KINETICS OF PHYTOCHROME CONVERSION: MULTIPLE PATHWAYS IN THE P_R TO P_{FR} REACTION, AS STUDIED BY DOUBLE-FLASH TECHNIQUE*

By Henry Linschitz and Volker Kasche†

DEPARTMENT OF CHEMISTRY, BRANDEIS UNIVERSITY, WALTHAM, MASSACHUSETTS

Communicated by Sterling B. Hendricks, July 12, 1967

The initial flash excitation studies on phytochrome showed that the interconversion between the two forms of the pigment is not a direct one-step photochemical transformation but takes place via a complex set of light and dark processes.[1] Four dark reactions can be distinguished in the conversion from the red (P_r) to the far-red (P_{fr}) absorbing forms, and two are seen in the reverse transformation. Absorption spectra corresponding to the various intermediate configurations were obtained and the rate constants measured at 0°C for all six distinguishable dark reactions. However, these experiments did not yet fully establish the relationships among the several steps in the interconversion. In particular, because of the difficulty of kinetic analysis of overlapping reactions, there remained some uncertainty whether the various stages correspond to sequential or parallel processes. In this paper, we describe further experiments on the phytochrome system using a "double-flash" technique. These studies provide very strong evidence that, as suspected earlier, the final three dark reactions observed in the $P_r \rightarrow P_{fr}$ conversion are indeed concurrent, parallel processes, in which three different intermediates transform independently to P_{fr}. To understand the design of these experiments, it is necessary to review briefly the findings of our earlier work.[1]

Flashing P_r causes an immediate (within 50 μsec) drop at the 664-nm peak and the formation of a new band at 695 nm, but there is no absorption increment at 724 nm, the P_{fr} peak. This initial configuration (R_1) undergoes a fast dark transformation ($k_1 = 6000$ sec^{-1} at 0°C) in which the 695-nm peak shifts to 700 nm (R_2 configuration). There is no change at 664 nm in the transformation $R_1 \rightarrow R_2$. Absorption at 724 nm then begins to develop, and proceeds through three well-defined stages, denoted by R_3, R_4, and finally P_{fr}. The rate constants for the transformations $R_2 \xrightarrow{k_2} R_3$, etc., are, respectively, about 50, 4, and 0.3 sec^{-1} (0°C). The steps $R_2 \rightarrow R_3$ and $R_3 \rightarrow R_4$ are accompanied by further decreases at 664 nm, but R_4 is isosbestic with P_{fr} at this wavelength. These results are summarized in reference 1, Figure 4.

If it could be shown that the final product, P_{fr}, is the *only* substance responsible for the 724-nm absorption at *each* step of absorbance development, it would follow necessarily that these three reactions are parallel and not sequential. The fact that the far-red absorption increments at stages R_3 and R_4 both appear at 724 nm is, of course, suggestive but (particularly since the spectra of all the substances involved lie so close together) not conclusive, in the absence of any further criteria of identity. Such an additional criterion is found in the characteristic bleaching reactions of P_{fr} itself.

The conversion of P_{fr} to P_r may be represented by the formal scheme $P_{fr} \xrightarrow{h\nu} FR_1 \rightarrow FR_2 \rightarrow P_r$.[1] Neither of the two intermediate configurations, FR_1 or FR_2, show any absorption at 724 nm. Thus, flashing P_{fr} itself causes an immediate bleaching at 724 nm, with no further change in absorbance at this wavelength due to subsequent dark reactions (ref. 1, Fig. 8). On the hypothesis that *all* 724-nm absorption is due to P_{fr}, a suitably timed far-red flash following initiation of the $P_r \rightarrow P_{fr}$ conversion should therefore cause an immediate partial bleaching of the developing 724-nm absorption, superimposed on its relatively slow, stepwise growth from R_2 to P_{fr}. Moreover, the transformation $FR_1 \xrightarrow{k_1'} FR_2$ ($k_1' \sim 1000$ sec^{-1} at 0°C) is very much faster than $R_2 \rightarrow R_3$ or $R_3 \rightarrow R_4$, and FR_2 has practically the same absorption at 664 nm as P_r itself.[1] Therefore, one may hope to observe a fast rise in 664-nm absorption resulting from the conversion of the 724-nm "transient" by the second, far-red flash superimposed on the continued slow bleaching at 664 nm accompanying the transformations of the R_2 and R_3 configurations. In solutions of low optical density, the fraction of light-sensitive molecules converted by a flash of given intensity should be independent of concentration, since in this range the light flux will be essentially constant throughout the solution and the processes considered here are all first-order in pigment concentration.[1] Thus, by working in dilute solution, any fractional bleaching at 724 nm caused by the second, far-red flash during the $P_r \rightarrow P_{fr}$ conversion, and any corresponding rise at 664 nm, may be quantitatively compared with the changes caused by a similar flash in an undoubtedly authentic sample of P_{fr} (as produced by steady red irradiation of the same solution or by standing after a red flash until all dark reactions are complete). The nature of the intermediate 724-nm absorption may therefore be ascertained at each stage of the $P_r \rightarrow P_{fr}$ reaction.

Materials.—Phytochrome extracts in 0.5 *M* sucrose–0.1 *M* phosphate buffer, prepared by Mr. B. C. Turner according to the procedures of Siegelman and Hendricks,[2] were generously provided to us by Dr. Hendricks and his group. The preparations were kept at −20°C, and seemed to undergo no deterioration in photoreversibility after storage for up to a year. The general pattern of light and dark transformations in the extracts studied here was identical to that observed with previous samples.

Apparatus and Procedures.—The apparatus used previously was slightly modified for double-flash operation. The four flash lamps were arranged as before, in two pairs of two series-connected lamps each, but a second spark gap, fired by a separate delay and trigger circuit, was introduced to provide a variable delay between the flashes from each pair of lamps. The two series-connected lamps in each set were placed opposite each other in the four-leaved reflector so that the sample was illuminated symmetrically from both sides, by both the first and second flashes. Red and far-red filters, made of dyed plastic,[3] were interposed between the sample and the first and second set of lamps, respectively. These filters were supported on a metal frame which fitted around the water-jacketed flash cell, within the reflector unit. The same timing pulse which fired the first (red) flash also triggered the oscilloscope sweep. An auxiliary (far-red) source was used, as before, to restore the phytochrome to the P_r form, before each measurement. Care was taken to charge the condensers always to the same voltage, to obtain flashes of reproducible intensity.

Blank Corrections and Treatment of Data.—The far-red filters, passing light mainly absorbed by P_{fr}, do not completely exclude light absorbed by P_r. Thus, the phytochrome solution after its preliminary transformation to "P_r" still contains a small steady-state amount of P_{fr} (about 2% of total pigment). Fractions of this are bleached by the far-red and red flashes. In addition, the far-red flash bleaches some P_r. Corrections must therefore be applied to the observed absorbance changes to isolate the effects due solely to the action of the far-red flash on the 724-nm material formed by the first red flash. These corrections are evidently most serious in the initial phases of the $P_r \rightarrow P_{fr}$ conversion when the developing 724-nm absorption does not yet greatly exceed that initially present, or when the increment at 664 nm corresponding to 724 nm bleaching by the second far-red flash is comparable to the direct bleaching at 664 nm caused by the same flash. The corrections are made as follows:

Let P_r° and P_{fr}° = concentrations of P_r and P_{fr}, respectively, in the initial steady state, before flashing;
α_r and α_{fr} = fractions of P_r and P_{fr} converted by the first, red flash;
β_r and β_{fr} = fractions of P_r and P_{fr} converted by the second, far-red flash;
ϵ_i = extinction coefficient of species "i" at 664 nm;
ϵ_i' = extinction coefficient of species "i" at 724 nm;
C_{fr} (t) = concentration of P_{fr} formed by the conversion of P_r by the first flash, as function of time t after this flash.

We take it, as indicated above, that P_r and the various substances in configurations R_1, R_2, and FR_2 have negligible absorption at 724 nm.[1] In addition, since the fractional conversions are small and the far-red filter is fairly effective, we neglect any possible photolysis by the far-red flash of transient species which do not absorb at 724 nm. On the hypothesis that *any* absorption at 724 nm is due *solely* to P_{fr}, the change in absorbance at this wavelength as function of time after the first red flash is (l = cell path length):

$$\Delta D_{724}(t) = [-\alpha_{fr}P_{fr}^\circ + C_{fr}(t)]\epsilon_{fr}'l. \tag{1}$$

The immediate absorbance change at 724 nm caused by the second, far-red flash at time t after the first flash is:

$$\Delta(\Delta D)_{724} = -\beta_{fr}[(1-\alpha_{fr})P_{fr}^\circ + C_{fr}(t)]\epsilon_{fr}'l, \tag{2}$$

and the corresponding fast absorbance change at 664 nm is:

$$\Delta(\Delta D)_{664} = \beta_r[(1-\alpha_r)P_r^\circ + \alpha_{fr}P_{fr}^\circ](\epsilon_{R_1}-\epsilon_r)l + \beta_{fr}[(1-\alpha_{fr})P_{fr}^\circ + C_{fr}(t)](\epsilon_r-\epsilon_{fr})l. \tag{3}$$

In equation (3), $P_{fr}^\circ \ll P_r^\circ$ and since α_r and α_{fr} are both considerably less than 1 (see below), the term in $\beta_r\alpha_{fr}P_f{}^\circ$ may be safely neglected.

In equations (2) and (3), we wish to isolate the absorbance changes involving $C_{fr}(t)$ from the total observed changes. If, in a blank experiment, the *initial* phytochrome solution is illuminated by the far-red flash *alone*, then the fast changes in absorption, S, at 724 and 664 nm are, respectively:

$$S_{724} = -\beta_{fr}P_{fr}^\circ\epsilon'l \tag{4}$$

and

$$S_{664} = \beta_r P_r^\circ(\epsilon_{R_1}-\epsilon_r)l + \beta_{fr}P_{fr}^\circ(\epsilon_r-\epsilon_{fr})l. \tag{5}$$

In equations (1) and (2), we take $\alpha_{fr} = \beta_{fr}$, since most of the actinic light effective on P_{fr} in the red flash is passed also by the far-red filter, and both flashes are of equal intensity. Therefore, from equation (2):

$$\begin{aligned} -\beta_{fr}C_{fr}(t)\epsilon_{fr}'l &= \Delta(\Delta D)_{724} - (1-\beta_{fr})S_{724} \\ &= \Delta(\Delta D)_{724,\ \text{corr.}}, \end{aligned} \tag{6}$$

and from equation (1):

$$\begin{aligned} C_{fr}(t)\epsilon_{fr}'l &= \Delta D_{724}(t) + \beta_{fr}P_{fr}^\circ\epsilon_{fr}'l \\ &= \Delta D_{724}(t) - S_{724} \\ &= \Delta D_{724}(t)_{\text{corr.}}, \end{aligned} \tag{7}$$

whence the effective β_{fr} at any time t, for the far-red transient alone, is:

$$\beta_{fr}(t) = \left[\frac{-\Delta(\Delta D)}{\Delta D(t)}\right]_{724,\ \text{corr.}}. \tag{8}$$

The average value of β_{fr} in these experiments was found to be about 0.29 (Table 1, below) which we take also for α_{fr}. From the ratio of the total ΔD_{724} produced by the red flash to the initial 664-nm absorbance, and using the relation $\epsilon_r = 1.55\ \epsilon_{fr}'$,[4] we find the average value of α_r in these experiments to be about 0.20. Therefore, in equation (3), we approximate the factors $(1 - \alpha_r)$ and $(1 - \alpha_{fr})$ by the over-all average of 0.75, and obtain (neglecting $\beta_r\alpha_{fr}P_{fr}^{\circ}$):

$$\begin{aligned}\beta_{fr}C_{fr}\,(t)(\epsilon_r - \epsilon_{fr})l &= \Delta(\Delta D)_{664} - 0.75S_{664}\\ &= \Delta(\Delta D)_{664,\ \text{corr.}}\end{aligned} \qquad (9)$$

The absolute value of the correction is small, and the error incurred in this averaging approximation is less than the experimental error in determination of the ΔD's themselves.

Results and Discussion.—Figure 1 shows examples of typical oscillograms taken in this work. The straight sweeps measure the light transmission prior to flashing the sample. The second sweeps, taken with the same base-line and gain, show the transmission as function of time after the red flash which initiates the $P_r \rightarrow P_{fr}$ reaction. The breaks in this second sweep correspond to delivery of the far-red flash at various times ("delay," Fig. 1) after the red flash.

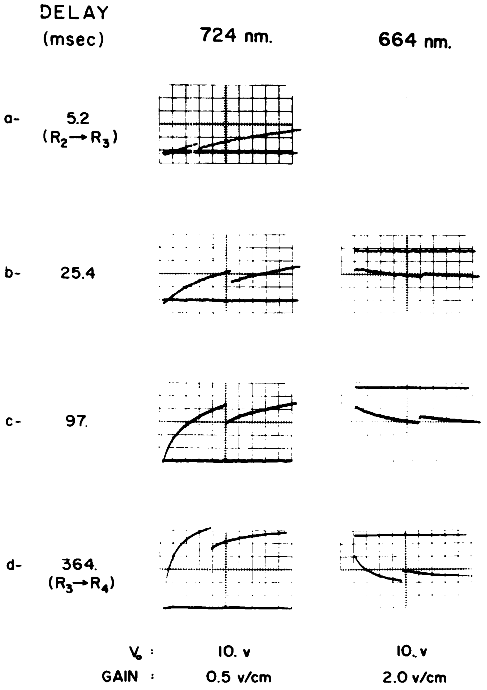


FIG. 1.—Typical double-flash oscillograms. *Increasing* light transmission corresponds to downward deflection. Base lines are displaced off scale at top of oscillogram, and photometer gain is adjusted to give initial 10.0-v signal. Straight sweeps, initial light transmission, before flash; second sweeps, triggered simultaneously with red flash, and far-red flash fired after indicated delay. Sweep speeds (msec/cm): *a*, 2.0; *b*, 5.0; *c*, 20; *d*, 100. Change in absorbance, $\Delta D = \log\,[10/(10-(\Delta y)(\text{gain}))]$, where Δy is upward vertical deflection in cm, measured from first sweep. Gain and wavelength of observation as indicated. Sample no. GO-17 (Table 1), pre-irradiated with far-red light before each oscillogram. In oscillograms (*a*) and (*d*), the predominant processes are $R_2 \rightarrow R_3$ and $R_3 \rightarrow R_4$, respectively.

It is apparent that the far-red flash causes a sharp and immediate bleaching at 724 nm at *all* stages of development of absorption. Correspondingly, the second flash causes a rise in absorbance at 664 nm, but with a rounding off of the trace (Fig. 1, 664, *b* and *c*). Both these effects are qualitatively to be expected if the material bleached at 724 nm by the far-red flash is indeed P_{fr}. The sharp drop in the traces at 724 nm is similar to the immediate bleaching, characteristic of P_{fr} (see ref. 1, Fig. 8), and the rise with rounding off at 664 nm corresponds to the conversion of P_{fr} to FR_1, followed by the rapid dark formation of FR_2 (ref. 1, Figs. 9c and 10).

Table 1, which includes the experiments illustrated in Figure 1, summarizes typical quantitative results. Measurements are given of the amount of absorbance growth (corrected) at 724 nm at the time of the far-red flash (ΔD_{724}), the absorbance changes directly caused by the far-red flash [$\Delta(\Delta D)$] at these various times, and the correction parameters (S and β). At 724 nm, where the samples have low absorbance, the precision of the photometric data is estimated (conservatively) at about ± 0.001 for ΔD's less than 0.05, or about 2 per cent for larger ΔD's. Comparable errors also arise from the irreproducibility of flash intensity and, for small conversions involving relatively large corrections, from variations in the extent of initial adjustment of the pigment to the P_r form. At 664 nm, where the sample absorbance is high, absolute photometric precision is somewhat less. Other sources of error at 664 nm include the estimation of the peak $\Delta(\Delta D)_{664}$ at the end of the fast dark process and correction of this $\Delta(\Delta D)$ for the accompanying slow bleaching associated with the transformation of R_2 to R_3. The over-all uncertainty of the $\Delta(\Delta D)_{664}$ values is estimated to be about ± 0.002.

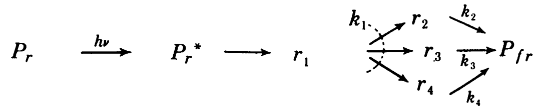
TABLE 1

EFFECTS OF FAR-RED FLASH ON TRANSIENTS IN $P_r \rightarrow P_{fr}$ CONVERSION(a)
(TEMPERATURE = 0.4°)

Sample specifications	Time (msec)	ΔD_{724}(c) corr.	$-\Delta(\Delta D)_{724}$(d) corr.	$\Delta(\Delta D)_{664}$(e) corr.	$\left[\frac{-\Delta(\Delta D)}{\Delta D}\right]_{724}^{(f)}$ corr.	$\frac{-\Delta(\Delta D)_{724}{}^{(f)}}{\Delta(\Delta D)_{664}}$ corr.
No. GO-15	5.2	0.045	0.011	0.010	0.24	1.10
Abs(664) = 1.06(b)	10.6	0.066	0.017	0.017	0.26	0.97
β_{fr} = 0.26	36.0	0.097	0.025	0.024	0.25	1.04
S_{724} = −0.003	67.	0.102	0.027	0.025	0.27	1.09
S_{664} = −0.005	150.	0.104	0.029	0.029	0.28	1.01
	302.	0.121	0.029	0.031	0.24	0.95
	Steady(g)	0.281(h)	0.079(h)		0.28	1.00(i)
	Steady	0.383	0.097		0.25	1.00
No. GO-17	5.2	0.020	0.0054		0.27	
Abs(664) = 1.69(b)	9.4	0.030	0.0081		0.27	
β_{fr} = 0.30	25.4	0.062	0.018	0.020	0.30	0.92
S_{724} = −0.005	97.	0.114	0.034	0.034	0.30	1.01
S_{664} = −0.004	364.	0.165	0.045	0.048	0.27	0.94
	Steady(g)	0.482(h)	0.146(h)		0.30	0.95(i)
	Steady	0.334	0.103		0.31	0.95

(a) ΔD values rounded to nearest 0.001 unit.
(b) In 5-cm cell.
(c) $\Delta D_{724,\text{corr.}} = \Delta D_{724} - S_{724}$.
(d) $-\Delta(\Delta D)_{724,\text{corr.}} = -\Delta(\Delta D)_{724} + (S_{724})(1 - \beta_{fr})$.
(e) $\Delta(\Delta D)_{664,\text{corr.}} = \Delta(\Delta D)_{664} - 0.75S_{664}$.
(f) Using unrounded $\Delta(\Delta D)$ data.
(g) Sample was partially converted to P_{fr} by auxiliary red light, and then exposed only to far-red flash.
(h) No corrections necessary, since only total ΔD's are used.
From independent spectrophotometric measurement (Cary, 14).

The diameter of the cylindrical flash cell (5-cm length) is 1 cm, so that the sample absorbance in the transverse direction of flash excitation, and at the ΔD_{724} values of Table 1, is very low. Under these conditions, as explained previously, the fraction bleached by the far-red flash is expected to be constant, if only a single substance is being converted. Table 1 demonstrates, within the precision stated above, that the fractional conversion at 724 nm $[-\Delta(\Delta D)/\Delta D]_{724}$ is indeed constant at all stages of the growth of far-red absorbance following initiation of the $P_r \rightarrow P_{fr}$ reaction. Moreover, this constant fractional conversion is equal to that produced by the same far-red flash, acting on authentic P_{fr} ("steady" values in Table 1). In addition, Table 1 shows that the ratio of the bleaching at 724 nm to the rise at 664 nm is constant at all stages of development of far-red absorption, and is equal, within experimental error, to the ratio observed spectrophotometrically for the over-all $P_r \rightarrow P_{fr}$ conversion. The photo-bleaching characteristics of the transients responsible for developing absorption at 724 nm in the three phases of the reaction,[1] $R_2 \xrightarrow{k_2} R_3$, $R_3 \xrightarrow{k_3} R_4$, and $R_4 \xrightarrow{k_4} P_{fr}$ are thus identical with P_{fr} itself. We may reasonably conclude that only one substance, P_{fr}, is responsible for absorption at 724 nm. The three dark reactions must therefore be concurrent processes. Using the small letter "*r*" to denote transient *substances*, in distinction to "*R*" for observed *stages* of the reaction, the over-all $P_r \rightarrow P_{fr}$ conversion must involve such schemes as:



In this scheme, the configuration R_1 corresponds to the substance r_1, R_2 is a mixture of r_2, r_3, and r_4, R_3 is a mixture of r_3, r_4, and P_{fr} formed from r_2, and R_4 is comprised of r_4 and additional P_{fr} formed from r_3. The first-order rate constant, k_1, is the sum of the three first-order constants leading from r_1 to r_2, r_3, and r_4, respectively. Other possible variants on this scheme have been discussed earlier.[1] In addition, the kinetics do not yet rule out the further possibility of cross-reactions among the transient substances. Thus, r_3 and r_4 may transform to P_{fr} via the shorter-lived r_2, and the observed constants k_3 and k_4 (which are much slower than k_2) may represent the rates of these cross-reactions. The appearance of similar reaction patterns in the dark transformations of rhodopsin intermediates has also been noted.[1, 5, 6]

Summary.—At 0°, flash excitation of phytochrome in its 664-nm absorbing form (P_r) causes the development of absorption at 724 nm in three well-defined stages. By means of a second suitably timed far-red flash, the photo-bleaching properties of the 724-nm absorber are shown to be constant throughout the entire conversion, and identical to P_{fr} itself. The simplest interpretation is that these three dark reactions are concurrent, parallel processes, leading to a single substance, P_{fr}.

---

* This research was supported by a grant (no. AT (30-1)-2003) from the U.S. Atomic Energy Commission to Brandeis University. Preparation of this paper was assisted by an NIH Specali Fellowship to one of us (H.L.).

† NATO fellow, Department of Chemistry, Brandeis University, 1964–1965; present address: The Gustaf Werner Institute, University of Uppsala, Uppsala, Sweden.

[1] Linschitz, H., V. Kasche, W. L. Butler, and H. W. Siegelman, *J. Biol. Chem.*, **241**, 3395 (1966).

[2] Siegelman, H. W., and S. B. Hendricks, *Federation Proc.*, **24**, 863 (1965).

[3] The far-red filter was Rohm and Haas, plastic no. FRF-700.

[4] Butler, W. L., S. B. Hendricks, and H. W. Siegelman, *Photochem. Photobiol.*, **3**, 521 (1964).

[5] Linschitz, H., V. S. Wulff, R. G. Adams, and E. W. Abrahamson, *Arch. Biochem. Biophys.*, **68**, 233 (1957).

[6] Grellmann, K. H., R. Livingston, and D. Pratt, *Nature*, **193**, 1258 (1962).