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MECHANISM OF PHYTOCHROME ACTION IN THE CONTROL OF BIOSYNTHESIS OF ANTHOCYANIN IN *BRASSICA OLERACEA*

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Key Word Index—*Brassica oleracea*; Cruciferae; red-cabbage; anthocyanin; phytochrome; membrane permeability; phenylalanine ammonia lyase.

Abstract—The synthesis of anthocyanin in red-cabbage is very sensitive to control by light, R/FR reversibility being effected by exposures of 5 min duration. The demonstration of this control does not depend upon a preceding irradiation of high-intensity light but depends upon the duration of incubation in darkness subsequent to irradiation. R/FR reversibility is well shown in seedlings kept in darkness for 48 hr after exposure but after 120 hr this reversibility is no longer evident. This is due to the fact that a further synthesis of anthocyanin occurs in unexposed seedlings and in FR and R/FR treated material in the period from 48 to 120 hr but does not occur in the R treatment after 48 hr. Reagents such as *n*-propanol which are believed to increase membrane permeability, greatly increase anthocyanin synthesis in dark grown material. *n*-PrOH also reverses the effect of 5 min FR irradiation but, by contrast with R light, does not promote PAL activity. It is concluded that the limitation to synthesis in material unexposed to light is substrate availability at the site of flavonoid biosynthesis, rather than the level of PAL activity. The evidence presented supports the hypothesis that R/FR reversible phytochrome action involves the control of the passage of substrate through a membrane to the site of anthocyanin biosynthesis.

INTRODUCTION

IT IS well known that anthocyanin synthesis in a wide range of tissues is promoted by light and that there are at least two photoreactions involved in its control.[1] These are, (a) a low-energy R/FR reversible phytochrome controlled reaction, and (b) a "high-energy" reaction. The light stimulated synthesis of anthocyanin characteristically exhibits a lag-phase of about 6 hr duration,[2] followed by a linear period of relatively rapid synthesis.

One hypothesis which has been advanced to explain the influence of phytochrome on photomorphogenetic phenomena such as the synthesis of flavonoids, rests upon the established R/FR reversible stimulation of phenylalanine ammonia lyase (PAL) (E.C. 4.3.1.5) activity.[3] The activity of this enzyme generally rises after a short lag-phase of about 1 hr duration and a peak is reached a few hr later.[4] However, there are some R/FR reversible phenomena, such as the movements of *Mimosa* leaflets, in which the period between irradiation and response is as short as a few min.[5] This has led to an alternative hypothesis that phytochrome affects membrane permeability.[6–8]

[1] SIEGELMAN, H. W. (1964) *Biochemistry of Phenolic Compounds* (HARBORNE, J. B., ed.), p. 437, Academic Press, London.
[2] FURUYA, M. (1968) *Prog. Phytochem.* **1**, 347.
[3] ATTRIDGE, T. H. and SMITH, H. (1967) *Biochim. Biophys. Acta* **148**, 805.
[4] SMITH, H. and ATTRIDGE, T. H. (1970) *Phytochemistry* **9**, 487.
[5] FONDEVILLE, J. C., BORTHWICK, H. A. and HENDRICKS, S. B. (1966) *Planta* **69**, 357.
[6] HENDRICKS, S. B. and BORTHWICK, H. A. (1967) *Proc. Nat. Acad. Sci. U.S.* **58**, 2125.
[7] JAFFE, M. J. and GALSTON, A. W. (1967) *Planta* **77**, 135.
[8] SMITH, H. (1970) *Nature* **227**, 665.

The work reported here was carried out to investigate the validity of both the above hypotheses with respect to the low-energy phytochrome control of anthocyanin synthesis in red-cabbage seedlings. The PAL hypothesis was tested by comparing the development of activity of the enzyme with the progress of anthocyanin accumulation after R and/or FR treatment. The permeability hypothesis was tested by the use of reagents which are believed to affect membranes. This approach stemmed from the knowledge that dimethylsulphoxide (DMSO)[9] and *n*-propanol (*n*-PrOH)[10] are valuable reagents in the assay of certain enzymes in intact plant tissue, their effects being considered to involve increases in membrane permeability.[9,10] These usages of such reagents raised the possibility that at lower concentrations than employed in the enzyme assays, effects on membranes might be experimentally induced without causing irreversible damage. Further, if these treatments were comparable in duration to R/FR treatments, it should be possible to combine the two approaches in order to determine whether the effects of such reagents and FR are mutually reversible.

TABLE 1. EFFECT OF DURATION OF DARK INCUBATION ON ANTHOCYANIN SYNTHESIS IN *Brassica oleracea*

Time after treatment (hr)	Anthocyanin content (A 525 nm)				
	Dark	R	FR	R/FR	FR/R
48	0·29 ± 0·02	0·36 ± 0·01	0·27 ± 0·02	0·33 ± 0·01	0·39 ± 0·03
120	0·35 ± 0·01	0·37 ± 0·04	0·34 ± 0·03	0·38 ± 0·03	0·39 ± 0·04

Seedlings were grown for 2 days in darkness prior to 5 min exposure to R and/or FR. The figures are means of 16 observations and standard errors are shown.

RESULTS

Preliminary work revealed that red-cabbage seedlings synthesize a limited amount of anthocyanin when grown in complete darkness but the quantity produced is only about 20% of that formed in continuously illuminated material over a 7 day period of growth. Very little pigment is formed in either instance in the first 2 days of growth but during the third day a marked increase in synthesis takes place. For this reason 2-day-old dark grown seedlings were selected for the present study. Chromatographic examination revealed the same major anthocyanin in cotyledons and hypocotyl; this was a glycoside of cyanidin. Harborne[11] reported the pigment of red-cabbage to be cyanidin 5-glucoside, 3-sophoroside (in acylated form).

Exposure of seedlings to 5 min R light promotes a small but highly significant increase in anthocyanin synthesis during a subsequent 48 hr period of dark incubation and the influence of R is completely nullified by 5 min exposure to FR (Table 1). The operation of a typical low-energy photoreversible phytochrome control is thereby established. However, if the seedlings are left in darkness until 120 hr after the light exposures, little difference in anthocyanin contents is evident between the treatments (Table 1). Seedlings which received an ultimate exposure to R light synthesized no more anthocyanin in the period 48–120 hr. By contrast, unexposed seedlings and those which received an ultimate exposure to FR, synthesized a further amount of anthocyanin and as a consequence the R/FR reversibility was masked. While the changes in behaviour of the material during prolonged

[9] DELMER, D. P. and MILLS, S. E. (1969) *Plant Physiol.* **44**, 153.
[10] JAWORSKI, E. G. (1971) *Biophys. Res. Commun.* **43**, 6.
[11] HARBORNE, J. B. (1963) *Phytochemistry* **2**, 85.

dark incubation are of some interest, it is the intention in the present communication to confine attention to the initial phase of synthesis.

The time course of synthesis during the early hours of dark incubation (Fig. 1) reveals the existence of a lag-phase of *ca* 6 hr duration.[12] The most significant observations are that synthesis occurs in R and unexposed material over approximately the same time period and at different rates (Fig. 1). Thus the R light induced promotion of synthesis occurs at a time when the tissue has an inherent tendency to produce pigment. After R/FR treatment, no more pigment is produced up to 30 hr than in the dark control.

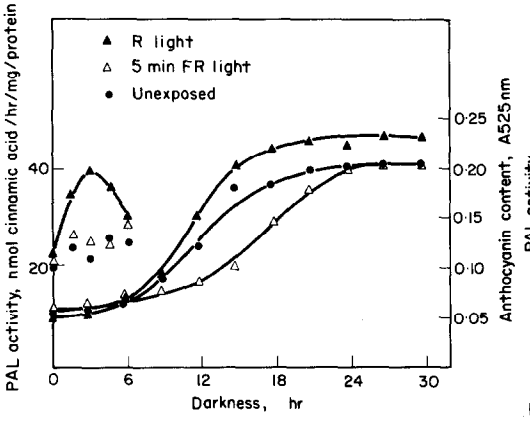

FIG. 1. INFLUENCE OF R, R/FR AND DARKNESS ON ANTHOCYANIN SYNTHESIS AND PAL ACTIVITY IN *Brassica oleracea*.
2-Day-old dark grown seedlings were exposed for 5 min to R light or 5 min R followed by 5 min FR or unexposed to light. Changes in PAL and anthocyanin level were followed during subsequent dark incubation. The PAL data are means of 4 observations and those of anthocyanin are means of 5.

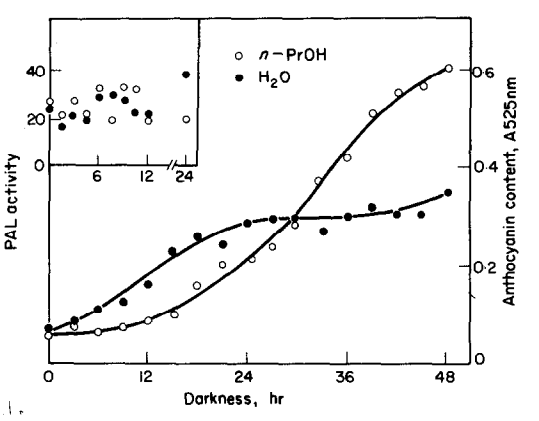

FIG. 2. INFLUENCE OF *n*-PrOH ON ANTHOCYANIN SYNTHESIS AND PAL ACTIVITY IN *Brassica oleracea*.
2-Day-old dark grown seedlings were treated for 15 min with 1% *n*-PrOH or water. Changes in PAL and anthocyanin level were followed during subsequent dark incubation. The PAL data are means of 4 observations and those of anthocyanin are means of 5. PAL activity—units as Fig. 1.

A small increase in PAL activity precedes the appearance of anthocyanin in the R treated material but no significant change in PAL activity is detectable in R/FR treated or unexposed seedlings. The time sequence is therefore consistent with the increase in anthocyanin synthesis following R exposure being a result of the increase in PAL activity. However, the fact that some increase in anthocyanin synthesis occurs in treatments which do not exhibit an increase in PAL activity must raise doubts about the nature of the limitation of synthesis.

To test the hypothesis that phytochrome control may involve changes in membrane permeability, the effects of PrOH and DMSO were investigated over a broad concentration range. Both of these reagents, when applied to 2-day-old dark grown seedlings, were found to promote anthocyanin synthesis quite markedly at concentrations which did not visibly affect either the condition or growth of the seedlings in any way over a period of 4 days in darkness. The optimal concentration of PrOH was 0·2%, which increased synthesis by 54%, and for DMSO was 1·5% which was as effective as PrOH. It was then found that the application of PrOH for only 15 min at an optimal concentration of 1% increased

[12] FURUYA, M. (1968) *Prog. Phytochem.* **1**, 347.

anthocyanin synthesis by 54% during 3 days growth in darkness. The time course following 15 min treatment with PrOH (Fig. 2) shows the magnitude of its effect and reveals that, by comparison with untreated dark grown seedlings, anthocyanin synthesis continues throughout the first 30 hr. The lag phase is longer in the PrOH treatment, which might imply some initial inhibitory effect of PrOH. The data for PAL (Fig. 2) show very clearly that the PrOH promoted increase in anthocyanin synthesis is not preceded by any increased activity of this enzyme.

TABLE 2. EFFECT OF PROPANOL ON PHYTOCHROME CONTROLLED ANTHOCYANIN SYNTHESIS IN *Brassica oleracea*

Treatment	Anthocyanin content (A 525 nm)				
	Dark	R	FR	R/FR	FR/R
Water	0·39 ± 0·01	0·57 ± 0·03	0·40 ± 0·03	0·41 ± 0·01	0·57 ± 0·03
PrOH before light	0·67 ± 0·01	0·72 ± 0·04	0·47 ± 0·02	0·50 ± 0·03	0·79 ± 0·02
PrOH after light	0·67 ± 0·01	0·71 ± 0·02	0·66 ± 0·04	0·67 ± 0·01	0·79 ± 0·02

Seedlings were grown for 2 days in darkness and then treated with either 1% PrOH or H_2O for 15 min before or after exposure to R and/or FR. Period of dark incubation subsequent to treatment was 48 hr. The figures are means of 5 observations and standard errors are shown.

The fact that PrOH stimulates pigment synthesis when the duration of treatment is as short as 15 min made it possible to investigate the relationship between its effect and those of short periods of exposure to R and FR. Propanol and 5 min R together produce a greater stimulation of anthocyanin synthesis than either treatment induces on its own and the effect is similar irrespective of the sequence of the treatments (Table 2). However, if PrOH treated material is subsequently treated with 5 min FR irradiation, very little more pigment is formed than with FR alone. On the other hand, if the FR irradiation precedes the PrOH treatments, a good deal more pigment is produced than with FR alone. These observations suggest that PrOH and R light both promote synthesis and that either can reverse the effect of FR. These data are consistent with the notion that the effects of R and FR irradiation are upon membrane permeability.

TABLE 3. EFFECT OF EXOGENOUSLY APPLIED SHIKIMIC ACID AND CINNAMIC ACID ON ANTHOCYANIN SYNTHESIS IN *Brassica oleracea* IN PRESENCE AND ABSENCE OF PrOH

Treatment	Anthocyanin content (A 525 nm)	Treatment	Anthocyanin content (A 525 nm)
Water	0·38	PrOH	0·47
Shikimic acid	0·39	PrOH + shikimic acid	0·63
Cinnamic acid	0·47	PrOH + cinnamic acid	0·73

Seedlings were grown for 2 days in darkness and then treated with H_2O or 1% PrOH for 15 min before transfer to the substrate solutions in which they were left for a further 3 days in darkness. Concentration of shikimic acid was 20 mg/l and that of cinnamic acid was 200 mg/l. The figures are means of six observations.

In the further assessment of the nature of the limitation of dark synthesis, the influence of exogenously applied substrate is of some importance. The data in Table 3 concern the effect of the flavonoid precursors shikimic and cinnamic acids, applied to dark grown material at what were found to be optimal concentrations, in the presence and absence of PrOH. It is very clear that neither of these substrates brings about a marked increase in pigment production when applied in water. PrOH increases synthesis substantially and

either substrate in the presence of PrOH promotes a greater production of anthocyanin than PrOH on its own. These data are consistent with the concept that PrOH facilitates the entry of substrate and that the limitation to synthesis is substrate availability.

DISCUSSION

The results presented above demonstrate that anthocyanin synthesis in red-cabbage is very sensitively controlled by light, a period of 5 min R irradiation being sufficient to promote a significant increase in pigment production. This synthesis is clearly controlled by the low-energy phytochrome system. Whether or not this phytochrome control can be demonstrated is shown to be a function of the duration of incubation subsequent to the light treatment(s). When too long a period of dark incubation is employed, a further synthesis occurs in those treatments in which there had been no ultimate exposure to R light (Table 1). It is interesting to note that it is not necessary to provide a period of high-intensity light before the brief R and/or FR irradiation in order to demonstrate R/FR reversibility (see Smith[13]).

R/FR reversibility in respect of 5 min irradiations has also been recently reported for anthocyanin synthesis in turnip seedlings by Schneider and Stimson,[14] though their material produced less pigment than red-cabbage, in mustard seedlings by Mohr,[15] and in our laboratory by French[16] for betacyanin synthesis in *Amaranthus caudatus*.

Studies on PAL in the present work reveal that, as shown by others[4,17] in flavonoid synthesising tissues, R light promotes an increase in activity of the enzyme and this effect is reversed by FR. The increase in PAL activity occurs before a detectable increase in anthocyanin content and therefore R/FR control of this enzyme activity could be responsible for the control of pigment production. However, the fact that unexposed seedlings exhibit some increase in anthocyanin synthesis at the time that this occurs in the R treatments but show no increase in PAL activity, raises the possibility that substrate rather than PAL limits the rate of anthocyanin synthesis in dark grown material.

It is obviously of the utmost importance to clarify the question of the nature of the limitation to dark synthesis in unexposed material, since it is vital to a decision as to the validity of the hypotheses of phytochrome control. Very little is known about the site of flavonoid synthesis in the cell, or whether there are pools of substrate which are separated from the enzymes concerned in synthesis. The new approach, employing reagents which increase membrane permeability, demonstrates that they increase anthocyanin synthesis substantially in unexposed seedlings. On the other hand there is no evidence that such treatments with PrOH stimulate PAL activity in the unexposed seedlings at any time during the lag-phase of anthocyanin synthesis, nor in the early hours of pigment production. We interpret these results to indicate that in material unexposed to light, substrate is limited at the site of synthesis. This could be the case if the enzyme were separated from the bulk of the substrate by a membrane. R/FR control of anthocyanin synthesis could then be explained by the assumption that phytochrome occurs in this membrane and that the molecular configuration of phytochrome controls the rate at which substrate passes through the membrane to the site of flavonoid synthesis. Evidence to support this view

[13] SMITH, H. (1972) *Phytochrome* (MITRAKOS, K. and SHROPSHIRE, W., eds.), p. 446, Academic Press, London.
[14] SCHNEIDER, M. and STIMSON, W. (1972) *Proc. Nat. Acad. Sci. U.S.* **69**, 2150.
[15] MOHR, H. (1972) *Lectures on Photomorphogenesis*, p. 10, Springer, Berlin.
[16] FRENCH, C. J. (1972) Ph.D. Thesis, University of Manchester.
[17] ENGELSMA, G. (1970) *Acta Bot. Neerl.* **19**, 403.

comes from the fact that a brief treatment with PrOH reverses the inhibitory effect of a previous exposure to FR.

Further support for the above view comes from the results of feeding shikimic acid and cinnamate to seedlings in the dark. These precursors stimulate anthocyanin synthesis considerably only when the seedlings have been treated with PrOH. This implies that these substances only reach the site of synthesis in quantity when the membrane is sufficiently permeable to them.

If the above hypothesis is correct, the promotion of PAL activity by R light may not be of functional significance in respect of brief periods of irradiation. However, it is not suggested that light promoted increases in PAL activity are without significance in circumstances where large additional quantities of photosynthetically derived substrate are available and where the membranes are fairly permeable to the substrates.

The nature of the membrane separating the enzymes of flavonoid biosynthesis from their substrates can only be a matter for speculation. However, it seems worth pointing out that organelles which are clearly visible with the optical microscope, and which contain high concentrations of red pigment, have been recognised for many years and called anthocyanophores.[18] These structures are present in the anthocyanin containing cells of red-cabbage and have been reported in a few other species.

That the effect of propanol is not unique is evident from the fact that DMSO too increases pigment synthesis in dark grown seedlings of red-cabbage (p. 817). Further, we have some preliminary evidence that DMSO and FR effects are to some extent mutually reversible. Jaffe[19] has demonstrated that acetylcholine (ACh) will replace the effect of R light in reducing the formation of secondary roots in mung bean seedlings, in inducing increased H^+ efflux and causing the root tips to adhere to a negatively charged glass surface. However, it does not appear that this worker has reported any mutually reversible relationship between ACh and FR light. In the present material ACh at a concentration of 8 mM increased anthocyanin synthesis in dark grown seedlings by 27% but no evidence is as yet forthcoming that this compound displays a mutually reversible relationship with FR.[20]

EXPERIMENTAL

Plant materials and growth conditions. Seeds of red-cabbage (*Brassica oleracea* L. cv. Stockley's Giant Red) (Hurst, Gunson, Cooper and Taber Ltd), were sown 40 to a Petri dish on two 9 cm circles of filter paper moistened with 4 ml dist. H_2O. They were grown in the dark at 25 ± 0·5° at high humidity.

Light sources and treatments. Red light exposures were carried out in a chamber illuminated by four 13 W warm white Atlas tubes, employing two sheets of red cinemoid No. 14 and one sheet of orange No. 5 cinemoid (Strand Electrics). The energy at seedling height in this chamber was 0·10 mW/cm^2. FR exposures were given in a cabinet illuminated by twenty-two 60 W Crysel Co. tubes and a filter system comprising two sheets of blue cinemoid No. 20 and two of orange No. 5 with a 10 cm deep water-screen between the lights and filters. The energy at seedling height was 0·42 mW/cm^2. Both chambers were housed in a dark room and all manipulations of material were carried out under a safelight with No. 39 green cinemoid.[21]

Anthocyanin extraction, assay and chromatography. The anthocyanin in samples of 10 seedlings was completely extracted using 10 ml 1% aq. HCl in three successive aliquots of 5, 2 and 3 ml. In the first aliquot the tissue was kept in a boiling water-bath for 20 min to facilitate the extraction of the pigment. The use of 1% methanolic HCl[22] proved unsatisfactory for the present material, as it had also been for radish seedlings,[23] since brown coloration developed which interfered with the anthocyanin assays. The extracts were filtered through GF/A paper

[18] BLANK, F. (1947) *Bot. Rev.* **13**, 241.
[19] JAFFE, M. J. (1970) *Plant Physiol.* **46**, 768.
[20] PECKET, R. C. and HATHOUT BASSIM, T. A. unpublished results.
[21] GRILL, M. and VINCE, D. (1964) *Planta* **63**, 1.
[22] SIEGELMAN, H. W. and HENDRICKS, S. B. (1957) *Plant Physiol.* **32**, 393.
[23] COSTIGAN, W. H. (1970) Ph.D. Thesis, University of Manchester.

and the absorbance determined at the E_{max} of the anthocyanin (525 nm). The bulk of the anthocyanin in the seedlings was in the cotyledons but some was in the hypocotyls. Separate extraction of the two organs gave no evidence for marked differences in response between them in the experiments reported here and for this reason only total values are given. In common with most anthocyanin forming materials, the pigment is superficial in location, being confined to the epidermal and sub-epidermal cells. Chromatographic separations of acid hydrolysed extracts of anthocyanin were carried out, with cyanidin as a reference, on paper in H_2O–HOAc–conc. HCl (10:30:3)[24] and in BuOH–2N HCl (1:1, upper).[25] Unhydrolysed 1% HCl extracts were chromatographed in *n*-BuOH–HOAC–H_2O (12:3:5).[26]

Propanol treatment. Short duration treatments were carried out by the transfer of seedlings in the dark room to a fresh Petri dish containing 4 ml of a 1% soln of the reagent followed by return to the original dish. It was found that with the age of seedling employed no significant damage was done to the material during this operation.

Enzyme extraction and assay. PAL activity was assayed using a modification of the methods of Attridge and Smith.[3,23] A sample of 25 seedlings was taken, and the shoot tissue crushed in a chilled hammer press. A 3 ml aliquot of borate buffer (0·1 M, pH 9·0) was added, the preparation filtered through glass fibre paper, the filtrate passed through a Sephadex G25 column equilibrated with the extraction buffer and 2·5 ml of extract collected. All assays were carried out at 38° using a total of 2·5 ml (enzyme 0·5 ml; reduced glutathione 8·57 μmol in 0·05 ml buffer; phenylalanine 114 μmol in 0·95 ml buffer; borate buffer 1 ml). Production of cinnamate was followed over the initial linear period by measuring the absorbance (A) at 290 nm[27] against a similar mixture without substrate. The identity of the product was ascertained by plotting an absorption spectrum of an ether extract of reaction mixture after 6 hr incubation and comparing with that of an authentic specimen (E_{max} 270 nm in Et_2O). For the determination of specific activity, protein determinations were carried out by the Folin phenol method.[28]

Not all the experiments reported here were carried out on the same batch of seed and some of the differences in magnitude of response are a reflection of this.

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[26] SMITH, I. and SMITH, M. (1965) *Paper and Thin-layer Chromatography and Electrophoresis* (SMITH, I. and FEINBURG, J. G., eds.), p. 155, Shandon Scientific, London.
[27] ZUCKER, M. (1965) *Plant Physiol.* **40,** 779.
[28] LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J. (1951) *J. Biol. Chem.* **193,** 265.