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INCREASED PHENYLALANINE AMMONIA-LYASE ACTIVITY DUE TO LIGHT TREATMENT AND ITS SIGNIFICANCE FOR THE MODE OF ACTION OF PHYTOCHROME*

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Abstract—Short irradiations with red light lead to marked increases in phenylalanine ammonia-lyase levels, which are probably mediated through the phytochrome system. Continuous illumination with white, blue or far-red light leads to two peaks in extractable enzyme activity, one at *ca.* 4–8 hr, and a later peak at *ca.* 12–14 hr. These changes can be correlated with the changes in flavonoid concentration. The increases in enzyme activity are preceded by a lag phase of 60–90 min under all light treatments. The rate constants of phytochrome "decay" under the various treatments are linearly related to the rate constants of the early increases in enzyme activity, indicating that phytochrome "decay" may be an integral part of the mechanism of action of phytochrome.

INTRODUCTION

IT HAS been shown that irradiation of dark-grown Alaska pea seedlings leads to complex changes in the flavonoid pattern.[1-5] These changes can be resolved into two photosystems; one mediated through phytochrome and leading to rapid but small increases in KGC† and QGC levels, and the other requiring continuous illumination and resulting in much larger changes in flavonoid levels occurring after about 18 hr.[5] In several other species in which continuous illumination leads to increases in flavonoid or phenol contents, changes in the extractable activity of phenylalanine ammonia-lyase (PAL) (E.C. 4.3.1.5) have been observed.[6-11]

In a preliminary communication,[11] we reported that in Alaska peas the photoactivation of phytochrome by a short period of red light was sufficient to cause large increases in extractable PAL activity. The increases in PAL activity were red/far-red reversible, and were saturated at low energies of red light, indicating that phytochrome was probably the sole photoreceptor. The discovery of a later rise in flavonoid content dependent on continuous illumination stimulated us to investigate the effect of such treatments on PAL activity. It

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† KGC, kaempferol-3-*p*-coumaroyltriglucoside; QGC, quercetin-3-*p*-coumaroyltriglucoside; P_R, red-light absorbing form of phytochrome; P_{FR}, far-red light absorbing form of phytochrome.

[1] M. FURUYA and R. G. THOMAS, *Plant Physiol.* 39, 634 (1964).
[2] F. E. MUMFORD, D. H. SMITH and P. G. HEYTLER, *Biochem. J.* 91, 517 (1964).
[3] W. BOTTOMLEY, H. SMITH and A. W. GALSTON, *Nature* 207, 1311 (1965).
[4] W. BOTTOMLEY, H. SMITH and A. W. GALSTON, *Phytochem.* 5, 117 (1966).
[5] H. SMITH and D. B. HARPER, *Phytochem.* 9, 477 (1970).
[6] M. ZUCKER, *Plant Physiol.* 40, 779 (1965).
[7] G. ENGELSMA, *Planta* 75, 207 (1965).
[8] F. DURST and H. MOHR, *Naturwissenschaften* 53, 531 (1966).
[9] H. SCHERF and M. H. ZENK, *Z. Pflanzenphysiol.* 56, 203 (1967).
[10] C. NITSCH and J. P. NITSCH, *Compt. Rend.* 262, 1102 (1966).
[11] T. H. ATTRIDGE and H. SMITH, *Biochim. Biophys. Acta* 148, 805 (1967).

was considered that if PAL were the controlling enzyme (or one of a small number of controlling enzymes), mediating the effects of light on flavonoid synthesis, then the changes in flavonoid levels under continuous illumination should, at least to a degree, be reflected in changes in PAL levels. It will be seen that this prediction was largely borne out.

This paper deals mainly with the responses of PAL activity to light and its relationship with phytochrome action. A succeeding paper discusses the role of PAL in the control of flavonoid synthesis.[12]

RESULTS

Effect of Brief Red Irradiation on PAL Activity

Figure 1 shows that irradiation of 7-day dark-grown pea seedlings with 5 min weak red light (200 kergs/cm^2) brings about a marked increase in the activity of extractable PAL.

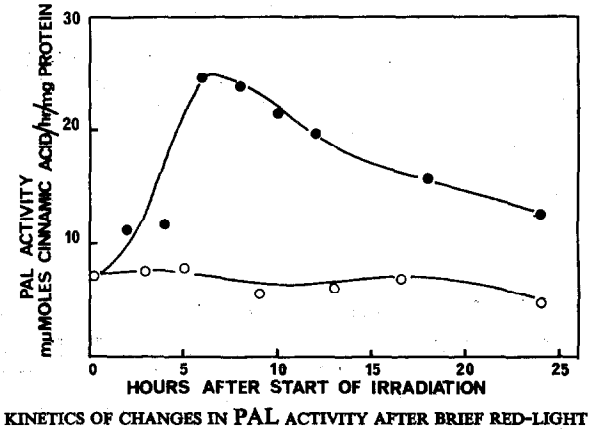

FIG. 1. THE KINETICS OF CHANGES IN PAL ACTIVITY AFTER BRIEF RED-LIGHT IRRADIATION. Seedlings grown in the dark for 7 days were treated with 15 min red light (solid symbols) or maintained in darkness (open symbols), and determinations of PAL activity made at intervals over 24 hr.

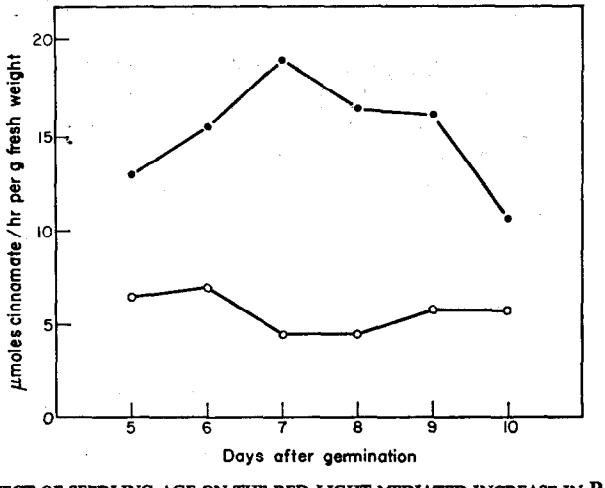

FIG. 2. THE EFFECT OF SEEDLING AGE ON THE RED-LIGHT MEDIATED INCREASE IN PAL ACTIVITY. Seedlings were grown in the dark for various periods and given 15 min red light 10 hr before the extraction times denoted by the symbols. Solid symbols, red-light treated; open symbols, dark controls.

[12] D. B. HARPER, D. J. AUSTIN and H. SMITH, *Phytochem.* **9**, 497 (1970).

The rise reaches a peak at about 6–8 hr, after which the activity falls. Over a period of 36–48 hr the level of extractable activity returns to that maintained over this period in dark-grown terminal buds. In order to determine the limits of reliability of this system, it was decided to investigate the response of peas grown for varying periods in darkness, to a brief irradiation with red light. Figure 2 shows that from 5 to 9 days of dark growth, red-light treatment brings about an increase in extractable PAL activity. In some experiments of this nature a possible endogenous rhythm of response to red light was detected, having a period of about 24 hr. However, this result has not been consistently achieved.

The response to red-light treatment is not immediate. In all experiments a lag phase of 60–90 min has been observed, as is exemplified by the detailed kinetics given in Fig. 3. In this case the results are expressed as differences between the light-treated and the dark-controls extracted at the same time.

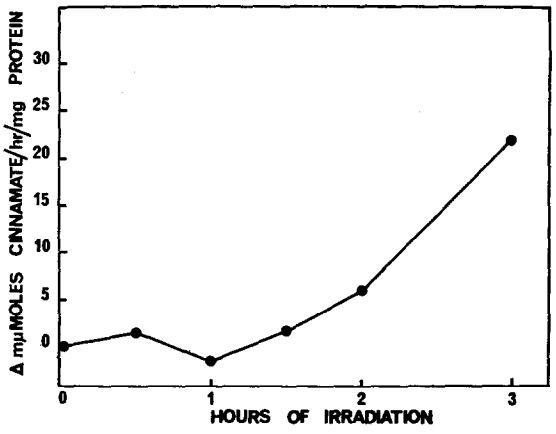

FIG. 3. THE LAG-PHASE IN THE RED-LIGHT MEDIATED INCREASE IN PAL ACTIVITY.

Seedlings grown for 7 days in darkness were treated with 15 min red light and determinations of PAL activity made at subsequent intervals over a period of 3 hr. Each point represents the difference between treated and control samples.

The Effects of Continuous Illuminations

If 7-day dark-grown seedlings are subjected to continuous illumination with white fluorescent, blue or far-red light, two components of increase in extractable PAL activity can be observed. Figure 4 shows the effect of continuous white light in which an early peak at 6–8 hr is followed by a much greater peak at around 12 hr. Figure 5 shows the behaviour in blue light, in which the early peak is the greater, but which is still followed by a prominent shoulder at approximately 12 hr. In far-red light (Fig. 6) an early shoulder is again followed by a peak at 12 hr. It should be noted that continuous white light leads to very much greater increases in extractable PAL activity than do any of the other irradiation treatments used. It is possible that the greater energy of the white source may be responsible; no attempts have as yet been made to assess the role of light energy in these responses.

When more detailed measurements are made of the early part of these responses (Fig. 7), it can be seen that there is in all cases a lag phase similar to that observed under the effect of brief red irradiations.

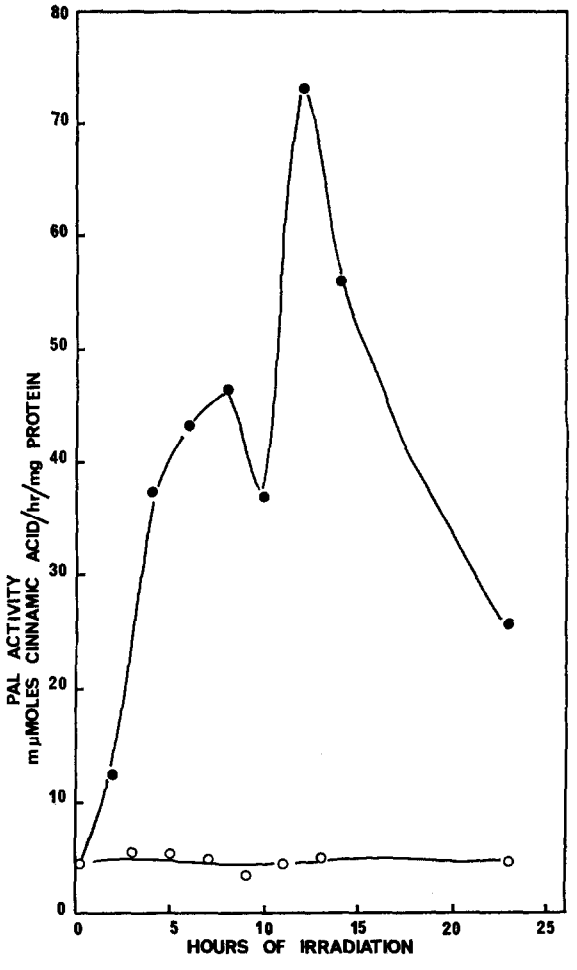

FIG. 4. THE EFFECT OF CONTINUOUS WHITE-LIGHT IRRADIATION ON THE LEVELS OF PAL ACTIVITY IN TERMINAL BUDS.

The procedure was as in the caption to Fig. 1, except the seedlings were placed under continuous white light. Solid symbols, light treated; open symbols, dark controls.

Phytochrome Decay and the Early Increase in PAL Activity

On close examination of the early kinetics it can be seen that the rise in extractable enzyme activity after the lag phase is very nearly linear under all treatments. However, the rate of increase of enzyme activity differs markedly between the various wavelength sources. Furthermore, it was considered possible that the early rises in activity under continuous illumination may be phytochrome mediated. Consequently, attempts have been made to relate the rates of increase of enzyme activity to various parameters of phytochrome.

Figure 8 gives the observed "decay" curves for phytochrome as established by the light sources used in the enzyme experiments. These results agree reasonably well with published reports.[13] A point of dissimilarity is the lag in "decay" under blue light. However, the existence of this lag of phytochrome decay in *Pisum* tissues under the effect of blue light has

[13] R. E. KENDRICK and B. FRANKLAND, *Planta* **82**, 317 (1968).

been confirmed by Clarkson (personal communication). The light sources used in this investigation set up the following percentages of phytochrome in the P_{FR} form: red, 80 per

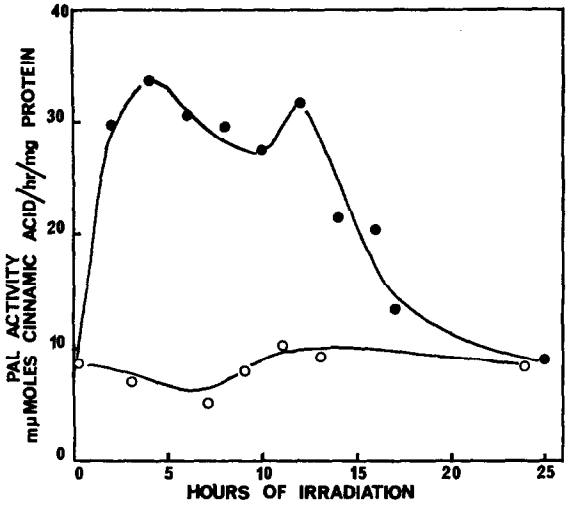

FIG. 5. THE EFFECT OF CONTINUOUS BLUE-LIGHT IRRADIATION ON THE LEVEL OF PAL ACTIVITY.
The procedure was as in the caption to Fig. 1 except the seedlings were placed under continuous blue light. Solid symbols, light treated; open symbols, dark controls.

cent; white fluorescent, 82 per cent; blue, 22 per cent; and far-red, 4·5 per cent. It has not been possible to establish any direct relationships between these photostationary states and the rate of increase in enzyme activity. However, if the rate constants of phytochrome decay

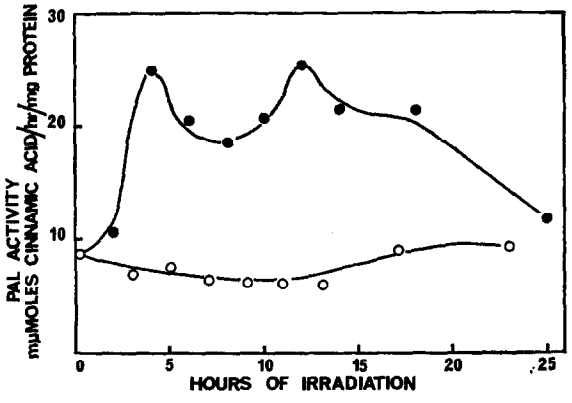

FIG. 6. THE EFFECT OF CONTINUOUS FAR-RED IRRADIATION ON THE LEVEL OF PAL ACTIVITY.
The procedure was as in the caption to Fig. 1 except the seedlings were placed under continuous far-red light. Solid symbols, light treated; open symbols, dark controls.

(calculated by the method of Kendrick and Frankland[13]) are plotted against the rate constants for the linear portions of the early increases in enzyme activity, then an apparent relationship emerges (Fig. 9). The rate constants for enzyme increases used in compiling Fig. 9 are averages of all the information available. The significance, or otherwise, of this relationship is discussed below.

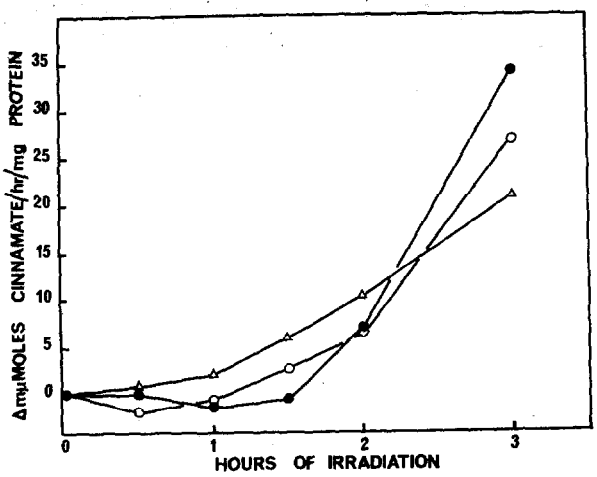

FIG. 7. EARLY KINETICS IN THE RISE OF PAL ACTIVITY UNDER CONTINUOUS WHITE, BLUE AND FAR-RED LIGHTS.

Enzyme activities were determined at intervals over 3 hr after placing 7-day dark-grown seedlings in continuous white (●), blue (○), or far-red (△) light.

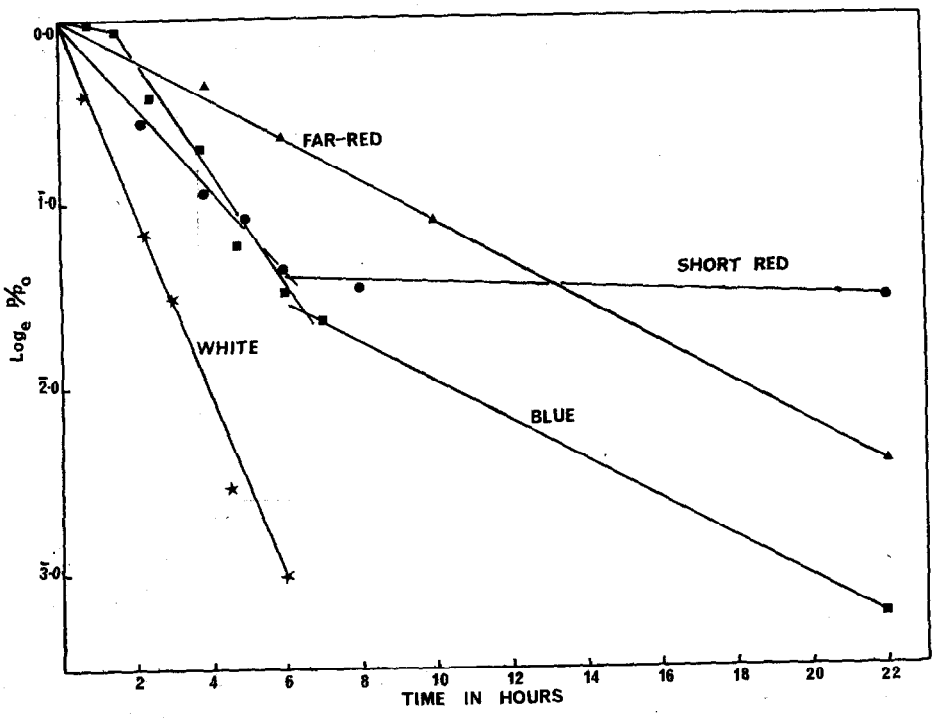

FIG. 8. THE LOSS OF TOTAL PHYTOCHROME IN TERMINAL BUDS.

Occurring upon exposure of 7-day dark-grown seedlings to 15 min of red (short red), or to continuous white (white), blue (blue) or far-red (far-red) illumination.

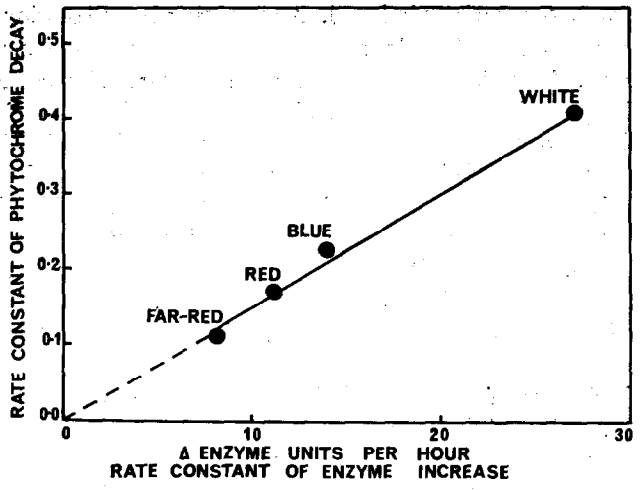

FIG. 9. LINEAR PLOT OF THE RATE CONSTANT OF PHYTOCHROME DECAY AGAINST THE RATE CONSTANT OF INCREASE IN ENZYME ACTIVITY BROUGHT ABOUT BY EXPOSURE OF 7-day DARK-GROWN SEEDLINGS TO 15 min OF RED (RED), OR TO CONTINUOUS WHITE (WHITE), BLUE (BLUE), OR FAR-RED (FAR-RED) LIGHT. THE ENZYME DATA REPRESENT MEAN VALUES FROM FOUR (RED, WHITE), AND THREE (BLUE, FAR-RED) EXPERIMENTS.

DISCUSSION

It is clear from these results that there are two components in the rise in PAL activity of the terminal buds under the influence of continuous illumination. All the light treatments used brought about an early rise in extractable enzyme activity which reached a peak (or shoulder) at 6–8 hr, followed by a further increase reaching a maximum at 12–14 hr. In all cases the second peak was followed by a decline in specific activity, eventually returning to the steady-state level maintained in dark-grown controls over the experimental period.

These changes in enzyme activity are closely correlatable with the changes in the end-products of the pathway, i.e. KGC and QGC. In the preceding paper,[5] it has been shown that a single irradiation with red light brings about small and transient increases in KGC and QGC, whilst continuous illumination with blue and white light lead to much larger increases occurring at 16–18 hr. Furthermore, the intracellular concentrations of KGC and QGC fall slowly after 18 hr, returning to the concentrations found in dark-grown tissues by about 36 hr. Thus there is very close correlation between the kinetics of the enzyme changes and those of the flavonoid changes, suggesting that PAL may have a regulatory role in the control of flavonoid biosynthesis. This question is considered more fully along with precursor incorporation evidence in a succeeding paper. [12]

The fact that the increases in PAL and flavonoids are out of phase by a few hours could be taken to indicate the existence of a characteristic induction and repression sequence. However, it should be stressed that, although evidence for the participation of enzyme synthesis in the responses of PAL levels to light treatment has been adduced in other tissues,[6–8] such evidence has not been obtained as yet for peas. Moreover, it would seem unlikely that a rise consisting of two separate components as found in the peas could be due to a simple induction of enzyme synthesis. Such a situation would only be possible if PAL existed in two isoenzymes, as suggested by Havir and Hanson,[14] and Minamikawa and Uritani,[15]

[14] E. A. HAVIR and K. R. HANSON, *Biochemistry* **7**, 1896 (1968).
[15] T. MINAMIKAWA and I. URITANI, *J. Biochem.* **57**, 678 (1965).

and the two forms were sequentially induced. A more simple explanation, however, would be that the two increases were mediated through separate mechanisms, e.g. enzyme activation and enzyme synthesis. Indeed, the abrupt nature of the early rise in PAL activity upon completion of the lag phase is more suggestive of enzyme activation than of enzyme synthesis.

The early rise in activity under continuous illumination follows closely similar kinetics to the rise brought about by a brief irradiation with red light, a rise which has been shown to be a straightforward, phytochrome-mediated response.[11] Thus, on kinetic grounds alone, it appears likely that the early rise in enzyme activity under continuous illumination is also phytochrome-mediated. However, it seems probable that the later increases under continuous illumination are not directly phytochrome-mediated. The much greater response to white light may be due to its higher energy content, although this aspect has not, as yet, been investigated.

A characteristic feature of these light-induced increases in enzyme activity is the lag phase of 60–90 min. A lag of similar magnitude has been observed by Mohr and his colleagues[16] in the photoinduction of PAL in *Sinapis alba* seedlings, where it has been taken to represent the time required for the de-repression of the gene for PAL (amongst others). Mohr *et al.*[16] have also shown that pre-irradiation of seedlings with far-red light leads to the elimination of the lag phase when the seedlings are later re-illuminated, suggesting that the gene is permanently de-repressed by the first illumination treatment. In similar experiments with peas, in which a brief red-light illumination followed by several hours of darkness was followed by a further red-light treatment, no further increase in PAL activity was detectable (unpublished results). Thus the situation in peas appears to be quite different from that in *S. alba*. This lack of a second increase in PAL may have been due to the observed decrease in rate of response to red light in de-etiolated pea tissues demonstrated by Fox and Hillman.[17]

Figure 7 shows clearly that the rate of the early, linear, rise in PAL activity is in some way dependent on the quality of illumination given. That intensity is probably not involved is shown by the fact that the rate of enzyme increase after a brief irradiation with red light is greater than with continuous far-red of very much higher energy. Furthermore, it has been demonstrated previously[11] that the early rise is saturated at low energies of red illumination. Since it seemed likely that the early rises were predominantly phytochrome mediated, it was argued that some relationship may exist between the rates of enzyme increase and some parameter of phytochrome. It is well known that lights of varying wavelength distributions establish different proportions of total phytochrome as P_R and P_{FR}.[18] Furthermore, since P_{FR} is known to "decay" to a spectrophotometrically undetectable form, probably through an enzyme-catalysed reaction,[19] the rate of loss of total detectable phytochrome is related to the quality of light treatment given. When the decay constants of phytochrome under the various light treatments are plotted against the rate constants of enzyme increase (Fig. 9), it is seen that they are apparently directly related. It must be admitted, of course, that only four points are available and that the observed relationship may well be fortuitous. However, if the relationship is real, it could well be of significance for the mode of action of phytochrome.

The nature of the "decay" process is not known, except that it is a thermochemical, metabolic reaction, leading to loss of spectrophotometrically detectable photoreversibility.[18, 19] This change could range from a complete degradation of the molecule to a

[16] H. MOHR, C. HUAULT, H. LANGE, L. LOHMANN, I. RISSLAND and M. WEIDNER, *Planta* **83**, 267 (1968).
[17] L. R. FOX and W. J. HILLMAN, *Plant Physiol.* **43**, 1799 (1968).
[18] W. L. BUTLER, H. C. LANE and H. W. SIEGELMAN, *Plant Physiol.* **38**, 514 (1963).
[19] L. H. PRATT and W. R. BRIGGS, *Plant Physiol.* **41**, 467 (1966).

relatively small change in its electronic configuration leading to differences in spectrophotometric properties. If a causal relationship between "decay" and metabolic action does exist, perhaps the simplest hypothesis is that P_{FR} is used up as it brings about the biochemical change. This hypothesis has been put forward previously.[20] Perhaps a more attractive alternative is that P_{FR} is not the active form, but that the active form is produced as a result of the "decay" process. In this case the metabolic responses to phytochrome photoactivation would be rate-limited by the rate of production of the active form by "decay" giving the relationship shown in Fig. 9. However, as is common with hypotheses of phytochrome action, contradictory evidence already exists. Dooskin and Mancinelli[24] have recently shown that coleoptile elongation in *Avena*, which comes under the control of phytochrome, is not related to the rate of decay of phytochrome.

EXPERIMENTAL

Plant Materials and Growth Conditions

Seeds of *Pisum sativum* var. Alaska were obtained from Carters, Wimbledon, England and growth conditions were as previously reported.[5]

Light Sources and Treatments

The sources of red, blue and white light were as previously reported.[5] The far-red light source consisted of 15 × 100 W single coil tungsten bulbs filtered through 10 cm running water, 1 layer of Cinemoid (Strand Electrics, London) No. 5A Deep Orange and one layer of No. 20 Deep Blue (Primary). The intensity at plant height was 5000 ergs cm^{-2} sec^{-1} and 90% of the radiant energy was above 735 nm. The temperature of the continuous illumination cabinets was maintained at 25° ± 1° by a refrigerated air-flow system.

Enzyme Extraction and Assay

Enzyme extraction and assay were carried out as reported previously,[11] with the exception that glutathione was omitted from both extraction buffer and reaction mixture. Protein assays were made by the biuret method.[21]

In Vivo *Phytochrome Determinations*

The phytochrome content of terminal buds was measured using a dual wavelength difference spectrophotometer (ASCO Ratiospect, R-2). Standard samples of 100 terminal buds were packed to 1 cm depth in a cylindrical cuvette of 12 mm dia., and were maintained at 0° by placing the cuvette in an ice bath. All manipulations were carried out under the green safe-light. Measurements of total phytochrome were determined from the change in absorbancy between 725 and 800 nm with alternating 2 min exposures of far-red (725 nm) and red (665 nm) light.[22] The values were divided by 0·8 to correct for incomplete conversion of P_R to P_{FR} by red light,[23] and results expressed as proportions of initial total phytochrome (P/P_0).

Acknowledgements—The authors are grateful for the support of research grants from the Science Research Council and the University of London Central Research Fund. T. H. A. is a recipient of a S.R.C. Postgraduate Studentship.

[20] W. S. HILLMANN, *Ann. Rev. Plant Physiol.* **18**, 301 (1967).
[21] E. LAYNE, in *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. III, p. 447, Academic Press, New York (1957).
[22] W. L. BUTLER and H. C. LANE, *Plant Physiol.* **40**, 13 (1965).
[23] W. H. KLEIN, J. L. EDWARDS and W. SHROPSHIRE, JR., *Plant Physiol.* **42**, 264 (1967).
[24] R. H. DOOSKIN and A. L. MANCINELLI, *Bull. Torrey bot. Club* **95**, 474 (1968).