Long-lived Intermediates in Phytochrome Transformation I: In Vitro Studies[1]

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Abstract. Irradiation of phytochrome solutions with a high-intensity mixed red and far red light source causes measurable absorbancy increases at 543 nm. Evidence is presented that these absorbancy increases are caused by accumulation of intermediates on the P_R to P_{FR} pathway with relatively slow thermal decay constants. Kinetic analysis of the decay signals is consistent with the interpretation that the signals represent simultaneous independent and parallel decay of 2 species by first order kinetics to P_{FR}. If actinic light intensity is kept constant and exposure time changed, the relative amounts of the 2 components change, with proportionately more of the rapidly decaying species present following short exposure times. If the amount of the intermediates is decreased by decreasing actinic light intensity at constant exposure time, however, the relative amounts of the 2 remain constant. The Q_{10} for intermediate decay following illumination is approximately 2.0, while that for complete phototransformation of the pigment in either direction is very close to 1.0. Incomplete transformation of P_R to P_{FR}, caused by overlapping absorption of the 2 forms, is shown by the presence of intermediates (indicating cycling of the pigment) in continuous red light. Such intermediates do not appear in continuous far red, indicating a rate of pigment cycling below detection by the available instrumentation.

The elegant flash photolysis experiments by Linschitz and his co-workers have revealed several fundamental aspects of phototransformation of the pigment phytochrome (9, 10). First, transformation in both directions involves the formation and decay of a number of spectrally distinguishable intermediates. Second, the P_R to P_{FR} pathway involves different intermediates than the reverse photoreaction. Third, concurrent parallel dark reactions lead to the formation of the final product in both directions.

Finally, at least 2 intermediates on the P_R to P_{FR} pathway have rate constants for decay to P_{FR} at least 10-fold smaller than the rate constants for any of the other intermediates on either pathway. Low temperature studies by Pratt and Butler (12) and by Cross *et al.* (7) suggest that an early event in P_R transformation is the formation of an intermediate which can be photoreversibly driven back to P_R, but there is no evidence that more than the initial photoreaction in transformation of P_R to P_{FR} in a forward direction is light-driven. Spruit has also observed phytochrome intermediates at liquid nitrogen temperature (14–17) but interpretations of his data are complicated by possible interference in the spectral measurements by protochlorophyll, chlorophyll, or both.

It seemed possible that irradiations of phytochrome solutions with a high intensity mixture of

red and far red light might lead to the accumulation, under steady-state conditions, of significant amounts of the longer-lived intermediates on the P_R to P_{FR} pathway. Difference spectra determined for the various intermediates by Linschitz et al. indicated that if such intermediates accumulated in reasonable amounts, they should be detectable under suitable spectrophotometric conditions, enabling one to study their kinetics and some of their properties. Experiments bearing out these predictions are described in the present paper. A preliminary report of this work has appeared elsewhere (1).

Materials and Methods

Phytochrome was isolated and partially purified from 5-day-old dark-grown oat seedlings as described previously (4). Briefly, the harvested shoots were ground in tris buffer, the extract filtered through cheesecloth and centrifuged to remove particulate material, the supernatant passed through sephadex G-50, and the excluded material adsorbed on calcium phosphate gel (brushite). The phytochrome was then eluted with 0.4 M phosphate buffer, the active fractions combined and precipitated by adding an equal volume of saturated ammonium sulfate, centrifuged, and the pellet redissolved in 0.001 M phosphate buffer. All operations were carried out at 4° under dim green light, and the pH maintained at 7.8. Phytochrome recovery from the initial extract was better than 50 %, with approximately 5-fold purification. Phytochrome was assayed using a Ratiospect R-2 difference spectrophotometer (Agricultural Specialty Company, Incorporated), and activity is expressed below as the sum of the optical density differences inducible at 660 and 730 nm by red and far red light $[\triangle(\triangle OD)]$.

For studying phytochrome intermediates, a special double beam spectrophotometer described in detail elsewhere (8) was used. Light from a 650 w tungsten-iodine lamp was passed through 27 mm of water and then through appropriate filters to obtain high intensity actinic red or far-red light or a mixture of the two. For red light, a broad band interference-type filter (Balzers K6) and a Corning 2403 glass filter were used (intensity at sample: 7.7×10^5 erg cm^{-2} sec^{-1}); for far red light, a Schott RG10 filter (intensity at sample: 10^6 erg cm^{-2} sec^{-1}); for mixed red and far red light, a Corning 2030 filter (intensity at sample: 1.3×10^6 erg cm^{-2} sec^{-1}). The sample was contained in an ice-jacketed lucite cell 2 cm thick and 3 cm in diameter, closed top and bottom, and completely filled with the phytochrome solution. Beneath the sample, above the photomultiplier tube was a filter combination which excluded any of the actinic light but allowed light of wavelength less than 580 nm to pass through. The measuring beam was obtained from a 6 volt tungsten lamp, with the light suitably collimated and passed through a Balzer interference filter transmitting

maximally at 543 nm. Its intensity was below 300 ergs cm^{-2} sec^{-1}. A thermistor inserted into the sample allowed continuous monitoring of sample temperature with a Yellow Springs Instruments Telethermometer. Unless otherwise stated, sample temperature varied between 4 and 5°. The phototube output was recorded with an oscillographic recorder, and the system was calibrated so that recorder signal could be directly converted into absorbancy changes. Light intensities were measured with an Eppley 8 junction bismuth-silver thermopile and a Hewlett-Packard microvoltmeter. An approximate correction for infrared was obtained by measuring light intensity in all cases with and without Polaroid XRN5X55 filter which transmitted light only beyond about 800 nm. The value obtained with the Polaroid filter was subtracted from the value without to determine total energy below 800 nm for each actinic light source.

Phytochrome samples contained photoreversibility ranging from 1.8 to 3.2 $\triangle(\triangle OD)$ in a total volume of 25 ml. Loss of activity during an experiment never exceeded 10 %, and a fresh sample was always used on any given day.

Results

Accumulation of Intermediates Under Cycling Conditions. A typical oscillographic recorder tracing of the absorbancy change caused by the mixed red-far red actinic beam is shown in Fig. 1. The difference spectra obtained by Linschitz et al. (10) suggest that the long-lived intermediates on the P_R to P_{FR} pathway should absorb more than P_{FR} at 543 nm (although their measurements were not carried out below about 575 nm). The sample illustrated had been preïlluminated with the actinic light to bring the pigment into approximate photostationary equilibrium before the measurement was made. Therefore, if the actinic beam were to cause accumulation
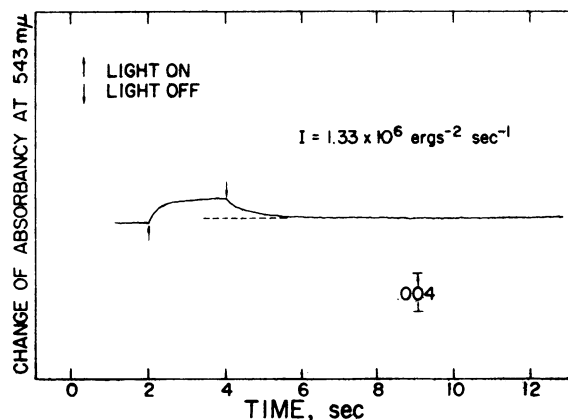

Fig. 1. Absorbancy change at 543 nm induced by high intensity mixture of red and far red light in phytochrome solution.
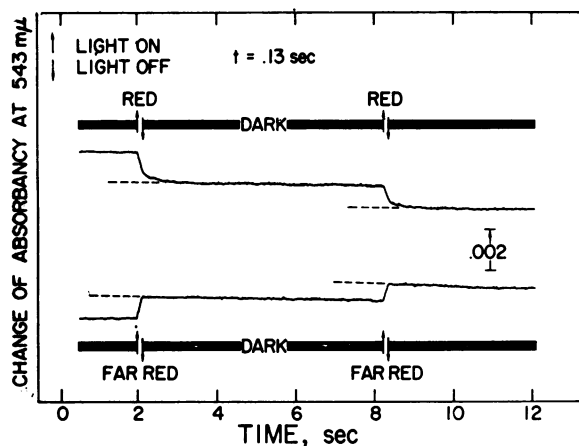
FIG. 2. Absorbancy changes induced at 543 nm by brief red light flashes on P_R (top) or brief far red light flashes on P_{FR} (bottom). Curves placed far closer together than they actually were to save space.

of these long-lived intermediates, one should see an absorbancy increase upon illumination, and then a decrease when the light is turned off and intermediates decay to P_{FR}. Fig. 1 clearly shows the anticipated signal.

It was then necessary to clarify 2 questions: first, since the purification of the phytochrome was not great, one had to determine that the signal indeed came from phytochrome and not some form or forms of chlorophyll; second, should the signal turn out to be phytochrome, one had to determine whether the intermediates observed were indeed those on the P_R to P_{FR} pathway. The following experiments actually bear on both questions.

First, the sample was transformed by red light to P_{FR}, and a series of brief flashes of far red light
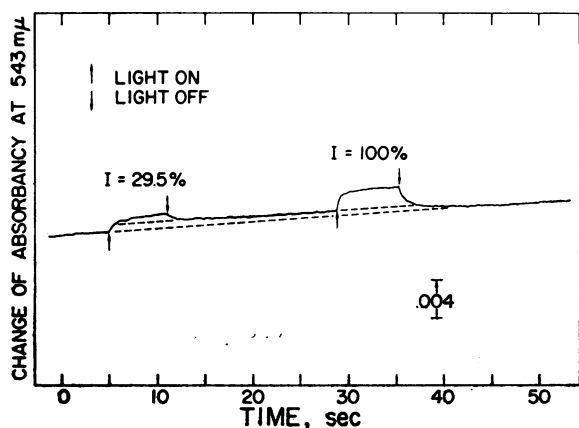

FIG. 3. Shift in base line caused by irradiation of phytochrome sample alternately with high and low intensity mixed red and far red light. Sample had received 6 sec illumination at 100 % intensity just prior to start of record shown.

given (0.13 sec at 6 sec intervals). A portion of the tracing is shown at the bottom of Fig. 2. As one would expect, the far red flashes administered to P_{FR} brought about an absorbancy increase at 543 nm. However, the absorbancy change stops abruptly with the termination of the flash, and there is no evidence of any spectral change in the dark. The converse experiment is shown at the top of Fig. 2. P_R was administered similar flashes of red light. In this case, there is clearly a dark decay following the end of the light flash (see dashed lines). Furthermore, the amount of dark decay decreased linearly with the amount of P_R remaining after the flash, and the decay rate was roughly similar to that shown in Fig. 1. The experiments suggest both that the Fig. 1 signal was from phytochrome, and that it represented events on the P_R to P_{FR} pathway.

A second kind of experiment is illustrated in Fig. 3. It was reasoned that the higher the light intensity, the higher the steady-state level of intermediates should be. If these intermediates really decayed to P_{FR}, then the ratio of P_R to P_{FR} should be lower after a high intensity saturating actinic dose than after a low intensity dose. The assumption was that regardless of intensity, the ratio of P_R to P_{FR} should be constant, and only the percentage of pigment as intermediate present at steady state should be changed by intensity. Since P_{FR} absorbs less than P_R at 543 nm, the base line after a high intensity exposure should be lower than the base line following a low intensity exposure. Intensity was therefore altered using neutral density filters. Fig. 3 shows one typical record. The base line could be reversibly shifted many times simply by altering the actinic beam intensity.

It is possible to calculate from experiments such as that shown in Fig. 3 the percentage of total phytochrome present as intermediates under any given conditions of illumination. Complete transformation of phytochrome from P_R to P_{FR} at 543 nm in this experiment yields a lowering of the baseline of 115.5 mv [of which only 80 % can actually be directly measured, see (5)]. The baseline shift between high and low intensity treatments (Fig. 3) averaged 5.46 mv (15 measurements). The difference in signal heights for intermediate between high and low intensity averaged 17.18 mv (9 measurements). It should be remembered that a 5 % shift in the baseline represents a 10 % change in P_{FR} (or P_R) concentration, since one form increases in absorbancy as the other decreases. Thus 17.18 mv of intermediate equals $(2 \times 5.46/115.5) \times 100 \%$ of total phytochrome, or approximately 9.5 %. By simple ratio, it can be determined that the 25.15 mv representing intermediates with the actinic intensity at 100 % is equivalent to 12.9 % of the total phytochrome, while the 7.97 mv representing intermediates with the actinic intensity at 29.5 % is equivalent to 4.4 % of the total phytochrome.
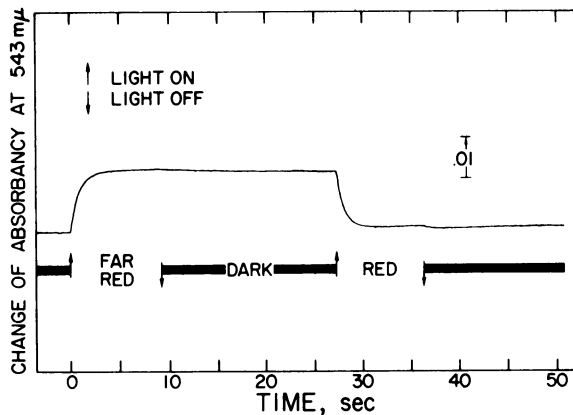
FIG. 4. Absorbancy changes induced by saturating far red and then red light at 543 nm. Sample of phytochrome received saturating red treatment just prior to start of record shown. Note small decrease in absorbancy immediately following end of red, but not far red, exposure.

A final kind of experiment suggesting that the observed signals represented intermediates of phytochrome transformation is shown in Fig. 4. A sample of P_{FR} was first irradiated with far red light until no further change could be observed. Upon termination of illumination, there is no further spectral change except a slow drift down caused by the measuring beam. The sample was then irradiated with red light until no further spectral change could be observed. Upon termination of illumination, a small but distinct dark decay is observed. Since P_{FR} absorbs substantially at 660 nm, it is well known that complete transformation of P_R to P_{FR} is not possible, and, in fact, with oat phytochrome, one obtains a $P_R:P_{FR}$ ratio of about 20:80 (5). Thus one would expect to see some consequences of cycling with red light, including at least a low level of intermediates. On the other hand, far red light brings about a far more complete transformation of
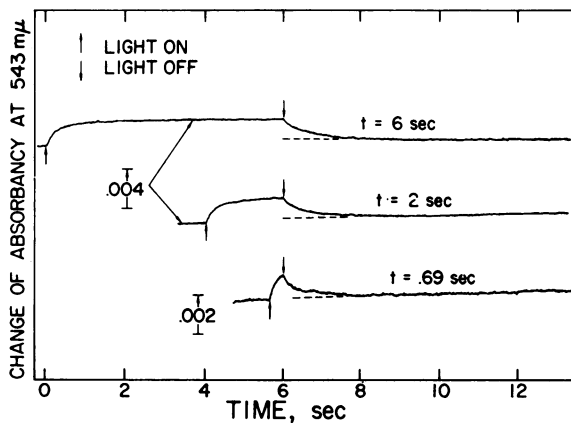
P_{FR} to P_R, so little cycling should be observed under far red illumination and no intermediates should be observed. Experiments such as that shown in Fig. 4 support this prediction.

As will be shown below, the decay of intermediates can be resolved into 2 simultaneous independent first order components. The rate constants for these 2 components fit well with those calculated by Linschitz et al. (10) for the 2 slowest intermediates on the P_R to P_{FR} pathway. Thus all available evidence indicates that the signals observed in the present experiments are indeed these intermediates.

Kinetic Studies on Intermediate Decay. In preliminary attempts to analyse the decay of the observed intermediates, light intensity was kept constant and exposure time was varied between 0.125 and 24 sec (a manual shutter was used, and exposure time calibrated with an oscilloscope). Some typical records are shown in Fig. 5. It is clear that the decay half time is substantially shorter with the shorter exposures. In Fig. 6, decay half times for 2 separate experiments are plotted against amount of spectrally detectable intermediate at the end of the light period.
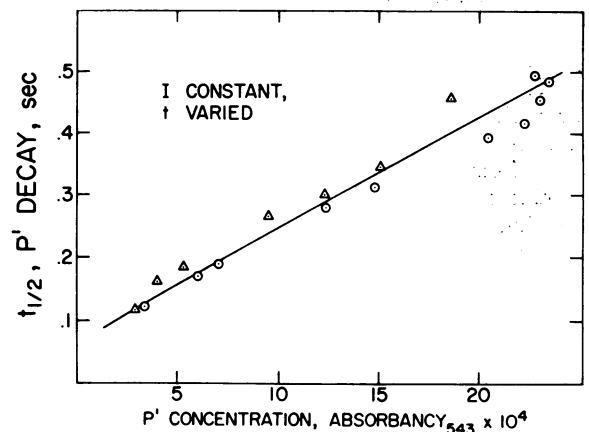

FIG. 6. Measured half times for signal decay plotted against signal height. Details in text. Signal height varied by keeping intensity constant and varying exposure time.

Each point represents the average measurement from a minimum of 6 signals. It is clear that simple first or second order kinetics cannot account for the results, since with first order kinetics, decay half time should be independent of concentration of intermediates, and with second or higher order, half time for decay should decrease with increasing concentration.

In another series of experiments, intermediate concentration was varied by keeping exposure time constant, and varying actinic beam intensity using neutral density filters. Typical records are seen in Fig. 7. In these experiments, it is clear that half time does not vary as a function of intermediate
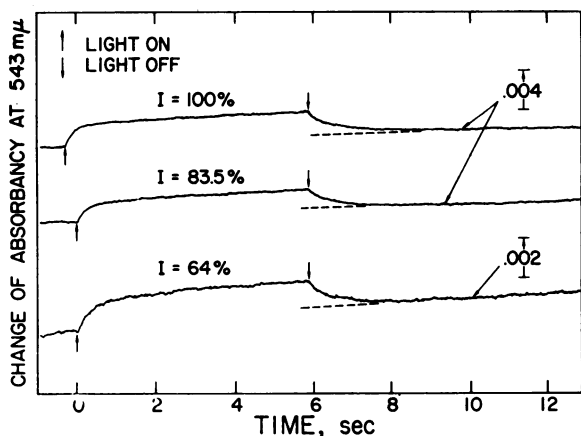

FIG. 5. Absorbancy changes at 543 nm induced by different exposure times with high intensity mixed red and far red light.

FIG. 7. Absorbancy changes at 543 nm induced by similar exposure times of different intensities of mixed red and far red light.

FIG. 9. Semilog plots of signal decay following 2 different exposure times at full intensity of mixed red and far red light. Upper solid line: actual data (average of 6 decay curves); dashed line: extrapolation to time zero of log-linear portion of original data; lower solid line: arithmetic differences between upper solid line and dashed line. P' indicates intermediate concentration, with subscripts t, 1, and 2 referring to total intermediate, rapidly decaying form, and slowly decaying form, respectively. τ values are reciprocals of rate constants. Details in text.

concentration, and Fig. 8 shows half time plotted against concentration under 2 different conditions of exposure time. Apparent first order kinetics are obtained.

The resolution of the apparent discrepancy is found by plotting log of absorbancy decay against time, as has been done in Fig. 9 for 2 different exposure times at full actinic beam intensity. The upper solid curves represent the average measurements of at least 6 decay curves. They start to decline in a non-log-linear fashion, but then eventually assume the typical log-linearity expected for a first order reaction. Extrapolation of the straight portion of the curve (dashed line) and replotting the arithmetic difference between the actual data and the extrapolated curve produces a second straight line. Thus, the decay observed can be shown to fit simultaneous decay of 2 independent components, in agreement with the parallel pathways proposed by
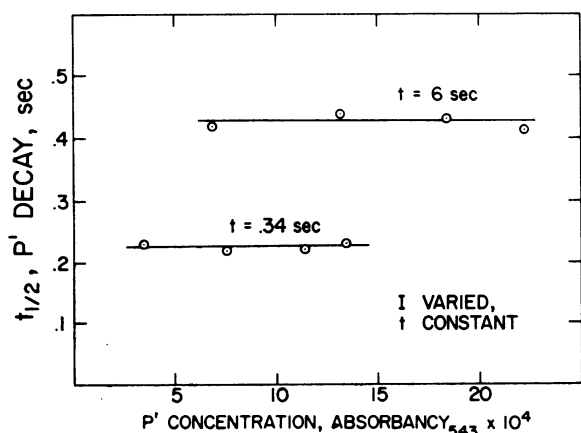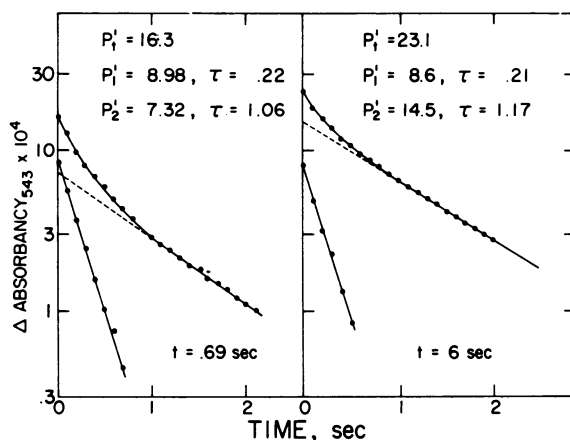
Linschitz et al. (9, 10). The zero time intercepts yield values for the initial relative amounts of each of the 2 species. τ is the reciprocal of the rate constant in each case. The shorter exposure (Fig. 9, left) shows a higher proportion of the fast-decaying species, while the longer exposure shows substantially more of the slow decaying species (Fig. 9, right). The relative amounts of the 2 components, P'_1 and P'_2 for various exposure times with full intensity actinic light, calculated as in Fig. 9, are shown in Fig. 10. P'_t represents total intermediate. The faster component, P'_1 builds up rapidly, and then decreases somewhat, while the slower component, P'_2

FIG. 8. Measured half times for signal decay plotted against signal height for 2 different exposure times at 4 different intensities each.
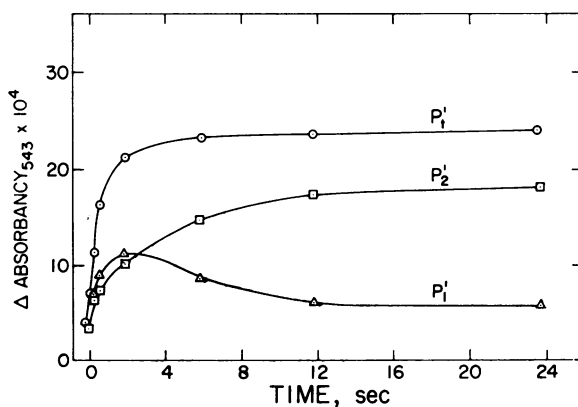

FIG. 10. Relative amounts of total intermediate (P'_t), rapidly decaying (P'_1) and slowly decaying (P'_2) forms plotted against length of exposure to mixed red and far red light, 100 % intensity.

Table I. *Relative Amounts and Decay Constants for Phytochrome Intermediates Produced by Different Irradiation Times*

Exposure time	% P'$_1$ (rapid)	% P'$_2$ (slow)	τ.P'$_1$	τ.P'$_2$	Temp.
sec					deg
		Experiment 1			
0.125	47	53	0.13[1]	0.69	4.3
0.36	57	43	0.18	0.85	4.0
0 73	57	43	0.23	0.90	4.2
2.0	44	56	0.22	0.86	4.8
		Experiment 2			
0.13	56	44	0.13	0.63	3.7
0.34	53	47	0.14	0.87	3.7
0.69	55	45	0.22	1.06	3 8
2.0	52	48	0.26	1.18	4.2
6.0	37	63	0.21	1.17	4.2
12 0	26	74	0.17	. . .	4.2

[1] Accuracy of measurements for short exposure times when the signal was small or for the longest measurements where baseline drift could be substantial is less than for intermediate exposure times. Each data set is the average of at least 6 decay curves.

builds up gradually to a stable level. Table I shows the percentage of each component as a function of exposure time and also shows the consistency of the τ values obtained under a variety of conditions.

A similar analysis of curves obtained by keeping exposure time constant but varying intermediate concentration by varying intensity showed little change in the relative amounts of the 2 components, as one would expect from the apparent first order nature of the kinetics as shown in Fig. 8.

Effect of Temperature on Transformation Versus Intermediate Decay. On 2 occasions, the sample was allowed to warm up slowly and 6 sec actinic exposures at full intensity were given at intervals. Temperature was monitored continuously. The half times for decay are plotted against temperature for one of these experiments in Fig. 11. The Q_{10} near 2 clearly indicates the thermal nature of the reaction.
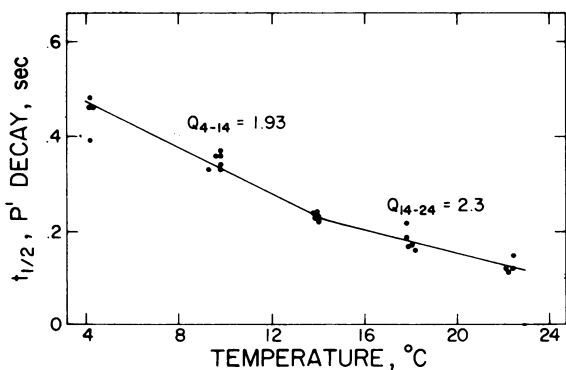

FIG. 11. Half times for signal decay, 543 nm, following 6 sec 100 % intensity mixed red and far red light at different temperatures.

It should be pointed out that these half times represent both components, and no effort was made to resolve differential effects of temperature on the 2. The rate of complete transformation of the pigment in both directions was measured on the same samples to obtain Q_{10}'s for the over-all reaction. The data are shown in Fig. 12. Over-all transformation is clearly temperature-independent in both directions under our conditions, and decay of long-lived intermediates therefore cannot be the limiting step in transformation.
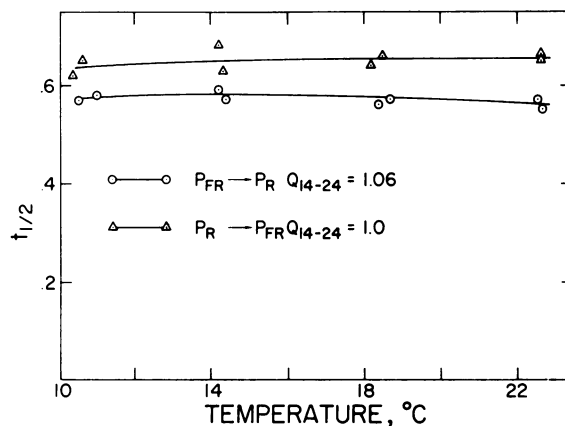

FIG. 12. Half times for P_R to P_{FR} transformation (triangles) and P_{FR} to P_R transformation (circles) at different temperatures.

Discussion

The above results are consistent with 2 possible interpretations for P_R to P_{FR} conversion. The first interpretation, presented independently by Linschitz's group (9, 10) and by Briggs and Fork (1) is that a single initial species of P_R, upon suitable excitation, follows a pathway which eventually splits, leading to at least 2 long-lived intermediates which decay independently and in parallel to P_{FR}. Briggs and Fork proposed a possible light-mediated formation of 1 of the intermediates from the other, prior to their independent decay to P_{FR}, but this pathway can be eliminated by the careful double flash experiments of Linschitz and Kasche (9). It seems clear that the 2 intermediates arise by dark reactions. However, the question of their ultimate origin remains unanswered. The results are wholly consistent with a scheme in which there are 2 initial species of P_R. Upon excitation, both follow closely similar pathways until the formation of the final intermediates which then decay with rate constants differing by a factor of about 5 to discrete final species of P_{FR}. A survey of the literature reveals a number of reasons why the second interpretation should be given serious consideration. Mumford and Jenner (11) mention in a footnote that they have some evidence for aggregation of oat

phytochrome (molecular weight approximately 60,000) into higher molecular weight species. Correll et al. (6) report similar aggregation phenomena with phytochrome from rye. Finally, Briggs et al. (4) show by gel filtration chromatography the presence of large and small molecular weight species of oat phytochrome early in the purification procedure. Thus both the studies done in Linschitz' laboratory (7, 9, 10) and those reported in the present paper were probably done on a mixture of molecular weight species.

A second complication is raised by some spectral observations by Briggs et al. (4). Native phytochrome in oats, in vivo, shows an absorption maximum for P_R at 667 nm. Material carefully isolated under dim green light retains this absorption maximum. However, if the phytochrome is transformed to P_{FR} and allowed to remain overnight at 4°, the absorption maximum shifts to 660 nm. Linschitz et al. (10) report that the material which they used for flash photolysis had a maximum for P_R at 664 nm, and could easily have been a mixture of 667 and 660 nm material. The same complication applies to the present work. Thus our experiments and those of the Linschitz group may well have been done on a complex mixture of phytochrome molecules, differing in molecular weight, and also differing in absorption properties. Unpublished observations in the laboratory of W. B. show that both large and small molecular weight forms of phytochrome can have their absorption maxima at 667 nm, so the spectral shift cannot be simply correlated with molecular size.

None of the work mentioned so far, however, provides an answer to a crucial question: do any of the current studies on phytochrome intermediates have any bearing on phytochrome transformation as it occurs in the living cell or is one simply studying artifactual mixtures? Three lines of evidence support the first alternative. First, Pratt and Butler (12) and Cross et al. (7) have observed intermediates in isolated phytochrome preparations at low temperature which appear remarkably similar to intermediates reported by Spruit (14–17) to be present in vivo in etiolated pea epicotyl tissue. Second, Purves and Briggs (13) have presented dosage-response curves for phytochrome transformation in vivo which are consistent with the interpretation that there are 2 kinetically distinct species of phytochrome within the tissue of oats, peas, corn, and cauliflower. Finally, Briggs and Fork have studied formation and decay of intermediates in vivo in oats, and find decay kinetics which, like those reported in the present paper, can be resolved into 2 kinetically distinct first order components (2, 3).

It would be useful to study kinetic properties of intermediates in phytochrome preparations in which one was certain of having a single spectral form and material of a single molecular weight. Unfortunately, to date, it has not been possible to do so in

a meaningful way. The only form which will remain reasonably stable under the cycling conditions of the present experiments is material of approximately 60,000 molecular weight, absorbing maximally at 660 nm—in other words already spectrally altered from the native material. Further experiments are currently in progress to attempt to stabilize the various possible kinds of phytochrome obtained during the isolation procedure, but thus far, the results have not been successful.

The identity of the proposed long-lived intermediates discussed here with the 2 slowest ones on the P_R to P_{FR} pathway proposed by Linschitz et al. (10) seems reasonable on the basis of comparative rate constants and information regarding their position in the complex series of reactions involved in cycling phytochrome from 1 form to the other. It should be pointed out that the intermediates are sufficiently long-lived to participate in some biochemistry, and the possibility that they could participate in certain kinds of phytochrome responses in plants cannot be rigorously excluded at the present time.

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