Temperature and pH Studies on Phytochrome *in Vitro**

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ABSTRACT: Phytochrome from oat seedlings was studied over a range of temperatures from −50 to +15° and in aqueous and mixed aqueous-buffered solutions of pH 4.7–8.6. The results show that phytochrome *in vitro* behaves as a thermochromic as well as a photochromic protein, particularly at lower temperatures and in the pH range 6.0 and below. Furthermore, the rate at which phytochrome undergoes a spontaneous reaction during prolonged dark periods is strongly dependent upon temperature and pH. A model involving three forms of phytochrome is proposed to account for these results. Two of the forms are the well-established species having their first absorption maxima at λ 663 and 727 nm, respectively, whereas the third form exhibits a relatively weak and broad absorption at approximately λ 650 nm. Analysis of the model shows that the rate of the dark reaction is insensitive to temperature at certain specific pH values. It is suggested that this temperature compensation in the phytochrome dark reaction may be physiologically important. It is also suggested that phytochrome may act as a thermoregulator as well as as a photoreceptor in plants.

Phytochrome is believed to be the photoreceptor which mediates many of the red–far-red light responses in plant growth and development (Borthwick *et al.*, 1952a,b; Hendricks *et al.*, 1956; Butler *et al.*, 1959; Hendricks and Borthwick, 1959; Borthwick and Hendricks, 1960; Siegelman and Butler, 1965; Hillman, 1967). Under red (660 nm) irradiation phytochrome exists predominately in a form, FR, which has an absorption maximum at 727 nm; under far-red (727 nm) light in a second form, R. which absorbs at 663 nm. In the literature FR and R have usually been designated P_{FR} and P_R, respectively. There is also evidence that FR spontaneously reverts to R in darkness.

$$R \underset{\text{far-red light or darkness}}{\overset{\text{red light}}{\rightleftharpoons}} FR$$

Considerable information concerning the photolysis of phytochrome has been obtained by Linschitz *et al.* (1966, 1967). However, despite numerous studies definitive work on the dark reaction is still lacking (Hillman, 1967). In the previous *in vitro* studies with phytochrome isolated from oats, one of us found that at pH 7.8 the dark reaction is first order with respect to phytochrome and that the rate is highly temperature dependent (Mumford, 1966). This paper describes the effect of temperature and pH on the course of the dark reaction *in vitro* and also our observation that FR is in equilibrium with a previously undetected form of phytochrome which predominates at low temperature and low pH.

Experimental Section

Phytochrome Preparation. The phytochrome used in this work was extracted from *Avena sativa* L. cv Garry (oat) seedlings and purified by a published procedure (Mumford and Jenner, 1966). It had a specific activity of 200–300 units/g of protein.

Aqueous Solution Studies. Dark reaction measurements (Table I) were made essentially as has been described in detail previously (Mumford, 1966). Acetate buffers were used in the 4.5–5.5 pH range, phosphate in the 5.9–7.9 range, and Tris in the 7.3–8.6 range. Spectra

TABLE I: Effect of pH on Rate of Phytochrome Dark Reaction at 10°.

Buffer System[a]	pH	Dark Period, t (sec)	% FR at Time, t	k_{ob}[b] (sec^{-1})
Acetate	4.7	6,120	60.3	82.1×10^{-6}
		7,080	57.1	79.0×10^{-6}
Acetate	5.1	63,840	12.7	32.4×10^{-6}
		15,360	62.4	31.8×10^{-6}
Acetate	5.5	7,620	84.5	16.6×10^{-6}
		63,600	40.5	14.2×10^{-6}
Phosphate	5.9	65,700	69.9	5.47×10^{-6}
		166,560	48.9	4.29×10^{-6}
Phosphate	6.9	67,200	89.5	1.65×10^{-6}
		168,360	80.0	1.31×10^{-6}
Tris	7.3	73,680	91.4	1.19×10^{-6}
		171,960	83.3	1.05×10^{-6}
Phosphate	7.9	70,320	89.1	1.61×10^{-6}
		170,760	82.7	1.1×10^{-6}
Tris	8.6	76,560	90.8	1.23×10^{-6}
		173,160	80.3	1.26×10^{-6}

[a] Ionic strength = 0.025. [b] $k_{ob} = (1/t) \ln [FR]_0/[FR]_t$.

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were scanned at intervals while the sample remained in the sample compartment, sometimes for periods of 2–3 days for one dark reaction run. The amount of photolysis produced by the analysis beam of the spectrophotometer was negligible at moderate scanning rates. In the pH 4.7–5.0 range the phytochrome sample lost 10% photoreversibility during the dark reaction measurements. The loss of photoreversibility at other pH valves was negligible. As in the previous study, the decrease in absorbance at 727 nm and the increase in absorbance at 663 nm during the dark reaction were essentially equivalent.

Low-Temperature Studies. A three-component solvent containing equal volumes of glycerol, ethylene glycol, and water is liquid at temperatures down to and below −78°. Solutions of phytochrome in glycerol–ethylene glycol–water were prepared by first dissolving the phytochrome in phosphate buffer adjusted to the desired pH. One part of this buffered solution was added to two parts of a 1:1 mixture of glycerol and ethylene glycol. The solvents were reagent grade and not purified further except to remove dissolved oxygen by bubbling nitrogen gas through the 1:1 mixture for approximately 18 hr just before use. The final mixing process and the transfer into the absorption cell also were carried out under nitrogen. The "pH" (with quotation marks) of the glycerol–ethylene glycol–water solution was defined as the pH of the undiluted aqueous buffer at 25°.

Spectra were measured in a modified Cary Model 11 spectrophotometer. For studies below −15°, a special 5-cm path-length Pyrex dewar absorption cell was employed made by H. S. Martin and Sons, Evanston, Ill. The sample container was an integral part of the dewar flask. A heat-transfer liquid surrounded the sample and was chilled in turn by a coil of copper tubing through which cold nitrogen gas was passed. The temperature was controlled by the rate at which the cold nitrogen passed through the coil; the temperature was measured by a thermocouple placed next to the sample container.

Results and Discussion

Low-Temperature Spectra of Phytochrome. Initial attempts to study the photolysis intermediates of phytochrome in glycerol–ethylene glycol–water mixed solvent at low temperature, indicated that we were not observing merely a simple kinetic system of intermediates and final product. The absorption near the 727-nm maximum of FR disappeared at low temperature and reappeared slowly as the temperature was raised. The results were interpreted as an equilibrium phenomenon involving a third stable form of phytochrome, FRH. Since the equilibrium was shifted by changes in pH as well as temperature, the equilibrium equation finally was expressed as

$$FR + H^+ \rightleftharpoons FRH \qquad (1)$$

where the charge on the protein is deliberately eliminated for simplicity. This model provided the most reasonable interpretation of our data over the temperature range −50 to +25° and pH range 5.0–8.0.

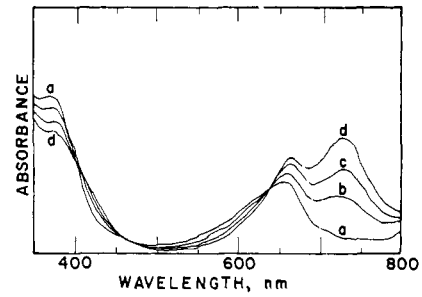

FIGURE 1: Absorption spectra of phytochrome in glycerol–ethylene glycol–water mixed aqueous solvent, "pH" 6.9. (a) −41.3, (b) −32.9, (c) −28.0, and (d) −22.0°.

Figure 1 is an example of the type of phytochrome temperature-dependent spectra observed. The sample initially was converted to the FR form at 0° with 655 nm light from a Bausch & Lomb high-intensity, grating monochrometer and a tungsten (quartz–iodine) source. With a 3-mm exit slit, the band pass was 20 nm. The sample temperature was lowered to −41° and the sample was left to come to equilibrium overnight. The following day spectra were taken (in order) at −41.3, −32.9, −23.0, and −28.0°. The spectra were scanned several times at each temperature to ensure that equilibrium conditions had been established.

The composite spectra (Figure 1) show three distinct isosbestic points at 400, 465, and 637 nm. This evidence is the most concise proof we can offer for a simple two-component equilibrium, *e.g.*, eq 1 above. The FR species has a maximum at 727 nm whereas FRH is associated with a lower peak near 655 nm and with enhanced adsorption below 400 nm. FRH also appears to be nonabsorbing (or nearly so) at 727 nm so that one may use the optical density at this wavelength as a measure of FR concentration

$$[FRH]/[FR] = (A(727)_{max} - A(727))/A(727) \qquad (2)$$

Following this assumption, the van't Hoff equation is plotted in Figure 2 using these and similar data at various buffered pH values. The equilibrium constant is expressed as

$$K = \frac{[FRH]\gamma_{FRH}}{[FR]\gamma_{FR^aH^+}} \qquad (3)$$

$$\log K = \frac{-\Delta H^\circ}{2.3RT} + \frac{\Delta S^\circ}{2.3R} \qquad (4)$$

$$\log\left(\frac{\Delta A(727)}{A(727)}\right) = \frac{-\Delta H^\circ}{2.3RT} + \frac{\Delta S^\circ}{2.3R} - \text{pH} - \log\left(\frac{\gamma_{FRH}}{\gamma_{FR}}\right) \qquad (5)$$

The slopes of the lines that best fit the data measured at various pH values are approximately parallel and yield

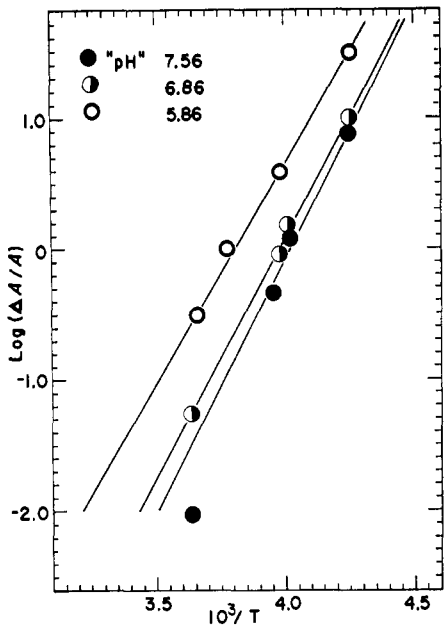

FIGURE 2: Van't Hoff equation plot showing the temperature dependence of the phytochrome equilibrium, FR + $H^+ \rightleftarrows$ FRH, in glycerol–ethylene glycol–water mixed aqueous solvent. The optical density (A) is measured at the peak of the FR spectrum, 727 nm.

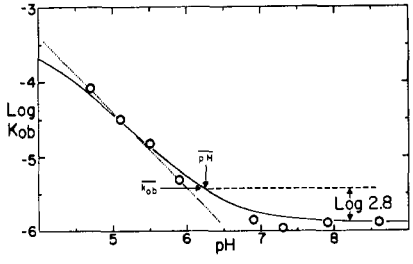

FIGURE 3: The pH effect on the phytochrome dark reaction rate at 10°. The solid line is a theoretical fit to the experimental points (open circles). The limiting slope in the lower pH range is −1.0. The pH for temperature compensation of the dark reaction graphically is found to be $\overline{pH}$ = 6.3; the rate constant, $\bar{k} = 3.8 \times 10^{-6}$ sec^{-1}.

an enthalpy value for the reaction FR + $H^+ \longrightarrow$ FRH of $\Delta H° = -21 \pm 2$ kcal/mole.

From the intercept of the van't Hoff equation plot and the estimated pH values of the glycerol–ethylene glycol–water solutions, we find that

$$\Delta S° - 2.3R \log \gamma_{FRH}/\gamma_{FR} = -56 \pm 7 \text{ eu}$$

In addition to the uncertainty (standard deviation) expressed above, there is also the problem of assigning a "pH" value to the mixed solvent employed. The "pH" values given are those for the phosphate buffer at 25° and no attempt has been made to correct for the effect of the added organic solvent.

The hydrogen ion involvement, however, in the FR–FRH equilibrium does seem reasonably well established from the evidence presented in Figure 2 as well as other data. The three lines through the data points are displaced vertically in the expected direction and are roughly in agreement with the change in "pH" between one data set and another. As suggested above, greater precision in "pH" values may result in a more exacting study of the phytochrome equilibrium. Nevertheless, approximate pK values in both H_2O and glycerol–ethylene glycol–water for the acid, FRH, are calculated to be 4.55 at 0°, 3.95 at 10°, and 3.4 at 20°.

One interesting aspect of the approximate FR–FRH entropy term is its relatively large negative value, −56 eu, which implies that FRH is a significantly more ordered or a higher crystalline state of the protein than FR. Since a hydrogen ion is implicated in the FR–FRH transition, hydrogen-bond formation between appropriate side-chain groups of the long polypeptide molecule might provide the added energy to force the protein into conformation.

The Effect of pH. The effect of pH on the dark reaction of aqueous phytochrome is shown in Table I. The reaction appears to be first order in phytochrome to within our experimental error, and the dark reaction is independent of pH from 6.9 to 8.6. However, as the pH is lowered below 6, the rate of the FR to R reversion is markedly accelerated, and there appears to be a linear relationship between pH and the logarithm of the observed reaction rate (Figure 3).

These results seem best explained by a mechanism involving two kinetic processes instead of one (eq 6 and

$$FR \xrightarrow{k_1} R \qquad (6)$$

$$FRH \xrightarrow{k_2} R \qquad (7)$$

7) and the equilibrium eq 1. By comparison, the equilibrium will be assumed to be *fast* in relation to the two dark reaction rates, k_1 and k_2.

If C is the total phytochrome concentration, we may assume that

$$C = [R] + [FR] + [FRH] \qquad (8)$$

Eliminating the variable [FRH] from eq 3 and 8 we get

$$[FR] = \frac{C - [R]}{1 + Ka_{H^+}} \qquad (9)$$

Finally, the rate equation can be written directly using eq 6 and 7.

$$\frac{d[R]}{dt} = k_1[FR] + k_2K[FR]a_{H^+}$$

$$= \frac{k_1 + k_2Ka_{H^+}}{1 + Ka_{H^+}}(C - [R]) \qquad (10)$$

If k_{ob} is the observed rate constant measured in the laboratory at 727 nm or some other convenient wavelength

$$\frac{d[FR]}{dt} = -k_{ob}[FR] \qquad (11)$$

and

$$k_{ob} = (k_1 + k_2Ka_{H^+})/(1 + Ka_{H^+}) \qquad (12)$$

By making a realistic assumption from the data, we say that $k_2 >> k_1$. It follows, therefore, that the denominator of the observed rate constant can be made to approach unity at high and intermediate pH levels, *i.e.*, $1 >> Ka_{H^+}$, while both terms in the numerator are important, *i.e.*, $k_1 \sim k_2 K a_{H^+}$. In the simplified form then, the observed rate constant is given by

$$k_{ob} \cong k_1 + k_2 K a_{H^+} \quad (13)$$

Above pH 7, we find that the low hydrogen concentration eliminates the second term and

$$k_{ob} \cong k_1 = \text{constant} \quad (14)$$

However, below pH 6, the pH dependence is given approximately by

$$k_{ob} \cong k_2 K a_{H^+} \text{ or}$$
$$\log k_{ob} \cong \log k_2 K - \text{pH} \quad (15)$$

The mechanism, therefore, is in agreement with the pH insensitivity of the rate above pH 7 and with the linear relationship between log k_{ob} and pH below pH 6. It predicts that as the pH is lowered farther the rate will again level off and become independent of pH when pH $<<$ pK. This region is difficult to explore experimentally because of the denaturation of phytochrome at low pH.

For example in Figure 3, the solid line gives the solution at 10° of the complete dark reaction rate eq 12 where $k_1 = 1.4 \times 10^{-6}$ sec^{-1}, $k_2 = 4.5 \times 10^{-4}$ sec^{-1}, and $K = 9 \times 10^3$ l. mole^{-1}. The plot shows good agreement with the experimental points. The slope of the dotted line in Figure 3 is -1.0 and definitely suggests a single proton involvement in the FR–FRH equilibrium. For instance, if two protons were involved in the equilibrium, FR + 2H$^+$ $\rightleftarrows$ FRH, the slope of the line would be -2.0 and in obvious disagreement with our data.

The Effect of Temperature on the Phytochrome Dark Reaction. In earlier work on the phytochrome dark reaction at pH 7.8, the rate of the dark reaction, k_1, increased by an order of magnitude for a 16° increase in temperature (Mumford, 1966).

$$k_1 = A_1 e^{-E_1/RT} \quad (16)$$

where $A_1 = 1.06 \times 10^{13}$ sec^{-1} and $E_1 = 24.4$ kcal/mole.

In solutions of low pH, however, we have now observed precisely the opposite effect, namely a *negative* pseudo-energy of activation. The data plotted in Figure 4 is for the temperature range of -6 to $+15$°. These results may be interpreted in terms of the mechanism involving FRH as follows.

Assume the FRH dark reaction (eq 7) is an energy-activated process

$$k_2 = A_2 e^{-E_2/RT} \quad (17)$$

where A_2 and E_2 are unknowns that we would like to evaluate from available data. The complete functional form of the observed rate constant (eq 12) therefore is known since we already know the temperature-dependent terms, k_1 and K. Using the simplified form (eq 13) for moderate pH conditions

$$k_{ob} \cong A_1 e^{-E_1/RT} + A_2 a_{H^+} e^{\Delta S^\circ/R - (\Delta H^\circ + E_2)/RT} \quad (18)$$

Now, E_1 and E_2 must be positive numbers while ΔH° we know already to be negative. Thus, if the sum $\Delta H^\circ + E_2$ is negative too, we would have a situation similar to the one we have observed. The negative pseudo-energy of activation for the observed rate constant arises from the second k_{ob} term in which a negative change of enthalpy for the reaction

$$\text{FR} + \text{H}^+ \rightarrow \text{FRH}$$

dominates the temperature factor.

To evaluate the unknowns, we rearrange the equation and take the logarithms of both sides

$$\ln (k_{ob} - k_1) = -(\Delta H^\circ + E_2)/RT + \ln A_2 - 2.3(\text{pH}) + \Delta S^\circ/R \quad (19)$$

Plotting the left-hand side *vs.* reciprocal temperature, we find slope $= -(\Delta H^\circ + E_2)/R$ and $-(\Delta H^\circ + E_2) = 13.8 \pm 2$ kcal/mole from our best data. Knowing ΔH°, we find the activation energy, $E_2 = 7.2 \pm 2$ kcal/mole.

Temperature Compensation in the Phytochrome Dark Reaction. It is possible to derive an analytical expression for complete temperature compensation, a small temperature range over which k_{ob} does not change. By set-

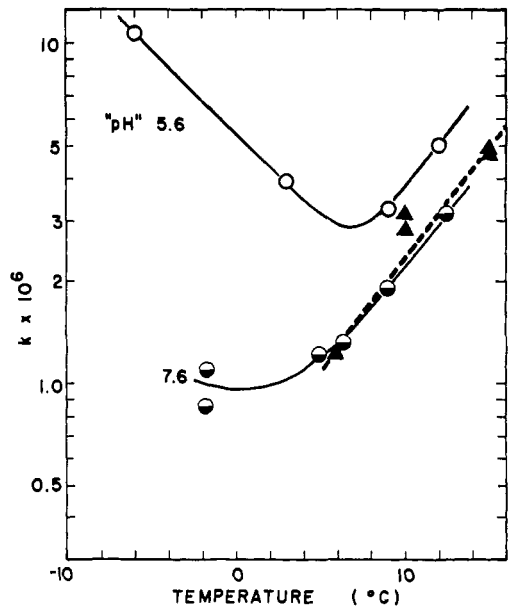

FIGURE 4: Temperature dependence of the phytochrome dark reaction rate constant in glycerol–ethylene glycol–water solvent, "pH" 5.6, ○; and 7.6, ◒; and in buffered water, "pH" 7.6, ▲.

ting its first derivative with respect to temperature equal to zero, we find that the temperature and pH are now dependent variables. We will note the temperature compensation condition with a "bar."

$$\frac{d\bar{k}_{ob}}{dT} = \bar{f}(\bar{T}, \bar{pH}) = 0 \qquad (20)$$

Using this equation to substitute back into the original expression for k_{ob}, we get

$$\bar{k}_{ob} = \frac{E_2 + \Delta H - E_1 + \Delta H}{E_2(1 + K_e a_H{}^+) + \Delta H} k_1$$

$$\bar{k}_{ob} \approx (1 - \frac{E_1}{E_2 + \Delta H})k_1 \text{ (moderate temperature and pH)} \qquad (21)$$

$$\boxed{\bar{k}_{ob} \approx 2.8k_1} \qquad (22)$$

Thus, if oat phytochrome at pH > 7 has a half-life of 9 hr at 25° as previously suggested (Mumford, 1966), the half-life should be 3.2 hr at a lower pH where there is complete temperature compensation. Working backwards, one also finds that the pH at which temperature compensation is reached at 25° is approximately 6.2.

Another direct application of this type of model calculation is shown in Figure 3 where the compensation conditions are indicated with arrows. The temperature compensation pH (here at 10°) is found graphically to be 6.3.

As an attempt to visualize the total behavior of k_{ob} with temperature and pH, a three-dimensional graph was prepared (Figure 5). The vertical coordinate is in units of log k_{ob} from −6.0 to −3.5. The horizontal axes are labeled temperature (−10 to +30°) and pH (4.0–8.0). The following kinematic parameters were used (by a computer) to generate k_{ob} values systematically over the entire surface

$$k_1 = 1.06 \times 10^{13} \exp(-12,300/T)$$
$$k_2 = 210 \exp(-3630/T)$$
$$K = 5.6 \times 10^{-13} \exp(10,600/T) \qquad (23)$$

On the lower right portion of the model (Figure 5), the process

$$FR \xrightarrow{dark} R$$

is illustrated as k_1 dominates. Next to the right-hand slope is the "central valley," a region in which opposing trends cancel each other and temperature compensation of k_{ob} is realized. A center line down the central valley is the geometric counterpart to the temperature compensation eq 20. Finally to the left of the valley is the negative activation energy region and plateau where the FRH species dominates the phytochrome's dark reaction behavior.

Speculation on the Physiological Significance of These Observations. It was originally suggested that the FR → R reversion in darkness might serve as a time-measuring reaction in photoperiodically sensitive plants (Butler, 1960; Hendricks *et al.*, 1962; Hendricks, 1963). Experimental support for this concept has not been obtained, and in fact, a number of observations argue against the "reversion-as-timer" hypothesis (Lang, 1965; Hillman, 1967). Initial work on the phytochrome dark reaction *in vitro* (Mumford, 1966) also does not support the "reversion-as-timer" proposal since the dark reaction appeared to be highly temperature sensitive while time measurement in the photoperiodic reaction is largely temperature independent (Bunning, 1967). Our present *in vitro* work shows, however, that at a pH of around 6.2 the phytochrome dark reaction is temperature compensated. Therefore we can speculate that the FR → R dark reversion *in vivo* is temperature compensated provided that phytochrome is localized in a slightly acidic environment in the cell. Although information on the location of phytochrome in the cell is lacking, the half-life of FR *in vivo* in the *Avena* mesocotyl is around 2.5 hr at room temperature (Edwards and Klein, 1964). This is much closer to the *in vitro* half-life of 3.2 hr found here at pH 6.2 and 25° than that of 9 hr found previously at pH values above 7.

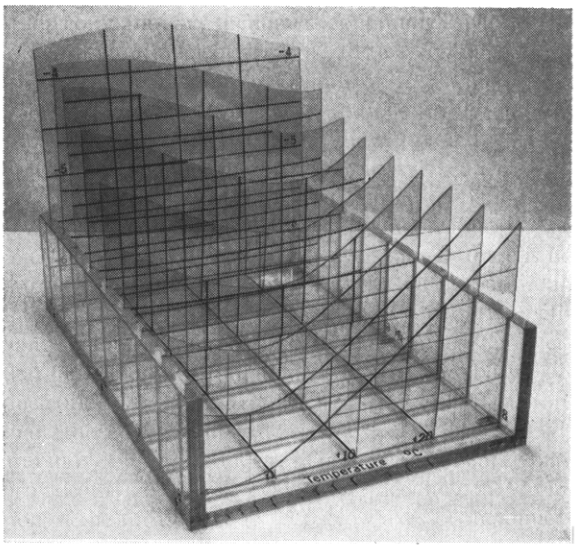

FIGURE 5: A three-dimensional representation of the model discussed in the text governing the rate constant of the phytochrome dark reaction, FR → R, as a function of the temperature and pH. The logarithm of the observed rate constant (measured in sec^{-1}) is plotted along the vertical axis covering the range log k_{ob} = −3.5 to −6.0. See text for further discussion.

Currently there are two main schools of thought concerning the mechanism of phytochrome action. Hendricks and Borthwick (1967) have proposed that phytochrome acts by somehow controlling cell permeability. The experiments of Lange *et al.* (1967) and Carr and Reid (1966) on the actinomycin inhibition of phytochrome-mediated responses, however, suggest that phytochrome in the FR form produces its effect through differential gene activation. Whatever the mechanism, it seems that when phytochrome is in the FR form inhibitors of flowering are synthesized in the leaf and transported to the meristematic regions (Lang, 1965; Leshem and Koller, 1964).

One result of FR action is an increase in the phenylalanine deaminase, an enzyme which catalyzes the deamination of α- phenylalanine to *trans*-cinnamic acid (Attridge and Smith, 1967; Lange *et al.* (1967). The cinnamic acids, of course, are intermediates in the biosynthetic pathways that lead to flavonoids, anthocyanins, and other plant phenolics. Interestingly, the level of these phenolics is reported to be higher in noninduced plants (where FR predominates) than in induced plants (Konishi and Galston, 1964; Taylor, 1965; Zucker *et al.*, 1965). It has been postulated that phenolics could regulate flowering and other growth responses by controlling indoleacetic acid levels through appropriate inhibitor or cofactor effects on the indoleacetic acid–oxidase system. It remains to be proved, though, that the level of phenolics in plants has anything directly to do with any growth response.

As was pointed out above, the biological clock in photoperiodic plants probably involves more than the FR→R dark reversion. Nevertheless, it would seem that reversion is essential to the flowering process because the flow of flowering inhibitor generated as long as FR is present must be checked before induction can proceed. Our present work shows that FR can revert to R by either of two pathways depending upon the pH and temperature (eq 6 and 7). It seems probable that the pathway through FRH, which would predominate at pH values of 6 and below, is of considerable biological importance since the rate of reversion through this intermediate is rapid enough to produce an appreciable FR reversion in physiologically significant periods of darkness. It is also tempting to speculate that phytochrome may play a role in thermoinduction as a result of the FR $\rightleftarrows$ FRH equilibrium being shifted in favor of FRH at low temperatures.

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References

Attridge, T. H., and Smith, H. (1967), *Biochim. Biophys. Acta 148*, 805.

Borthwick, H. A., and Hendricks, S. B. (1960), *Science 123*, 1223.

Borthwick, H. A., Hendricks, S. B., and Parker, M. W. (1952b), *Proc. Natl. Acad. Sci. U. S. 38*, 929.

Borthwick, H. A., Hendricks, S. B., Parker, M. W., Toole, E. H., and Toole, V. K. (1952a), *Proc. Natl. Acad. Sci. U. S. 38*, 662.

Bunning, E. (1967), The Physiological Clock, 2nd ed, New York, N. Y., Springer-Verlag.

Butler, W. L. (1960), *Sci. Am. 203*, 560.

Butler, W. L., Norris, K. H., Siegelman, H. W., and Hendricks, S. B. (1959), *Proc. Natl. Acad. Sci. U. S. 45*, 1703.

Carr, D. J., and Reid, D. M. (1966), *Planta 69*, 70.

Edwards, J. L., and Klein, W. H. (1964), *Plant Physiol. 39*, 50.

Hendricks, S. B. (1963), *Science 141*, 21.

Hendricks, S. B., and Borthwick, H. A. (1959), *Proc. Natl. Acad. Sci. U. S. 45*, 344.

Hendricks, S. B., and Borthwick, H. A. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 2125.

Hendricks, S. B., Borthwick, H. A., and Downs, R. J. (1956), *Proc. Natl. Acad. Sci. U. S. 42*, 19.

Hendricks, S. B., Butler, W. L., and Siegelman, H. W. (1962), *J. Phys. Chem. 66*, 2550.

Hillman, W. S. (1967), *Ann. Rev. Plant Physiol. 18*, 301.

Konishi, M., and Galston, A. W. (1964), *Phytochemistry 3*, 559.

Lang, A. (1965), *in* Encyclopedia of Plant Physiology, Vol. 15, Part 1, Ruhland, W., Ed., Berlin, Springer-Verlag, p 1380.

Lange, H., Bienger, I., and Mohr, H. (1967), *Planta 76*, 359.

Leshem, Y., and Koller, D. (1964), *Ann. Botany (London) 28*, 569.

Linschitz, H., and Kaasche, V. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 1059.

Linschitz, H., Kaasche, V., Butler, W. L., and Siegelman, H. W. (1966), *J. Biol. Chem. 241*, 3395.

Mumford, F. E. (1966), *Biochemistry 5*, 522.

Mumford, F. E., and Jenner, E. L. (1966), *Biochemistry 5*, 3657.

Siegelman, H. W., and Butler, W. L. (1965), *Ann. Rev. Plant Physiol. 16*, 383.

Taylor, A. O. (1965), *Plant Physiol. 40*, 273.

Zucker, M., Nitsch, C., and Nitsch, J. P. (1965), *Am. J. Botany 52*, 271.