural similarity to phenylalanine. Thus it was important to confirm that this was allosteric inhibition. Consequently, three commonly used desensitization techniques were applied to PAL (Table 4). It was possible to partially desensitize PAL to quercetin inhibition with all treatments, resulting in approximately a 16% increase in PAL activity in comparison to the untreated, inhibited enzyme, whereas partial desensitization to kaempferol inhibition could only be achieved with the $HgCl_2$ treatment. Since gallic acid also acted as a mixed inhibitor for PAL, desensitization treatments were applied to the enzyme with respect to gallic acid inhibition. Here, too, partial desensitization could only be achieved with the $HgCl_2$ treatment.

TABLE 3. EFFECT OF VARIOUS COMPOUNDS ON PAL ACTIVITY

Compound	Concentration (mM)	PAL activity as a % of the control	Compound	Concentration (mM)	PAL activity as a % of the control
Control	—	100	Tyrosine	2·5	87
Cinnamic acid	1	9	Shikimic acid	7·5	100
D-Phenylalanine	1	33	Gallic acid	1·25	77
o-Coumaric acid	1	13	Vanillic acid	7·5	100
p-Coumaric acid	7·5	95	Quercetin	1·25	34
Caffeic acid	7·5	97	Kaempferol	0·25	60
Ferulic acid	6·25	91	Coumarin	1·25	85

PAL was extracted from the hypocotyls of 3-day-old dark-grown gherkin seedlings, pretreated with 4 hr white light (39·6 W m^{-2}), by $(NH_4)_2SO_4$ precipitation (30–70% saturation). Enzyme activity was estimated in the presence of various compounds, and the results expressed as a % of the control.

Further verification of the desensitization effect with respect to quercetin is shown in Fig. 4 where it was possible to partially desensitize PAL over a range of quercetin concentrations.

TABLE 4. EFFECTS OF DESENSITIZATION TREATMENTS ON THE RESPONSE OF PAL TO A VARIETY OF INHIBITORS

Treatment	Activity as % control				
	Kaempferol 0·25 mM	Kaempferol 0·5 mM	Gallic acid 1·5 mM	Gallic acid 1·75 mM	Quercetin 1·25 mM
None	64	30	84	83	24
Incubation	66	27	85	82	40
$HgCl_2$	80	45	100	96	36
Freezing and thawing	62	31	79	80	31

PAL was extracted from the hypocotyls of 3-day-old dark-grown gherkin seedlings, pretreated with 4 hr white light (39·6 W m^{-2}), by $(NH_4)_2SO_4$ precipitation (30–70% saturation). An aliquot of enzyme was either incubated at 45° for 30 min (incubation) or incubated at room temp. for 1 hr in a final concentration of 10^{-4} M $HgCl_2$ and then desalted through a Sephadex G25 column ($HgCl_2$) or frozen and thawed at room temp. 3× (freezing and thawing) or left untreated. Enzyme activity was estimated, in the presence of various inhibitors of PAL, and the results expressed as a % of the respective control.

DISCUSSION

Some of the properties of PAL extracted from light-treated gherkin hypocotyls have been investigated. In general agreement with other workers[1,3,5] no metal ion requirement for PAL could be demonstrated (Table 1). There were, however, indications that PAL in this plant tissue was a sulphydryl enzyme, similar to PAL in barley,[1] pea buds[12] and tobacco[5] but in marked contrast to PAL in potatoes[2] and maize[3] (Table 2).

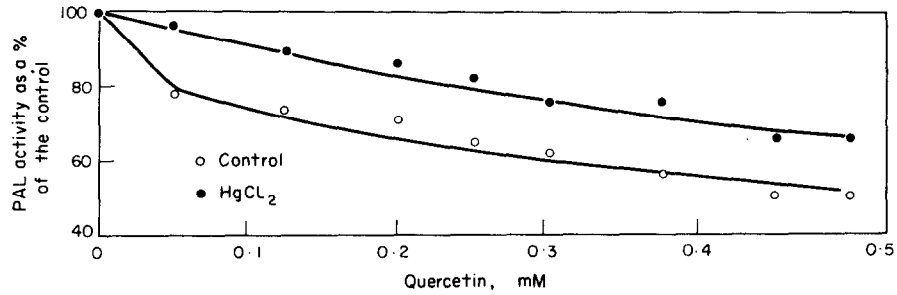

FIG. 4. THE PARTIAL DESENSITIZATION OF PAL TO QUERCETIN INHIBITION.
PAL was extracted from the hypocotyls of 3-day-old gherkin seedlings, pretreated with 4 hr white light (39·6 W m^{-2}), by $(NH_4)_2SO_4$ precipitation (30–70% saturation). One aliquot of the enzyme was incubated with either 10^{-6} M $HgCl_2$ (final concn) or H_2O for 1 hr at room temp. and then desalted through a Sephadex G25 column. Enzyme activity was estimated over a range of quercetin concentrations, using untreated or $HgCl_2$ treated enzyme, and the data cast as a % of the control.

An investigation into the effects of a range of substrate concentrations on the *in vitro* activity of PAL showed that the enzyme did not exhibit exact Michaelis–Menten kinetics. Instead, the Lineweaver–Burk plot was curvilinear and concave downwards. From these results two K_m values were determined, K_m^H was $2{\cdot}9 \times 10^{-4}$ M and K_m^L was $4{\cdot}3 \times 10^{-5}$ M. These values were of the same order of magnitude reported in other plant tissues.[3,6] At least two hypotheses can explain these unusual kinetics: (a) PAL has two substrate binding sites which differ in their affinity for substrate and interact with each other, or (b) there are two isoenzymes of PAL with differing affinities for phenylalanine. However, evidence has been obtained from gel filtration (Figs. 1 and 2), polyacrylamide gel electrophoresis (Fig. 3) and sucrose density gradient centrifugation that only one species of PAL, with a MW of 316000, exists in gherkin hypocotyls. Thus we conclude that the first hypothesis satisfies our kinetic data.

Similar kinetics to those reported above have been obtained with glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle,[14] alkaline phosphatase from *E. coli*,[15] and PAL from a variety of sources.[3,4,6,12] It has been suggested by Levitzki and Koshland,[16] that these are the kinetics of regulatory enzymes exhibiting negative homotropic cooperativity. Criteria for the recognition of negative homotropic interactions were also constructed by Levitzki and Koshland.[16] These include: (a) the substrate saturation plot looks qualitatively like a Michaelis–Menten curve; (b) the R_s value is greater than 81; (c) the double reciprocal plot is concave downwards; and (d) the Hill coefficient is less than 1. In the case of PAL from gherkin hypocotyls all of these criteria are satisfied: the substrate saturation curve is not shown here but is qualitatively similar to a Michaelis–Menten

[14] CONWAY, A. and KOSHLAND, D. E. (1968) *Biochemistry* **7**, 4011.
[15] SIMPSON, R. T. and VALLEE, B. L. (1970) *Biochemistry* **9**, 953.
[16] LEVITZKI, A. and KOSHLAND, D. E. (1969) *Proc. Nat. Acad. Sci. U.S.A.* **62**, 1121.

curve; the R_s value is 85·7; the double reciprocal plots are concave downwards and the Hill coefficient is 0·65. We therefore concluded that PAL from gherkins exhibits negative homotropic interactions.

Further experiments with inhibitory substances appear to support this conclusion. It was established that the *in vitro* activity of PAL was inhibited not only by products of the reaction or related compounds but also by later metabolites on the biosynthetic pathways leading from the PAL reaction. Cinnamic acid, the product of the reaction, and the related compound *o*-coumaric acid were both competitive inhibitors of the enzyme.

Cinnamic acid has been reported many times in the literature to be an effective inhibitor of PAL in various plant tissues,[1,3–5,7,8,12] whereas the inhibition of PAL by *o*-coumaric acid has only been reported in tobacco.[5] *In vivo* the inhibition of PAL by cinnamic acid could be a very useful property since it could well be a means of regulating the flow of carbon into either protein synthesis or phenolic biosynthesis. Similarly the inhibition of PAL by *o*-coumaric acid could also be advantageous *in vivo*. *o*-Coumaric acid is a precursor of coumarins (a possible end-product of the reaction catalyzed by PAL) and could well regulate the metabolism of this compound by exerting its effect on PAL. On the other hand, there is no evidence that the observed *in vitro* effects of these substances have any parallel in the cell and thus no conclusions relating to their *in vivo* importance can be made.

Evidence for the regulatory role envisaged for PAL can be adduced from inhibitor studies with the end-products quercetin and kaempferol and also gallic acid. These substances acted as mixed inhibitors of PAL, thereby suggesting that the inhibitor is binding at a site on the enzyme molecule other than the catalytic site. This is supported by the results of the desensitization experiments in which it was possible to partially desensitize PAL to inhibition by quercetin, kaempferol and gallic acid (Table 4, Fig. 4). The low degree of desensitization, however, suggests that the inhibitors have non-specific inhibitory effects in addition to any possible regulatory affects, which may lead to irreversible damage to the enzyme.

It is possible from these results that PAL controls flavonoid biosynthesis. However, it is also quite conceivable that quercetin and kaempferol are not allosteric effector molecules *in vivo* but are merely structurally related to other substances which endogenously control PAL activity.

In conclusion, it can be stated that there is strong evidence in gherkins that PAL is a regulatory enzyme exhibiting negative homotropic cooperativity. The regulatory importance of such negative interactions is not yet known,[16] although it is possible that they act to maintain relatively high rates of reactions even at very low substrate concentrations. Such a mechanism may serve a useful purpose in circumstances when the substrate is toxic, by keeping the cellular levels of the substrate low. In the case of PAL, however, there seems no reason to suppose that the substrate, phenylalanine, is particularly toxic and thus it is difficult to propose a physiological function for the peculiar behaviour of this enzyme.

EXPERIMENTAL

Preparation of plant material. *Cucumis sativus* L. var Venlo Pickling seeds were obtained from Suttons, Reading. Seedlings were grown in Petri dishes on filter paper, moistened with H_2O, in the dark for 72 hr at 25°.[17] After this time they were treated with white light (39·6 W m^{-2}) for 4 hr.

Ammonium sulphate precipitation of PAL from gherkin hypocotyls. After light treatment 24 g fr. wt of hypocotyls were subjected to a cooled steel hammer press, the resulting solidified material being taken up in 65 ml 1 mM

[17] ENGELSMA, G. and MEIJER, G. (1965) *Acta Botan. Neerl.* **14**, 54.

glutathione in 0·1 M borate buffer pH 8·8 and filtered through Whatman GF/A glass fibre paper. $(NH_4)_2SO_4$ was added to the soln with stirring (20 min) to give 30% saturation and then centrifuged at 7100 *g* for 10 min.[18] The pellet was discarded and the supernatant was brought to 70% saturation by the addition of more $(NH_4)_2SO_4$. Centrifugation was then carried out at 7100 *g* for 10 min, the pellet this time being dissolved in the minimal vol. of cold extraction buffer and desalted through Sephadex G25. The protein fraction collected could then be stored frozen for a number of weeks without appreciable loss of PAL activity.

Enzyme assay for PAL. The assay employed was a radioactive assay similar to that of Koukol and Conn[1] and details have been published earlier.[19]

Gel filtration. Sepharose 4B was suspended in 0·1 M borate buffer pH 8·8 and allowed to sediment in a column of 1·1 cm i.d. to 25 cm high. This was then kept in a cold room at 3°.

Polyacrylamide gel electrophoresis. 7% Polyacrylamide gels were pre-run for 1 hr at 3 mA/tube in a running buffer composed of 0·6 g Tris and 3·88 g glycine to a total vol. of 1 l. (pH 8·3). Samples (0·1 ml) containing 15% sucrose (w/v) were then loaded onto each gel and run at 3 mA/tube for 3 hr. Each gel was cut horizontally into 0·8 mm slices and incubated in a reaction mixture for 4 hr. Cinnamic acid was extracted into 3 ml toluene, 2 ml of this being added to 10 ml scintillation fluid and counted in a Packard Tri-Carb Liquid Scintillation Counter.

Sucrose density gradient centrifugation. The method employed was essentially that of Martin and Ames[20] with suitable modifications. Linear gradients of 5–20% sucrose in 0·1 M borate buffer pH 8·8 were made to a total vol. of 16 ml in two 17-ml cellulose nitrate tubes. After equilibration for several hours at 2–4° 0·6 ml of enzyme was pipetted on top of each gradient and overlayed with liquid paraffin to prevent any evaporation during the run. Centrifugation was carried out using a Beckman L2-65 Preparative Ultracentrifuge with an SW 27 rotor run at 27000 rpm at 2°. After centrifuhation 4 drop fractions (0·1 ml) were collected from each gradient. All fractions from one gradient were assayed for PAL, whilst every third fraction from the second gradient was assayed for protein.

Protein determination. Protein was determined by the method of Lowry *et al.*[21]

Acknowledgements—The authors are grateful to the Science Research Council for a Research Scholarship (S.E.I.) and a Research Grant (H.S.) for the support of this work. Mr. Brian A. Hemmings is also thanked for his collaboration in some of the experiments.

[18] GREEN, A. A. and HUGHES, W. L. (1955) in *Methods in Enzymology* (COLOWICK, S. P. and KAPLAN, N. O., eds.), Vol. I, p. 67, Academic Press, New York.
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[21] LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J. (1951) *J. Biol. Chem.* **193,** 265.