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Low-Temperature Absorption and Circular Dichroism Studies of Phytochrome†

Michael J. Burke,* Douglas C. Pratt, and Albert Moscowitz

ABSTRACT: At temperatures between +4° and −70°, phytochrome in 66% glycerol can exist in four forms: P_R, P_{BL}, P_{FR}, and A. These forms are interconverted *via* the thermal and photochemical reactions depicted in eq 1–5. The absorption spectra of P_R and P_{FR} between +4° and −70° are quite similar to those at room temperature, although the circular dichroism (CD) spectra show a more significant temperature dependence. P_{BL} has a weak absorption band (relative to P_R) and a large negative CD band centered at about 660 nm. Intermediate A has an absorption band and a negative CD band centered at 658 nm. Theoretical calculations have been made of the variation in absorption intensities as a function of molecular geometry, and the results compared with experimental data. Such comparisons suggest that the phytochrome chromophore has an extended conformation in P_R, P_{FR} and A, and a folded conformation in P_{BL}. The transformations from extended to folded conformations require cis–trans isomerizations.

The plant pigment phytochrome exerts a controlling influence over important aspects of plant growth and development, such as flowering, seed germination, and stem elongation (Hendricks and Borthwick, 1965). It is a chromoprotein which has an absorption maximum at approximately 660 nm after exposure to far-red light, and at 730 nm after exposure to red light. The phytochrome form that absorbs at 730 nm (P_{FR}) is more susceptible to standard denaturants than the form absorbing at 660 nm (P_R), thus indicating substantially different protein conformations in P_R and P_{FR} (Butler *et al.*, 1964). Chromatographic and spectral properties of the isolated prosthetic group indicate that it is a bilatriene similar to certain bile pigments and algal chromophores (Siegelman *et al.*, 1966); however, its exact structure and conformation have not been established. The photoconversion of P_R to P_{FR} at low temperatures has been studied by Spruit (1966a, 1966b), Cross *et al.* (1968), Pratt and Butler (1968), and Anderson *et al.* (1969). Their results indicate that the absorption of a photon chemically changes the bilatriene prosthetic group, and a sequence of thermal reactions ensues. By varying the temperature, one can stop the sequence at a number of intermediate stages. In this paper we extend the study of the thermal reactions occurring above −70°. In addition, we report the low-temperature absorption and circular dichroism (CD) spectra of P_R, P_{FR}, and two intermediates. An analysis which correlates the absorption spectra to the prosthetic group conformation is included.

Materials and Methods

The procedure for phytochrome isolation is patterned after one described by Siegelman and Firer (1964). Phytochrome is extracted from the coleoptiles of 5-day-old etiolated oat (*Avena sativa*, Clintland variety). Harvested tissue (1.5 kg) is added to 1 l. of aqueous 0.1 M Tris buffer (pH 8) containing 0.1 M thioglycol and 0.002 M EDTA, and ground

† From the Departments of Botany and Chemistry, University of Minnesota, Minneapolis, Minnesota 55455. *Received June 2, 1972.* This work was supported by Grant-in-Aid GB-29329 from the National Science Foundation.

* Author to whom correspondence should be addressed at the Department of Horticulture, University of Minnesota, St. Paul, Minn. 55101.

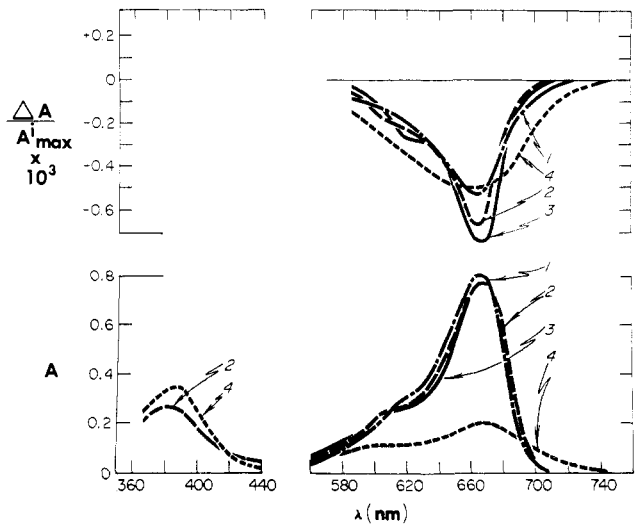


FIGURE 1: The absorption and CD spectra of P_R and the bleached intermediate (P_{BL}) in 66% glycerol buffer. P_R at +4° (1); P_R at −45° (2); P_R at −70° (3); a mixture containing primarily P_{BL} at −45° formed by illumination (600–700 nm) of P_R at −45° (4).

with a mortar and pestle. After filtration through cheesecloth, the solution is precipitated by addition of 1.0 volume of saturated ammonium sulfate solution previously adjusted to pH 7.5 with ammonium hydroxide. The precipitate is dissolved in a standard buffer (pH 7.8) used throughout these studies which contains 0.01 M phosphate–0.1 M thioglycol–0.002 M EDTA.

The dissolved precipitate is dialyzed against the standard buffer and applied to a calcium phosphate column which is equilibrated with the same buffer. The column is washed with the standard buffer and eluted with a pH 7.8 buffer containing 0.4 M phosphate–0.1 M thioglycol–0.002 M EDTA. The phytochrome-containing fraction is precipitated with ammonium sulfate, and dissolved in and dialyzed against the standard buffer. This dialyzed solution is applied to a DEAE-cellulose column equilibrated with the standard buffer, washed with the same buffer, and eluted with a linear gradient starting with the standard buffer and ending with a pH 7.8 buffer containing 0.25 M phosphate–0.1 M thioglycol–0.002 M EDTA. As with the eluate from the calcium phosphate column, the phytochrome-containing fraction is collected, precipitated with ammonium sulfate, dissolved in, and dialyzed against standard buffer. This phytochrome solution is concentrated 10 times by dialysis against standard buffer and glycerol. The final glycerol concentration of the solution is 66% by volume.

Approximately 1 ml of phytochrome having an absorbance of 1.0 at 665 nm is obtained with the above procedures. The absorbance at 280 nm is between 10 and 20 times larger than the absorbance at 667 nm; therefore, phytochrome is probably less than 10% of the total protein isolated. In the spectral region examined, phytochrome is the only absorbing species. The final phytochrome solutions are stored at −20° until needed. Dim green illumination is used throughout the isolation.

Biliverdin and mesobiliverdin were gifts from the University Medical Unit, Northwestern Hospital, Minneapolis, Minn. The chloroform and methanol solvents used with these bile pigments are Spectral Grade.

Absorption is measured with a Cary Model 15 recording spectrophotometer. CD spectra are measured with a Jasco recording dichrograph. The CD data are expressed as $\Delta A/A_{max}^{i}$, where A_{max}^{i} is the maximum absorbance of the initial phytochrome solution as P_R at 667 nm and at +4°, and ΔA is the difference in absorbance for left minus right circularly polarized light. Cells with 1-cm light path designed to maintain a constant temperature (±2°) in the range −120° to +4° are used in these studies. In any given experiment the same low-temperature cell is used for the absorption and CD spectra to assure that conditions are as identical as possible. Light for photochemical irradiation of samples is provided either by external light sources or the light source and monochrometer of the Cary and Jasco instruments.

Results

Phytochrome Thermal and Photochemical Reactions. The absorption and CD spectra of P_R are shown in Figure 1. At +4° the CD spectrum reported here is similar to the spectra obtained at 25° by Hopkins and Butler (1970), and at 25° by Kroes (1968).

A bleached intermediate (P_{BL}) is formed when P_R is irradiated at −45° (Figure 1) (Cross *et al.*, 1968). The formation of this intermediate *via* a photochemical reaction (reaction 1)

$$P_R \xrightarrow[-45^\circ]{\lambda\ (600\text{–}700\ nm)} P_{BL} \qquad (1)$$

having a single product and a single reactant is indicated by isosbestic points observed at 695 and 415 nm. The P_{BL} spectrum between 300 and 800 nm is independent of the wavelengths of illumination (range 600–700 nm) used to form it. This fact coupled with the observation that the P_{BL} spectrum cannot be altered by illumination at wavelengths greater than 700 nm where only P_{BL} absorbs light, indicates that reaction 1 is a unidirectional photochemical reaction. For a single allowed electronic transition, the CD band has the same shape as the corresponding absorption band in the sense that their behavior as a function of wavelength differs only by a constant multiplicative factor (Moscowitz, 1965). The mirror image relationship between the absorption and the fluorescence bands in the long-wavelength region (Correll *et al.*, 1968) suggests that the absorption band is due to a single electronic transition. Therefore, the difference in the shape of the absorption and the CD bands of P_{BL} (Figure 1) indicates that in addition to P_{BL} another entity is present, possibly an impurity. Indeed the difference in band shape for the absorption and CD curves of P_{BL} can be accounted for if 8–10% of our phytochrome is denatured P_R which does not undergo photochemical or thermal transformations.

P_{FR} is obtained when P_{BL} is warmed to +4° (Figure 2 and reaction 2). At +4° our CD spectrum of P_{FR} is similar to that

$$P_{BL} \xrightleftharpoons{+4^\circ} P_{FR} \qquad (2)$$

published by others at 25° (Hopkins and Butler, 1970; Kroes, 1968). It has been suggested that the positive band is from P_{FR} and the negative band is from P_R (Hopkins and Butler, 1970). P_{FR}, although stable at +4°, is unstable at lower temperatures (Anderson *et al.*, 1969). When P_{FR} is allowed to stand several hours in the dark at −35° a new absorption spectrum is obtained (Figure 3, curve 4). We can account for this new spectrum if we assume that P_{FR} is in equilibrium with P_{BL}, an equilibrium that is only slightly disturbed by the relatively slow formation of a red-absorbing pigment. If estimated as P_R, this pigment is usually less than 2% of the

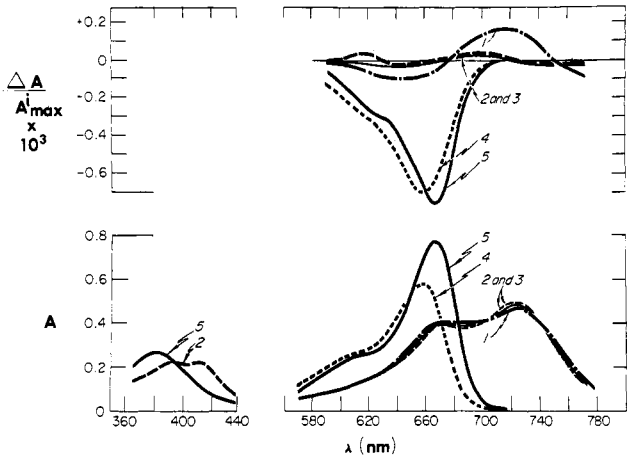


FIGURE 2: The absorption and CD spectra of P_{FR}, intermediate A, and P_R in 66% glycerol. P_{FR} is formed by warming P_{BL} to +4° (1); P_{FR} at −45° (2); P_{FR} at −70° (3); A at −70° formed by illumination (640–800 nm) of P_{FR} at −70° (4); P_R (formed by warming A to +4°) at −70° (5). At wavelengths greater than 700 nm the CD noise level is about $\pm 0.15 \times 10^{-3}$.

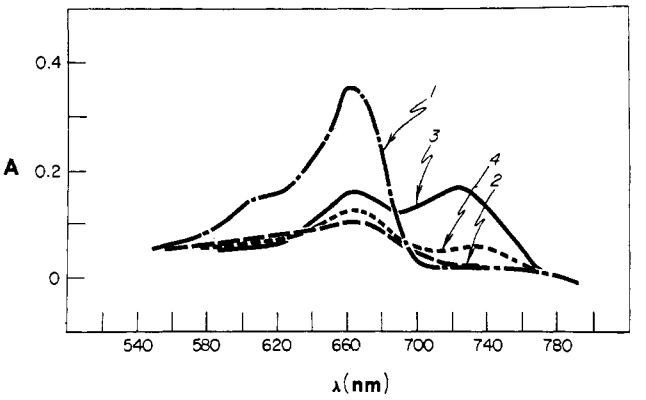


FIGURE 3: The change in the absorption spectrum in the conversion of P_{FR} to P_{BL} and P_R in 66% glycerol buffer. P_R at −35° (1); P_{BL} at −35° formed by illumination of P_R with red light at −35° (2); P_{FR} (formed by warming P_{BL} to +4°) at −35° (3); and the spectrum obtained from P_{FR} in the dark at −35° after 2.5 hr (4). No further absorption change occurs after 2.5 hr. Warming (4) to +4° gives P_{FR}.

total phytochrome present (reaction 3). The mechanism of

$$P_{BL}(P_{FRH}) \underset{}{\overset{-35°}{\rightleftharpoons}} P_{FR} \qquad (3)$$

$$(?P_R)$$

leakage to P_R is uncertain and can be either a dark reaction (Pike and Briggs, 1972) and/or a photochemical reaction initiated by the light used in recording the absorption spectra. The equilibrium constant for the interconversion of P_{BL} to P_{FR} is approximately 0.25. In light of this equilibrium, we propose that the intermediate P_{FRH} described by Anderson *et al.* (1969), which also has reduced extinction coefficient and is formed from P_{FR}, is identical with P_{BL}. This similarity was first suggested by Borthwick *et al.* (1969). These observations are not compatible with the suggestion of Cross *et al.* that P_{BL} on warming forms a significant amount of P_R. The possible formation of P_R from P_{BL} might occur but as discussed above the P_R formed would be less than 2% of the total photochrome.

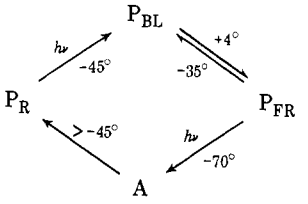
A red-absorbing pigment (A) is obtained when P_{FR} is illuminated at −70° (Figure 2). Isosbestic points at 672 and at 393 nm indicate that the conversion involves a single product and a single reactant. The total loss of absorbance above 710 nm, regardless of the illuminating wavelength between 640 and 800 nm, indicates that a unidirectional photochemical reaction is responsible for the spectral changes (reaction 4).

$$P_{FR} \xrightarrow[-70°]{\lambda\ (640\text{–}800\ nm)} A \qquad (4)$$

P_R is formed very slowly from A at −45° and rapidly at +4° (reaction 5 and Figure 2).

$$A \xrightarrow{>-45°} P_R \qquad (5)$$

The reaction scheme shown in eq 6 summarizes the result discussed above. (For clarity the broken lines of reaction 3 have been omitted.) The oscillator and rotational strengths for these phytochrome intermediates have been computed



(6)

from the spectra shown and are collected in Table I. The oscillator strength f provides some measure of the nature of the electronic charge rearrangement associated with an electronic transition. Experimentally it is roughly proportional to the area under an absorption band and is given exactly by the following relationships

$$f = +4.32 \times 10^{-2} \int (\epsilon(\lambda)/\lambda^2)d\lambda \qquad (7)$$

$$f \cong +7.65 \times 10^{-2} \epsilon_{max} \Gamma/\lambda_{max}^2 \qquad (8)$$

The analogous quantity for CD is rotational strength and is given by the following relationship

$$R = +0.248 \int (\Delta\epsilon(\lambda)/\lambda)d\lambda \qquad (9)$$

$\epsilon(\lambda)$ is the extinction coefficient and $\Delta\epsilon(\lambda)$ is the difference in the extinctions coefficients for left minus right circularly polarized light. The rotational strength is given in Debye–Bohr magnetons (DBM), the wavelength is nanometers. Here Γ is the bandwidth as defined by the equation for a Gaussian curve $\exp[-(\lambda - \lambda_0)^2/\Gamma^2]$. The approximate relationship (8) is derived under the assumption of a Gaussian band shape.

Extinction Coefficient of P_R. Experimental values for the extinction coefficient of P_R at 667 nm (ϵ_{667}^{Pr}) are reported to be between 16,400 and 76,000 (Mumford and Jenner, 1966; Correll *et al.*, 1968). Correll *et al.* (1968) point out that the reported experimental values are at best minimum values. Recently, evidence for an even larger value for this extinction coefficient (110,000) has been suggested to us (W. Briggs, unpublished data, 1972). An additional criterion for helping to

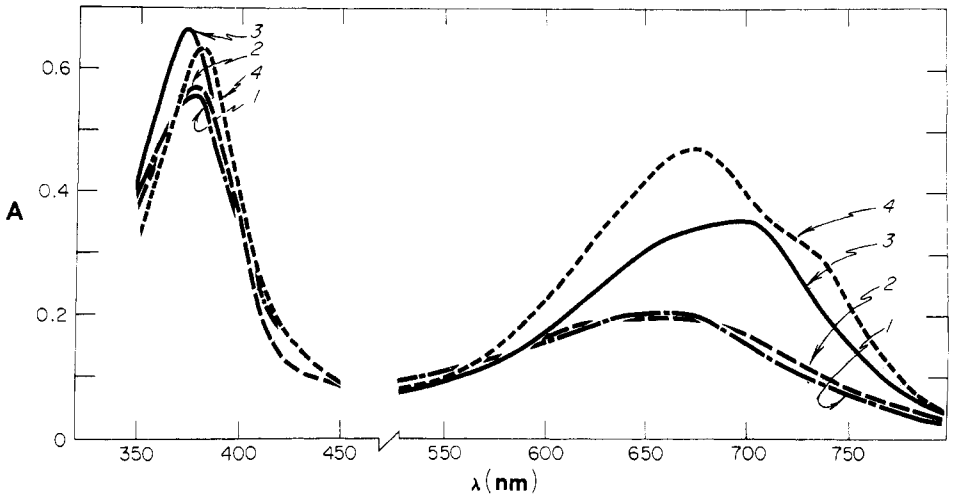


FIGURE 4: The absorption spectra of biliverdin. Biliverdin in 90% methanol–10% chloroform (1); in chloroform (2); in 90% methanol–10% chloroform plus a trace of HCl (added as a gas) (3); and chloroform plus a trace amount of HCl (4).

TABLE I: The Measured Oscillator Strength (f) and Rotational Strength (R) for Phytochrome Intermediates, Degraded Phytochrome, Mesobiliverdin, and Biliverdin.

Compound	Temperature (Solvent)	f_1[a] (700-nm Band)	f_2[a] (380-nm Band)	f_2/f_1	R_1[a] (700-nm Band) (DBM)
P_R	−70° (66% glycerol)	0.90	0.62	0.69	−1.8
P_{FR}	−70° (66% glycerol)	0.97	0.50	0.52	
A	−70° (66% glycerol)	0.75	0.62	0.83	−1.5
P_{BL}	−45° to −70° (66% glycerol)	0.20	0.85	4.3	−2.7
Degraded phytochrome[b]	+25° (5% formic acid)	0.40	1.01	2.5	
Mesobiliverdin	+25° ($CHCl_3$)	0.23	0.86	3.7	
	+25° ($CHCl_3$–trace HCl)	0.42	0.93	2.2	
Biliverdin	+25° ($CHCl_3$ or CH_3OH)	0.21	0.78	3.7	
	+25° ($CHCl_3$–trace HCl)	0.41	0.86	2.1	
	+25° (CH_3OH–trace HCl)	0.29	0.90	3.1	

[a] The concentration of phytochrome is determined using 150,000 for the extinction coefficient of P_R at 667 nm (ϵ_{667}^{Pr}). The rotational strength is in Debye–Bohr magnetons. The reason for selecting this large value for ϵ_{667}^{Pr} is given in the next section. [b] The oscillator strengths are calculated from the spectrum given by Fry and Mumford (1971).

evaluate this extinction coefficient is suggested by comparison of the absorption spectrum of P_{BL} with those of model compounds.

The absorption spectra of biliverdin in various solvents are shown in Figure 4. The oscillator strengths computed from these spectra along with the oscillator strengths of degraded phytochrome and mesobiliverdin are given in Table I. It will be noted that P_{BL}, mesobiliverdin, and biliverdin in neutral solution have similar (to within 15%) oscillator strength ratios f_2/f_1 for the 700- to 380-nm transition. One expects that the absolute values for f_1 and for f_2 in Table I should also agree. The absolute values obtained with P_{BL} are brought into agreement with the values for mesobiliverdin and biliverdin in neutral solution when the concentration of phytochrome and therefore P_{BL} is determined using ϵ_{667}^{Pr} equal to 150,000. We therefore suggest that 150,000 is a more reasonable value for ϵ_{667}^{Pr}. The data of Table I show a similar correlation in acid media between the spectral data of degraded phytochrome and the model bile pigments.

Hückel Molecular Orbital Calculations. In the present section we attempt to correlate the intensity of the absorption spectra with the conformations of the prosthetic group. We are interested only in distinguishing qualitatively between folded and extended conformational forms. Hence, for our purposes, it will be sufficient to limit ourselves to simple Hückel calculations for planar conformations. This type of calculation is described in detail in Suzuki (1967), and we use the parameters for heteroatoms indicated there.

We have considered the 16 bilatriene tetrapyrroles of Figure 5. These molecules are similar to the phytochrome chromophore proposed by Rüdiger and Correll (1969). There are 64 possible planar cis–trans geometric isomers for each tetrapyrrole, and we have considered them all. The value of the resonance integral was chosen in each case such that the long wavelength transition falls at 700 nm. This value of the resonance integral also gives two roughly degenerate bands in the 380-nm region. The calculated oscillator strengths were scaled by a factor of 0.5 (Suzuki, 1967; Mulliken and Rieke, 1941).

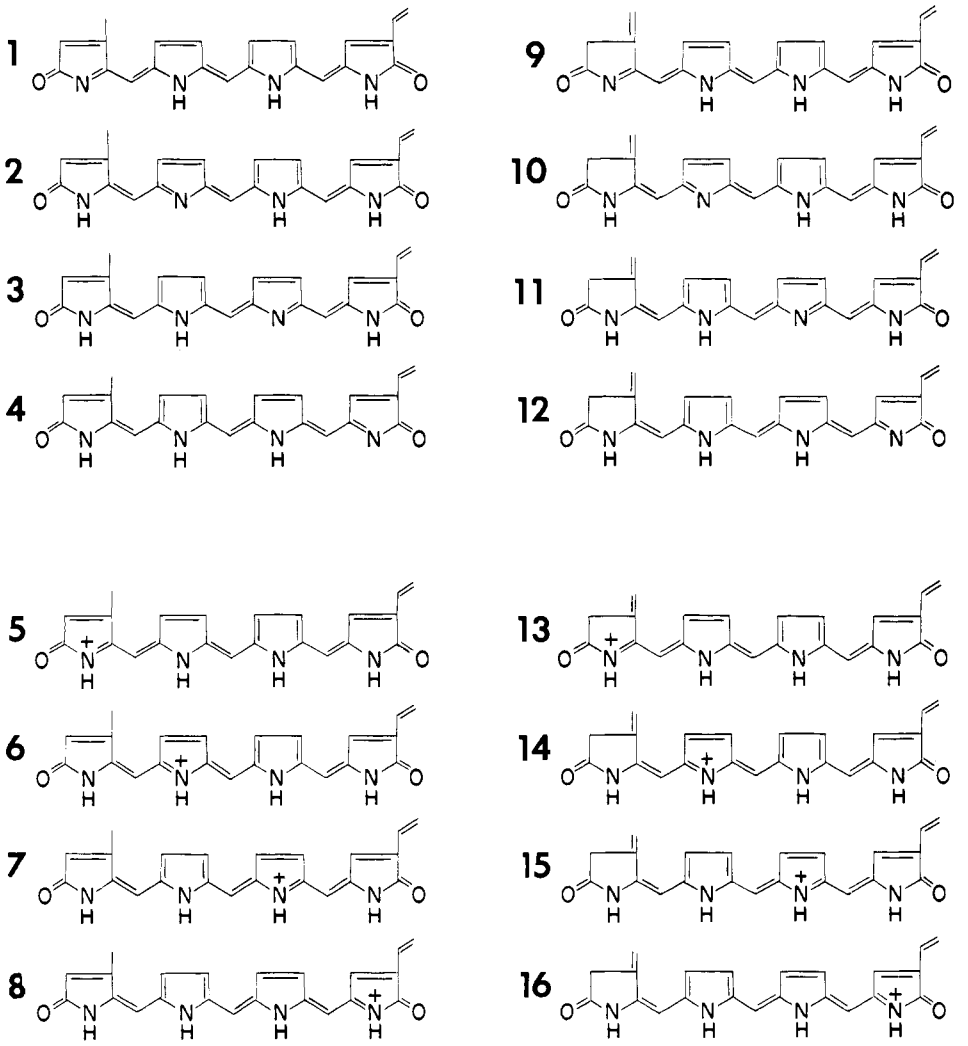


FIGURE 5: The 16 tetrapyrrole molecules used in the Hückel molecular orbital calculations.

The Hückel calculations indicate that the oscillator strength for the long wavelength transition is an increasing function of the molecular extension, while the oscillator strengths for the other two transitions are decreasing functions of molecular extension (Figure 6). The molecular extension depends primarily on the cis–trans geometric state of the molecule.

Discussion

Several observations should be made with regard to the differences between the absorption intensities of P_{BL} and the absorption intensities of P_R, P_{FR}, and A. First, the 700-nm oscillator strengths of P_R, P_{FR}, and A are each approximately four times larger than that of P_{BL}. Further, the maximum calculated variation in the 700-nm oscillator strengths among the chemical structures shown in Figure 5 is indicated by the vertical bars to be smaller than 50% for any given cis–trans arrangement of the chemical bonds (Figure 6). It follows that only a structural change which includes a cis–trans geometric isomerization will explain the observed fourfold difference in the 700-nm oscillator strengths between P_{BL} and the other phytochrome derivatives. In addition, the ratio f_2/f_1 of P_{BL} and of degraded phytochrome implicates for these compounds the more folded isomer types A, B, and C in Figure 6. *Thus, our considerations point to a chromophore with an extended form in P_R, P_{FR}, and A, and to a chromophore of a more folded nature in P_{BL} and the degraded phytochrome.*

The large rotational strength and folded conformation of P_{BL} certainly suggest a dissymmetric conformation (Deutsche *et al.*, 1969). In other dissymmetric tetrapyrroles with folded conformations some structures have been indicated to be helical (Moscowitz *et al.*, 1964). Such may be the case here for P_{BL}.

These present calculations cannot address themselves to the wavelength shifts which are of the size observed in the photoconversion of phytochrome. The long-wavelength transition maxima of P_R and P_{FR} differ by a meager 1500 cm^{-1} (80 nm in the 700-nm region) and with molecules of this type a shift of this magnitude can be attributed to solvent and protein effects or to cis–trans geometry effects, etc. Indeed the long-wavelength absorption band of biliverdin is strongly influenced by the solvent in the spectral range between 650 and 730 nm (Figure 4).

Conclusion

Photochemical mechanisms proposed for the conversion of phytochrome between P_R and P_{FR} include isomerization of the prosthetic group by internal oxidation–reduction (Siegelman and Butler, 1965; Crespi *et al.*, 1968), lactim–lactam

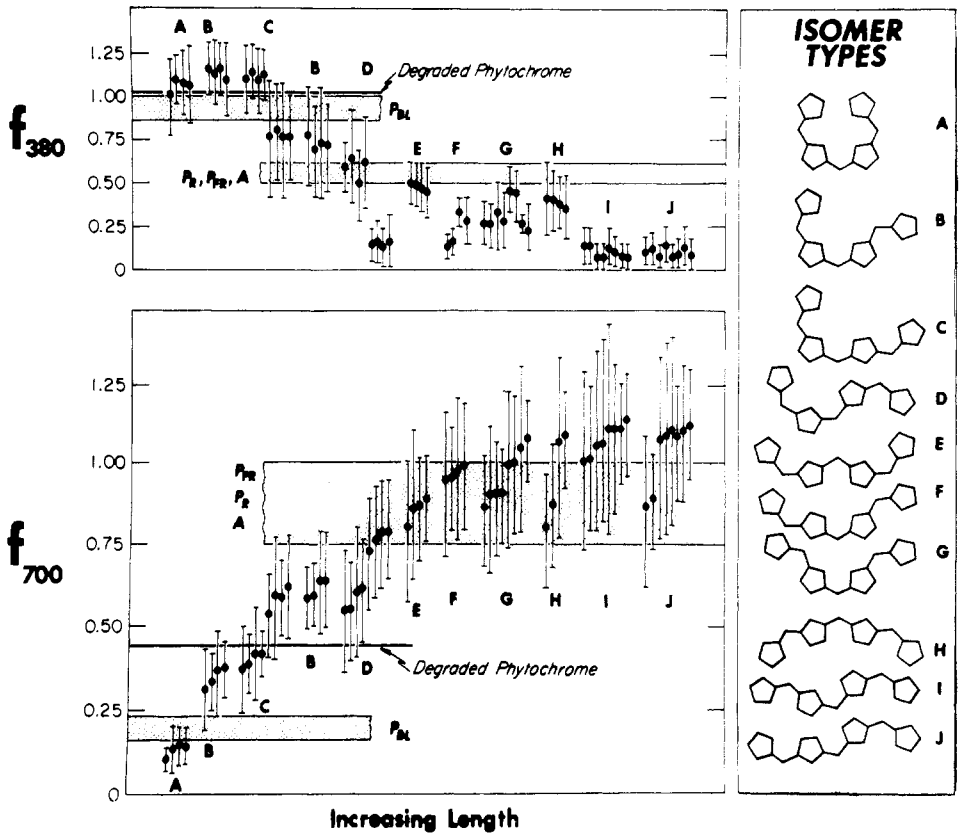


FIGURE 6: The calculated oscillator strength for the 700-nm region transition (f_1) and the calculated total oscillator strength for the 380-nm region transitions (f_2). Each vertical bar represents one of the possible 64 geometric isomers. The length of the bar represents the range of oscillator strengths predicted for the compounds in Figure 5. The isomers are grouped into categories A, B . . . . The skeletal structure for each category is given on the right. The experimental oscillator strengths measured for P_R, P_{FR}, A, P_{BL}, and degraded phytochrome are indicated by shaded areas.

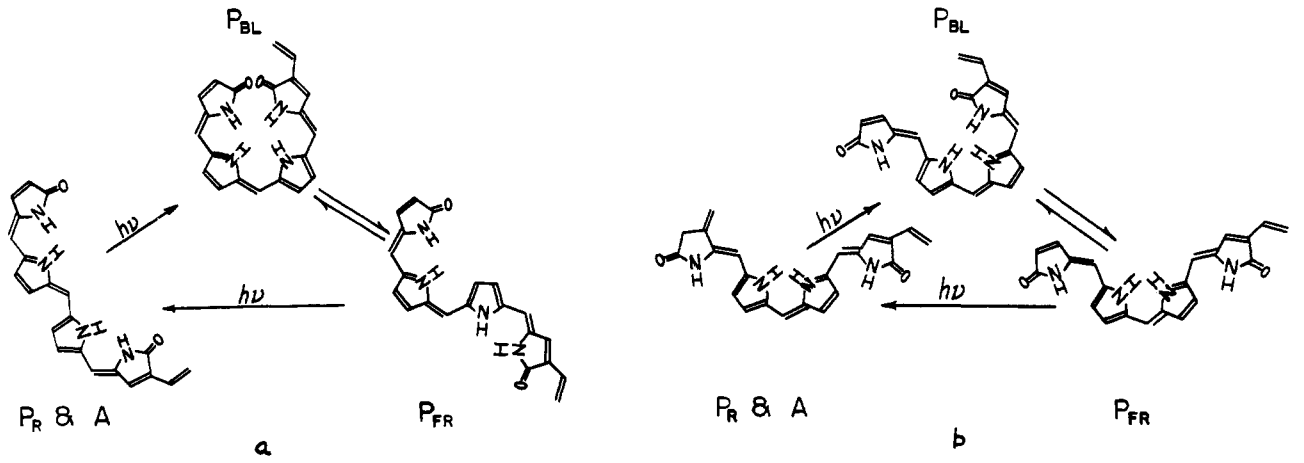


FIGURE 7: Two possible mechanisms for the transformations in phytochrome. The photon provides the large amount of energy needed for isomerization about double bonds. Only thermal energy is necessary for isomerization about single bonds.

isomerization (Siegelman and Butler, 1965), and cis–trans geometric isomerization (Suzuki and Hamanaka, 1969; Siegelman and Butler, 1965). *The analysis of oscillator strengths implicates a cis–trans geometric isomerization; however, this isomerization is not necessarily the photochemical event.* There are two types of cis–trans isomerization mechanisms which are compatible with the above reaction scheme 6. An example of the first type which has photochemical and thermal cis–trans geometric isomerizations is shown in Figure 7a. An example of the second type which has only thermal cis–trans geometric isomerizations is shown in Figure 7b. The important features of any possible sequence must be: the folded nature of P_{BL}; the extended nature of P_R, P_{FR}, and A; and the feasibility of transforming from one conformation to the next.

In phytochrome, both the protein and its prosthetic group are of the order of tens of angstroms in extension (Correll *et al.*, 1968). Hence, it seems likely that prosthetic group geometrical alterations of the type discussed in this paper must

cause or be caused by significant changes in the protein conformation.

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Circular Dichroism Study of the Effects of Magnesium Perchlorate and Temperature on the Solution Conformation of Uridine 5′-Monophosphate, Uridine 3′-Monophosphate, Uridine, and Uridylyl-(3′→5′)-uridine†

Carl Formoso

ABSTRACT: The circular dichroism (CD) spectra of uridine 5′-monophosphate, uridine 3′-monophosphate, uridine, and uridylyl-(3′→5′)-uridine were obtained under a variety of solution conditions generated by adjusting magnesium perchlorate concentration and temperature. The CD results for the monomers are discussed in comparison to recent nuclear magnetic resonance (nmr) results. Both the CD and nmr of these compounds are sensitive to temperature and salt concentration, indicating that conformational changes are induced by these solution conditions. Evidence is presented which indicates that the CD of the monomers is primarily affected by the position of the base moiety relative to the furanose ring. The CD results clearly show that the effects of increasing temperature are quite different from the effects of increasing magnesium perchlorate concentration. Discrepancies with the nmr results are discussed. From an examination of the uridylyl-(3′→5′)-uridine CD spectra, with proper accounting for monomer conformational processes, it is concluded that the conformation of the dinucleoside phosphate achieved by high magnesium perchlorate concentration is similar to the conformation achieved by high temperature. Based on the conformation-perturbing effectiveness of magnesium perchlorate as a function of temperature, it is suggested that magnesium perchlorate acts both by direct binding of the Mg^{2+} ion and by disruption of solvent structure.

Recent nuclear magnetic resonance studies (Prestegard and Chan, 1969; Schleich *et al.*, 1972) have indicated that conformational changes occur in uracil nucleosides and nucleotides upon addition of certain salts to solution, and with temperature. These conformational changes are thought to involve both the position of the base relative to the sugar

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