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An Analysis of Phytochrome-mediated Anthocyanin Synthesis

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ABSTRACT

Phytochrome (far red form) alone can mediate anthocyanin synthesis in the mustard seedling (*Sinapis alba* L.). Complete photoreversibility and reciprocity, for both red and far red light exposures over a period of at least 5 minutes, demonstrate this phytochrome involvement.

The duration of the initial lag-phase is constant (about 3 hours at 25 C) for seedlings more than 30 hours old and is specific for the system, being independent of the dose or quality of light. Since a complete reversal by far red of a red light induction is possible only during a 5 minute period, phytochrome (far red form) obviously mediates anthocyanin synthesis during the lag-phase although the actual synthesis of pigment can proceed only after the lag-phase is overcome. We suggest that phytochrome (far red form) exerts a double function during the initial lag-phase. It mediates both the build up of a biosynthetic potential ("capacity") and anthocyanin synthesis. However, the sequence of events leading to anthocyanin is arrested at some intermediate stage until this "capacity" is built up after 3 hours. Once "capacity" is achieved it does not decay readily. Therefore, no significant "secondary lag-phase" occurs if the seedling, under appropriate conditions, is reirradiated after an intervening dark period. The rate or extent of synthesis for both anthocyanin and lipoxygenase, previously reported (32), are functions of the amount of phytochrome (far red form). No "phytochrome paradoxes," *i.e.*, nonrational relationships between the amount of phytochrome (far red form) and rate or extent of response, were detected. This fact suggests that the mustard seedling is especially well suited for investigating the biophysical and molecular mechanisms of phytochrome action.

Since the well known report by Arthur (1), the effect of light on anthocyanin synthesis has attracted the attention of many plant physiologists (5, 21, 37). However, the general mechanism of this light effect has not yet been formulated. This failure is due partly to the descriptive nature of many reports and, in the main, because light appears to exert its influence through different photochemical mechanisms which probably are mixed in the usual experimental approaches. Recently, (*e.g.*, 2, 6, 7, 9, 10, 11, 35, 40) it had to be concluded that at least two photochemical reactions or steps were involved and that these mechanisms and interactions are by no means clear.

The mustard seedling (*Sinapis alba L.*) has a number of advantages for attacking directly this question: Can anthocyanin synthesis be mediated exclusively without the interference of any other photochemical mechanism? These advantages are that (24) anthocyanin synthesis by light is predominantly controlled by phytochrome without the requirement of any prolonged "high energy pretreatment" (*e.g.*, 2, 6, 10, 35), and the mustard seedling does not produce significant amounts of anthocyanin in complete darkness. Seedlings can easily be handled under standardized conditions, and a great amount of information, including spectrophotometric measurements of P_{fr},[2] is available on this system (26). While the mustard seedling forms five anthocyanins, the aglycon is always cyanidin (17). At 25 C the stored fat and protein of the cotyledons allow development in total darkness without any indications of starvation for at least 72 hr after sowing (19).

Furthermore, it was hoped that a detailed analysis of the initial lag-phase (*i.e.*, the duration between the onset of light and the first appearance of anthocyanin) would improve our knowledge of "phytochrome action." A final important question is whether the spectrophotometrically detectable phytochrome content and the rate or degree of anthocyanin synthesis are rationally related. In other words, do the data force us toward so-called paradoxes (nonrational relationships) (*e.g.*, 4, 18, 22)? Phytochrome-mediated control of lipoxygenase synthesis (32) in the mustard seedlings leads to no "paradoxes." If the same result were obtained for phytochrome-mediated anthocyanin synthesis, the previous conclusion is greatly strengthened that the mustard seedling is very well suited to relate the spectrophotometrically detectable phytochrome content to the extent or to the rate of responses.

MATERIALS AND METHODS

Two different seed populations of white seeded mustard (*Sinapis alba L.*) were used; namely, seed of a progeny of the original sample, sample 1, (24) and seed of a new large sample bought from a commercial grower, sample 2. Quantitative differences were observed but both seed samples responded in essentially the same way. Selection of seeds and treatment of seedlings followed the standard techniques developed previously (26) in our laboratory. The seedlings were grown at 25 C in the dark, the light treatment was started 36 hr after sowing, which is taken as time zero in the figures. Sowing was done under weak green safelight (27) to prevent any light influence on the soaked seeds.

For irradiation, the standard red (29) and far red (26) sources were used at an irradiance of 67.5 μw/cm^2 ± 10% (red) and 350 μw/cm^2 ± 10% (far red) or fractions of them (29). If required, the experiments were performed in an interference filter monochromator system (30). In this case red light was generated by an interference filter (Schott & Gen., Mainz) of the IL-type (single line interference filter) (λ_{max}: 658 nm; bandwidth: 12 nm), while far red light was generated

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[2] Abbreviation: I: irradiance; P_{fr}: phytochrome (far red form).

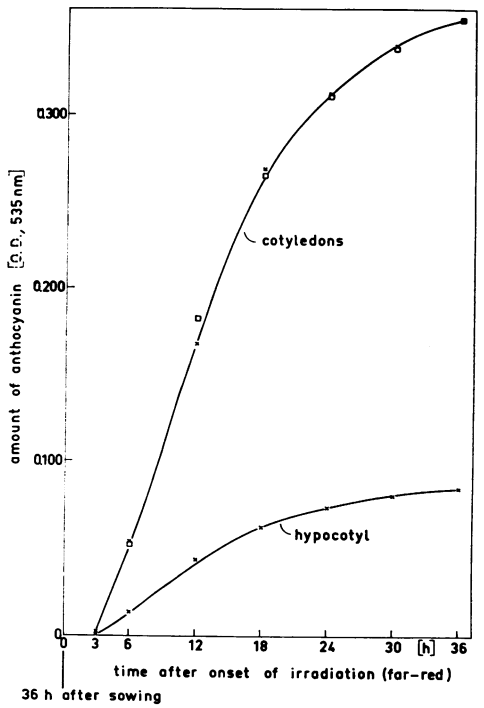

FIG. 1. The accumulation of anthocyanin in cotyledons and hypocotyl under continuous far red light. If the lower curve is multiplied by the factor 4.2 the upper curve results (squares). (Seed sample 2.)

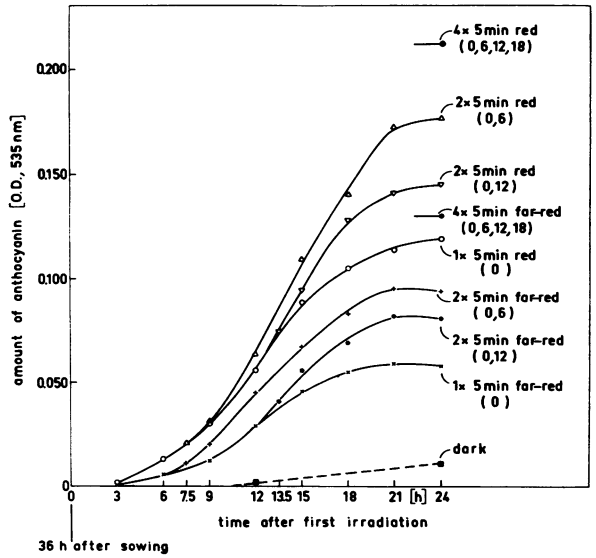

FIG. 2. The time courses of anthocyanin accumulation after one or more repeated brief irradiations (5 min each) with red or far red. The numbers in parentheses indicate the time (in hours) when the irradiation was given (seed sample 2).

by an interference filter of the AL-type (single band interference filter) (λ_{max}: 756 nm; bandwidth: 21 nm).

The radicle, which never contains anthocyanin, was removed from the seedlings and hypocotyl and cotyledons (designated "seedling") were extracted as a unit. Anthocyanin was extracted from 23 seedlings placed into 20 ml of extraction solution (propanol-HCl-H_2O = 18:1:81 vol%), and then the extraction vials were immersed in boiling water for 1.5 min. For full extraction the seedlings remained about 24 hr in the extraction medium at 25 C in the dark. Extracts were centrifuged for 40 min at about 5000*g* and the absorbance was measured at 535 nm and 650 nm. The A values at 535 nm were corrected for scattering (S_{535}) using the A values at 650 nm (A_{650}) and Rayleigh's formula. Thus, since there is no absorption by anthocyanin at 650 nm,

$$\text{corrected } A_{535} = A_{535} - 2.2\,A_{650}$$

Experimentally, it was verified that this equation can be applied to the extracts. Values in the figures and tables are means of 16 parallel (8 independent) experiments. The standard error is of the order of 2 to 5%.

The question of whether or not the cotyledons and the hypocotyl could be extracted as a unit was tested. Cotyledons or hypocotyls were extracted separately and Figure 1 indicates that they can be treated as a unit. The lower curve (hypocotyl) can be transformed into the upper curve (cotyledons) by multiplication using the factor 4.2. This result confirms earlier data by Schopfer (36). Different findings by Grill and

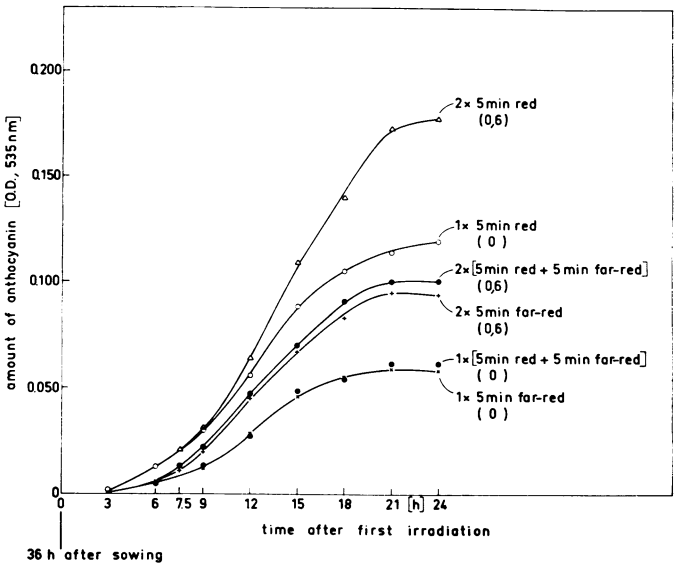

FIG. 3. Test for reversibility. The time courses of anthocyanin accumulation after one or two brief irradiations (5 min each) with red, far red, or red followed by far red (indicated by +). The numbers in parentheses indicate the time (in hours) when the irradiation was given (seed sample 2).

Table I. *Induction-Reversion Experiments of Mustard Seedling (Sinapis alba L.)*

Conventional induction-reversion experiments demonstrate the involvement of phytochrome in light-mediated anthocyanin accumulation of the mustard seedling (*Sinapis alba* L.). Extraction of anthocyanin was performed 24 hr after the first irradiation. The amount of anthocyanin is represented by the A values at 535 nm (see "Materials and Methods") (seed sample 2).

No. of Irradiations for Induction and Reversion	Time at Which the 5 Min Irradiations were Given	5 Min Red	5 Min Red Followed Immediately by 5 Min Far Red	5 Min Far Red
	hr			
1 ×	0 = 36 hr after sowing	0.120	0.062	0.058
2 ×	0, 6	0.178	0.100	0.094
2 ×	0, 12	0.146	0.087	0.081
4 ×	0, 6, 12, 18	0.212	0.127	0.130

Vince (9) with turnip seedlings indicate that anthocyanin data from one system may not be extrapolated readily to other systems.

The rate of anthocyanin *accumulation* is assumed to represent the rate of anthocyanin *synthesis*. About 70 hr after sowing (compare Fig. 10, 34 hr after zero time) the anthocyanin level tends to decrease. This destruction of anthocyanin is indicative of a turnover (17). Beyond this point, this assumption is no longer justified. Also, since the mustard seedling nearly linearly increases fat-free dry matter (19), symptoms of starvation are improbable. For these reasons the experiments are limited to the period from 36 to 72 hr after sowing.

Table II. *Test of the Reciprocity Law with Red and Far Red Light*

Test was performed over time period of 5-min exposure. Irradiation was performed at time zero (*i.e.*, 36 hr after sowing). The time of extraction was 24 hr after irradiation. The interference filter monochromator system (see "Materials and Methods") was used in these experiments. To isolate the far red the Plexiglas filter combination of the standard far red source was used. The red light was generated by an interference filter (λ_{max}: 658 nm, see "Materials and Methods"). By I_{red} or $I_{far\ red}$ we designate the irradiances of the corresponding red or far red standard sources. The amount of anthocyanin is represented by the A values at 535 nm. The values for far red are based on 32 parallel experiments, those for red on 16 (sample 2).

Irradiance I	Time of Irradiation		
	300 sec	30 sec	3 sec
$10 \times I_{far\ red}$	0.085	0.080	0.075
$1 \times I_{far\ red}$	0.081	0.077	0.065
$1/10 \times I_{far\ red}$	0.074	0.065	0.053
$1/100 \times I_{far\ red}$	0.065	0.051	
$10 \times I_{red}$	0.158	0.141	0.107
$1 \times I_{red}$	0.140	0.110	0.069
$1/10 \times I_{red}$	0.107	0.069	

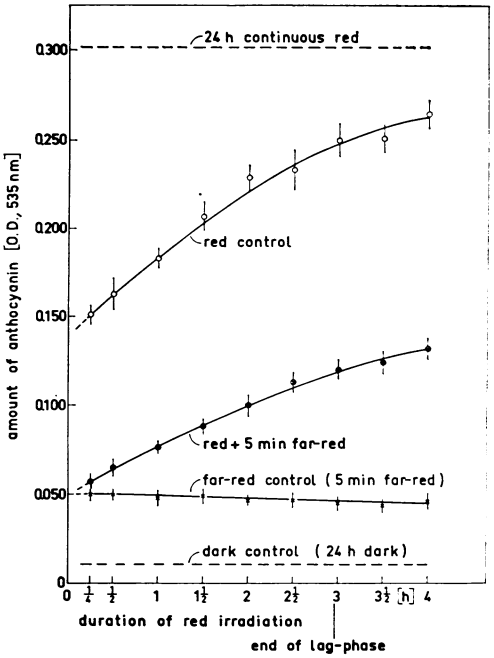

FIG. 5. Test for reversibility during the initial lag-phase. After irradiation with standard red light of varying duration (abscissa) the seedlings were irradiated for 5 min with long wavelength far red (756 nm; 700 μw/cm^2) and placed in the dark. Extraction was performed 24 hr after onset of the red light. The "red" controls received only red light of varying durations, the "far-red" controls received only 5 min far red each at the times indicated (seed sample 2).

RESULTS

Experiments within the Range of Reciprocity. If dark grown seedlings are irradiated at time zero (*i.e.*, 36 hr after sowing) with 5 min of red or far red light, there is a lag-phase of about 3 hr followed by sigmoid kinetics of anthocyanin accumula-

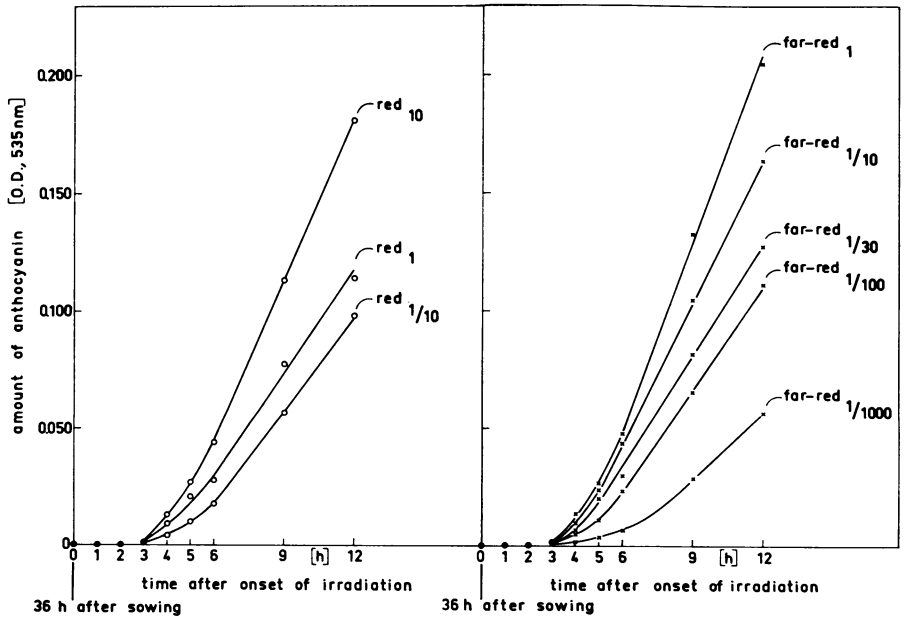

FIG. 4. Initial lag-phase and time courses of anthocyanin accumulation under continuous red and far red light as a function of irradiance. "Red," denotes the standard red irradiance (67.5 μw/cm^{-2}). "Far-red," denotes the standard far red irradiance (350 μw/cm^{-2}). (Seed sample 1.)

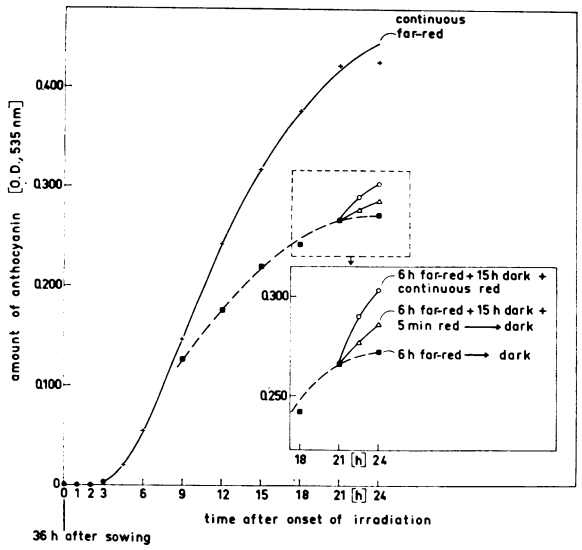

FIG. 6. An experiment to demonstrate that a primary irradiation (6 hr far red) eliminates the lag-phase for the action of continuous red or 5 min red (insert) even if a long dark period (15 hr dark) is inserted between the primary and the secondary irradiation (seed sample 2).

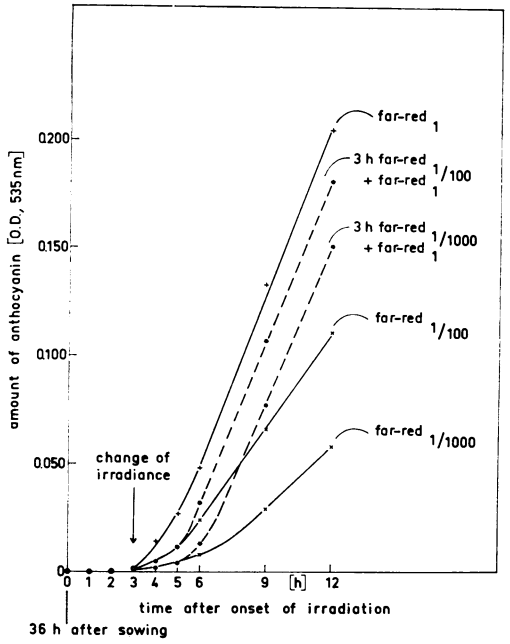

FIG. 7. The time courses of anthocyanin accumulation under continuous far red light in a typical "step up experiment". Three hours after the onset of far red the irradiances were increased by 100-fold (dashed curves). "Far-red$_1$" denotes the standard far red irradiance (350 μw/cm^2) (seed sample 1).

tion (Fig. 2). Repeated exposures to either red or far red light increase the rate of accumulation and the eventual level of anthocyanin.

Are the operational criteria for the involvement of phytochrome in this system fulfilled? These criteria require that the kinetics elicited by far red light (5 min at each treatment) be identical with the kinetics observed for exposure to red light, followed by far red light (5 min each). The data of Figure 3 and Table I clearly demonstrate that this requirement is fulfilled. The curves have nearly identical time courses and photoreversibility of an induction by 5 min of red light by immediately following with 5 min of far red is complete or at least nearly complete at every point of time within the 24-hr period. Note that the values for 2 × (0, 12) are smaller than for 2 × (0,6). The reason is that the second exposure is at a later time on the kinetic curve, and the rate of synthesis can no longer catch up. Such data emphasize that kinetic data must be available; and end point determinations are not sufficient and actually may be misleading. We conclude that the effect of light is due exclusively to the formation of P_{fr} and that P_{fr} is the physiologically active form of the phytochrome system for anthocyanin synthesis in *Sinapis*.

Direct *in vivo* measurements (12, 16) for the photostationary state of the phytochrome system established by far red indicate a considerable amount of P_{fr} (*e.g.*, about 3% at 720 nm). Thus, could the effect of far red on anthocyanin synthesis be attributed to the formation of P_{fr} as well? This question was asked experimentally in the following way: Within the range of reciprocity (*i.e.*, within the range where the degree of the response depends only on the dose [Einstein/cm^2], and not on the irradiance [Einstein/cm^2·sec]) the effect of red light can be attributed to the formation of P_{fr}. If reciprocity holds for far red light as well, it is extremely unlikely that far red light leads to the formation of more than one photoproduct, and the simplest conclusion is that this photoproduct is P_{fr}. The data of Table II indicate that for a period of at least 5 min reciprocity holds for both red and far red light. We conclude that this fact can be interpreted only by the hypothesis that even the highest irradiance has no other effect than the formation of P_{fr}. Therefore, no postulate of a cooperative or cycling effect between the two absorbing forms of phytochrome is needed, since there is no indication of any significant irradiance dependence during 5 min exposures. The value obtained with 10 times the standard irradiance (10·I·300 sec) cannot be interpreted unambiguously, because of technical limitations of available irradiance.

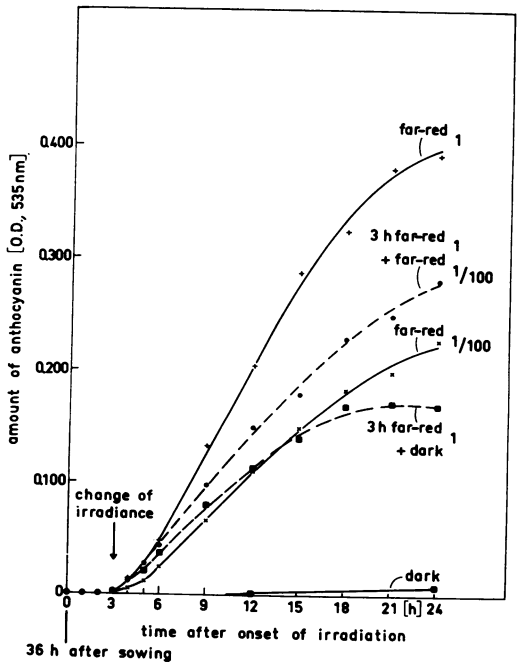

FIG. 8. The time courses of anthocyanin accumulation under continuous far-red light in a typical "step down experiment." Three hours after the onset of far red the irradiance 1 was decreased by a factor of 100 or the light was turned off (dashed curves). "Far-red$_1$" denotes the standard far red irradiance (350 μw/cm^2) (seed sample 1).

Two alternatives are considered. Either the photostationary state of the phytochrome system is not established fully after 5 min of either standard irradiance (1·I·300 sec) or an irradiance dependency comes into play. In any case up to the dose (1·I·300 sec) reciprocity holds for red as well as for far red light. Therefore, we conclude that the effects of 5 min red or far red light (Figs. 2, 3) can be fully and exclusively attributed to the formation of P_{fr}.

The Problem of the Initial Lag-Phase. The initial lag-phase (*i.e.*, the time lag between the onset of light and the first appearance of anthocyanin) is always close to 3 hr irrespective of the light treatment (Figs. 1–4). Thus, the duration of the lag-phase depends neither on the dose applied within the range of reciprocity (*i.e.*, on the amount of P_{fr} formed) nor on the rate at which anthocyanin is formed under various irradiances of continuous irradiation. The length of the initial lag is constant, specific for the system and independent of the dose, irradiance or quality of light. This result is by no means trivial. In the case of auxin-induced elongation of *Avena* coleoptile segments (31), the lag period before a steady state rate of growth is reached is dependent on the hormone concentration. On the other hand, however, the induction lag of the *E. coli* lac operon (3) does not lengthen as the induction level is lowered. In other words, no change in induction lag with inducer level is found. Neither the time of first appearance of β-galactosidase in excess of the basal levels nor the value of the intercept of the straight line part of the induction curve with the basal level is increased.

The following question arises: Is P_{fr} inactive in mediating anthocyanin synthesis during the 3 hr of the initial lag? Experimentally, is an induction of anthocyanin synthesis by red light reversible for a period of 3 hr? To test this question, induction was performed with the standard red source. The reversion, however, was done with 5 min of the wavelength 756 nm at an irradiance of 700 μw/cm² in order to keep the level of far red induction as low as possible. Extraction was performed 24 hr after onset of the red light (Fig. 5). If extraction was made 12 hr after onset of the red light a qualitatively identical pattern of curves was obtained, indicating that the time of extraction is not relevant for this problem. Complete (or nearly complete) reversal is probably only possible on the order of a few minutes after the onset of light. On the other hand, the rate of escape from reversibility is neither rapid nor is the escape a total one, even after 4 hr. This fact indicates that there is a continuous requirement for P_{fr} during the whole period of anthocyanin accumulation. In addition, P_{fr} clearly mediates anthocyanin synthesis during the lag-phase in spite of the fact that the actual synthesis of the pigment can proceed only after the lag-phase is overcome.

In the case of a secondary irradiation (28) (*e.g.*, 12 hr light-18 hr dark-light of the same quality and irradiance) no significant lag-phase can be detected. The same fact is true even if the secondary irradiation differs in quality and irradiance. The only requirement seems to be that the rate of anthocyanin synthesis mediated by secondary irradiation does not exceed the one mediated by the primary irradiation. A primary irradiation (Fig. 6) with 6 hr far red eliminates the lag-phase for the action of continuous red or 5 min red given at the end of a long intervening dark period. Therefore, we conclude that during the initial lag-phase P_{fr} exerts two functions:

(1) A potential for biosynthesis of anthocyanin (= capacity) is built up under the influence of P_{fr}.

(2) Anthocyanin synthesis is mediated by P_{fr} but the sequence of events is arrested at some intermediate stage until the "capacity for biosynthesis", required for the final manifestation of anthocyanin synthesis, is eventually built up.

The *time* required to build up this capacity is determined by the system. It is about 3 hr under our standard conditions (25 C). The *degree* of capacity, however, is a function of the primary light treatment. For a "step up experiment" (Fig. 7), *i.e.*, a change from low to high irradiance, there is a considerable delay before the higher rate of synthesis becomes apparent. The additional lag seems to be somewhat less than the initial lag, possibly for the reason that some capacity was already built up under the influence of the lower irradiance. If the capacity for a given irradiance is once built up it does not readily decay. There is no lag-phase for anthocyanin or enzyme induction (Ref. 28 and Fig. 6) if the irradiance of the secondary exposure is not changed to higher values. But even if the capacity is built up and maintained, the rate of anthocyanin synthesis depends on irradiance. For "step down experiments", (Fig. 8) within about 3 hr the rate of anthocyanin accumulation adjusts nearly to values found with the continuous lower irradiances. This dependency on irradiance under conditions of continuous irradiation is a characteristic of the

Table III. *Calculated Estimates of the Amount of P_{fr} Present in the Cotyledons of the Mustard Seedling*

Estimates were made at various times after a 5 min red irradiation at time zero (upper line) or after a 5 min red irradiation at time zero and a 5 min far red irradiation at time t. The amount of P_{fr} at various times $[P_{fr}]_t$ is always expressed as a fraction of the total amount of phytochrome spectrophotometrically measured at time zero $[P_{total}]_{t=0}$.

Time after First Irradiation	0.25 hr	3 hr	6 hr	12 hr
	%	%	%	%
$[P_{fr}]_t$ in % of $[P_{total}]_{t=0}$				
After 5 min red-irradiation at time zero	65	5	0.3	0
After 5 min red-irradiation at time zero plus 5 min far red irradiation at time t[1]	2.1	0.8	1.3	1.7

[1] t = time after first irradiation.

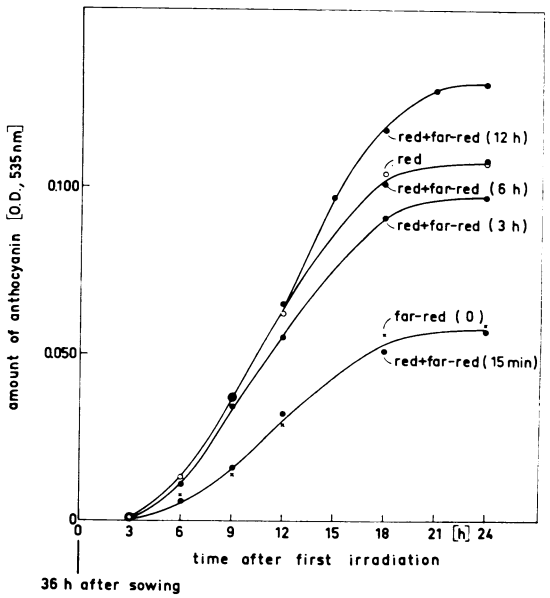

FIG. 9. The time courses of anthocyanin accumulation after one or two brief irradiations (5 min each) with red or far red. Every sample (except the far red control, fr [0], which received 5 min far-red instead) received 5 min red at time zero. The numbers in parentheses indicate the time when a second irradiation (5 min far red) was given (seed sample 2).

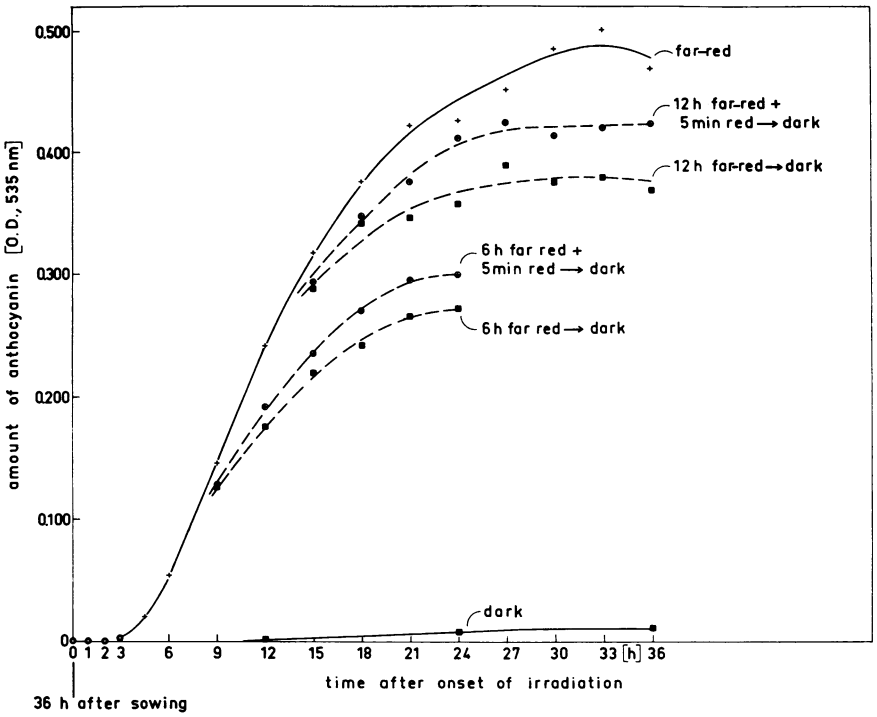

FIG. 10. The time courses of anthocyanin accumulation in experiments where the standard far red was turned off 6 or 12 hr after the onset of irradiation. The samples were either placed into the dark immediately or they received 5 min red before the transfer to darkness (see sample 1).

so-called "high intensity reactions". According to Hartmann (14, 15) this effect can be attributed to the phytochrome system as well. And indeed, we have not found any data which could not be reconciled with the hypothesis advanced and supported by Hartmann (13) that the effects of continuous light in the wavelength range above 550 nm can be fully attributed to phytochrome.

"Phytochrome paradoxes" (4, 18, 20) are situations where no rational relationships between the spectrophotometrically detectable phytochrome *in vivo* and the rate or extent of physiological responses can be derived. For example, a most dramatic effect was recently reported by Klein (22). In bean seedlings (*Phaseolus vulgaris*, cv. Black Valentine) growth, anthocyanin accumulation and increases in two enzymes induced by a 10 min red irradiation were stopped by a 7 min far red irradiation given 17, 24, or 48 hr after the red light induction.

Estimates were calculated from Marme's data (23) of the amount of P_{fr} present in the seedlings at various times after a 5 min red irradiation given at time zero followed by either dark or 5 min far red irradiation at time t. The decay constant (half-life) of P_{fr} in both cotyledons and hypocotyl is 45 min at 25 C. The amount of P_{fr} at various times $[P_{fr}]_t$ (Table III) is always expressed as a fraction of the total amount of phytochrome spectrophotometrically measured at time zero $[P_{total}]_{t=0}$. It is assumed that the standard red source establishes a photostationary state $[P_{fr}]/[P_{total}] = 0.8$ whereas the standard far red source establishes a value of $[P_{fr}]/[P_{total}] = 0.03$. These values are adjusted for *de novo* synthesis of P_r from Marme's data (23) but since these latter data are not very precise and the possible existence of a small stable fraction of P_{fr} (38) has been neglected the estimates in Table III are "order of magnitude" values. Satisfactory agreement between prediction (Table III) and experimental result (Fig. 9) exists. The important feature is the shift from reversion by far red to induction by far red. This latter phenomenon becomes obvious 12 hr after the red treatment.

The anthocyanin synthesizing system responds (Fig. 10) to changes of the P_{fr} level, exerted by 5 min red after the termination of continuous far red, in precisely the way one would expect from Figure 2. An increase of P_{fr} rapidly leads to an increase in the rate of anthocyanin accumulation.

DISCUSSION

The questions outlined in the introduction can now be answered as follows: (*a*) Within the range of reciprocity the influence of red or far red light on anthocyanin synthesis of the mustard seedling is exclusively due to the formation of P_{fr}. It is very probable (38, 39), but not proven in the same stringent sense, that even under continuous irradiation with red or far red light the effect of light is exclusively exerted through the phytochrome system. (*b*) The initial lag-phase (3 hr) has a partial escape from reversibility, which can be demonstrated as early as 15 min after the onset of red light. This fact is interpreted as indicative of a rapid action of P_{fr} for mediating anthocyanin synthesis. On the other hand, there are many indications (*e.g.*, Figs. 2, 9, 10) that the rate of anthocyanin synthesis is controlled by the level of P_{fr} during the whole period of synthesis. This conclusion is also supported by the fact (Fig. 5) that even 4 hr after the onset of red light the escape from reversibility is only in the order of 50%. (*c*) Finally, a most important result is the fact that for anthocyanin synthesis in the mustard seedling no "phytochrome paradoxes" could be observed. Since the same conclusion was reached with respect to phytochrome-mediated control of lipoxygenase synthesis in the mustard seedling (32), the mustard seedling seems to be especially well suited to investigate the biophysical and molecular mechanisms of phytochrome action.

It has been shown (8, 25, 33) that the enzyme phenylalanine ammonia-lyase can be induced by phytochrome in the mustard seedling. Phenylalanine ammonia-lyase catalyzes the formation of *trans*-cinnamic acid from phenylalanine. This reaction is probably the first step of a biosynthetic sequence which leads from phenylalanine to the phenyl-propane moiety of the flavonoids, including the anthocyanidins. Since the next

enzyme in the reaction sequence, *trans*-cinnamic acid 4-hydroxylase, which catalyzes the formation of *p*-coumaric acid from *trans*-cinnamic acid, can also be induced by phytochrome as demonstrated by Russell (34) with peas, it is not improbable that in course of time the mediation by light of anthocyanin formation in the mustard seedling can be fully understood in terms of phytochrome-mediated enzyme induction. Thus anthocyanin synthesis, mediated by phytochrome, is a promising model system to investigate step by step the molecular mechanism of developmental processes. However, it must again be emphasized that kinetic data must be obtained. End point determinations alone are not sufficient and actually may be misleading in the interpretation of this process.

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