Phytochemistry, 1970, Vol. 9, pp. 2287 to 2290. Pergamon Press. Printed in England.

THE EFFECT OF LIGHT ON THE ACTIVITY OF ENZYMES OF THE AROMATIC PATHWAY IN PEAS AND MUNG BEANS

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(*Received* 27 *February* 1970)

Abstract—Red-light has no effect on the activity of 5-dehydroquinase dehydrate or shikimate: NADP oxidoreductase in dark-grown seedlings of mung beans, but both enzymes double in pea seedlings 20 hr after irradiation. Total phenylalanine ammonia lyase activity also doubles in both species after light treatment, but in mung bean exists in two forms, only one of which is photoinducible. 5-Dehydroquinate synthetase, 3-enolpyruvylshikimate-5-phosphate synthetase and shikimic acid kinase have been demonstrated in extracts from both species, the latter for the first time in higher plants.

SEVERAL workers have proposed that the key reaction controlling the biosynthesis of cinnamic acids, lignin and flavonoids in a wide variety of plants is the irreversible deamination of L-phenylalanine to *trans*-cinnamic acid.[1-8] This reaction is catalysed by L-phenylalanine ammonia lyase (PAL) which has been shown to undergo a light-mediated induction in several plant species.[9] Since phenylalanine is required also for the synthesis of protein and plastoquinone, the lyase reaction is obviously an important branch point in the overall metabolism of the amino acid.

It seemed probable to us, in view of the complexity of the control mechanisms of the aromatic pathway in bacteria and fungi,[10-13] that other enzymes of aromatic metabolism, as well as PAL, might be involved in control of the biosynthesis of flavonoid and related compounds in higher plants. It also appeared possible that more than one form of PAL might exist. This would enable control of the formation of either lignin, flavonoids or other phenylpropanoid compounds to be rapidly achieved. Indeed, two forms of PAL have been shown to exist both in the tubers of white potato (*Solanum tuberosum*)[9] and the roots of sweet potato (*Ipomoea batatas*)[14] but in neither case was it suggested that they might have different functions. We have investigated the effect of red-light on the activity of PAL and on several enzymes of the aromatic pathway in dark-grown pea (*Pisum sativum* var. Alaska) and mung

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[1] M. ZUCKER, *Plant Physiol.* **40**, 779 (1965).
[2] G. ENGELSMA, *Planta* **75**, 207 (1967).
[3] I. RISSLAND and H. MOHR, *Planta* **77**, 239 (1967).
[4] T. H. ATTRIDGE and M. SMITH, *Biochem. Biophys. Acta* **148**, 805 (1967).
[5] H. SCHERF and M. H. ZENK, *Z. Pflanzenphysiol.* **56**, 203 (1967).
[6] P. H. RUBERY and D. H. NORTHCOTE, *Nature* **219**, 1230 (1968).
[7] L. L. CREASY, *Phytochem.* **7**, 441 (1968).
[8] W. GRISEBACH and W. BARZ, *Naturwiss.* **56**, 203 (1967).
[9] E. A. HAVIR and K. R. HANSON, *Biochem.* **7**, 1896 (1968).
[10] H. TRISTAM, *Sci. Progr. Lond.* **56**, 449 (1968).
[11] C. H. DOY, *Rev. Pure Appl. Chem.* **18**, 41 (1968).
[12] S. I. AHMED and N. H. GILES, *J. Bacteriol.* **99**, 231 (1969).
[13] M. B. BERLYN and N. H. GILES, *J. Bacteriol.* **99**, 222 (1969).
[14] T. MINAMIKAWA and I. URITANI, *Agri. Biol. Chem.* **29**, 1021 (1965).

bean (*Phaseolus mungo*) seedlings. We have found that two separable forms of PAL exist in the mung bean seedlings, only one of which is inducible with red-light. Different forms of the enzyme apparently do not exist in the pea.

RESULTS

Our aim was to investigate, in dark-grown seedlings of peas and mung beans, the effect of red-light on the activity of several enzymes of the aromatic pathway. These were 5-dehydroquinate synthetase (DHQ synthetase), 5-dehydroquinate dehydratase (DHQase), shikimate-NADP oxidoreductase (DHS reductase), shikimate kinase (SA kinase), 3-enolpyruvylshikimate 5-phosphate synthetase (EPSP synthetase) and L-phenylalanine ammonia lyase (PAL). With the exception of SA kinase, all these enzymes have been previously detected in higher plants, including mung beans and peas.[4, 15]

Attridge and Smith[4] had previously shown in dark-grown pea buds that while the activity of PAL increased greatly after red-light treatment, the level of DHS reductase remained unchanged. They, like Rissland and Mohr,[3] used an unfractionated direct buffer extract as a source of enzyme. Most other workers[1, 2, 5–7] investigating the photoinduction of PAL, have used extracts prepared from acetone powders of the plant material. Both these procedures could give rise to errors in comparative studies: the first because of the possible presence of inhibitors[16] or unknown amounts of co-factors; and the second because of differential denaturation of the different enzymes being examined. We therefore decided to overcome these difficulties as far as possible in our experiments, by making a direct extract of the tissue and purifying the enzymes by a simple routine procedure. This involved treatment of the initial extract with protamine sulphate, and subsequent fractionation of the soluble extract with ammonium sulphate. We used two arbitrary cuts of 0–50% and 50–75% in all the investigations reported here. The enzyme activities in the dialysed soluble fraction from these cuts were estimated by standard procedures.[12, 13, 17, 18]

Using these methods, however, we have not found it possible to obtain repeatable quantitative estimations of the activity of three of the enzymes under study which would enable us to determine the effect of light with the desired degree of certainty. DHQ synthetase and EPSP synthetase were easily detected in the 50–75% and 0–50% cut respectively of both dark- and light-treated peas and mung beans, but their activity varied in an arbitrary fashion from one preparation to another.

SA kinase activity was weakly present in the 0–50% cut from both species when assayed by measuring the production of anthranilic acid fluorimetrically in the presence of an extract of the *arom 5* mutant of *Neurospora crassa*.[17] Attempts to increase the sensitivity of this method by the addition of L-phenylalanine and L-tyrosine to drive the initial product into the tryptophan pathway did not give any advantage. Using the more direct method with ^{14}C-labelled DL-shikimate,[13] however, the presence of the enzyme was unambiguously demonstrated in extracts of dark-grown pea seedlings. Unfortunately we did not try this method until the end of our series of experiments, so that comparisons with other extracts could not be made. However, this is the first report of DHS kinase in higher plants.

When examining the quantitative changes in the activities of the other three enzymes (DHQase, DHS reductase and PAL) we found that light increased the total extractable

[15] O. L. GAMBORG, *Can. J. Biochem.* **44**, 791 (1966).
[16] M. R. YOUNG, G. H. N. TOWERS and A. C. NEISH, *Can. J. Botany* **44**, 341 (1966).
[17] N. H. GILES, M. E. CASE, C. W. H. PARTRIDGE and S. I. AHMED, *Proc. Natl. Acad. Sci. U.S.* **58**, 1453 (1967).
[18] N. H. GILES, C. W. H. PARTRIDGE, S. I. AHMED and M. E. CASE, *Proc. Natl. Acad. Sci. U.S.* **58**, 1930 (1967).

protein by 60–70% (Table 1). Hence when we calculated the specific activities of the enzymes on this basis, we found, not surprisingly, that in several cases their activity actually decreased after light treatment. Since it appeared unlikely that the true specific activities would alter in this way and it seemed likely that much of the newly formed protein was concerned with other activities, we have expressed the activities in Table 1 on fr. wt. basis.

We found that in extracts of either mung bean or pea seedling both DHQase and DHS reductase are concentrated mainly in the 0–50% ammonium sulphate cut (Table 1). It can be seen from these results that the overall activities of these two enzymes in the mung bean were hardly affected by light. In the pea, on the other hand, the activity of both enzymes in the 0–50% fraction approximately doubled in the light-treated material (Table 1).

The total activity of PAL in these tissues was much lower than that of the other two enzymes and was distributed between the two salt fractions. In pea seedlings, light treatment

TABLE 1. THE EFFECT OF RED LIGHT ON THE ACTIVITY OF VARIOUS ENZYMES IN PEA AND MUNG BEAN SEEDLINGS

		Activity (I.U./g fr. wt. × 10^3) Ammonium sulphate fraction			
		0–50%		50–75%	
Source	Enzyme	Dark	Light	Dark	Light*
Pea†	DHQase	15·6	35·7	1·0	0·9
	DHS reductase	131	252	30·8	14·4
	PAL‡	170	328	59·1	115
Mung bean†	DHQase	26·8	30·2	1·1	1·0
	DHS reductase	103	110	3·3	44
	PAL	3·3	64·0	65·0	62·1

* Seedlings were grown in moist, washed, vermiculate at 27 ± 1° in the dark for 6 days. One batch was then given 10 min red-light (330 erg cm^2 sec^{-1}). The plants were harvested 20 hr later, the top 5 cm being used for enzyme extraction.

† Total protein in the fractions increased after light treatment from 1·74 to 2·74 mg/g fr. wt. in pea and from 2·68 to 4·60 mg/g fr. wt. in mung bean.

‡ Activity in I.U./g fr. wt. × 10^6.

increased activity equally in each fraction, to about twice that for the dark control. In mung bean, on the other hand, the enzyme was unequally distributed in the ammonium sulphate fractions from the dark-grown plants, nearly 95% being in the 50–75% cut. After irradiation, the activity in this fraction hardly changed, whereas the activity in the 0–50% fraction increased nearly 20-fold (Table 1).

DISCUSSION

We believe that the results present above show that the control of the biosynthesis of flavonoids, lignin and related compounds, is probably more complex than has hitherto been supposed. In mung bean there are plainly two separable PAL enzymes and only the one which is precipitable with 0–50% ammonium sulphate is light inducible. It may be that these two forms are allosteric.[6,9] We found that the rate of synthesis of soluble phenylpropanoid compounds (mainly chlorogenic acid) in light-treated mung bean seedlings (28 nM/min/g fr. wt.) is well within the compass of the activity of PAL at the level found in dark tissue

(69 nM/min/g fr. wt. total). On this basis we assume that the 50–75% enzyme may be mainly responsible for the pathway leading to caffeic acid and suggest that the 0–50% fraction of PAL in mung is perhaps responsible for controlling the transformation of phenylalanine into the lignin precursors. Lignification is known to increase rapidly on illumination.

The fact that PAL is not separable in extracts from pea seedlings does not mean that only one form exists there. It is possible that our routine procedures are inadequate for the separation of distinct species.

However, the fact that PAL, from both mung bean and peas, shows a much lower activity than the other enzymes examined (Table 1) indicates that this enzyme perhaps plays a more central role in regulating the biosynthesis of secondary aromatic compounds arising from phenylalanine. However, as we have pointed out elsewhere,[19] it may be equally true that there are routes to lignin and the flavonoids which do not involve phenylalanine. Much more work is required before this question can be satisfactorily settled.

EXPERIMENTAL

Materials. Peas were obtained from Asgrow Ltd., New Haven, Connecticut, U.S.A., mung beans were purchased locally. Both were soaked overnight in tap-water and planted in moist, washed, vermiculite. Further details of growing conditions and light treatments are given in Table 1.

Methods. The harvested plant material was frozen at −20° overnight and then ground with 0·1 of its weight of washed sand in an Omnimix macerator in 0·1 M phosphate buffer (pH 7·4) containing 5×10^{-4} dithiothreitol (1 ml/g). Polyvinylpyrrolidone (50 mg/g) was added in some preparations but did not appear to offer any advantage. Subsequent fractionations were carried out as described by Ahmed and Giles.[12]

Enzymes were assayed as described by Giles and his co-workers for the pre-aromatic enzymes[12,13,17,18] or for PAL by the method of Rissland and Mohr.[3] Protein was estimated by the biuret[20] method. Phenolic compounds were extracted into MeOH and estimated spectrophotometrically using suitable standards.

Acknowledgement—We wish to thank Professors A. W. Galston and N. H. Giles for helpful discussions.

[19] T. SWAIN and C. A. WILLIAMS, *Phytochem.* **9**, 2115 (1970).
[20] L. C. MORKASCH and R. W. MCGILVERY, *J. Biol. Chem.* **221**, 909 (1956).