Studies on the Phytochrome Dark Reaction *in Vitro**

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ABSTRACT: The spontaneous reversion of the far-red absorbing form of phytochrom to the red absorbing form in darkness has been investigated using partially purified phytochrome from *Avena sativa* L. cv Clintland. The reaction was first order with respect to phytochrome and the rate was highly temperature dependent. Extrapolation of data obtained at lower temperatures *in vitro* suggested that at 25° the far-red absorbing form of phytochrome has a half-life of about 9 hr.

The action spectra of a number of morphological changes in plant development including germination, flowering, and dormancy suggest that they are all controlled by a single light-sensitive pigment, which exists in two readily interconvertible forms (Borthwick *et al.*, 1952a,b; Hendricks *et al.*, 1956; Butler *et al.*, 1959; Hendricks and Borthwick, 1959; Borthwick and Hendricks, 1960). The one form, P_R, generated by far-red (725 mμ) irradiation has an absorption maximum at 665 mμ; the other form, P_{FR}, produced by red (665 mμ) irradiation has a maximum at 725 mμ.

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

Initial *in vivo* studies indicated that P_{FR} could revert spontaneously to P_R in darkness (Butler *et al.*, 1963; DeLint *et al.*, 1963; Edwards and Klein, 1964; Furuya and Hillman, 1964; Hopkins and Hillman, 1964), and it was suggested that this reversion might serve as a time-measuring reaction in photoperiodically sensitive plants (Butler, 1960; Hendricks *et al.*, 1962; Hendricks, 1963). Re-examination of some of this work (Siegelman and Butler, 1965) showed that the apparent P_{FR} to P_R reversion observed in dark grown seedlings was really just a dark destruction of P_{FR}. However, Hillman (1964) has recently demonstrated that in *Cyanara scolymus* (globe artichoke) and *Pastinaca sativa* (parsnip), at least, a true P_{FR} to P_R reversion in darkness can occur.

Previous *in vitro* investigations of the P_{FR} dark reversion have not provided definitive information as to the nature of the reaction. Bonner (1962) found that partially purified phytochrome from peas underwent a slow transformation from P_{FR} to P_R in the dark but the absorbancy drop at 725 mμ (ΔA_{FR}) was less than the increase at 665 mμ (ΔA_R); the Beltsville group found a similar result with phytochrome extracted from barley (Hendricks *et al.*, 1962). These absorbancy changes would be expected to be equivalent, since the ratio $\Delta A_{FR}/\Delta A_R$ for the light-induced conversion of P_{FR} to P_R is 1. More recently it was reported that freshly prepared oat phytochrome showed no spontaneous P_{FR} to P_R reversion in the dark (Butler *et al.*, 1964). After aging or mild denaturation, however, a degree of dark reversion was noted, but again the absorbancy changes at 665 and 725 mμ were unequal. This paper describes our observations on the course of the phytochrome dark reaction *in vitro*.

Experimental Section

Phytochrome Preparation. Partially purified phytochrome was obtained from 5-day-old etiolated *Avena sativa* L. cv Clintland seedlings by the method of Siegelman and Firer (1964). After fractionation on "Brushite" and Sephadex G-200 columns the phytochrome had a specific activity of 10–20 units/g of protein. We have defined the phytochrome unit as that quantity of phytochrome which when dissolved in 1 ml of solution will give a ΔA (absorbancy P_R − absorbancy P_{FR}) at 665 mμ of 1 in a 1-cm path. Protein was determined from the nomograph prepared by E. Adams (California Corp. for Biochemical Research, Los Angeles 63, Calif.) which is based on the extinction coefficients for enolase and nucleic acid given by Warburg and Christian (1942).

Dark Reaction Studies. The studies were carried out in 0.01 M phosphate buffer, pH 7.8, which was 0.05 M in 2-mercaptoethanol. Best results were obtained when the phytochrome solutions were kept anaerobic. For this reason the buffer was made up with freshly boiled water that was cooled under nitrogen, and the phytochrome solutions were also prepared and loaded into the spectrophotometer cells in a nitrogen atmosphere.

Spectra were taken with a Cary recording spectrophotometer fitted with a jacketed sample holder adapted to accept a 10 × 100 mm cylindrical Pyrex cell (Aminco Corporation, Silver Spring, Md.). The sample holder was maintained at a desired temperature by use of a "Forma-Temp" constant temperature bath (Forma Scientific, Inc., Marietta, Ohio). During the runs the

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TABLE I: Phytochrome Dark Reaction.

Temp (°C)	Photoreversible Phytochrome: Before Dark Reaction $\Delta A_{665m\mu}$	Before Dark Reaction $\Delta A_{725m\mu}$	After Dark Reaction $\Delta A_{665m\mu}$	After Dark Reaction $\Delta A_{725m\mu}$	Dark Period (hr)	Dark Reaction: Increase in A at 665 mμ	Dark Reaction: Decrease in A at 725 mμ
10	0.146	0.145	0.145	0.147	17	0.029	0.026
	0.145	0.147	0.143	0.141	40	0.053	0.048
	0.143	0.141	0.138	0.139	66	0.071	0.068
15	0.138	0.139	0.141	0.142	18	0.038	0.039
	0.141	0.142	0.137	0.138	44	0.078	0.073

sample compartment was purged with dry nitrogen to prevent condensation on the optical surfaces.

The phytochrome samples were irradiated with light obtained by passing the output of a 1000-w G.E. PH/1M/P12P lamp through a Corning 2408 cutoff filter (cut off at 600 mμ) and then through a 661 mμ Bausch and Lomb interference filter for red light or through a 732 mμ interference filter for far-red light. Irradiation (4 min) with these light sources was adequate to saturate the phytochrome solutions used in this work.

In the dark reaction experiments the amount of phytochrome was first determined by comparison at 665 and 725 mμ of the absorbance of P_R and P_{FR} obtained by 4-min far-red and 4-min red irradiation, respectively. P_{FR} was then allowed to stand in darkness at a constant temperature for the desired time and the absorbance at 665 and 725 mμ then measured immediately and again after 4-min red irradiation. Comparisons of the absorbances obtained indicated the extent of the dark reaction. The amount of phytochrome at the end of each dark reaction was also determined by comparison of the absorbances of P_{FR} and P_R at 665 and 725 mμ.

Results

Results obtained at 10 and 15° in typical runs are shown in Table I. The decrease in absorbance at 725 mμ and the increase in absorbance at 665 mμ during the dark reaction were essentially equivalent. In addition the P_{FR} to P_R reversion occurred without loss of photoreversible phytochrome. The data also show that the P_{FR} to P_R reversion was temperature dependent.

The P_{FR} dark reaction was first order with respect to phytochrome, since, as shown in Figure 1, a plot of the log of concentration P_{FR} against time produced a straight line. Accordingly, the specific reaction rate constants, k, were calculated from the equation

$$k = \frac{2.303}{t} \log \frac{C_0}{C}$$

Results of calculations made from data obtained at 6, 10, 15, and 19° are shown in Table II. Comparison of the k values at the various temperatures shows that the Q_{10} for the spontaneous P_{FR} reversion in the dark is 3–4.

Direct determination of the phytochrome dark reac-

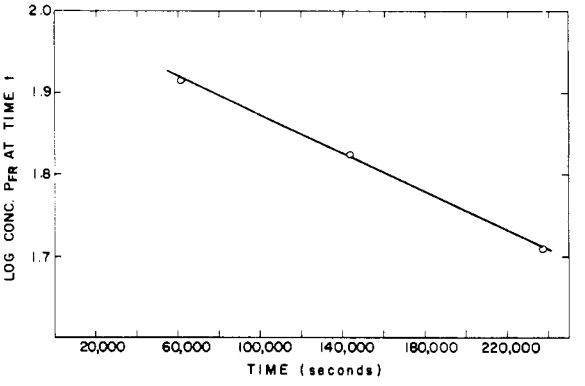

FIGURE 1: Plot of log concentration P_{FR} at time t *vs.* t (seconds) for spontaneous reversion of P_{FR} to P_R in darkness at 10°.

TABLE II: Specific Reaction Rate Constants of Phytochrome Dark Reaction.

Temp (°C)	Dark Period (sec)	% P_{FR} at Time t (C)	k
6	72300	91.4	1.21×10^{-6}
10	61200	82.3	3.16×10^{-6}
	144000	66.6	2.82×10^{-6}
	237600	51.4	2.80×10^{-6}
15	64800	72.5	4.95×10^{-6}
	158400	46.7	4.80×10^{-6}
19	7200	91.7	11.50×10^{-6}
	25200	75.2	11.20×10^{-6}

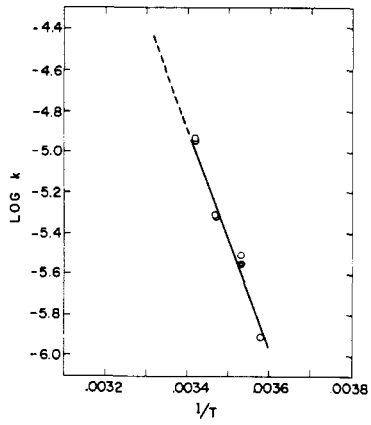

FIGURE 2: Plot of log of the specific reaction rate constants for the spontaneous reversion of P_{FR} to P_R in darkness (Table II) *vs.* $1/T$.

tion rate at temperatures above 19° was not possible, due to extensive denaturation of the protein at the higher temperatures. However, by extrapolation from an Arrhenius plot (Figure 2) the specific reaction rates at higher temperatures could be calculated. For example, at 25° a specific reaction rate of 20.9×10^{-6} sec^{-1} was obtained. The P_{FR} *in vitro* half-life in darkness at 25°, then, would be about 9 hr.

The slope of the Arrhenius plot (Figure 2), −5330, corresponds to a heat of activation, ΔH_a, of 24,400 cal for the P_{FR} to P_R reversion.

Discussion

This investigation shows that the far-red absorbing form of phytochrome will spontaneously revert to the red absorbing form in darkness; further, the reversion does not involve a loss of photoreversible phytochrome. In this respect the reversion *in vitro* appears to be similar to that found *in vivo* in *Cyanara scolymus* and *Pastinaca sativa* by Hillman (1964), but differs from the reversion observed in dark grown seedlings where the P_{FR} dark reaction was accompanied by disappearance of spectrophotometrically detectable phytochrome (Butler *et al.*, 1963; Chorney and Gordon, 1964; Furuya and Hillman, 1964; Hopkins and Hillman, 1964; Siegelman and Butler, 1965).

The discrepancy between our results and those found previously *in vitro* with oat phytochrome (Butler *et al.*, 1964) may be due to our use of buffer which was essentially free of oxygen. When no precaution was made to rid the system of oxygen we frequently, but not always, observed that at the end of the dark period the increase of absorbance at 665 mμ was up to 50% greater than the decrease at 725 mμ. However, other factors must be involved in the pea (Bonner, 1962) and the barley (Hendricks, 1962) systems as we were also unable to obtain an unequivocal P_{FR} reversion in partially purified phytochrome from these sources.

Edwards and Klein (1964) have reported that the rate of the P_{FR} to P_R *in vivo* reversion in darkness is much slower at 4° than at 25°. Our *in vitro* data are in agreement with this finding and indicate that the phytochrome dark reaction has a Q_{10} of between 3 and 4. Edwards and Klein have also observed that the rate of the *in vivo* dark reaction varies considerably from tissue to tissue. For example, at 25° the P_{FR} half-life in the bean hook was 40 min, but in the *Avena* mesocotyl it was 2.5 hr. These are both considerably shorter than the half-life of 9 hr calculated for oat phytochrome *in vitro*. A possible explanation of the differences in half-life found *in vitro* and *in vivo* is that plant tissues contain substances which catalyze the phytochrome dark reaction and these are lost during phytochrome purification. The different nyctoperiods required by photoperiodic plants may reflect the relative concentration of these catalysts in various plant species. Work directed toward finding such catalysts in plant extracts is in progress.

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