Absorption Spectra of Phytochrome Intermediates

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Z. Naturforsch. **40 c**, 109–114 (1985); received October 8, 1984

Avena sativa L., Native Phytochrome, Low Temperature Spectra, Chromophore Absorption Bands, Oscillator Strength

Native phytochrome (124 kdalton) was isolated from etiolated seedlings of *Avena sativa* L. cv. Pirol (Baywa, Munich). From low temperature spectra (0 °C to −165 °C) of phytochrome samples irradiated either with red or far-red light, spectra of intermediates were calculated. Both absorption bands of the phytochrome chromophore were reported. On the pathway $P_r \rightarrow P_{fr}$, native phytochrome does not form a bleached intermediate (meta-Rb) in contrast to degraded phytochrome.

An additional intermediate, meta-Rc ($\lambda_{max} = 725$ nm), is formed instead. On the reverse pathway only two intermediates were found, lumi-F and meta-F. Oscillator strengths' values were calculated and interpreted in terms of chromophore conformation. A scheme of intermediates for native phytochrome is presented.

Introduction

Phytochrome, the photoreceptor for many photomorphoses in higher plants, exists in the inactive P_r form and the physiologically active P_{fr} form. The mutual conversion of both involves several intermediate steps which have mainly been characterized by their difference spectra [1–7]. We have recently obtained the first data on absolute spectra of intermediates [8]. This was achieved by determination of absolute absorption spectra of irradiated phytochrome at low temperature. From the amount of intermediates produced at a defined temperature, the absolute spectrum of the intermediate(s) can be determined. So far, these experiments were only performed with partially degraded phytochrome (118 kdalton), furthermore only a limited spectral region (500–800 nm) was included [8].

We wish to report here data for the intermediates of native phytochrome (124 kdalton). We also extend the measurements to the near UV region (to 300 nm) to include the second absorption band of the phytochrome chromophore.

Abbreviations: Lumi-R, meta-Ra, meta-Rb, meta-Rc, P_{bl}, phytochrome intermediates in the pathway from P_r to P_{fr}; P_r, red absorbing phytochrome; P_{fr}, far-red absorbing phytochrome; lumi-F, meta-F, intermediates in the pathway from P_{fr} to P_r; PMSF = Phenylmethylsulfonylfluoride; EDTA, ethylenediaminotetraacetate.

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Materials and Methods

Native oat phytochrome (124 kd) was isolated from 3.0 days old etiolated oat seedlings (*Avena sativa* L., cv. Pirol, Baywa, Munich) according to the method of Vierstra and Quail [9]. Harvested shoots were extracted with 100 mM Trisbuffer, containing 50% (v/v) ethylene glycol and 4 mM PMSF, in P_{fr}-form [9]. After poly-(ethylene)imine precipitation and concentration with recrystallized ammonium sulfate, phytochrome was adsorbed on hydroxyapatite. Elution was achieved with 100 mM potassium phosphate buffer (instead of 20 mM) containing 2 mM PMSF, pH = 7.8. Final purification was achieved, after transformation of phytochrome into the P_r-form, by chromatography at first on Affi-Gel Blue and then on Biogel A-1.5. This material showed a purity-index A_{280}/A_{667} of 1.7. It was precipitated with ammoniumsulfate and dissolved in 100 mM potassium phosphate buffer, pH = 7.8, containing 14 mM 2-mercaptoethanol and 5 mM EDTA, diluted with glycerol (final concentration 66% v/v). In this state it could be stored without spectral changes at −190 °C. Absorbance at 667 nm differed between 0.2 and 0.4. This material was, according to SDS gel electrophoresis (see below), more than 85% pure and had a moleculare weight of 124 ± 2 kdalton.

SDS-polyacrylamide disk gel electrophoresis [10, 11] with 6% acrylamide was used to establish purity of the material. Tris-buffers were used according to [12]. Gels were stained with Coomassie Brillant

Blue. Molecular weight standards were obtained from SIGMA (Myosine 205 kdalton, β-Galactosidase 116 kdalton, Phosphorylase B 97,4 kdalton, Bovine serum Albumin 66 kdalton).

Spectra were recorded on a dual wavelength spectrophotometer with head-on photomultiplier next to the sample (SIGMA ZWS-II, Sigma Instruments, Berlin), connected on line with an 8110 electronic recorder with data processing and storage capability (Bascom-Turner Instruments, Mass., USA). The reference beam was fixed either to 480 nm (measuring range 300 to 500 nm) or 780 nm (measuring range 500 to 800 nm). Both wavelengths proved to be isosbestic points. The reference channel was used for automatic calibration to any changes in the optical properties of the sample. The spectra were corrected for the stored baseline, which had been determined in a reference sample containing only buffer. For the measuring range 300 to 500 nm the UV-lamp was used, cut-off filters (WG 305, Schott, Germany) inserted in both channels. For the range 500 to 800 nm the VIS-lamp was used, cut-off filters (reference channel OG 550, measuring channel GG 395; Schott, Germany) were inserted. Spectra were recorded at a rate of 2 nm per second. To eliminate noise and drift, spectra were averaged from at least 5 measurements.

The oscillator strength f was calculated by the following equation [13]:

$$f = 4.32 \times 10^{-2} \int \frac{\varepsilon(\lambda)}{\lambda^2} \, d\lambda \, .$$

Integration was performed from 500 to 800 nm (first absorption band) or from 340 to 500 nm (second absorption band). The oscillator strength of the second absorption band is only approximative because protein absorption interfered somewhat at lower wavelengths. There was no need to include wavelengths below 340 nm because difference spectra showed no changes below 340 nm.

The phytochrome solutions were placed in a conventional plastic micro-cuvette (pathway 1 cm) or in an alumin-cell with quartz windows. They were cooled down in a cuvette holder with a stream of cold nitrogen gas.

Irradiation of the samples was achieved with a light source (Volpi Intralux 150 H, Volpi GmbH, Denzlingen, Germany) connected with a fiber-glass light conductor and filters as follows: red irradiation was performed with an interference filter (660 nm, half width 10.1 nm, 50% peak transmission, fluence rate 4.1 $W m^{-2}$), far-red irradiation with a cut-off filter (RG 715, Schott, fluence rate 3.3 $W m^{-2}$).

Difference spectra and spectra of pure P_r, P_{fr} and intermediates were calculated with the same equipment. For lumi-R, calculation was based on $\varepsilon_{693} = 190000$ (see [8]). The amount of meta-Ra and meta-Rc was determined *via* the corresponding amount of P_{fr} produced by rapid thawing. The amount of lumi-F and meta-F was equated with the amount of P_{fr} disappearing.

Results and Discussion

Pathway $P_r \rightarrow P_{fr}$

The first intermediate of irradiated P_r which can be stabilized below −100 °C is lumi-R [8]. We confirmed that the spectrum of red-irradiated native P_r is identical from −120 °C to −165 °C. For the calculation of the absolute spectrum of lumi-R (as for all other intermediates) we chose the temperature −140 °C. The spectrum of lumi-R of native phytochrome (Fig. 1) turned out to be identical with that of lumi-R of partially degraded phytochrome [8]. Upon transition from P_r to lumi-R,

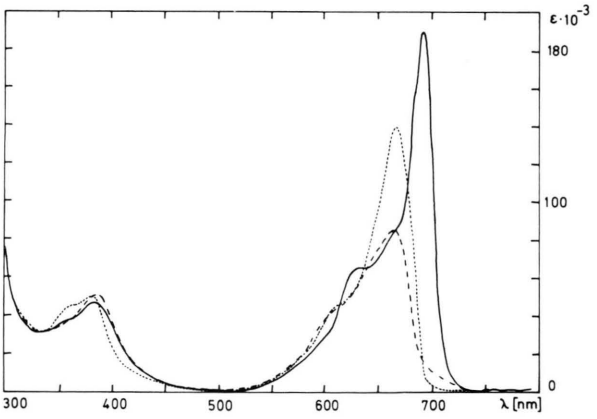


Fig. 1. Absorption spectra of P_r (. . .), lumi-R (——) and meta-Ra (---) at −140 °C. Solvent: 100 mM potassium phosphate buffer, pH 7.8, containing 14 mM 2-mercaptoethanol and 5 mM EDTA, diluted with glycerol (final concentration 66% (v/v)). The spectrum of lumi-R was calculated from the absorption difference spectrum P_r minus irradiated P_r at −140 °C, using an average ε_{693} (lumi-R) = 190000 [8]. The spectrum of meta-Ra was obtained by saturating irradiation of P_r at −85 °C, cooling down the sample to −140 °C and extrapolating for quantitative photoconversion. For details see text.

a small but significant shift occurred in the second absorption band (from 380 to 384 nm) whereas the shift in the first absorption band was more pronounced (from 667 to 693 nm). The first absorption band had a more pronounced fine structure in lumi-R than in P_r.

The next intermediate called meta-Ra [1, 8] can be stabilized at −65 °C to −85 °C. In this range absorption above 690 nm (arising either from lumi-R for lower temperatures or meta-Rc – see below – for higher temperatures) becomes minimal. To exclude any effect of temperature upon the calculated spectra, we cooled the phytochrome solution after saturating red irradiation at −85 °C down to −140 °C for recording of the spectrum. The amount of photoconversion was determined in two ways: rapid thawing of the irradiated sample yielded 35% P_{fr}. Alternatively the spectrum of meta-Ra was calculated with the absorption coefficient of lumi-R at 665 nm because it had been shown that no change in absorption occurs at this wavelength for the step from lumi-R to meta-Ra [8]. The calculated spectrum of pure meta-Ra (Fig. 1) differs from P_r and lumi-R in the first absorption band but it is very similar to P_r and lumi-R in the second absorption band.

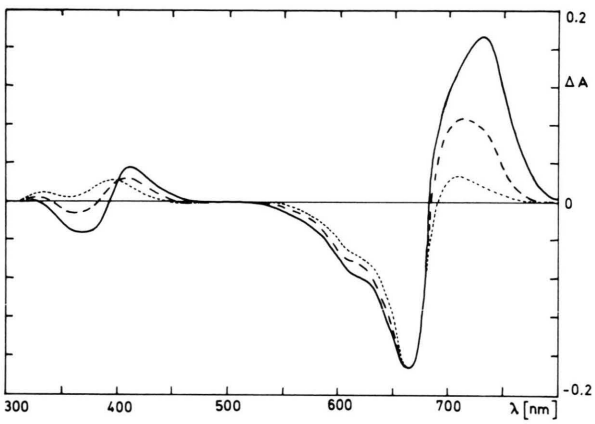


Fig. 2. Absorption difference spectra of red irradiated phytochrome (formation of intermediates) minus unirradiated P_r at −27 °C: (---) P_r (124 kdalton) 2 min irradiated (saturating), (...), P_r (114/118 kdalton) 15 min irradiated (saturating). For comparison the absorption difference spectrum P_{fr} (124 kdalton) minus P_r at −27 °C (——) is included. All spectra are normalized with respect to a constant amount of P_r photoconverted. Solvent as in Fig. 1.

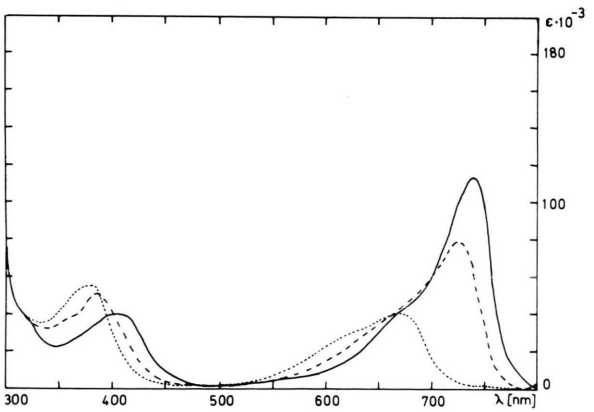


Fig. 3. Calculated absorption spectra of pure P_{fr} (——), meta-Rc (---) and meta-Rb (...) at −140 °C. For details of the calculaion see [8, 15]. Solvent as in Fig. 1.

Irradiation at higher temperature yields different results for native and partially degraded phytochromes. In Fig. 2, difference absorption spectra are recorded of P_r irradiated at −27 °C minus P_r unirradiated for native and partially degraded phytochrome. For comparison, the difference absorption spectrum for P_{fr} minus P_r at −27 °C is also recorded. As can be seen, the typical bleaching effect due to formation of meta-Rb (= P_{bl}) [1] is only obtained for degraded phytochrome. In native phytochrome, a new absorption peak centered at about 710–720 nm appears instead. A similar peak (P_{710}) has been described for a phytochrome intermediate *in vivo* [14]. We call this additional intermediate meta-Rc. After determination of the amount of meta-Rc (*via* the amount of P_{fr} produced by thawing), it's absorption spectrum was calculated (Fig. 3). The calculated spectra of pure P_{fr} and meta-Rb are also included for comparison. The general shape of the absorption curve of meta-Rc is similar to that of meta-Ra but the first absorption band is bathochromically shifted (from 663 to 725 nm). It can be deduced from Fig. 3 that meta-Rc has the same extinction coefficient at 667 nm as P_{fr} and as meta-Rb. A constant absorbance at this wavelength during the last steps of P_{fr} formation was also found in flash light experiments [3–5]. Meta-Rc exists at temperatures between −50 °C and −25 °C. At higher temperatures, formation of P_{fr} takes place. It cannot be excluded that our irradiated phytochrome sample contained some meta-Rb besides meta-Rc. Prolonged red irradiation (10 min) at −27 °C leads to some bleaching at > 700 nm (data

not shown) which can be interpreted as partial formation of meta-Rb. This bleaching is fully reversible in the dark within a few minutes. This means that meta-Rb can be formed by irradiation of meta-Rc but reverses to this intermediate in the dark.

Pathway $P_{fr} \rightarrow P_r$

Far-red irradiation of native P_{fr} at $-140\,^{\circ}C$ photoconverts P_{fr} into intermediate(s) only to the extent of 50%. The rest of P_{fr} remains photochemically inactive. This is not due to a photoequilibrium between P_{fr} and the first intermediate lumi-F because no P_{fr} is formed back by red irradiation. A previous report of (partial) formation of P_{fr} from lumi-F [8] is erraneous; the absorbance increase at 700 nm was probably due to formation of lumi-R from residual P_r. Due to the lacking absorption of lumi-F at 730 nm, it is anyhow unlikely that a presumed photoequilibrium should contain as much as 50% P_{fr}. It has to be concluded that the phytochrome sample although extensively purified is heterogenous, at least with regard to it's chromophore (*e.g.* chromophore conformation which could be frozen at $-140\,^{\circ}C$). Heterogeneity of P_{fr} has repeatedly been reported for the kinetics of dark reversion [16–18] although this had been explained with partially degraded phytochrome.

The absorption spectrum of the intermediate formed at $-140\,^{\circ}C$ is shown in Fig. 4. The absorption maximum is found at 673 nm, *i.e.* bathochromically shifted compared to P_r. A product with similar spectral properties had previously been obtained with partially degraded phytochrome [8, 13], [19]. Because the same spectral properties are also found in the first product of flash photolysis of P_{fr} [6, 20], we call this intermediate lumi-F. Far-red irradiation of native P_{fr} at temperatures between $-60\,^{\circ}C$ and $-80\,^{\circ}C$ yields another intermediate which is also formed by warming up lumi-F to this temperature. Consequently, this intermediate is called meta-F. Phototransformation of P_{fr} into meta-F is quantitative contrary to the situation with lumi-F (see above). The absorption maximum of meta-F (660 nm) (Fig. 4) is hypsochromically shifted compared to P_r. There are no other spectroscopically differing intermediates on the pathway $P_{fr} \rightarrow P_r$ in accordance with flash light experiments [6]. At temperatures above $-45\,^{\circ}C$, P_r is formed.

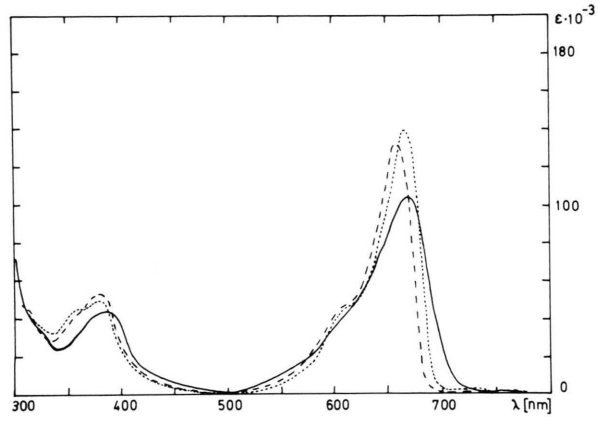


Fig. 4. Absorption spectra of P_r (...), lumi-F (——) and meta-F (- - -) at $-140\,^{\circ}C$. For details of the calculation see [8]. Solvent as in Fig. 1.

Structure and conformation

In Table I, spectral data of phytochrome forms and intermediates of both pathways are compiled. Essential for the discussion of chromophore structure and conformation are not only λ_{max} and ε-values but also the oscillator strength values for both absorption bands (f_1 and f_2). A low ratio f_2/f_1 is typical for an extended conformation, a high ratio f_2/f_1 is typical for a closed conformation of bilatrienes [13, 21–23]. From the data of Table I, an extended conformation can be deduced for the chromophore in P_r, P_{fr} and (even more pronounced) lumi-R. The conformation of the chromophore in meta-Ra and meta-Rc should be less extended but not as much closed as in meta-Rb. However, this interpretation of the experimental data has to be considered as preliminary because the influence of other parameters (*e.g.* proton transfer, see [24]) upon the oscillator strength ratio is not yet established with precision.

It can be concluded from the data of Table I that the chromophore in meta-Rc behaves as if it were something between meta-Rb and P_{fr}. The closed chromophore of meta-Rb has been considered a free tetrapyrrole whereas the extended chromophore of P_{fr} exhibits strong protein-chromophore interaction [13, 24]. Consequently, chromophore-protein interaction should be present in meta-Rc but weaker than in P_{fr}. This type of chromophore-protein interaction might be even still weaker in partially degraded phytochrome where meta-Rb

Table I. Spectral data of phytochrome forms (measured at −140 °C in glycerol-buffer solution). Data are based on calculated spectra of pure species as shown in Figs. 1, 3, 4.

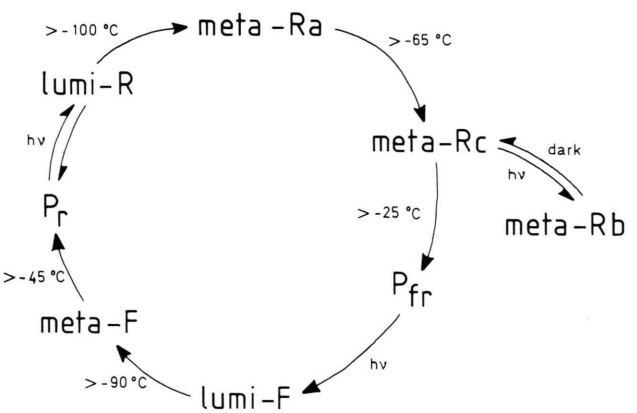
Species	1st Absorption band			2nd Absorption band			
	λ_{max}	ε	f_1	λ_{max}	ε	f_2	f_2/f_1
a) Pathway P_r to P_{fr}							
P_r	667	140 000 [a]	0.87	380	50 000	0.96	1.10
Lumi-R	693	190 000	1.09	384	47 000	0.94	0.86
Meta-Ra	663	86 000	0.73	386	52 000	1.00	1.37
Meta-Rb (P_{bl})	665	40 000 [a]	0.45	380	57 000	1.03	2.29
Meta-Rc	725	80 000	0.71	387	50 000	1.03	1.45
P_{fr} [b]	741	119 000	0.83	403	41 000	0.91	1.10
b) Pathway P_{fr} to P_r							
P_{fr} [b]	741	119 000	0.83	408	41 000	0.91	1.10
Lumi-F	673	105 000	0.88	388	44 000	0.90	1.02
Meta-F	660	133 000	0.86	381	55 000	0.99	1.15
P_r	667	140 000	0.87	380	50 000	0.96	1.10

[a] [8].
[b] Pure, calculated from photoequilibrium P_{fr}, containing 86% P_{fr} [25].

($=P_{bl}$) is formed instead of meta-Rc and where absorption of P_{fr} resembles more that of meta-Rc than that of native P_{fr}. In this context it is important that P_{fr} is formed already at lower temperature in native phytochrome than in partially degraded phytochrome (data not shown). The occurrence of meta-Rc therefore seems to facilitate P_{fr} formation.

Spectral changes on the pathway $P_{fr} \rightarrow P_r$ are less pronounced than on the reverse pathway. The corresponding ε- and f-values of P_{fr}, lumi-F, meta-F and P_r are very similar to each other. The ratio f_2/f_1 remains constant within the limits of error of determination. The main observable features are only spectral shifts of both absorption bands. This is surprising because the multiple changes occurring on the pathway $P_r \rightarrow P_{fr}$ have to be anyhow reversed to obtain the same P_r back. It is however possible that not all changes are spectroscopically detectable on the way back.

The following scheme summarizes the observations on low temperature spectroscopy of native phytochrome (124 kdalton) in 66% glycerol/buffer solution. The bleached intermediate meta-Rb is not included in the main pathway because it was only observed as a transient during irradiation of meta-Rc. The indicated temperatures are the approximate limits of stability of the respective intermediate. The scheme cannot directly be compared with schemata of kinetic data. Parallel pathways with spectrally identical intermediates which have differing kinetic properties [5, 7, 20] can principally not be detected by steady-state low-temperature measurements.



Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg. We thank Mr. U. Reiff for assistance in the SDS gel electrophoresis. We thank Dr. P.-S. Song for helpful discussions and the NATO for a travel grant (SA 5205RG 545/83).

[1] R. E. Kendrick and C. J. P. Spruit, Photochem. Photobiol. **26,** 201–214 (1977).
[2] S. E. Braslavsky, J. J. Matthews, H. J. Herbert, J. De Kok, C. J. P. Spruit, and K. Schaffner, Photochem. Photobiol. **31,** 417–420 (1980).
[3] Y. Shimazaki, Y. Inoue, K. T. Yamamoto, and M. Furuya, Plant Cell Physiol. **21,** 1619–1625 (1980).
[4] M. M. Cordonnier, P. Mathis, and L. H. Pratt, Photochem. Photobiol. **34,** 733–740 (1981).
[5] L. H. Pratt, Y. Shimazaki, Y. Inoue, and M. Furuya, Photochem. Photobiol. **36,** 471–477 (1982).
[6] Y. Inoue, K. Konomi, and M. Furuya, Plant Cell Physiol. **23,** 731–736 (1982).
[7] L. H. Pratt, Y. Inoue, and M. Furuya, Photochem. Photobiol. **39,** 241–246 (1984).
[8] W. Rüdiger and F. Thümmler, Physiol. Plantarum **60,** 383–388 (1984).
[9] R. D. Vierstra and P. M. Quail, Biochemistry **22,** 2498–2505 (1983).
[10] U. K. Laemmli, Nature **227,** 680–684 (1970).
[11] K. Weber, J. R. Pringle, and M. Osborn, Methods Enzymol. **26,** 3–27 (1972).
[12] R. D. Vierstra and P. M. Quail, Proc. Natl. Acad. Sci. USA **79,** 5272–5276 (1982).
[13] M. J. Burke, D. C. Pratt, and A. Moscowitz, Biochemistry **11,** 4025–4031 (1972).
[14] C. J. P. Spruit and R. E. Kendrick, Photochem. Photobiol. **18,** 145–152 (1973).
[15] F. Thümmler and W. Rüdiger, Physiol. Plantarum **60,** 378–382 (1984).
[16] D. L. Correll, J. L. Edwards, and W. Shropshire jr., Photochem. Photobiol. **8,** 465–475 (1968).
[17] M. Negbi, D. W. Hopkins, and W. R. Briggs, Plant Physiol. **56,** 157–159 (1975).
[18] L. H. Pratt, Photochem. Photobiol. **21,** 99–103 (1975).
[19] L. H. Pratt and W. L. Butler, Photochem. Photobiol. **8,** 477–485 (1968).
[20] H. Linschitz, V. Kasche, W. L. Butler, and H. W. Siegelman, J. Biol. Chem. **241,** 3395–3403 (1966).
[21] G. Blauer and G. Wagnière, J. Amer. Chem. Soc. **97,** 1949–1954 (1975).
[22] Qu. Chae and P. S. Song, J. Amer. Chem. Soc. **97,** 4176–4179 (1975).
[23] H. Suzuki, T. Sugimoto, and K. Ishikawa, J. Phys. Soc. Japan **38,** 1110–1118 (1975).
[24] W. Rüdiger, P. Eilfeld, and F. Thümmler, in Optical Properties and Structure of Tetrapyrroles (edited by G. Blauer and H. Sund), W. de Gruyter, Berlin, in press.
[25] R. D. Vierstra and P. M. Quail, Plant Physiol. **72,** 264–267 (1983).