Dark Transformations of Phytochrome in vivo. II [1]

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Previous work (4) showed that the phytochrome in dark-grown seedlings was present entirely as the red-absorbing form, P_R. If the seedlings were irradiated with red light and returned to darkness, the P_{FR} formed by the red light, decayed over the course of several hours. After the decay reactions had gone to completion, approximately 20 % of the original amount of phytochrome remained and it was again all P_R. It was assumed that the initial irradiation with red light converted essentially all of the phytochrome to P_{FR} and that during the dark period approximately 20 % of the P_{FR} reverted to P_R, while 80 % of the P_{FR} was either destroyed or altered so that it was no longer photoreversible. The dark conversion of P_{FR} to P_R had been indicated earlier by physiological experiments and had been implicated in the timing mechanism of photoperiodism.

More recent work on purified solutions of phytochrome showed that red light does not convert all of the P_R to P_{FR} (3, 10). P_{FR} absorbs appreciably in the red region of the spectrum so that red light establishes a photostationary state. This was confirmed in experiments on the denaturation of phytochrome with parachloromercuribenzoate (5) which destroyed the absorbance of P_{FR} but had little effect on the absorbance of P_R. A calculation, based on absolute absorption spectra and kinetic data on the rate of conversion of P_R and P_{FR}, showed that the photostationary state in red light consisted of 81 % P_{FR} and 19 % P_R (3). The fact that about 20 % of the P_R was not converted by irradiation with red light alters the previous conclusion that 20 % of the P_{FR} reverted to P_R in the dark. Most of the P_R which was found in the seedlings several hours after the brief irradiation was due to the P_R which was not converted initially. The decay of P_{FR} in the dark-grown seedlings was due mainly to the destruction of P_{FR}; little of the P_{FR} reverted to P_R.

The time course for the apparent conversion of P_{FR} to P_R (4) was that of the destruction of P_{FR}. As P_{FR} disappeared, an irradiation with red light would reestablish the photostationary state and show the presence of some P_R which had not been apparent previously. The previous measurements of dark transformations of phytochrome in dark-grown seedlings were valid measurements of the loss of P_{FR} but not of the dark reversion of P_{FR} to P_R. A recent paper by De Lint and Spruit (7) also shows the loss of phytochrome from dark-grown seedling tissue. They assumed that red light did not convert all of the P_R and concluded that the measurements showed a loss of phytochrome but not dark reversion.

A true dark conversion of P_{FR} to P_R was measured in the curds of cauliflower (4). In this tissue, the total amount of phytochrome remained constant after an irradiation so that the dark conversion of P_{FR} could be studied apart from the destruction of P_{FR}. In the present paper, the dark destruction and the dark conversion of P_{FR} have been studied separately in an effort to define these processes better.

Materials and Methods

The experiments on the destruction of P_{FR} were made with coleoptile sections of maize seedlings, including the primary leaves. The seedlings were grown on moist pads of cellulose in complete darkness at 26° and were harvested 3 to 3½ days after planting. The coleoptiles were about 12 mm long and were completely filled with the primary leaves. Harvesting and preparation of the samples were carried out in the dim green light. The coleoptile sections were removed from the seedlings just prior to the experiment. At various times throughout the experiment a 2-g sample was removed, cut into small segments, and placed in a 19-mm-diameter cell for the photometric measurement of phytochrome.

The phytochrome in the coleoptile segments was assayed with a dual-wavelength difference photometer as described previously (4). In the present work, however, the instrument measured the optical-density difference, Δ OD, between 730 and 800 mμ, rather than the difference between 660 and 730 mμ, in order to eliminate the optical-density changes due to protochlorophyll transformations. Both the relative amounts of P_{FR} and the total phytochrome could be determined. After a given treatment, measurement of the sample would give a certain Δ OD reading, R_1, on the instrument. The sample would then be irradiated with the actinic source of far-red light to convert any P_{FR} present to P_R and decrease the Δ OD reading to R_2. The amount of P_{FR} would be equal to $k\,(R_1 - R_2)$, where k is a constant of proportionality. The sample would then be irradiated with the actinic source of red light which converts 80 % of the P_R to P_{FR} and gives a Δ OD reading, R_3. The total amount of phytochrome would be equal to $1.25\,k\,(R_3 - R_2)$. The

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difference between Δ OD readings is referred to as $\Delta(\Delta$ OD).

The actinic sources consisted of a tungsten lamp with either a 650-mμ interference filter for red light or a plastic filter (Westlakes Plastics, FRF-700) for far-red light. These sources gave maximal photoconversions of phytochrome.

The effect of different atmospheres on the destruction of P_{FR} was examined. A series of connected Erlenmyer flasks, which contained coleoptile sections, was flushed for 30 minutes with the desired gas mixture and sealed. Each flask contained 1 sample so that a sample could be removed for measurement without disturbing the remaining samples. At zero time, the samples were either given a saturating exposure to red light and returned to darkness or placed in continuous light. At various times thereafter a sample was removed for measurement. The atmospheres used were air, N_2, mixtures of 2.5, 5, 7.5 and 10 % O_2 in N_2, and a mixture of 90 % CO and 10 % O_2.

The rate of O_2 uptake by the tissues was determined by direct Warburg manometry at 25°. One-half-g samples of intact coleoptile sections with 0.1 ml of water were placed in 20 ml Warburg flasks without center wells. Two-tenths ml of 15 % KOH was placed in a side arm.

The effect of azide and cyanide on the destruction of P_{FR} and the rate of respiration were also examined. For measurements of phytochrome, intact coleoptiles were immersed in 10^{-3} M NaN_3, 10^{-3} M KCN or potassium phosphate buffer, pH 7.0, for 15 minutes, then drained and blotted and placed in petri dishes. A watch glass containing 4.8 M KCN in 0.5 M KOH was also placed in the petri dish with the sample treated with cyanide. The samples were irradiated with red light and placed in darkness. The amount of P_{FR} was determined 2 hours later. The effect of the inhibitors on respiration was determined by placing coleoptile segments in 3 ml of phosphate buffer and 10^{-3} M azide or cyanide in the Warburg flasks. In the experiment with cyanide, the side arm contained 0.4 ml of 4.8 M KCN in 0.5 M KOH.

Measurements of the dark conversion of P_{FR} to P_R were made on the outer portion of cauliflower heads purchased in the market. The outer 2 mm of the curds was cut from the head and crumbled. One-g samples were placed in cells 12.5 mm in diameter to give a thickness of 11.2 mm. The samples were given a saturating exposure of red light, placed in darkness, and measured for P_{FR} and total phytochrome at various times thereafter.

Results

Destruction of P_{FR}. The destruction of photoreversible P_{FR} was measured in coleoptile sections from dark-grown maize seedlings. These measurements could be made either by irradiating the coleoptiles with a single, saturating exposure of red light and measuring the amount of P_{FR} present after various periods of darkness or by placing the coleoptiles in continuous red or white light and measuring the amount of total phytochrome present after various periods of irradiation. Previous work (4) showed that the loss of total phytochrome in continuous light was due solely to the destruction of P_{FR} and that the rate of loss of total phytochrome was the same for all light sources which maintained at least 10 % of the phytochrome as P_{FR}.

Dependence on Oxygen. Maize coleoptiles were equilibrated with different mixtures of O_2 and N_2. At zero time they were given a brief irradiation with red light, which converted 80 % of the P_R to P_{FR}, and returned to darkness. Samples were taken after 2, 3, and 4 hours and the amount of P_{FR} was determined. The results are shown in figure 1. The rate of loss

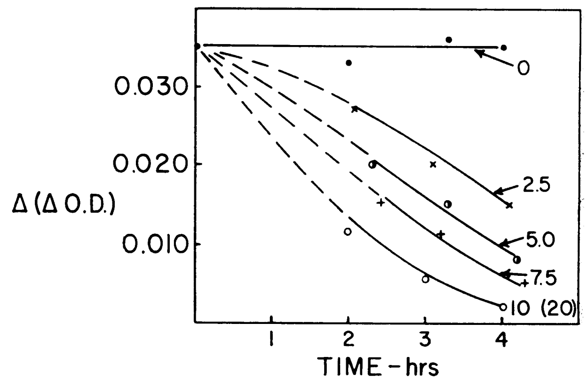

FIG. 1. The dark decay of P_{FR} in maize coleoptiles in atmospheres of different O_2 content after an irradiation with red light. The % O_2 is indicated on the curves. The rate of decay in air was the same as that in 10 % O_2.

of P_{FR} was independent of O_2 at concentrations between 20 and 10 % but at O_2 concentrations below 10 % the rate decreased.

After the dark decay of P_{FR} had gone to completion, measurement showed that 20 % of the original amount of phytochrome remained and it was present as P_R. This represented the P_R which was not converted by the initial irradiation with red light. If there had been a significant reversion of P_{FR} to P_R during the dark period, more than 20 % of the initial amount of phytochrome would have been found as P_R.

The rate of O_2 uptake by intact corn coleoptiles was measured in different concentrations of O_2. The rate of O_2 uptake (fig 2) also decreased at O_2 concentrations below 10 %, probably due to a diffusional limitation. Yokum and Hackett (12) demonstrated a diffusional limitation on respiration at much higher O_2 concentrations with tissue suspended in buffer. Ohmura and Howell (9) showed that even small amounts of water (0.1 ml in a Warburg flask) reduced the rate of respiration of corn scutella in air, presumably by limiting the diffusion of O_2 in the tissue. The intact coleoptile sections used in the

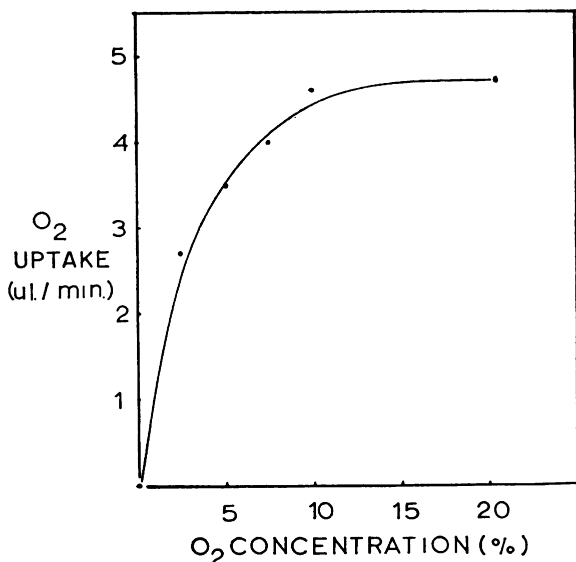

FIG. 2. The rate of O_2 uptake by maize coleoptiles at different concentrations of O_2.

present work were moist, turgid, and completely filled with primary leaves, and the rates of O_2 uptake per g of tissue were relatively high so that a diffusional limitation in atmospheres containing less than 10 % O_2 is not unreasonable. A comparison of figures 1 and 2 suggests a parallelism between the rate of decay of P_{FR} and the rate of respiration.

Effect of Respiratory Inhibitors. The effects of CO on the destruction of P_{FR} and the rate of respiration were examined by placing the coleoptiles in an atmosphere of 90 % CO and 10 % O_2. The curve for CO (dark) in figure 3 was measured in the same way as the curves of figure 1, i.e., one saturating exposure to red light followed by darkness. CO slowed the rate of dark decay of P_{FR}.

The effect of CO on respiration is shown in figure 4. The rate of respiration in the dark in 90 % CO and 10 % O_2 was about 40 % of the rate in 90 % N_2 and 10 % O_2. The inhibitory effect of CO on respiration could be partially reversed by illuminating the coleoptiles with strong white light. In the light (fig 4) the rate of respiration in CO was about 70 % of the uninhibited rate. Warburg (11) originally showed that the CO-cytochrome oxidase complex was broken by light.

The inhibitory effect of CO on the decay of P_{FR} was also reversed by light (fig 3). This was shown by placing the coleoptiles in continuous light at zero time and measuring the loss of total phytochrome. The results of figure 3 show that when the coleoptiles were in continuous white light the rate of loss of phytochrome in the CO atmosphere was almost as great at it was in 90 % N_2 and 10 % O_2. However, if the coleoptiles in the CO atmosphere were irradiated continuously with red light ($\lambda > 600$ mμ), the effect of CO on the loss of phytochrome was not altered by the light. Red light does not reverse the inhibitory effect of CO on respiration because it is not absorbed by the CO-cytochrome oxidase complex.

NaN_3 and KCN partially inhibited the decay of P_{FR} and the rate of O_2 uptake. One-half as much P_{FR} was lost in 2 hours of darkness when the seedlings were treated with 10^{-3} M azide or cyanide as compared with the control. The rate of O_2 uptake was also decreased 50 % by the 2 inhibitors.

Dark Reversion of P_{FR}. Dark-grown seedlings were not suitable for the study of the dark reversion of P_{FR} to P_R because P_{FR} was destroyed more rapidly than it was converted to the stable P_R. It was shown previously, however, with phytochrome in the curds of cauliflower, that P_{FR} reverted to P_R in the dark without loss of total phytochrome (4). Figure 5 shows the dark conversion of P_{FR} in cauliflower curds. At zero time, the samples were given a saturating exposure to red light and returned to darkness. At

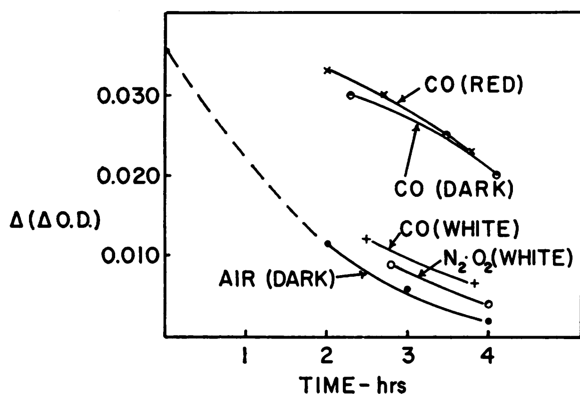

FIG. 3. The decay of phytochrome in maize coleoptiles in 90 % CO and 10 % O_2 and in 90 % N_2 and 10 % O_2. The curve for air (dark) was taken from figure 1. The decay of P_{FR} in the dark was measured in the curves marked (dark). The decay of total phytochrome in continuous light was measured in the curves marked (white) and (red).

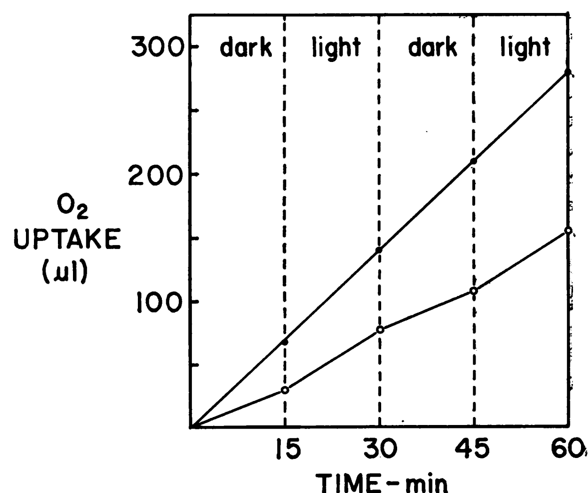

FIG. 4. O_2 uptake by maize coleoptiles; ○, in 90 % CO and 10 % O_2 and ●, in 90 % N_2 and 10 % O_2 in the light and in the dark.

various times thereafter a sample was measured for relative amounts of P_{FR} and total phytochrome. The total amount of phytochrome in a sample remained constant throughout the experiment. The fraction present as P_{FR}, however, fell from its initial value of 80 % (assuming the photostationary state set by red light is the same for cauliflower phytochrome as it is for seedling phytochrome) as P_{FR} was converted in the dark to P_R. At room temperature, the dark conversion appears to have 2 phases: a relatively rapid initial phase followed by a very slow phase which may continue for 24 hours or more. The fraction of the phytochrome converted during the initial phase varies with different heads of cauliflower. At low temperature, near 0°, the dark reversion is very slow, such that about 25 % of the P_{FR} may revert to P_R in 24 hours. Unlike the dark decay of P_{FR} in dark-grown seedlings, the dark reversion of P_{FR} in cauliflower was not affected by a lack of O_2. Experiments in which cauliflower samples were kept in air and in N_2 showed that the dark reversion of P_{FR} in N_2 paralleled that in air throughout the experiment. In the experiment shown in figure 5, the sample that was held in N_2 in the dark for 17 hours after an irradiation with red light showed the same amount of dark reversion as the sample in air (fig 5).

In dark-grown seedlings held in N_2 (fig 1), P_{FR} was quite stable in the dark over the course of the measurements. Thus, the dark reversion of P_{FR} to P_R was not observed even when the decay processes were inhibited.

Phytochrome Synthesis. Phytochrome is synthesized in dark-grown seedlings as the seedlings grow. The synthesis of phytochrome in seedlings which had received no light was compared against the synthesis in seedlings which received 4 hours of red light. Dark-grown corn seedlings, 3 to 3½ days old, were selected for uniformity and divided into 3 equal samples of 18 seedlings each. One sample was harvested and divided into coleoptile plus primary leaf sections and mesocotyl sections. Both sections were weighed and assayed for phytochrome. The other 2 samples were returned intact to pads of moist cellulose. One sample was placed in the dark for 24 hours. The other sample was irradiated 4 hours with red light and placed in darkness 24 hours. The weight and phytochrome content of the coleoptile and mesocotyl sections of these 2 samples were determined. The amount of phytochrome in the tissue immediately after

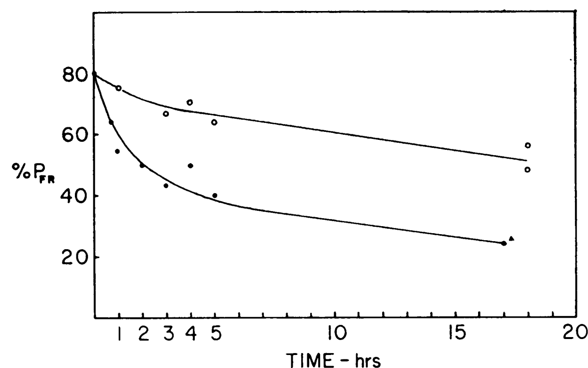

Fig. 5. The percentage of the phytochrome in cauliflower curds present as P_{FR} vs time after an irradiation with red light. ●, sample in air at room temperature; ○, sample in air at 0°; △, sample in N_2 at room temperature (single point at 17 hr).

the 4-hour irradiation was measured on seedlings not included in the 3 uniform samples. Total phytochrome, in relative units, in the various fractions was calculated as the weight of tissue times the $\Delta(\Delta$ OD) reading. The phytochrome measurements were made on 1-g samples in cells 12.5 mm in diameter. The results are presented in table I. Most of the weight increase associated with the 24 hours of growth occurred in the coleoptile plus primary leaf sections and was due primarily to the leaf growth. The leaf growth was greater in the sample which received 4 hours of light than in the dark control, but the increase of phytochrome during the 24-hour period was less. In all cases the phytochrome accumulated as P_R. The 4-hour irradiation period not only decreased the phytochrome content of the tissue to scarcely detectable level but also decreased the synthesis of phytochrome during subsequent growth in the dark. The tissue which had received 4 hours of light was obviously more mature than the dark control. The leaves had pushed through the coleoptiles and were green and partially unfurled.

Discussion

The concentration of phytochrome in dark-grown seedlings is much higher than that found in other plant tissues. When the seedlings are placed in light, the amount of phytochrome drops drastically to a level difficult to detect photometrically, particularly in the

Table I. *Growth and Phytochrome Synthesis in 3 to 3½-Day-Old, Dark-Grown Corn Seedlings*

		0 hr			24 hr		
		Wt g	$\Delta(\Delta OD)$	Total P (rel)	Wt	$\Delta(\Delta OD)$	Total P
Dark control	coleoptile	2.3	0.024	0.055	4.6	0.017	0.078
	mesocotyl	4.0	0.007	0.028	5.0	0.006	0.030
Irrad 4 hr red light	coleoptile	...*	< 0.001	0.001–0.002	5.4	0.002	0.011
	mesocotyl	...*	0	0	4.2	0	0

* Assumed to be the same as the dark control.

presence of increasing amounts of chlorophyll. The phytochrome does not disappear entirely, however. Seedlings and mature green plants are sensitive to low energies of red and far-red light after many hours of illumination and phytochrome has been extracted from the leaves of a number of mature green plants (8). Apparently, a small fraction of the phytochrome in dark-grown seedlings is different, or potentially different, from the rest. This small fraction might be more stable by virtue of different binding sites which might, or might not, be related to its presumed enzymatic activity. The question arises as to whether all of the phytochrome in dark-grown seedlings is physiologically functional or whether the only active phytochrome is the small amount that remains in continuous illumination.

The phytochrome which we measure in commercial heads of cauliflower is apparently that which is not destroyed in the light. The destructive processes may have occurred earlier. This phytochrome shows dark reversion of P_{FR} to P_R which can be studied without competition from the destructive processes. The rate of dark reversion of P_{FR} in this tissue is such that some P_{FR} may remain for 24 hours or longer after an irradiation with red light. The rate of dark reversion probably varies among different tissues, but rates of this order are consistent with some physiological observations. Physiological observations on lettuce seed indicated that P_{FR} reverted to P_R in the dark over the course of 24 to 48 hours (2). Observations on flowering, however, indicated that effective dark reversion could occur in 15 minutes (1). These differences might be related to the fast and slow phases of dark reversion. De Lint, et al. (6) found that the effects of red light on mesocotyl elongation could be partially reversed by far-red light up to 10 hours after the red irradiation. We would suggest that only a small fraction of the phytochrome of dark-grown seedlings is active in controlling the growth responses of the seedlings and that the dark reversion of this fraction of the phytochrome, which has not been measured directly, is similar to the dark reversion of phytochrome in heads of cauliflower.

The data on the synthesis of phytochrome in dark-grown seedlings were taken to determine if the prolonged sensitivity to far-red light following a red irradiation might be a manifestation of phytochrome synthesis rather than of dark reversion. Table I shows that phytochrome synthesis is associated with growth and that the synthesis occurs primarily in tissue growing by cell division (primary leaves) rather than by cell expansion (mesocotyl). This suggests that, with the tissue irradiated with red light, the synthesis occurred in new tissue; not the tissue that had been irradiated. Furthermore, the newly synthesized phytochrome appeared as P_R, not P_{FR}. These data do not indicate that the prolonged sensitivity to far-red light is due to phytochrome synthesis. It should also be noted that no net synthesis of phytochrome occurs in heads of cauliflower where dark reversion of P_{FR} to P_R has been measured.

The synthesis of phytochrome, which occurs in young etiolated tissue undergoing cell division is analogous to the large amount of phytochrome in dark-grown seedlings. The rate of synthesis as well as the amount present appears to be related to etiolation. The data of table I show that the irradiation with red light not only decreased the amount of phytochrome present but also decreased the rate at which phytochrome was synthesized subsequent to the irradiation.

Summary

The loss of phytochrome from dark-grown maize seedlings was measured following irradiation with red light. Most of the P_{FR} in this tissue was destroyed while showing little, if any, dark reversion to P_R. The rate of decay of P_{FR} was correlated with the rate of respiration, measured as O_2 uptake. Inhibiting respiration by lowering the O_2 concentration or by adding respiratory inhibitors such as CO, cyanide, and azide also inhibited the destruction of P_{FR} in dark-grown seedlings.

The tissue from heads of cauliflower showed phytochrome which was not destroyed in the light. The P_{FR} in this tissue reverted to P_R in the dark without loss of total phytochrome. The dark reversion of P_{FR} to P_R was not affected by lack of O_2.

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