Phytochemistry, 1973, Vol. 12, pp. 961 to 973. Pergamon Press. Printed in England.

REVIEW ARTICLE
PHENYLALANINE AMMONIA LYASE

EDITH L. CAMM and G. H. NEIL TOWERS
Botany Department, University of British Columbia, Vancouver, British Columbia, Canada

(*Received* 10 *October* 1972. *Accepted* 21 *November* 1972)

Key Word Index—Plants; Fungi; phenylalanine ammonia lyase; enzymology; phenolic metabolism; controlling enzyme; review.

Abstract—The literature concerning the physiology and biochemistry of the enzyme phenylalanine ammonia lyase (PAL) (E.C. 4.1.1.5) from different organisms has been reviewed. Levels of the enzyme are affected by age, light, phytochrome, wounding, infection and growth modifiers. The possibility that PAL is involved in the control of phenolic metabolism has been critically examined.

PHENYLALANINE AMMONIA LYASE

PHENYLALANINE ammonia lyase (PAL) (E.C. 4.1.1.5) is currently the most studied enzyme concerned with secondary metabolism in plants. One reason for this attention by plant physiologists, biochemists and phytochemists is that, in a given tissue, the levels of the enzyme may fluctuate significantly in relatively short intervals of time in response to a wide variety of stimuli. Cinnamic acid, one product of the enzyme reaction, is the precursor of a large variety of secondary constituents, some of which, e.g. lignins, may account for an appreciable proportion of the total carbon in a plant. Not the least reason for the interest in this enzyme is, perhaps, the fact that it is relatively stable and very easily assayed by spectrophotometry,[1,2] with [^{14}C]-phenylalanine,[3] and even by GLC.[4] Current interest in this enzyme, discovered by Koukol and Conn in 1961,[1] is reflected in the discussions by Yoshida,[5] and Zucker.[6] We have attempted, therefore, to bring together some of the known facts regarding the physiology and biochemistry of PAL and also to evaluate some of the current hypotheses concerned with its fluctuating levels in plant tissues.

Enzymological Studies

The reaction catalyzed by PAL is the deamination of L-phenylalanine to yield *trans*-cinnamic acid and ammonia.[1] There are no cofactor requirements. Preparations from many plant sources also catalyze the deamination of L-tyrosine to *trans-p*-coumaric acid and ammonia, although always to a lesser extent. Tyrosine ammonia lyase (TAL) is sometimes considered to be a separate enzyme.[7–9] Phenylalanine, stereospecifically labelled with isotopic hydrogen at C-3 has been used to establish that the pro-*S* proton from C-3 of L-phenylalanine, together with ammonia, is eliminated in an antiperiplanar fashion from C-3

[1] KOUKOL, J. and CONN, E. E. (1961) *J. Biol. Chem.* **236**, 2692.
[2] ZUCKER, M. (1965) *Plant Physiol.* **40**, 779.
[3] SUBBA RAO, P. V., MOORE, K. and TOWERS, G. H. N. (1967) *Can. J. Biochem.* **45**, 1863.
[4] VAUGHN, T. H. and ANDERSON, R. A. (1971) *Biochim. Biophys. Acta* **244**, 437.
[5] YOSHIDA, S. (1969) *Ann. Rev. Plant Physiol.* **20**, 41.
[6] ZUCKER, M. (1972) *Ann. Rev. Plant Physiol.* **23**, 133.
[7] KINDL, H. (1970) *Physiol. Chem.* **351**, 792.
[8] MINIMIKAWA, T. and URITANI, I. (1965) *J. Biochem.* **57**, 678.
[9] NEISH, A. C. (1961) *Phytochemistry* **1**, 1.

to generate *trans*-cinnamic acid.[10–12] Reversibility of the enzyme reaction has been demonstrated,[3] and the equilibrium constant for potato PAL has been estimated as 4·7 at 30°, pH 6·8 and zero ionic state.[13] There are no indications from tracer studies, however, of the *in vivo* synthesis of phenylalanine from cinnamate.

So far the enzyme has been found in all green plants including the higher cryptogams,[14] the Basidiomycetes[15,16] and *Streptomyces*.[17,18] There have been no systematic examinations of the algae for PAL, and the only reported occurrence of the enzyme has been in *Porphyridium* (H. Kindl, pers. comm.). This has been confirmed by us.* Crude preparations of plant extracts may not always be reliable as indicators of the levels of PAL. Young and Neish[19] were unable to demonstrate PAL activity in *Chrysanthemum* extracts because of the presence of an endogenous inhibitor. In contrast, buffer extracts of bean acetone powder may give a positive test for PAL in the absence of added substrate[20] and the endogenous phenylalanine has to be removed by dialysis. Again, crude acetone powders are often good sources of 'bound' cinnamic acids and their release, as a result of autolysis or hydrolysis, may lead to spurious results especially in the usual spectrophotometric assays.

The Number of Isozymes

The MWs reported for PAL are 306 000 for maize;[21] 300 000 for mustard;[22] 226 000 for *Streptomyces verticillatus*[18] and 330 000 for potato.[13] The enzyme from mustard is unstable in dilute Tris buffer and aggregates to a form with a MW greater than 600 000.[22] The potato enzyme has been reported to exist in two forms, one species being twice the MW of the other, but these forms are not interconvertible.[13] The slower migrating band of PAL on electrophoretograms of preparations from *Sporobolomyces pararoseus*, also appears to be an aggregation product.[23]

On the other hand, two forms of PAL with different catalytic properties have been found in a number of tissues. In sweet potato the two forms are separable from TAL and show, moreover, differing sensitivities to phenolic inhibitors.[24] The two forms of PAL from *Quercus pedunculata*, separable on DEAE-cellulose, also differ in their sensitivities to phenolic compounds.[25] The PAL activity in preparations from light- and dark-grown mung bean seedlings appears in different ammonium sulfate fractions.[26] These indications of the pres-

* LANDYMORE, A. and TOWERS, G. H. N. unpublished.

[10] HANSON, K. R. and HAVIR, E. A. (1972) in *Recent Advances in Phytochemistry* (RUNECKLES, V. C. and WATKIN, J. E., eds.), Vol. 4, pp. 45–85, Appleton-Century-Crofts, New York.
[11] HANSON, K. R., WIGHTMAN, R. H., STAUNTON, J. and BATTERSBY, A. R. (1971) *Chem. Commun.* 185.
[12] IFE, R. and HASLAM, E. (1971) *Chem. Commun.* 2818.
[13] HAVIR, E. A. and HANSON, K. R. (1968) *Biochemistry* **7**, 1896.
[14] YOUNG, M. R., TOWERS, G. H. N. and NEISH, A. C. (1966) *Can. J. Botany* **44**, 341.
[15] BANDONI, R. J., MOORE, K., SUBBA RAO, P. V. and TOWERS, G. H. N. (1968) *Phytochemistry* **7**, 205.
[16] POWER, D., TOWERS, G. H. N. and NEISH, A. C. (1965) *Can. J. Biochem.* **43**, 1397.
[17] BEZANSON, G. S., DESATY, D., EMES, A. V. and VINING, L. C. (1970) *Can. J. Microbiol.* **16**, 147.
[18] EMES, A. V. and VINING, L. C. (1970) *Can J. Biochem.* **48**, 613.
[19] YOUNG, M. R. and NEISH, A. C. (1966) *Phytochemistry* **5**, 1121.
[20] RAHE, J. E., KUC', J. and CHUANG, C.-M. (1970) *Phytochemistry* **9**, 1009.
[21] MARSH, JR., H. V., HAVIR, E. A. and HANSON, K. R. (1968) *Biochemistry* **7**, 1915.
[22] SCHOPFER, P. (1971) *Planta* **101**, 339.
[23] PARKHURST, J. R. and HODGINS, D. S. (1971) *Phytochemistry* **10**, 2997.
[24] MINIMIKAWA, T. and URITANI, I. (1965) *J. Biochem.* **58**, 53.
[25] BOUDET, A., RANJEVA, R. and GADAL, P. (1971) *Phytochemistry* **10**, 997.
[26] AHMED, S. I. and SWAIN, T. (1970) *Phytochemistry* **9**, 2287.

ence of more than one enzyme in a given tissue are not general, however; PAL from light- and dark-grown buckwheat plants,[27] mustard plants,[22] and *Glycine max* tissue cultures[28] have been examined by electrophoresis and found to be the same. Similarly, the PAL in preparations from light- and dark-aged potato disks is not separable by gel filtration (Sephadex G200).[29]

TAL activity cannot be demonstrated in many PAL preparations and often the activity towards tyrosine, where it exists, is lost rapidly during purification.[1] In *Ustilago*, not only is TAL activity not present in crude or purified PAL preparations but *p*-coumaric acid is not formed when tyrosine is administered *in vivo*.[3] In the purification of wheat PAL the PAL/TAL ratio varied from 4 to 20[19] and in *Sporobolomyces roseus* from 1·35 to 5.[30] The pH optimum curves of PAL and TAL were also different[9,31] and, together with the above evidence, suggest that there may be two separate enzymes. In barley preparations, two peaks of activity, with very different ratios of PAL/TAL activity are resolved on Sephadex G200.[7] In contrast, a constant ratio of PAL/TAL activity was found in the purification of PAL from maize and both activities were lost at the same rate on treatment with $NaBH_4$.[32] TAL activity accompanied PAL activity throughout the 450-fold purification of the latter enzyme from *Sporobolomyces pararoseus*.[23] The differing pH optima for PAL and TAL at saturating substrate concentrations may be expected since they are determined by the ionization state of groups of the enzyme-substrate complex which varies with the substrate.[32] The question of how many enzymes is therefore not settled and is likely to remain unanswered until more work is done with highly purified preparations. If a generalization can be made it is that some PAL preparations display catalytic activity towards tyrosine.

Active Site

Cyanide and $NaBH_4$ irreversibly inhibit PAL from potato,[33,34] maize,[32] *Rhodotorula glutinis*,[35] tobacco[36] and *Streptomyces verticillatus*.[17] Hydrolysis of purified potato or maize PAL treated with tritiated $NaBH_4$ yields tritiated alanine.[32,34,37] The enzyme therefore has a dehydroalanyl residue at the active site, and, in this respect, resembles histidine ammonia lyase.[38]

K_m Values

Most reported K_m fall midway in the range $0{\cdot}3 \times 10^{-4}$ to $1{\cdot}5 \times 10^{-2}$ M (Table 1) although it has been noted with a number of preparations[9,21,34,39] that there is no single value for K_m. In a detailed study of the kinetics of highly purified PAL from potato, Havir and Hanson[34] have shown that the anomalous kinetics are replaced by Michaelis–Menton kinetics in the presence of D-phenylalanine, a competitive inhibitor. Since V_{max} is not reduced under

[27] AMRHEIN, N. and ZENK, M. H. (1971) *Z. Pflanzenphysiol.* **64,** 145.
[28] HAHLBROCK, K., KUHLEN, E. and LINDL, T. (1971) *Planta* **99,** 311.
[29] SACHER, J. A., TOWERS, G. H. N. and DAVIES, D. D. (1972) *Phytochemistry* **11,** 2383.
[30] CAMM, E. L. and TOWERS, G. H. N. (1969) *Phytychemistry* **8,** 1407.
[31] OGATA, K., UCHIYAMA, K., YAMADA, H. and TOCHIKURA, T. (1967) *Agr. Biol. Chem.* **31,** 600.
[32] HAVIR, E. A., REID, P. D. and MARSH, JR. H. V. (1971) *Plant Physiol.* **48,** 130.
[33] HANSON, K. R. and HAVIR, E. A. (1969) *Fed. Proc.* **28,** 602.
[34] HAVIR, E. A. and HANSON, K. R. (1968) *Biochemistry* **7,** 1904.
[35] HODGINS, D. S. (1968) *Biophys. Biochem. Res. Commun.* **32,** 246.
[36] O'NEAL, D. and KELLER, C. J. (1970) *Phytochemistry* **9,** 1373.
[37] HANSON, K. R. and HAVIR, E. A. (1970) *Arch. Biochem. Biophys.* **141,** 1.
[38] GIVOT, I. L., SMITH, T. A. and ABELES, R. H. (1969) *J. Biol. Chem.* **244,** 6341.
[39] ATTRIDGE, T. H., STEWART, G. R. and SMITH, H. (1971) *FEBS Letters* **17,** 84.

TABLE 1. CHARACTERISTICS OF PAL ISOLATED FROM VARIOUS ORGANISMS

Organism	pH optimum	K_m	Inhibitors	Specificity
Streptomyces verticillatus[17,18]	8·5–9·5	$1·6 \times 10^{-4}$	cinn, his, *p*-Fl, phe	Active with Fl- and Cl-phe, but not tyr
Rhodotorula glutinis[31,35,40,41]	9·0	$2·1 \times 10^{-3}$		Active with tyr
Rhodotorula texensis[31]	9·0	$1·5 \times 10^{-2}$		
Sporobolomyces roseus[30, unpublished data]	8·7	4×10^{-4}	cinn, *p*-coum	
Sporobolomyces pararoseus[23]				Active with phe and tyr at 450× purification
Ustilago hordei[3,42]	8·8	$4·5 \times 10^{-4}$	cinn, but not *p*-coum	Not active with tyr
Pteridium aquilinum[19]				Not active with tyr
Avena sativa[43]			*m*-, *p*-, *o*-, Fl-phe (competitive)	
Hordeum vulgare[1]	8·8–9·2	$(1·7 \pm 0·3) \times 10^{-3}$	cinn, tyr, *p*-coum	When fresh, active with L-tyr, *m*-tyr
Triticum aestivium[19]			L-tyr, D-tyr, *m*-tyr, *m*-Fl-phe and others	Active with tyr and many derivatives
Zea mays[21,32]		$2·7 \times 10^{-4}$–$1·1 \times 10^{-3}$	D-phe	Active with tyr
Ipomea batatas[8,24]	8·5–9·5	A $(1·01 \pm 0·04) \times 10^{-4}$	A. cinn, *p*-Fl-phe, tyr	
		B $(0·95 \pm 0·09) \times 10^{-4}$	B. the above plus *p*-coum, caff	
Pisum sativum[39]		high substr $8·0 \times 10^{-4}$ low substr $5·2 \times 10^{-5}$	querc and the *Pisum* flavonoids	
Nicotiana tabacum[36]	8·0–8·6	$2·2 \times 10^{-4}$	cinn, *o*-coum, *o*-tyr, querc	No activity with tyr
(tissue culture)[44]	8·8	3×10^{-5}	cinn, IAA, try scop	
Solanum tuberosum[10,11,13,34,35,37,45]	8·7	$0·38 \times 10^{-4}$–$2·6 \times 10^{-4}$	cinn, D-phe	No activity with tyr

Abbreviations. tyr—tyrosine; phe—phenylalanine; his—histidine; try—tryptophan; *p*-coum—*p*-coumarate; cinn—cinnamate; Fl-phe—fluorophenylalanine; Cl-phe—chlorophenylalanine; querc—quercetin; scop—scolopetin.

these conditions the behaviour of PAL is best explained in terms of allosteric interactions: while the enzyme may exist in two or more conformations with characteristic kinetic properties, the binding of D-phenylalanine causes a single form to predominate which displays simplified kinetics.

[40] OGATA, K., UCHIYAMA, K. and YAMADA, H. (1966) *Agric. Biol. Chem.* **30,** 311.
[41] OGATA, K., UCHIYAMA, K., and YAMADA, H. (1967) *Agric. Biol. Chem.* **31,** 200.
[42] SUBBA RAO, P. V. and TOWERS, G. H. N. (1970) in *Methods in Enzymology* (TABOR, H. and TABOR, C. W., eds.) Vol. XVIIA, p. 551, Academic Press, New York.
[43] HOPKINS, W. G. and ORKWISZEWSKI, J. A. J. (1971) *Can. J. Bot.* **49,** 129.
[44] INNERARITY, L. T., SMITH, E. C. and WENDER, S. H. (1972) *Phytochemistry* **11,** 83.
[45] HAVIR, E. A. and HANSON, K. P. (1970) in *Methods in Enzymology* (TABOR, H. and TABOR, C. W., eds.), Vol. XVIIA p. 575 Academic Press, New York.

Inhibitors

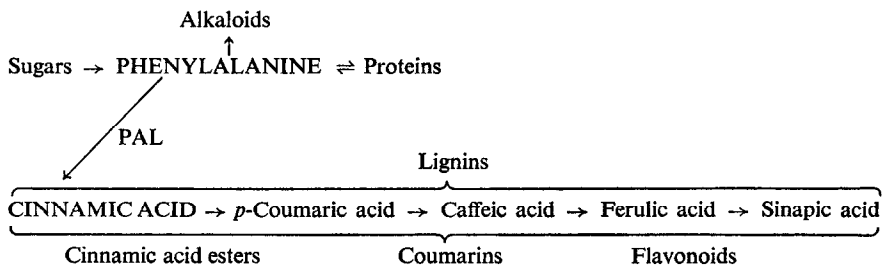
Enzyme preparations from barley[1] and *Rhodotorula*[31] are sensitive to sulfhydryl inhibitors whereas preparations from potato,[34] maize,[21] *Ustilago*[3] and *Streptomyces*[18] are not.

All enzyme preparations are strongly inhibited by cinnamic acid. Cinnamate is more effective as an inhibitor of PAL from potato[34] or tobacco[36] at pH 7·0 than at the pH optimum which is greater than 8.

p-Coumarate has been found to be an inhibitor where tested (see Table 1) except with preparations from *Ustilago* and these do not display any TAL activity Flavonoids such as kaempferol have been observed to inhibit the enzyme and may be examples of end product inhibitors. Although both isozymes of PAL from oak leaves are sensitive to cinnamate and *p*-coumarate one is more sensitive to benzoic acid derivatives and the other to cinnamic acid derivatives.[25] This is true of the two isozymes of sweet potato.[24] This may be important in the control of the synthesis of C_6–C_1 derived acids as opposed to the control of hydroxycinnamic acid formation.

Factors Influencing the Levels of PAL

Here it may be useful to consider a simple model to clarify the significance of some of the physiological work to be discussed.



Control of lignification or flavonoid synthesis could be effected by regulation of the levels of PAL or of phenylalanine production or both. Because cinnamic acid is the first compound committed strictly to the biogenesis of molecules such as flavonoids it may be expected that PAL has regulatory properties. This has yet to be determined. Nearly all the physiological studies to be discussed concern changes in PAL levels resulting from stimuli such as light and correlated changes in levels of specific phenolic compounds or of lignins. At least one attempt has been made to determine whether rates of phenylalanine production are affected by these stimuli[27] but the results were negative.

An increased level of PAL activity is often referred to in the literature as an induction of the enzyme although it is generally understood that an inducible enzyme is one which is synthesized *de novo* in response to an external stimulus. In order to demonstrate *de novo* protein or enzyme synthesis it is necessary to establish that the molecule is synthesized and not merely converted from an inactive to an active form during the period of putative induction.[46] The PAL isolated from light-treated *Xanthium* leaf disks fed [^{14}C]-arginine and [^{14}C]-isoleucine was shown to be radioactive[47] and could therefore be considered to have been synthesized *de novo*. Similarly deuterium is incorporated into PAL during phytochrome

[46] FILNER, P., WRAY, J. L. and VARNER, J. E. (1969) *Science* **165**, 358.
[47] ZUCKER, M. (1969) *Plant Physiol.* **44**, 912.

stimulation in mustard[48] and in potato disks during light stimulation.[29] Here one may refer to the phenomenon as a *de novo* synthesis of enzyme. It may be misleading, however, to refer to an induction of the enzyme until certain other facts are established. There, is evidence, based on cycloheximide treatments, that an inactivator of PAL is present in a number of plants such as potato,[49] mustard[50] and radish.[51] In *Xanthium* the 'induction' of PAL was found to be due to a decreased rate of inactivation of the PAL molecule.[52,53] Apparently, in this system there is a continuous production of inactivator and changes in PAL levels are a reflection of a change in rate of synthesis of the inactivator and not of an increased rate of synthesis of PAL.

Intrinsic Factors Causing Variations in PAL Levels

Wide variations in levels of PAL activity of plants have been recorded. Not only do they depend on the genotype[54] or plasmotype,[55] but also on the age and development, organ and even tissue of the plant.

During the germination of seedlings under constant conditions, PAL activity, originally low or absent rises to a peak and then declines. The pattern of these events varies considerably: in wheat, the maximum occurs about 7 days after germination[55a] in etiolated buckwheat 4 days,[56] in etiolated radish, 60 hr,[57] and in light and dark grown *Impatiens* seedlings, 4 days.[58,59] In light grown suspension cultures of parsley, the peak of activity coincides with the logarithmic phase of growth.[60] In *Tulipa* anthers enzyme levels are correlated with meiotic stages.[61] On storage, asparagus spears develop increasing levels of PAL but become less susceptible to the stimulation following excision.[62] Variation of response also occurs in radish and mustard seedlings; the lag before PAL formation after illumination decreases as the seedlings age, as does the maximum level of enzyme formed.[63,64]

The maximum specific activity of PAL in a plant depends on the part examined. Roots of etiolated buckwheat,[27] etiolated radish[57] and etiolated *Impatiens* seedlings[59] show relatively high levels of PAL, while the hypocotyls of these seedlings have a much lower level. Tissues in which PAL seems specifically localized include the tapetal layer of developing tulip and narcissus anthers[61] and the cambial and adjacent tissues in stems of young buckwheat.[65] At the subcellular level, PAL seems to be located in the glyoxysomes of castor bean endosperm[66] and in the peroxisomes of spinach.[67] In the potato tuber, however, the enzyme is

[48] SCHOPFER, P. and HOCK, B. (1971) *Planta* **96,** 248.
[49] ZUCKER, M. (1968) *Plant Physiol.* **43,** 365.
[50] WEIDNER, M., RISSLAND, I., LOHMANN, L., HUAULT, C and MOHR, H. (1969) *Planta* **86,** 33.
[51] KLEIN-EUDE, D., HUAULT, C. and ROLLIN, P. (1971) *Compt. Rend.* **273D,** 1276.
[52] ZUCKER, M. (1970) *Biochim. Biophys. Acta* **208,** 331.
[53] ZUCKER, M. (1971) *Plant Physiol.* **47,** 442.
[54] NITSCH, C. and NITSCH, J. P. (1967) *Compt. Rend.* **262D,** 1102.
[55] HACHTEL, W. (1972) *Planta* **102,** 247.
[55a] YOUNG, M. R. (1966) Ph.D. Thesis, Dalhousie University.
[56] AMRHEIN, N. and ZENK, M. (1970) *Z. Pflanzenphysiol.* **63,** 384.
[57] BELLINI, E. and VAN POUCKE, M. (1970) *Planta* **93,** 60.
[58] WEISSENBÖCK, G. (1971) *Z. Pflanzenphysiol.* **66,** 73.
[59] WEISSENBÖCK, G. (1972) *Z. Pflanzenphysiol.* **66,** 243.
[60] HAHLBROCK, K. and WELLMAN, E. (1970) *Planta* **94,** 236.
[61] WIERMANN, R. (1972) *Z. Pflanzenphysiol.* **66,** 215.
[62] GOLDSTEIN, L. D., JENNINGS, P. H. and MARSH, H. V. JR. (1971) *Plant Cell Physiol.* **12,** 657.
[63] DITTES, L., RISSLAND, I. and MOHR, H. (1971) *Z. Naturforsch.* **26B,** 1175.
[64] HUAULT, C., KLEIN-EUDE, D., ROLLIN, P. and BLONDEL, J. D. (1971) *Compt. Rend.* **273D,** 745.
[65] YOSHIDA, S. and SHIMOKORIYAMA, M. (1965) *Bot. Mag.* **78,** 14.
[66] RUIS, H. and KINDL, H. (1970) *Z. Physiol. Chem.* **351,** 1425.
[67] RUIS, H. and KINDL, H. (1971) *Phytochemistry* **10,** 2627.

absent from the corresponding microbodies.[68] In young sorghum,[69] buckwheat[27] and potato tuber (Camm, unpublished), although some PAL appears in the particulate fractions, most of the enzyme is soluble.

The Effect of Light

The effect of light on levels of PAL has received a great deal of attention as a result of Zucker's discovery[2] of the increased activity of PAL in potato slices incubated in light. The stimulatory effect of light in either etiolated or excised (wounded?) tissue has since-been shown to be quite general[47,52,53,60,70–77] although exceptions are also known. For example grapefruit peel,[78] excised axes of *Phaseolus vulgaris*,[79] some varieties of *Helianthus tuberosus*[54] and the roots of many plants,[56,80] are insensitive to light.

Phytochrome involvement was demonstrated in etiolated pea,[81] mustard[82] and radish seedlings[83] and *Helianthus tuberosus* tubers.[84] A short period of illumination with red light (R) followed by a return to darkness causes an increase in PAL activity. This response varies with the logarithm of the energy of the red illumination. A brief illumination with far-red (FR) suppresses the response to R. In addition to this effect of R and its reversibility by FR continuous FR illumination increases PAL levels in etiolated tissue.[57,81,85,86] In etiolated radish seedlings this increase is correlated with changes in the amount of phytochrome.[51,83,87,88] According to Hartmann,[89] under constant FR light, 3–5% of the phytochrome in a given plant material is present in the active, unstable form (Pfr), the remainder being maintained in a stable but inactive form (Pr). The maintenance of the low but significant amount of Pfr is believed to be responsible for the photo-morphogenetic effects of constant FR light. This hypothesis has been invoked by Durst and Mohr to explain their results on PAL activities in FR irradiated mustard and radish seedlings.[85] Bellini and Hillman,[90] however, also working with mustard and radish seedlings, were unable to show a correlation of PAL activity with Pfr. In their experiments, a R light programme maintains a measurable level of Pfr but the effect is a low enzyme level as in dark controls. Irradiation for 6 hr with FR light significantly increases the extractable activity of PAL although Pfr levels are very low. They

[68] RUIS, H. (1971) *Z. Physiol. Chem.* **352,** 1105.
[69] STAFFORD, H. A. (1969) *Phytochemistry* **8,** 743.
[70] AMRHEIN, N. and ZENK, M. (1970) *Naturwissenschaften* **57,** 312.
[71] CREASY, L. L. (1968) *Phytochemistry* **7,** 441.
[72] CREASY, L. L. (1971) *Phytochemistry* **10,** 2705.
[73] HAHLBROCK, K., EBEL, J., ORTMAN, R., SUTTER, A., WELLMANN, E. and GRISEBACH, H. (1971) *Biochim. Biophys. Acta* **244,** 7.
[74] SCHERF, H. and ZENK, M. (1967) *Z. Pflanzenphysiol.* **56,** 203.
[75] SCHERF, H. and ZENK, M. (1967) *Z. Pflanzenphysiol.* **57,** 401.
[76] SMITH, H. and ATTRIDGE, T. H. (1970) *Phytochemistry* **9,** 487.
[77] SMITH, H. and HARPER, D. B. (1970) *Phytochemistry* **9,** 477.
[78] RIOV, J., MONSELISE, S. P. and KAHAN, R. S. (1969) *Plant Physiol.* **44,** 631.
[79] WALTON, D. C. and SONDHEIMER, E. (1968) *Plant Physiol.* **43,** 467.
[80] RHODES, M. J. C. and WOOLTORTON, L. S. C. (1971) *Phytochemistry* **10,** 1989.
[81] ATTRIDGE, T. H. and SMITH, H. (1967) *Biochim. Biophys Acta.* **148,** 805.
[82] WEIDNER, M., RISSLAND, I. and MOHR, H. (1968) *Naturwissenschaften* **55,** 452.
[83] HUAULT, C., LARCHER, G. and MALCOSTE, R. (1971) *Compt. Rend.* **273D,** 1371.
[84] DURST, F. and DURANTON, H. (1970) *Compt. Rend.* **270D,** 2940.
[85] DURST, F. and MOHR, H. (1966) *Naturwissenschaften* **53,** 531.
[86] DURST, F. and MOHR, H. (1966) *Naturwissenschaften* **53,** 707.
[87] LARCHER, G., HUAULT, C. and MALCOSTE, R. (1971) *Compt. Rend.* **273D,** 2257.
[88] MALCOSTE, R., LARCHER, G. and HUAULT, C. (1971) *Compt. Rend.* **273D,** 1197.
[89] HARTMANN, K. M. (1967) *Z. Naturforsch.* **22b,** 1172.
[90] BELLINI, E. and HILLMAN, W. S. (1971) *Plant Physiol.* **47,** 668.

have concluded that the FR effect on their system cannot be explained solely by formation and maintenance of Pfr. In a rebuttal, Schopfer and Mohr[91] have claimed that as little as one 5-min irradiation of R light does, in fact, induce PAL formation and 'the operational criteria for the involvement of Pfr (ground state) in the induction of this response are fulfilled'. They state that the hypothesis (that Pfr is the effector of the high intensity reaction) cannot be tested by simply comparing spectrophotometrical data. Pfr (ground state) cannot be distinguished from Pfr (excited state), the effective species,[89] which is maintained at a low but effective level by FR light.

In addition to exhibiting R–FR sensitivity, many of the systems are also sensitive to blue light. *Helianthus tuberosus* slices, for example, form PAL when incubated in white, red (660–680 nm), and blue light (427 nm).[54] One variety, Piédallu 17, responded only to blue light suggesting that at least two photoreceptors are involved in the phenomenon.

In gherkin seedlings a long term irradiation with blue light results in a great stimulation of PAL activity.[92,93] Long term irradiations with R or FR also cause an increase but there is no R–FR induction–reversion.[94] Moreover, even tissue saturated with respect to R or FR light will respond to blue light. The specific photoreceptor for the blue wavelengths seems located in cotyledons, for while whole gherkin seedlings respond to R, FR or blue light, ecotylized seedlings are affected by R and FR light only. Even though phytochrome absorbs in the blue region of the spectrum,[89] Engelsma has invoked a non-phytochrome pigment, absorbing at 460 nm, which is activated in the high energy reaction.[94]

The problem of phytochrome involvement in PAL synthesis is not resolved. As we have seen there is substantial evidence, for phytochrome mediated stimulation in pea, buckwheat, mustard and radish tissue but in other plants this is not so and the response to blue light may involve a different pigment.

Effect of Wounding, Infection and Ethylene

An increased level of PAL upon wounding occurs in pea seedlings,[95] citrus fruit peel,[78] excised bean axes,[79] sweet potato,[8,24] swedes,[80] buckwheat[27,96] and gherkin.[97] This effect might be ascribed to the production of ethylene because wounding a tissue stimulates the endogenous production of ethylene and the effect of added ethylene on most tissues is to give rise to an increased production of PAL. In gherkin, however, excised but not intact hypocotyls are ethylene-sensitive.[98] The concentrations of ethylene that affect levels of PAL vary in different plants; in citrus fruit peel maximum stimulation occurs at 100 ppm ethylene[78] while pea seedlings require 10 ppm,[95] swedes 4 ppm[80] and sliced sweet potato around 5 ppm.[99] Although light is effective in increasing the levels of PAL over dark controls, in potato disks, added ethylene seems to have no effect (M. J. C. Rhodes personal communication); perhaps the endogenous levels are already saturating.

Citrus fruit peel shows an increase in PAL after γ-irradiation[100] as does pea after UV irradiation.[101] These effects might be a response to ethylene produced as a result of injury.

[91] SCHOPFER, P. and MOHR, H. (1972) *Plant Physiol.* **48,** 8.
[92] ENGELSMA, G. and MEIJER, G. (1965) *Acta Bot. Neerl.* **14,** 54.
[93] ENGELSMA, G. (1967) *Planta* **75,** 207.
[94] ENGELSMA, G. (1968) *Acta. Bot. Neerl.* **17,** 85.
[95] HYODO, H. and YANG, S. F. (1971) *Plant Physiol.* **47,** 765.
[96] AMRHEIN, N. and ZENK, M. (1968) *Naturwissenschaften* **55,** 394.
[97] ENGELSMA, G. (1968) *Planta* **82,** 355.
[98] ENGELSMA, G. and VAN BRUGGEN, J. M. H. (1971) *Plant Physiol.* **48,** 94.
[99] IMASEKI, H., UCHIYAMA, M. and URITANI, I. (1968) *Agr. Biol. Chem.* **32,** 387.
[100] RIOV, J., MONSELISE, S. P. and KAHAN, R. S. (1968) *Radiation Bot.* **8,** 463.
[101] HADWIGER, L. A. and SCHWOCHAU, M. E. (1971) *Plant Physiol.* **47,** 588.

Ethylene is also produced by plants following infection by a pathogenic organism. This may be responsible for triggering PAL production in bean leaves following infection with tobacco necrosis virus,[102] in tobacco infected with tobacco mosaic virus[103] or *Pseudomonas solanacearum*,[104] in sweet potato infected with *Ceratocystis fimbriata*,[8] and in soybean infected with *Helminthosporium carbonum*.[105]

It can be shown that ethylene formation in rice is inhibited by R light and restored by a subsequent exposure to FR.[106] Is it possible that the effectiveness of FR in certain systems, in stimulating PAL formation is because of the increased production of ethylene under these conditions?

The Effect of Hormones and Growth Modifiers

In *Avena*, *p*-fluorophenylalanine inhibits PAL *in vivo*.[43] An attempt has been made to link this inhibition to the increased elongation of *p*-fluorophenylalanine treated coleoptile segments, via changes in phenolic modifiers of IAA oxidase activity. IAA itself, and tryptophan, are inhibitors of PAL in tobacco tissue cultures although concentrations used to demonstrate inhibition are rather high (i.e. 1–3 mM).[44]

Gibberellin treatment increases PAL production and lignification in dwarf pea plants, but only when the plants are held in the light.[107] This differs from the elongation effect of gibberellin which is light independent. Although Abscisin 2 can act as a gibberellin antagonist, it too causes an increase in PAL levels in excised pea axes.[79] The PAL activity rises earlier to a higher level than in untreated plants.

The effect of 2,4-D has been tested in rose tissue culture.[108] While this auxin has no effect on PAL *in vitro*,[44] it can delay the formation of the enzyme *in vivo*.[108]

In each of the above cases, so little is known about the precise action of the applied modifier, that attempts to interpret at this time the effects of PAL involve largely unwarranted speculation.

Effects of Carbohydrate Levels

The status of the tissue with respect to its carbohydrate metabolism appears to be important. The increase in production of PAL in green leaf disks of strawberry, for example, requires light and CO_2.[109] The requirement for light can be replaced by floating the tissue on a solution of sucrose. Sucrose, glucose or fructose raise the level of PAL in green buckwheat leaf disks 50-fold; light stimulates PAL activity to a lesser extent.[27] The photosynthesis inhibitor DCMU, which has no effect on PAL induction in etiolated buckwheat tissue, completely inhibits the light effect in green tissue. Sucrose alone cannot be the limiting factor, for there is no increase of PAL in dark in sucrose-fed green leaf disks of *Xanthium*, although there is a significant increase in PAL levels when these disks are transferred to light.[47] These results, taken with others, e.g. the lack of correlation between PAL levels and levels of endogenous sugars in dark-grown buckwheat seedlings,[56] indicate that the relationship between PAL levels and carbohydrate metabolism are not obvious and that much more needs to be done before any useful interpretation of the data can be made.

[102] FARKAS, G. L. and SZIRMAI, J. (1969) *Neth. J. Plant. Path* **75**, 82.
[103] PAYNOT, M., MARTIN, C. and GIRAUD, M. (1971) *Compt. Rend.* **273D**, 537.
[104] PEGG, G. F. and SEQUEIRA, L. (1968) *Phytopath.* **58**, 476.
[105] BIEHN, W. L., KUC', J. and WILLIAMS, E. B. (1968) *Phytopath.* **58**, 1255.
[106] IMASEKI, H., PJON, C.-J. and FURUYA, M. (1971) *Plant Physiol.* **48**, 241.
[107] CHENG, C. K.-C. and MARSH, JR., H. V., (1968) *Plant Physiol.* **43**, 1755.
[108] DAVIES, M. E. (1972) *Planta* **104**, 66.
[109] CREASY, L. L. (1968) *Phytochemistry* **7**, 1743.

Kinetics of Formation and Decay of PAL

In many of the R–FR light experiments there is a lag before PAL levels start to increase. In very young etiolated radish seedlings a relatively long lag and in older seedlings a shorter one precedes the increase of enzyme.[64] This situation is similar to the pattern of induction of thymidine kinase in lily anthers;[110] if the inductive agents are applied before a critical period of enzyme inducibility there is a lag before enzyme production.

Rissland and Mohr[111] explain the lag in FR irradiated radish seedlings as the time necessary to activate the gene which codes for PAL. They showed, furthermore, that if a mustard seedling is irradiated with 12 hr FR and the PAL activity is then allowed to decay in the dark for 6 hr, a second FR illumination is effective without a lag, i.e. the gene(s) is still activated.

In nearly all cases of the light activated enzyme, the initial increase in activity is followed by a significant decline, even in the presence of continuous light. The final level depends on the tissue; in radish cotyledons the high activity drops back almost to the dark levels, while in the hypocotyls the activity remains higher.[57,63,64,83] This loss of enzyme can be prevented by the administration of the protein synthesis inhibitor, cycloheximide. Cycloheximide administered at the beginning of the inductive period prevents PAL formation; when administered later enzyme levels do not fall. This has been demonstrated with gherkin,[112] potato,[49] excised broad bean axes[79] and radish.[51]

Similar observations in potato have been interpreted as the sequential induction of the lyase and a lyase inactivating system.[49] From the work with cyclohexomide, it is inferred that PAL inactivation is dependent upon protein synthesis and perhaps on the synthesis of a specific protein or enzyme which inactivates or destroys PAL. In postulating a mechanism for the reaction of PAL, Havir and Hanson[34] have suggested that the enzyme activity could be controlled by the action of a specific dehydrogenase or transaminase acting on the carbonyl group of the active site.

In the above plants, the observed decay of PAL levels is due to decreased PAL synthesis coupled with PAL inactivation. If PAL synthesis were to continue unabated while the inactivator was being produced, the kinetic pattern would resemble that observed in *Xanthium* leaf disks in which the increase is followed by a plateau without any decrease in activity.[47] If these leaf disks are transferred to dark, however, enzyme activity quickly decays.[47] Measurements of the uptake of [^{14}C]-labelled amino acids into *Xanthium* PAL indicate that synthesis of the enzyme continues unabated in dark. It appears then that the rate of inactivation is increased in dark. Recognition by Zucker of this inactivating factor, which is involved in the regulation of PAL levels, is the beginning of an exciting new phase in studies of this enzyme. Undoubtedly its nature will be discovered before long and perhaps give us a better insight into other factors affecting lignification of flavonoid production.

Studies of the effect of low temperatures on the synthesis and inactivation of PAL in gherkin seedlings have given rise to another model of PAL control.[113–115] Irradiation of dark grown seedlings causes an increase in PAL production followed by a decline. If the seedlings are now given a cold treatment (4°) in dark followed by a temperature raise (25°) a second maximum in PAL activity is obtained. Treatment with cycloheximide prior to the transfer to higher temperature does not block the increase of PAL activity but causes a delay

[110] HOTTA, Y. and STERN, H. (1965) *J. Cell Biol.* **25,** 99.
[111] RISSLAND, I. and MOHR, H. (1967) *Planta* **77,** 239.
[112] ENGELSMA, G. (1967) *Naturwissenschaften* **54,** 319.
[113] ENGELSMA, G. (1969) *Naturwissenschaften* **56,** 563.
[114] ENGELSMA, G. (1970) *Planta* **90,** 133.
[115] ENGELSMA, G. (1970) *Planta* **91,** 246.

of the subsequent decline. Engelsma postulates that an inactive enzyme-inhibitor complex is formed at the higher temperature. Cold shock causes the relase of PAL from the complex. The model, however, is not as satisfactory as that proposed by Zucker. If active molecules of PAL are released at lower temperatures one would expect an increase in enzyme activity in time at low temperatures which does not appear to be the case.

Is PAL a Controlling Enzyme in Phenolic Metabolism?

If PAL levels were a limiting factor in phenolic biosynthesis, then treatments causing PAL to increase should also cause an increase in phenolic compounds.

Concomitant increases in levels of PAL and of phenolic compounds have been demonstrated in many plants and plant tissues. In gherkin seedlings changes in PAL levels after treatment with blue light,[92,93] long term R light,[94,116] wounding,[97] photoperiod[117] and temperature changes[113–115,118] are all reflected in the levels of accumulated hydroxycinnamic acids. Fresh potato tissue, devoid of PAL or chlorogenic acid, develops both on incubation in light.[2] Both PAL and chlorogenic acid production are inhibited by cycloheximide. Similar increases in PAL and chlorogenic acid have been described in ethylene-treated sweet potato[99] and swede tissues.[80] In the Actinomycete *Streptomyces verticillatus*, there is a correlation between cinnamide production and the levels of PAL.[17]

Although flavonoids are biosynthetically more removed from the simple phenolics mentioned above there are many examples of correlations between their increased production and increase in PAL. Naringenin in grapefruit,[119,120] leucoanthocyanins and catechins in strawberry leaf disks[71] and fruit[121] are examples. In buckwheat both PAL and anthocyanin production are affected in the same way by R or FR light.[75] In pea exposed to continuous light there are two peaks in PAL activity which are reflected in the levels of a quercetin glycoside.[76,77,122] Apiin and graveobioside-B formation after illumination of parsley cell cultures coincides with a peak in PAL activity.[60] This is true also of cell cultures of *Glycine max*.[28] In developing tulip and narcissus anthers, the content of several flavonoids peaks just after maxima in PAL levels occur.[61]

High PAL activity is often correlated with active lignification although non-lignifying tissues may also have very high specific activities of the enzyme. In bamboo shoots growing in light the regions of highest PAL activity are those undergoing most rapid lignification while in the very young shoot and in already lignified tissue there is little activity.[123] In asparagus spears the highest levels and specific activities of PAL (per unit protein) are in the basal portion which is rapidly becoming woody.[62] Highest concentrations of the enyme have been reported in sycamore stems in contrast to callus cultures of this species which appear to lack the enzyme.[124] Wound vessel formation in *Coleus* internode slices is also paralleled by an increase in PAL activity.[125]

PAL and lignification are not correlated in all plants. Very little or no PAL is present in lignifying *Eucalyptus* leaves,[126] while in buckwheat the highest specific activity (per g fr.

[116] ENGELSMA, G. (1967) *Planta* **77**, 49.
[117] ENGELSMA, G. (1969) *Acta Bot. Neerl.* **18**, 347.
[118] ENGELSMA, G. (1968) *Acta Bot. Neerl.* **17**, 499.
[119] MAIER, V. P. and HASEGAWA, S. (1970) *Phytochemistry* **9**, 139.
[120] THORPE, T. A., MAIER, V. P. and HASEGAWA, S. (1971) *Phytochemistry* **10**, 711.
[121] HYODO, H. (1971) *Plant Cell Physiol.* **12**, 989.
[122] HARPER, D. B., AUSTIN, D. J. and SMITH, H. (1970) *Phytochemistry* **9**, 479.
[123] HIGUCHI, T. (1966) *Agr. Biol. Chem.* **30**, 667.
[124] RUBERY, P. H. and NORTHCOTE, D. H. (1968) *Nature* **219**, 1230.
[125] RUBERY, P. H. and FOSKET, D. E. (1969) *Planta* **87**, 54.
[126] HILLIS, W. E. and ISHIKURA, N. (1970) *Phytochemistry* **9**, 1517.

wt) is found in the stem apex (although 86% of the total activity is found in the region of the stem where secondary development is taking place).[65] In *Periploca graeca* and in tobacco very high specific activities have been found in young tissues and in the terminal bud.[127] Since very young tissues are often conspicuously colored with anthocyanin, obviously there is a demand for products of cinnamate metabolism other than lignins in these tissues and this should be borne in mind when interpreting correlations in PAL levels and the levels of particular polyphenols.

The same caution should be applied to interpreting the results of investigations of hydroxycinnamic esters and flavonoids. In the following examples the results appear paradoxical until one remembers; (a) that the 'normal' amount of PAL present may be sufficient to account for the throughput of carbon into cinnamate derivatives, and (b) there is always more than one class of cinnamate derivatives produced in a given tissue: in gherkin, a short exposure to R light causes an increase in hydroxycinnamic acids without affecting PAL levels.[116] Gherkin seedlings held at 10° develop high PAL levels even in dark, but synthesis of hydroxycinnamic acid is still dependent upon light.[113–115] In *Melilotus alba* the amounts of PAL are the same in strains which contain high or low levels of *o*-hydroxycinnamic acid.[128] Administration of certain DNA intercalating compounds to *Pisum* leads to increased production of the isoflavonoid, pisatin with no concomitant increase in PAL.[129] Other compounds of this kind do just the opposite, i.e. lead to increase in the levels of PAL. Similar lack of consistent correlation is noted in pea treated with histones,[130] drugs,[131] or infected by fungi.[132,133]

Strain AGI of a carrot tissue culture produces flavonoids but has a lower level of PAL than strain AGID which does not synthesize these compounds.[134] In strawberry leaf disks flavonoid formation shows phytochrome reversibility whereas PAL formation does not.[71] Further, if phenylalanine is fed to the disks at a concentration of 0·0026 M significant changes occur in the hydroxycinnamic acid levels without any changes in PAL levels.[72] Zucker[2] has shown that potato disks cultured on phenylalanine in either light or darkness contained less than half as much enzyme as corresponding samples supplied only with water. Chlorogenic acid synthesis, however, is increased in disks maintained on phenylalanine indicating clearly that the normal 24-hr increase in PAL levels in potato disks maintained in light is in excess of that required for chlorogenic acid synthesis.

It would appear, from these examples, that PAL is not the only site of control of cinnamate metabolism in plants. Indeed, Swain and Williams[135] cast doubt on the exclusive role of phenylalanine as a flavonoid precursor, although other work[72] reaffirms the role. A recent investigation of control of flavonoid synthesis in parsley cell suspension cultures may clarify the situation.[60,73] These cultures do not produce flavone glycosides in the dark but when exposed to high intensities of white light they start producing apiin and graveobioside-B, the normal flavone glycosides of parsley. The first stage in the synthesis of these flavones is reflected in a marked increase in the levels of eight enzymes which are probably involved in their synthesis. These enzymes are PAL, cinnamic acid 4-hydroxylase, *p*-coumarate:CoA

[127] PAYNOT, M., MELIN, D. and MARTIN, C. (1971) *Compt. Rend.* **273D**, 749.
[128] KLEINHOFS, A., HASKINS, F. A. and GORZ, H. J. (1966) *Plant Physiol.* **41**, 1276.
[129] HADWIGER, L. A. and SCHWOCHAU, M. E. (1971) *Plant Physiol.* **47**, 346.
[130] HADWIGER, L. A. and SCHWOCHAU, M. E. (1970) *Biochem. Biophys. Res. Commun.* **38**, 683.
[131] HADWIGER, L. A. and SCHWOCHAU, M. E. (1972) *Biochem. Biophys. Res. Commun.* **46**, 71
[132] HADWIGER, L. A. (1968) *Neth. J. Plant Path.* **74**, suppl. 1, 163.
[133] HADWIGER, L. A., HESS, S. L. and VAN BROEMBSEN, S. (1970) *Phytopath.* **60**, 332.
[134] SUGANO, N. and HAYASHI, K. (1968) *Bot. Mag.* **81**, 371.
[135] SWAIN, T. and WILLIAMS, C. A. (1970) *Phytochemistry* **9**, 2115.

ligase, chalcone-flavanone isomerase, a glucosyl transferase, an apiosyltransferase, UDP-apiose synthetase and an *O*-methyltransferase. The first three enzymes have as substrates phenylpropanoid derivatives and the enzymes reach a peak and start to decline within 15 hr after the onset of continuous illumination. The remaining enzymes which are all concerned with flavonoid synthesis reach a maximum 24 hr after illumination and only then do the levels decline. Regulation of the levels of these two sets of enzymes thus appear to be different. So far this is the most complete study of flavonoid biosynthesis at the enzyme level and the parsley cell culture system holds some promise of being suitable for elucidating the mechanisms of control of flavonoid synthesis.

The phenomenon of co-ordinate control of groups of enzymes may be a general one. In buckwheat, wounding and incubation in light of hypocotyls affects cinnamate hydroxylase in much the same way as it affects PAL[70,96] although these enzymes appear to be located on different sub-cellular particles.[27] The levels of PAL and *p*-coumarate:CoA ligase increase dramatically in batch propagated cells of *Glycine max* shortly before the stationary phase is reached.[28] The study of all the enzymes along a specific pathway, e.g. flavonoid biosynthesis, is likely to lead to a more meaningful assessment of the level of control of the pathway by PAL. Results so far suggest that PAL acts as a primary control and that enzymes concerned with subsequent steps in phenylpropanoid metabolism display secondary control.,

In general, PAL seems to be extraordinary sensitive to the physiological state of the plant. Levels change as the plant germinates and develops; in mature plants or dormant tissue such as the potato tuber, it is found only in very low levels. These changes can be the natural ones occurring during growth, or they may follow wounding, infection drug or γ-ray damage, or activation of phytochrome. It has been noted that there are many examples where there does not appear to be a direct correlation between the level of PAL and the production of a specific phenolic compound. This would be the case if, for example, the supply of phenylalanine were insufficient to saturate the enzyme, or if an enzyme further along a pathway were draining off the intermediate measured under the experimental conditions.