*LOW-TEMPERATURE STUDIES ON PHYTOCHROME: LIGHT AND DARK REACTIONS IN THE RED TO FAR-RED TRANSFORMATION AND NEW INTERMEDIATE FORMS OF PHYTOCHROME**

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The existence of a number of transient intermediates in the interconversion of the red (P_r) and far-red (P_{fr}) forms of phytochrome has been demonstrated in this laboratory by flash-excitation technique.[1] It was also reported (Linschitz and Kasche,[1] Spruit[2]) that at low temperatures the various stages of the reaction can be slowed down or stopped and that the first photoproduct of P_r, thus stabilized, is photoconvertible back to P_r. In this paper, we present a more complete account of these low-temperature studies, including the discovery of two new transient forms of phytochrome. The new results, although in agreement with our earlier flash observations, extend and clarify those observations and lead to a reinterpretation of the detailed pattern of the $P_r \rightarrow P_{fr}$ reaction.

Materials.—Phytochrome extracts in 0.5 *M* sucrose–0.1 *M* phosphate buffer were kindly provided by Dr. S. Hendricks and were the same as those used earlier.[1, 3] Samples withdrawn from the Deepfreeze were clarified by centrifugation at 2° for 1 hr at 80,000 × *g* and made up to 60 vol % glycerol at 0°. The solutions were stored at −20° when not in use and showed excellent stability and photoreversibility.

Apparatus and Procedures.—The cryostat consisted of a cylindrical copper block soldered to the bottom of a brass can of the same diameter, and supported inside a Dewar flask fitted with plane windows. An axial channel to hold the sample cell and slots for passage of the spectrophotometer and cross-excitation beams were cut into the block. The sample cell was lowered into position through a tube soldered to the top of the cryostat block. Refrigerant baths (isopentane, CO_2-ethanol, or liquid nitrogen) were placed in the annular space between the central tube and outer can (above the copper block) and did not obstruct the light paths. For temperatures other than the CO_2 or liquid N_2 points, methanol cooled in a dry-ice chamber or nitrogen evaporated from a liquid N_2 Dewar was circulated through a copper coil immersed in isopentane. Sample temperatures were measured by a copper-constantan thermocouple dipped directly into the test solution just above the measuring beam. Absorption cells were made of 1-cm square Pyrex tubing sealed to long Pyrex tubes for handling in the cryostat. Cracking of the glycerol-buffer medium was not encountered above approximately −110°. At N_2 temperature, cracking was marked, but optical measurements could still be made without difficulty. The cross-beam, admitted through an aperture in the side of the spectrophotometer (Cary 14) cell compartment, was of sufficient diameter to illuminate the entire sample. The exciting source was a 1000-watt projection lamp, which was used in conjunction with condensing lenses and water and interference filters. When necessary, filters were also placed in the measuring beam, at the rear wall of the sample compartment, to protect the photocell. Absorption changes could thus be followed, even during cross-illumination, without interference from scattered light.

Phytochrome concentrations in this work corresponded to P_r absorbance values (664 nm) in the range 0.08–0.15, and all measurements were made with the Cary 0.0–0.2 slide wire. Slit width and gain were adjusted to give typical absorbance noise levels as shown in Figure 2 below. In preparing difference spectra, base-line shifts were minimized by referring measurements to initial absorbances at 800 nm for each spectrum.

Results and Discussion.—At liquid nitrogen temperature, the first and only

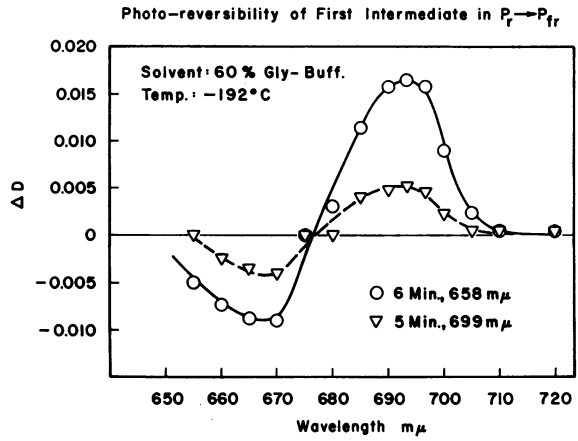

FIG. 1.—Difference spectra of P_r solution irradiated at nitrogen temperature, first with 658 nm light (O), then with 699 nm light (▽). Reaction, $P_r \underset{h\nu'}{\overset{h\nu}{\rightleftharpoons}} R_1$.

observable change following irradiation of P_r is an enhancement of absorption at about 692 and a drop at 664 nm. A typical difference spectrum is given in Figure 1. This photoproduct (R_1) is stable in the dark at temperatures below $-100°$, but reverts back to P_r photochemically (Fig. 1).

Warming the irradiated glass to about $-80°$ permits the first dark reaction to occur. The initial difference peak near 692 nm drops considerably and shifts slightly to longer wavelength (695–697). The new difference peak (R_2) is stable in the dark up to about $-60°$ but, like R_1, is photoconvertible back to P_r, as shown in Figure 2. The initial rise and leveling off of 695 nm absorption upon 638 nm illumination corresponds to formation of the R_1 configuration,[1] followed by establishment of a photostationary distribution of P_r, R_1, and R_2; the decrease in the dark is the reaction $R_1 \rightarrow R_2$ (occurring at a convenient rate at $-77°$); the final recovery in 699 nm light is the photoconversion $R_2 \rightarrow P_r$. The cycle can be repeated many times at $-77°$ without loss of reversibility.

Figure 3 illustrates the typical course of changes occurring as an irradiated P_r solution is allowed to warm slowly from $-70°$ to $0°$. The R_2 difference peak at

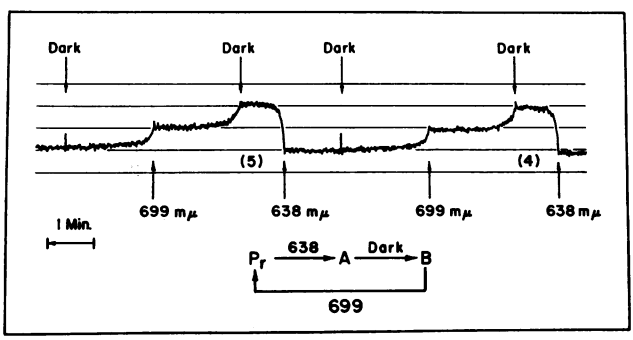

FIG. 2.—Spectrophotometer (Cary 14) tracing of absorption changes at 695 nm of P_r solution at $-77°$; cycle: 638 nm, dark, 699 nm, dark; time, increasing to left; absorbance scale, 0.005 per vertical division. The fourth and fifth cycles are shown. *A*, R_1; *B*, R_2.

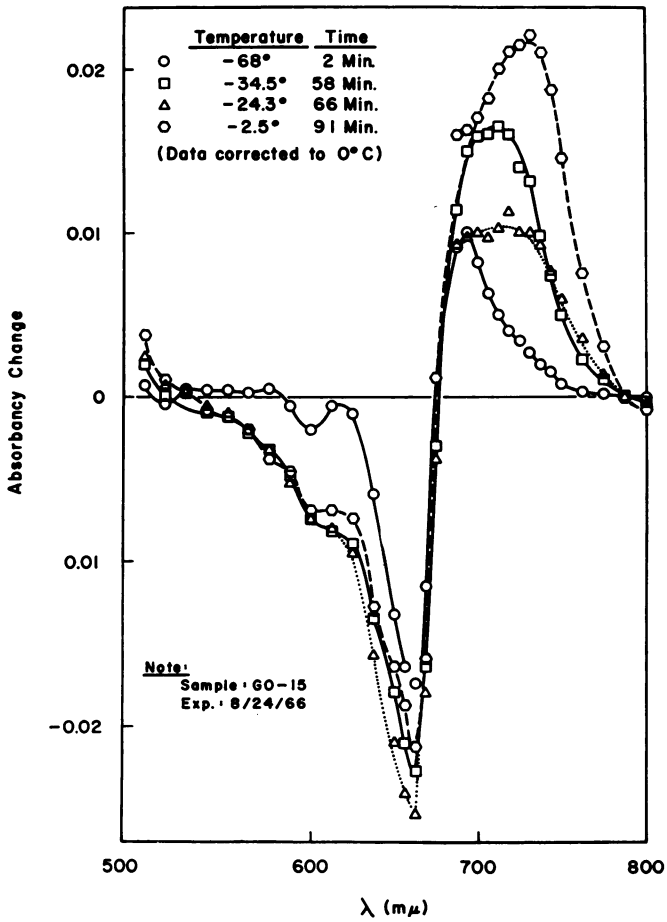

FIG. 3.—Absorption spectral changes in P_r upon slow warming in dark after irradiation at −68°. Indicated elapsed time between cessation of illumination and beginning of each (5-min) scan. First intermediate (O) is R_2. Spectra corrected for volume change with temperature.

about 695 nm first transforms, on warming above −50°, into a more intense band with a maximum at 710 nm (P_{710}) but with broad absorption extending to 780 nm. This is accompanied by some bleaching at 664 nm. On warming further to −25°, the 710-nm peak *drops*, as does the minimum at 664 nm, *but* far-red absorption is maintained. Bringing the sample to −2° results in a final far-red rise (P_{fr}) with a corresponding slight recovery at 664 nm also.

The unexpected *loss* of absorption at *both* 710 and 664 nm above −35° is probably correlated with the remarkable photobleaching phenomenon shown in Figure 4. Here P_r (curve *A*) is subjected to exhaustive irradiation (658 nm) at −35° until no further change is seen. The spectrum thus obtained (*B*) shows a very broad, low absorption, with maximum at about 650 nm and a possible secondary peak near 580 nm. We believe that this "bleached" species (P_{bl}) is the same

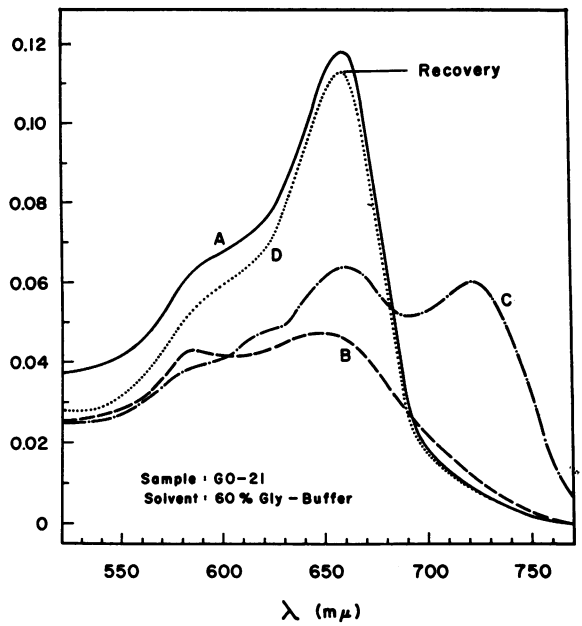

FIG. 4.—Formation of P_{bl} by irradiation of P_r (20 min, 658 nm) to constant spectrum, at −35°, and formation of P_r and P_{fr} on warming P_{bl}.

(*A*) Original spectrum irradiated at 723 nm, −35°.

(*B*) After irradiation with 658 nm, −35°.

(*C*) Warmed to 0°C (5 min in dark) and cooled to −35°.

(*D*) After irradiation with 723 nm, −35°.

intermediate responsible for the loss of absorption in the decay of P_{710} above −35°. Upon warming to 0°, P_{bl} yields *both* P_r and P_{fr} (curve *C*, Fig. 4). The formation of P_r from P_{bl} was shown in another experiment in which material at the curve *C* stage was irradiated at 0° with 658 nm light, with a resulting drop at 664 nm and corresponding rise at 725 nm. Table 1 summarizes the temperature ranges in which the various phytochrome intermediates are stable, or where the successive transformations occur.

Figure 5 presents an over-all reaction pattern for the conversion of P_r to P_{fr} based on both these low-temperature studies and our previous flash work at 0°. Direct evidence has been presented here for each light or dark reaction in the sequence $P_r \rightarrow R_1 \rightarrow R_2 \rightarrow P_{710} \rightarrow P_{bl} \rightarrow P_{fr}$. However, there should be further discussion of the accompanying *parallel* reaction pathway (shown in Fig. 5, dashed line) with rate constant k_2. This pathway is written on the basis of the following evidence, showing that the 710-nm difference band (Fig. 3, −35° spectrum), which extends as far as 780 nm, is composite and arises from P_{710} and P_{fr}:

(*a*) The absorbance at 710 nm changes with increasing temperature in a different manner than the absorbance at longer wavelengths. Thus, while the peak at 710 nm drops on warming from −35° to −25° (Fig. 3), absorbance beyond 730 nm remains unchanged or, if anything, slightly increases. If we attribute the drop at 710 to formation of P_{bl}, no far-red absorbance should remain (Fig. 4) if P_{710} were the only species present.

(*b*) The far-red portion of the 710-nm difference peak may be bleached by illumination with 723 nm light, and the difference spectrum for the bleaching corresponds to P_{fr}. A concomitant rise occurs at 660 nm. (At the temperature of this experiment, −42° the $P_{fr} \rightarrow P_r$ photoconversion is unimpeded.[4])

We conclude from these arguments that in the temperature range −70° to

TABLE 1. *Temperature effects in $P_r \rightarrow P_{fr}$ reaction (in 60% glycerol-buffer).*

Temperature	Stable product	Reaction	Half time
$>-100°$	R_1 (692)		
$-77°$		$R_1 \rightarrow R_2$	30 sec
$>-70°$	R_2 (695)		
$-55°$		$R_2 \rightarrow P_{710}$	3 min
$>-45°$	P_{710}		
$-35°$		$P_{710} \rightarrow P_{bl}$	10 min
$>-30°$	P_{bl}		
$-10°$		$P_{bl} \nearrow P_r$ $P_{bl} \searrow P_{fr}$	

$-50°$, P_{710} and P_{fr} must be growing in at comparable rates from their respective precursors (or precursor) which are (or is) present in the R_2 configuration.

Comparison between low-temperature and 0° flash experiments: The reaction pattern illustrated in Figure 5 is corroborated by the flash data at 0°.[1] The initial difference peak near 692 of the first photoproduct, and the initial dark reaction (drop in absorption and slight shift to longer wavelength), appear to be the same in both the low-temperature and 0° situations and we therefore use similar terminology, R_1 and R_2, for the two cases.[1] There may be a small displacement of the peaks to longer wavelength at the higher temperature (see ref. 1, Fig. 4). However, the next stage of reaction ($R_2 \rightarrow R_3$, ref. 1, Fig. 4) at 0° differs somewhat from the low-temperature transformation of R_2 in that, at 0°, a new peak develops at 725 but not at 710 nm, while the 698 peak of R_2 clearly persists. The double-flash technique shows that this 725 component has the photobleaching characteristics of P_{fr}.[3] The ensuing stage of reaction at 0° (R_4) shows no 698 peak but, instead, a well-marked shoulder at 710 nm and a further rise at 725 nm.[1] The simplest interpretation of these observations is that the initial growth of P_{fr} (k_2) is faster than that of P_{710} at 0°, whereas the two rates are comparable in glycerol-buffer medium at low temperature, as stated above. We had earlier assigned the 0° 710-nm shoulder of R_4 "probably" to a persistent 698-nm (R_2) band, shifted by the underlying developing P_{fr} absorbance, rather than to a

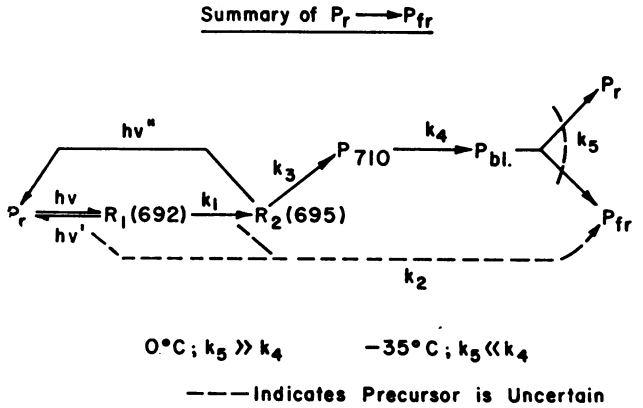

FIG. 5.—Reaction pattern for $P_r \rightarrow P_{fr}$ conversion. At 0°, P_{bl} stage is not seen.

separate species.[1] It now seems reasonable to identify this 0° shoulder with the P_{710} seen as a strong peak in the low-temperature situation.

The reaction pattern shown in Figure 5 differs from our previous interpretation[1, 3] in that *two* parallel pathways now appear instead of *three*. This change arises from our acceptance of P_{710} as an intermediate appearing sequentially in the $P_r \rightarrow P_{fr}$ reaction. The triple pathway proposed earlier was based on the appearance of three well-defined stages of development of 730 nm absorbance following flashing,[1] combined with constant far-red photobleaching, which corresponded to P_{fr} at all three stages as observed in the double-flash work.[3] The present interpretation assigns the second of these three stages (at 0°) to formation of P_{710} (k_3), with the initial P_{fr} absorbance riding on the rising P_{710} band. Comparison of the low-temperature (Fig. 4, −35°) and 0° difference spectra (ref. 1, Fig. 4, R_4) suggests also that the amount of P_{710} formed relative to P_{fr} (via the k_3 path) decreases as the temperature increases. This would account for the double-flash result, since only a relatively small part of the far-red absorbance would be assignable to P_{710} at 0°. The scheme given in Figure 5 provides for the four dark processes seen at 0°,[1] with similar interpretations for k_1 and k_2, but reassigns rate constants k_3 and k_4 to take into account the intermediate P_{710} now proposed.

No indication of the appearance of P_{bl} is seen at 0°; that is, there is no drop at 660 or 710 nm (nor any regeneration of P_r) in the final stages ($R_4 \rightarrow P_{fr}$) of the reaction. This could mean that either the rate of disappearance of P_{bl} (k_5) at 0° is much faster than its formation (k_4) from P_{710}, or that P_{bl} is bypassed altogether at 0°.

Remarks on P_{bl}: The quantitative conversion of P_r into P_{bl} by prolonged illumination at −35° can be understood from Figure 5. That fraction of irradiated P_r which reacts via the P_{710} pathway is stopped at the P_{bl} stage. The fraction of P_r transformed to P_{fr} via the k_2 path (open at −35°) is photoconverted back to P_r, since $P_{fr} \rightarrow P_r$ is not blocked at this temperature[4] and P_{fr} absorption overlaps that of P_r. This reconverted P_r is again excited and recycled. Since the only blocked step is k_5, all of the P_r eventually accumulates as P_{bl}. The precise nature of P_{bl} is as yet uncertain, but the apparently high-temperature coefficient of k_5 suggests that P_{bl} may be a reversibly denatured form of phytochrome. The formation of *both* P_r and P_{fr} from P_{bl} is striking, but the photoconversion of P_{fr} to P_r at temperatures far below −35°[4] indicates that P_{bl} is not a necessary intermediate common to both directions of the normal interconversion reaction.

Molecular interpretations: The formation of R_1 and its photoconversion back to P_r at liquid nitrogen temperature strongly indicates that the primary photochemical reaction of P_r is an isomerization of the chromophore moiety, as in the first step of rhodopsin photoconversion. Similarly, the photoconversion of R_2 back to P_r at CO_2 temperature suggests that R_2 differs from P_r mainly in an isomerized chromophore. The two-step H-atom migration within the chromophore, suggested by Siegelman *et al.*[5] for the $P_r \rightarrow P_{fr}$ reaction, seems to fit these experimental characteristics of the $P_r \rightarrow R_1 \rightarrow R_2$ interconversions. The later stages of the reaction, occurring at higher temperatures, may of course be protein

rearrangements. It is noteworthy that the reverse reaction of $P_{fr} \rightarrow P_r$ also occurs in at least two stages,[1] and is not impeded by temperatures as low as $-80°$.

The parallel pathways found in the $P_r \rightarrow P_{fr}$ conversion may arise from multiple reactions of the initially excited P_r, R_1, or R_2, or from two distinct initial populations of P_r.[2, 6, 7] Current studies of the factors controlling the relative probabilities of each pathway should clarify this question.

Summary.—Two new intermediate forms of phytochrome are described: P_{710}, obtained by warming P_r to $-40°$ after irradiating at lower temperatures, and P_{bl}, a low-absorbance form resulting from prolonged irradiation of P_r at $-35°$. Low temperature and 0° flash studies lead to a pattern for the $P_r \rightarrow P_{fr}$ conversion that includes two parallel pathways. The first two stages of the reaction are photoreversible at low temperature, an indication that the primary photoreaction is isomerization of the chromophore.

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