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# Phytochrome Chromophore Biosynthesis[1]

## BOTH 5-AMINOLEVULINIC ACID AND BILIVERDIN OVERCOME INHIBITION BY GABACULINE IN ETIOLATED *AVENA SATIVA* L. SEEDLINGS

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### ABSTRACT

**Etiolated *Avena sativa* L. seedlings grown in the presence of gabaculine (5-amino-1,3-cyclohexadienylcarboxylic acid) contained reduced levels of phytochrome as shown by spectrophotometric and immunochemical assays. Photochromic phytochrome levels in gabaculine-grown plants were estimated to be 20% of control plants, while immunoblot analysis showed that the phytochrome protein moiety was present at approximately 50% of control levels. Gabaculine-grown seedlings administered either 5-aminolevulinic acid or biliverdin exhibited a rapid increase of spectrophotometrically detectable phytochrome. Phytochrome concentrations estimated immunochemically did not similarly increase throughout treatment with either compound. Similar experiments with 5-amino[4-$^{14}$C] levulinic acid showed radiolabeling of phytochrome with kinetics that paralleled the spectrally detected increase. These results are consistent with (a) the intermediacy of both 5-aminolevulinic acid and biliverdin in the biosynthetic pathway of the phytochrome chromophore and (b) the lack of coordinate regulation of chromophore and apoprotein synthesis in *Avena* seedlings.**

Light excitation of covalently bound phytochromobilin, the linear tetrapyrrolic prosthetic group of phytochrome, drives a reversible photoconversion between Pr and Pfr—a process which initiates profound developmental changes in plants (18, 27). Because of the central role of phytochrome as a mediator of photomorphogenesis in plants, knowledge of the metabolic processes that regulate its biosynthesis is of great interest. Two convergent pathways contribute to the overall biogenesis of the phytochrome holoprotein. One involves the synthesis of the apoprotein and the other, the synthesis of the chromophore and its attachment to the apoprotein. By comparison with our understanding of the autoregulatory role of phytochrome on the expression of its own gene(s) (5, 6, 23, 24), comparatively little is known of the pathway of phytochromobilin synthesis in plants. There have been two reports which support the intermediacy of ALA[2] (2, 9) as well as a recent report that showed that phytochrome chromophore synthesis is not tightly coupled with apoprotein synthesis (14); however, direct experimental evidence for the chemical path of phytochromobilin biosynthesis is lacking.

The present work was undertaken to devise an *in vivo* experimental system for introduction of exogenous putative chromophore precursors into the prosthetic group of phytochrome. Our study has relied on the observation that gabaculine (5-amino-1,3-cyclohexadienylcarboxylic acid), an irreversible inhibitor of mouse brain $\gamma$-aminobutyric acid-$\alpha$-ketoglutaric acid transaminase (25), is a potent inhibitor of spectrally detectable phytochrome levels in etiolated corn, oat, and pea seedlings *in vivo* (9). Gabaculine's capacity to inhibit phytochrome levels in etiolated *Avena* seedlings has allowed us to assess the efficacy of two compounds, ALA and biliverdin, to overcome this inhibition.

## MATERIALS AND METHODS

**Reagents.** 5-Aminolevulinic acid hydrochloride, biliverdin, 2-mercaptoethanol, diethyldithiocarbamate, PMSF, BSA (type V) and Tris base were obtained from Sigma. Ethylene glycol, glycerol, and ammonium persulfate were obtained from Mallinckrodt. Electrophoresis-grade *N,N'*-methylenebisacrylamide, acrylamide, *N,N,N',N'*-tetramethylethylenediamine, SDS and 4-chloro-1-naphthol were purchased from Bio-Rad. 5-Amino [4-$^{14}$C]levulinic acid hydrochloride (53 mCi/mmol) was obtained from Amersham. Gabaculine was obtained from Fluka, PEI from Eastman Kodak, ultra pure $(NH_4)_2SO_4$ from Schwarz/Mann, 2,5-diphenyloxazole (PPO) from J. T. Baker, and bacto-agar from Difco. *N*-Methylmercaptoacetamide was synthesized and vacuum distilled (b.p. 48°C at 1.4 mm Hg) as described by Houghten and Li (12).

**Plant Material.** Oat seeds (*Avena sativa* L. cv Garry) were obtained from Stanford Seeds (Buffalo, NY). Following overnight imbibition at 4°C in distilled $H_2O$, oat seeds were germinated in complete darkness at 25°C on 100 mm × 15 mm Falcon Petri dishes (Becton Dickinson) containing 20 ml sterile 1% (w/v) agar in 15 mM Hepes buffer (pH 7.4). Ten grams of imbibed seeds were used per Petri dish. To germinate seeds in the presence of gabaculine, freshly prepared 15 mM gabaculine in 15 mM Hepes buffer (pH 7.4) was added to the cooling agar before solidification (approximately 50°C) to bring the final concentration to 1 mM gabaculine.

**Antisera.** Polyclonal rabbit antisera to phytochrome were obtained through immunization with denatured and native 124 kD *Avena* phytochrome preparations which had SAR values in TEGE buffer of 1.05 and 0.87, respectively (20). For denaturation, phytochrome (51 nmol) was dialyzed against 5% (v/v) acetic acid (3 × 1 L) at 5°C using a minimum of 4 h between buffer changes. A second dialysis was performed under argon in 300 ml 5% (v/v) acetic acid at 5°C with a minimum of 4 h between buffer changes. The denatured protein when analyzed by 6 to 9% (T) gradient SDS-PAGE migrated as a single species ($M_r$ 124 kD) and was stored as a lyophilized powder at −80°C. Immunization of New Zealand white rabbits (2 per antigen) was performed as described previously (19) and antiserum was stored at −80°C after addition of 0.02% (w/v) $NaN_3$. Native and dena-

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[2] Abbreviations: ALA, 5-aminolevulinic acid; PMSF, phenylmethylsulfonylfluoride; PEI, poly(ethyleneimine); SAR, specific absorbance ratio ($A_{668}/A_{280}$ for Pr); TEGE buffer, 50 mM Tris-HCl buffer (pH 7.8 at 5°C) containing 25% (v/v) ethylene glycol and 1 mM EDTA.

tured phytochrome antisera were used interchangeably in the present study.

**Extractions.** A modification of our previously described extraction procedure was developed in order to quantitate phytochrome levels in small amounts of etiolated *Avena* tissue (20). All procedures were carried out at 5°C under dim green light. The shoot tips from 20 oat seedlings (1–2 cm in length) were excised, weighed, and placed in 14 × 32 mm polyethylene vials (Dynalab, Rochester NY). After placing 4 stainless steel lock washers (No. 6) into each vial, the tissue was frozen in liquid $N_2$ and shaken on a Wig-L-Bug shaker (Crescent Dental Mfg. Co., Chicago, IL) for 15 s. Extraction buffer (50 mM Tris-HCl buffer, pH 8.3 at 5°C, containing 100 mM $(NH_4)_2SO_4$, 25% (v/v) ethylene glycol, 1 mM EDTA, 2 mM PMSF, 10 mM diethyldithiocarbamate, and 142 mM 2-mercaptoethanol) was then added to the pulverized tissue, using a v/w ratio of 3 to 1. The mixture was shaken for 45 s, cooled in liquid $N_2$, and then shaken an additional 45 s. The resulting mixture was centrifuged in a 1.5 ml tube in an Eppendorf microfuge at 15,600*g* for 30 min. A 10% (w/v) PEI solution (adjusted to pH 7.8 with HCl) was added to the supernatant (4 μl 10% PEI per 100 μl extract), and the mixture was centrifuged for 15 min. An $(NH_4)_2SO_4$ solution (330 g/L in 50 mM Tris-HCl [pH 7.9]) was added to the resulting supernatant to a final concentration of 209 g/L. The solution was thoroughly mixed and allowed to sit for 5 min before centrifuging for 15 min. The pellet was resolubilized in a volume of freshly prepared buffer equal to 80% of the PEI supernatant volume. Resolubilization buffer was identical to the extraction buffer except that 2-mercaptoethanol was reduced to 14 mM, diethyldithiocarbamate was omitted and the final pH was adjusted to 7.9. The extract was then clarified by centrifuging for 15 min, and this fraction was used for subsequent assays.

***In Vivo* Incorporation Experiments.** Following germination in the presence of 1 mM gabaculine as described above, the terminal 1 cm shoot tips of etiolated oat seedlings 1 to 2 cm in length were excised while submerged in 15 mM Hepes buffer (pH 7.4) to prevent the introduction of air bubbles into the vascular tissue. To determine the effect of putative chromophore precursors on levels of spectrally detectable phytochrome, 20 excised shoot tips were floated in 2 ml incubation buffer at 25°C in 35 mm × 10 mm Falcon culture dishes (Becton Dickinson) for up to 5.5 h in the dark and then extracted as described above. Incubation buffers consisted of 15 mM Hepes buffer (pH 7.4) containing the following: 0.1 mM gabaculine alone, 0.1 mM gabaculine plus 3 mM ALA, or 0.1 mM gabaculine plus 0.5 mM biliverdin. Hepes buffer alone was used as a control. For radiolabeling experiments, 75 excised shoot tips were floated in 2 ml 15 mM Hepes buffer (pH 7.4) containing 50 μCi [4-$^{14}$C]ALA (53 mCi/mmol) at 25°C. Fifteen shoots were extracted at each time point up to 5 h, and only crude extracts (*i.e.* without PEI and $(NH_4)_2SO_4$ precipitation) were analyzed by SDS-PAGE and fluorography.

**Immunoprecipitation.** Immunoprecipitations were performed on extracts purified through the $(NH_4)_2SO_4$ stage of the purification procedure described above. Rabbit antiphytochrome antiserum (7.5 μl) was added to 100 μl extract at 5°C, and the mixture was vortexed and incubated for 10 min at 5°C. A 10% (w/v) suspension of *Staphylococcus aureus* cells (Calbiochem) was then added (10 μl/100 μl extract). The mixture was vortexed, allowed to stand for 10 min, and then centrifuged for 2 min in 1.5 ml tubes in an Eppendorf microfuge. The pellet was washed twice with 50 mM Tris-HCl (pH 7.4) containing 200 mM NaCl, before SDS-PAGE analysis.

**Spectrophotometric Assay.** Clarified extracts were assayed for phytochrome by double difference measurements, $\Delta(\Delta A) = (A_{668}[Pr] - A_{668}[Pfr]) - (A_{730}[Pr] - A_{730}[Pfr])$, as described (20). Since the extracts typically had volumes of around 400 μl, a black masked microcell cuvette (2 mm width × 1 cm pathlength) was used for spectrophotometry. Spectral measurements on purified *Avena* phytochrome in our laboratory show that $A_{668}(Pr) = 0.86\ \Delta(\Delta A)$ (15). Using the molar absorption coefficient for Pr at 668 nm of $1.21 \times 10^5$ L mol$^{-1}$ cm$^{-1}$ per monomer (20) and a monomer molecular mass of 124 kD, the concentration of phytochrome in mg/ml was calculated according to the equation $c = 0.88\ \Delta(\Delta A)$.

**Protein Assay.** Aliquots for protein determination were frozen at −80°C until use. Protein determination was performed by a modification of the Bradford assay using BSA as a standard (26).

**SDS-PAGE.** Aliquots for SDS-PAGE analysis were diluted 1:2 (v/v) with 2 × SDS sample buffer (0.2 M Tris-HCl buffer, pH 6.8, containing 15% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 3% (w/v) SDS, and 0.01% (w/v) bromophenol blue), heated for 1 min at 100°C, and stored at −80°C until use. Further dilutions to reach desired protein loadings were made with 1 × SDS sample buffer. Discontinuous SDS-PAGE was performed using the Laemmli buffer system (16) on a 0.8 mm slab gel using a 3% stacking gel and a 7.5 to 15% (T) linear gradient resolving gel. Depending on the experiment, gels were either stained with silver (22), used for immunoblot analysis (see below), or stained with Coomassie blue and fluorographed (see below). For SDS-PAGE analysis of immunoprecipitates, washed precipitates were resuspended in 1 × SDS sample buffer. Mixtures were heated at 100°C for 1 min and centrifuged for 5 min in 1.5 ml tubes in an Eppendorf microfuge before analyzing the supernatant by SDS-PAGE as described above.

**Fluorography.** Coomassie blue stained gels were treated with 2,5-diphenyloxazole in acetic acid (28), and exposed to Kodak X-Omat AR x-ray film at −80°C.

**Immunoblot Analysis.** Electrophoretic transfer of proteins to nitrocellulose paper followed by immunochemical detection was performed by a modification of the procedure described by Towbin *et al.* (29). Transfer was carried out for 2 h at 250 mamp constant current using a custom built transblot apparatus with 10.5 cm between the electrodes. The BLOTTO milk buffer system was used for immunodevelopment (13). All incubations were performed at ambient temperature except for the primary antiserum incubations which were performed overnight at 5°C. Dilutions were 1:250 for the primary rabbit antiphytochrome antiserum and 1:1000 for the secondary horseradish peroxidase linked goat antirabbit antiserum (Cappel Labs). Color development was accomplished using 4-chloro-1-naphthol (11). Densitometric scanning of blots was performed with a Zeineh model SL-504-XL soft laser scanning densitometer (Bio Med Instruments) interfaced with a Hewlett Packard 3390A Reporting Integrator. A standardized dilution series of phytochrome using freshly prepared extracts from untreated *Avena* seedlings (20–150 ng phytochrome per lane, determined spectrophotometrically) was included on each blot for calibration purposes. Duplicate samples were scanned three times each to obtain an average value. Standard curves were used to estimate phytochrome concentrations in test extracts.

## RESULTS

**Gabaculine Inhibition of Phytochrome Levels in Etiolated *Avena* Seedlings.** Initial studies were undertaken to determine the efficacy of gabaculine inhibition of spectrally detectable phytochrome levels during germination of oat seedlings. Buffering of the 1% (w/v) agar support in the pH range from 6 to 8 was necessary for optimal germination when gabaculine was present. A 15 mM Hepes buffer (pH 7.4) proved effective for this purpose. In the presence of gabaculine, there was a decrease in the percentage of seeds that germinated, and these seedlings exhibited reduced root growth.

Since phytochrome concentrations are best correlated with seedling length rather than chronological age (3), we compared

the levels of extractable phytochrome from gabaculine-treated and untreated seedlings of the same length. Regardless of the 'tissue age,' when assayed spectrophotometrically, gabaculine-grown seedlings contained significantly less extractable phytochrome than their untreated counterparts. Maximum inhibition of phytochrome levels was observed in young seedlings (1-2 cm in length), and for this reason, our studies were conducted on young seedlings. Figure 1 shows representative far-red minus red difference spectra obtained from *Avena* seedlings germinated in the presence and absence of gabaculine. Gabaculine treatment afforded an 81% inhibition of the spectrally detectable levels of phytochrome (Table I). By comparison, gabaculine treatment had no measurable effect on the amount of total extractable protein nor did it lead to any obvious differences in protein patterns on SDS-polyacrylamide gels when compared with untreated plants (Fig. 2; *cf.* lanes 1 and 2, silver stained gel). Comparative immunoblot analysis shows that gabaculine treatment reduced the level of immunochemically detectable phytochrome by 40 to 50% compared to that in untreated plants (Fig. 2; Table I). Regardless of tissue age, immunoblot analysis always afforded a larger estimate for phytochrome concentration than that determined spectrophotometrically (complete data not shown).

**Phytochrome Resynthesis in Gabaculine-grown *Avena* Shoot Explants.** Using gabaculine-grown *Avena* seedling explants which have approximately 20% the spectral levels of phytochrome found in normal etiolated seedlings, we investigated the effect of exogenously supplied putative phytochromobilin precursors, ALA or biliverdin, on phytochrome levels. Initial experiments were done to establish the time course of phytochrome reaccumulation in the presence of each of these two compounds (Fig. 3). Both led to a rapid increase in the spectrophotometrically detectable level of phytochrome but with different kinetics. These increases were significant and rapid, representing 2.5- and 3.0-fold increases in the levels of extractable phytochrome within 5 h (normalized to total protein) for ALA and biliverdin, respectively (Fig. 3A; Table II). Control experiments with gabaculine or Hepes buffer alone (Table II) showed that the observed increases were indeed due to added ALA or biliverdin. By contrast with the spectral assay results, immunoblot analysis revealed little or no change in the level of phytochrome protein during the 5 h incubations of the excised shoot tips (Table II). Moreover, SDS-PAGE analysis showed that none of the above incubation conditions led to measurable changes in the composition of the extractable protein from *Avena* seedlings (data not shown). While ALA treatment produced only a 6% increase in immunochemically detectable levels of phytochrome, biliverdin treatment led to a 27% increase (Table II). Biliverdin treatment, however, led to a decrease in extractable protein per gram fresh weight (Fig. 3B) which offsets this apparent increase in total phytochrome. By comparison, no significant change in total protein extracted per gram fresh weight was observed for seedlings incubated in the presence of ALA.

**[$^{14}$C] ALA Feeding Experiments.** Labeling studies were undertaken to distinguish between a general stimulatory effect or a specific incorporation of ALA into the phytochrome molecule. Using the methodology devised for studying the resynthesis of phytochrome, gabaculine-grown *Avena* seedling explants were supplemented with [$^{14}$C]ALA, and the time course of $^{14}$C incorporation into total soluble protein was determined by fluorography of SDS-polyacrylamide gels (Fig. 4). Lane 1 shows a representative Coomassie blue stained protein pattern of crude extracts of gabaculine-grown plants incubated for 5 h with [4-$^{14}$C]ALA. The fluorographs shown in lanes 2 through 5 represent

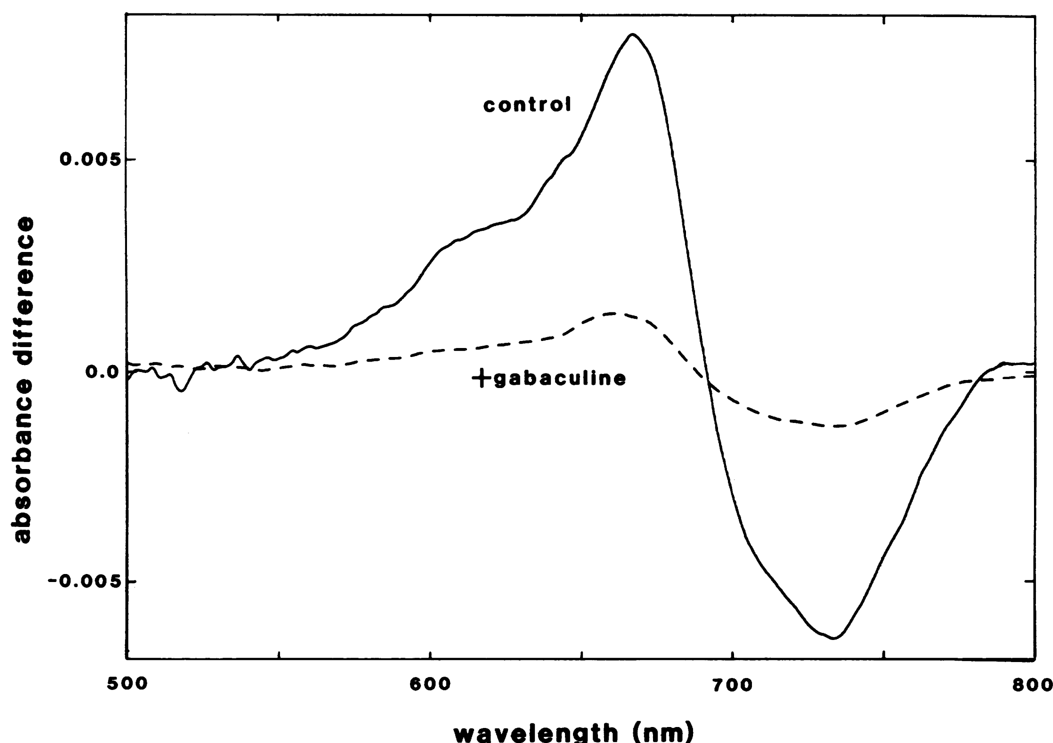



FIG. 1. Effect of gabaculine on spectrophotometrically detectable phytochrome levels of etiolated *Avena* seedlings. Far red minus red difference spectra of soluble extracts of the terminal 1 cm from 20 *Avena* seedlings (1-2 cm long) grown 3 to 4 d at 25°C on 1% agar in 15 mM Hepes buffer (pH 7.4) in the presence (- - -) or absence (——) of 1 mM gabaculine. Spectra were obtained at 5°C in 50 mM Tris-HCl buffer (pH 7.9) containing 100 mM $(NH_4)_2SO_4$, 1 mM EDTA, 14 mM 2-mercaptoethanol, and 2 mM PMSF. Spectra were normalized to the same protein concentration (1 mg/ml).

Table I. *Soluble Phytochrome Yields from Etiolated Avena Shoot Tips Grown in the Presence and Absence of Gabaculine*

Measurements were made on soluble extracts from the terminal 1.0 cm shoot tips of etiolated seedlings 1 to 2 cm in length as described under "Materials and Methods."

| Treatment | Spectral Assay[a] | | Immunoblot Assay[b] | |
|---|---|---|---|---|
| | *μg phytochrome/mg protein* | *% control* | *μg phytochrome/mg protein* | *% control* |
| Untreated | 12.9 (0.4) | 100 | 12.5 (0.3) | 100 |
| Gabaculine-grown[c] | 2.4 (0.2) | 19 | 7.6 (0.3) | 61 |

[a] Values shown represent the mean of the determinations from five different experiments, with the SE given in parentheses. [b] Values shown represent the mean from 2 to 3 separate experiments, with the SE given in parentheses. [c] Gabaculine-grown seedlings were obtained from *Avena* seeds germinated on 1% (w/v) agar containing 1 mM gabaculine in 15 mM Hepes buffer (pH 7.4) as described under "Materials and Methods."

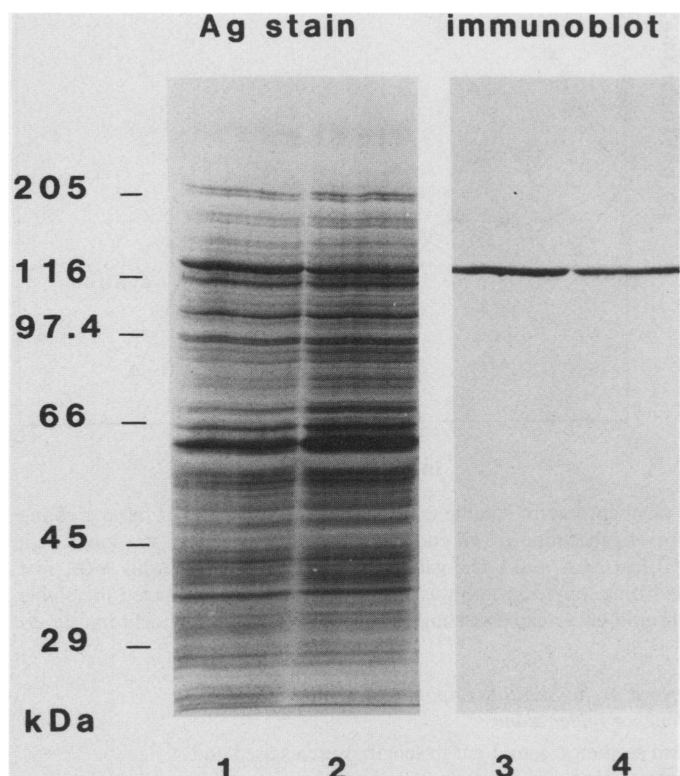



FIG. 2. Effect of gabaculine on immunochemically detectable phytochrome levels of etiolated *Avena* seedlings. Comparative silver-stained and immunoblotted discontinuous 5 to 15% (T) gradient SDS-polyacrylamide gels of partially purified soluble extracts of the terminal 1 cm shoot tips of 1 to 2 cm long etiolated *Avena* seedlings grown as described under "Materials and Methods" in the absence (lanes 1 and 3) and the presence (lanes 2 and 4) of 1 mM gabaculine. Sample loads were 30 μg total protein per well. The primary antisera used was obtained from rabbits immunized with native phytochrome (SAR 0.87) and was diluted 1:250.

different incubation times (1, 2, 3, and 5 h). Phytochrome specific incorporation is shown in lane 6 which is an immunoprecipitate of a 5 h time point. Besides phytochrome, five lower mol wt proteins show significant label incorporation. In addition, the time course of phytochrome labeling observed in Figure 4 parallels the observed spectral increase seen in Figure 3.

## DISCUSSION

The small scale procedure utilized in this work enabled reproducible extraction of phytochrome in amounts easily detectable by spectroscopic and immunochemical assays. To account for variability in extraction efficiency, phytochrome concentrations were normalized to total extractable protein. Utilizing this experimental protocol, we have confirmed and extended the observations of Gardner and Gorton (9) that gabaculine treatment elicits a pronounced reduction of spectrophotometrically detectable phytochrome levels in developing oat seedlings. In our hands, an inhibition of approximately 80% was observed which contrasts with the 55% inhibition reported by those authors for oat seedlings. Regardless of the reasons for the quantitative differences between the two reports, on a qualitative level, gabaculine clearly is a potent inhibitor of spectrally active phytochrome synthesis in developing *Avena* seedlings.

Immunoblot analyses yielded an estimate for phytochrome in gabaculine-grown plants which was significantly greater than that estimated spectrophotometrically. For the young seedlings used in these studies, the amount of phytochrome determined spectrophotometrically was 30 to 40% of that measured by immunoblot assay. In agreement with Jones *et al.* (14), these results suggest that excess apoprotein is present in gabaculine-grown seedlings and that synthesis of the apoprotein and chromophore are not tightly coupled in *Avena* seedlings. It is, however, possible that gabaculine treatment affords chromophore-bound phytochrome which is not photoreversible. Although gabaculine treatment leads to an apparent excess of apoprotein, on an absolute basis, the level of immunochemically detectable phytochrome in gabaculine-grown plants is 50 to 60% of the untreated control plants. This result contrasts with that observed in pea seedlings where the level of immunochemically detectable phytochrome in gabaculine-treated plants is the same as that in the untreated controls (14). It is possible that our result for oat seedlings is due to differential reactivity of the polyclonal antibodies towards the holoprotein *versus* the apoprotein, leading to an underestimate of phytochrome levels in gabaculine-treated plants. Alternatively, this reduced level of total phytochrome protein may indicate that chromophore and apoprotein synthesis are loosely coordinated, or that apoprotein and holoprotein are turned over at different rates.

The phytochrome re-synthesis experiments with gabaculine-grown seedlings administered ALA or biliverdin (Fig. 3; Table II) indicate that both compounds are taken up by the plant tissue and overcome inhibition by gabaculine. That no such spectral increase is seen in gabaculine-grown seedling explants incubated in buffer alone shows that these effects are not due to an adventitious interaction between gabaculine and ALA (or biliverdin) preventing gabaculine uptake or leading to its destruction. Several factors could account for the spectral increases observed: (a) the compounds serve as direct chemical precursors of the phytochrome chromophore, (b) they indirectly stimulate *de novo* synthesis of the chromophore by inducing new enzyme production, or (c) they stimulate detoxification pathways for gabaculine. The first interpretation is supported by two lines of evidence. First, the kinetics of phytochrome reappearance in

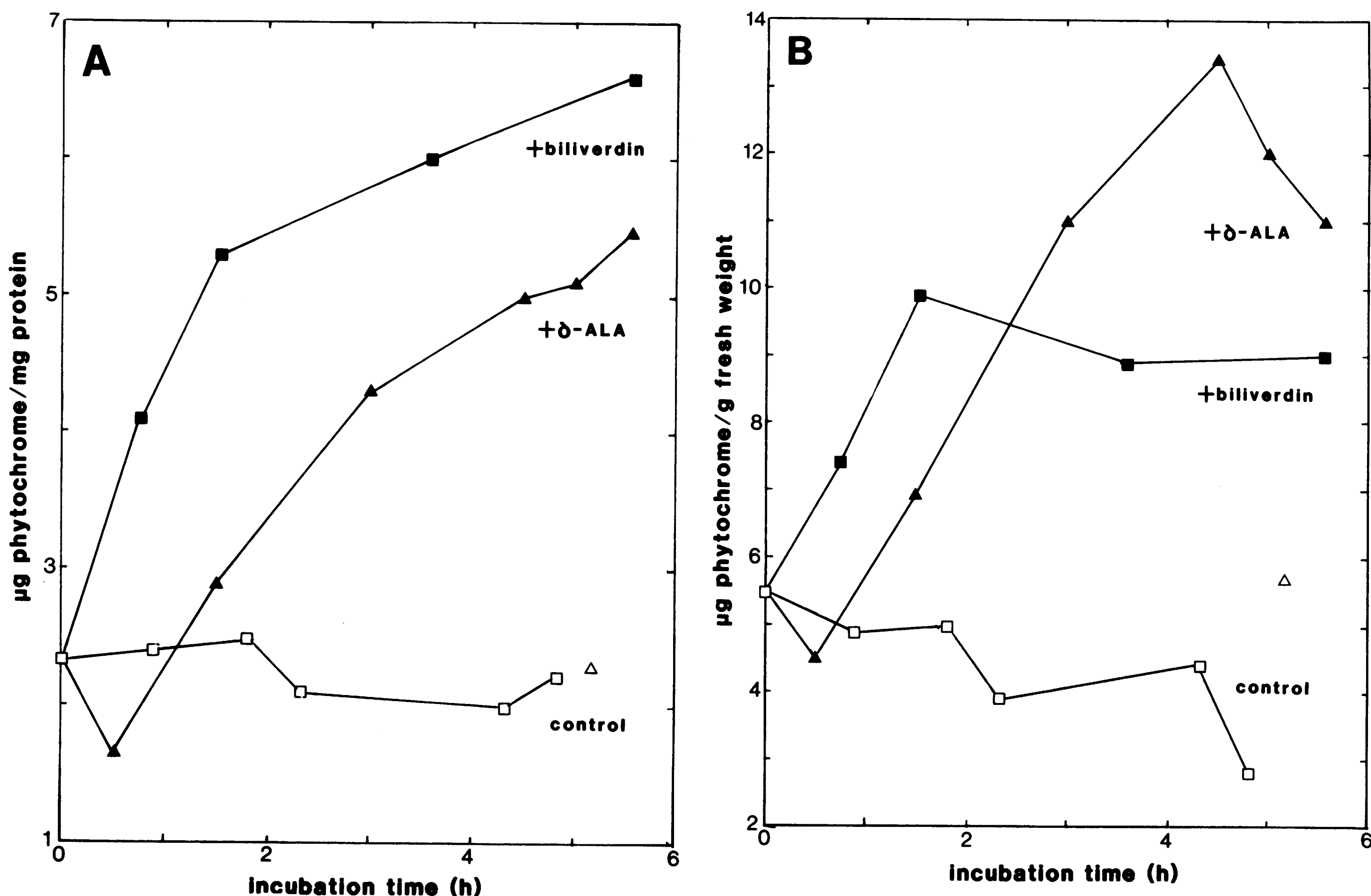



FIG. 3. Time course for expression of spectrophotometrically detectable phytochrome in soluble extracts of gabaculine-grown *Avena* seedling explants incubated with ALA or biliverdin. Excised terminal 1 cm shoot tips of gabaculine-grown etiolated *Avena* seedlings (1-2 cm long) were incubated at 25°C in darkness in 15 mM Hepes buffer (pH 7.4) containing 3 mM ALA + 0.1 mM gabaculine (▲), 0.5 mM biliverdin + 0.1 mM gabaculine (■), 0.1 mM gabaculine (□), and buffer only (△). At the indicated time, phytochrome was spectrophotometrically assayed in soluble extracts prepared as described under "Materials and Methods." A, Phytochrome levels expressed/mg extracted protein; B, phytochrome levels expressed/g fresh weight of tissue.

Table II. *Soluble Phytochrome Yields from Gabaculine-grown Avena Seedling Explants Incubated with ALA, Biliverdin, Gabaculine alone, or Buffer alone*

The terminal 1 cm shoot tips of gabaculine-grown *Avena* seedlings, 1 to 2 cm in length, were excised and incubated as described under "Materials and Methods." Values shown represent phytochrome levels in soluble extracts after a 5 h incubation period. All values shown were obtained from a representative experiment.

| Treatment[a] | Spectral Assay | | Immunoblot Assay | |
|---|---|---|---|---|
| | *μg phytochrome/mg protein* | *% control*[b] | *μg phytochrome/mg protein* | *% control*[b] |
| ALA (3 mM) | 5.7 | 45 | 9.1 | 73 |
| Biliverdin (0.5 mM) | 6.6 | 52 | 10.9 | 87 |
| Gabaculine alone | 2.2 | 17 | 8.6 | 69 |
| Buffer alone | 2.3 | 18 | 8.2 | 66 |

[a] All treatments, except that labeled Buffer alone, contained 0.1 mM gabaculine added to the 15 mM Hepes buffer (pH 7.4). See "Materials and Methods" section for full details. [b] Values are expressed as a percentage of the phytochrome levels found in untreated *Avena* seedlings germinated in the absence of gabaculine (*i.e.* 12.5 μg phytochrome/mg protein).

seedlings administered ALA exhibit a lag period not observed in the biliverdin treated tissue (Fig. 3). This lag is consistent with initial saturation of intermediate pools for heme and Chl biosynthetic pathways which share ALA as a common precursor. As predicted in Scheme I, biliverdin would not enter these pathways. Alternatively, the kinetic differences could reflect differences in uptake of the two compounds. This study does not directly address that question. The second line of evidence supporting direct incorporation of the exogenous compounds comes from the observed radiolabeling of phytochrome in seedlings administered [4-$^{14}$C]ALA (Fig. 4). Moreover, the time course of radiolabel incorporation parallels that of the spectral increases in phytochrome in tissue treated with ALA under similar conditions. Furthermore, even the initial lag seen in the spectral determination was observed in the radiolabeling experiment since prolonged film exposure revealed little detectable radiolabel incorporation into phytochrome within the 1st h of [4-$^{14}$C]ALA treatment.

It is conceivable that the observed radiolabeling of phytochrome by [4-$^{14}$C]ALA is due to nonspecific incorporation of

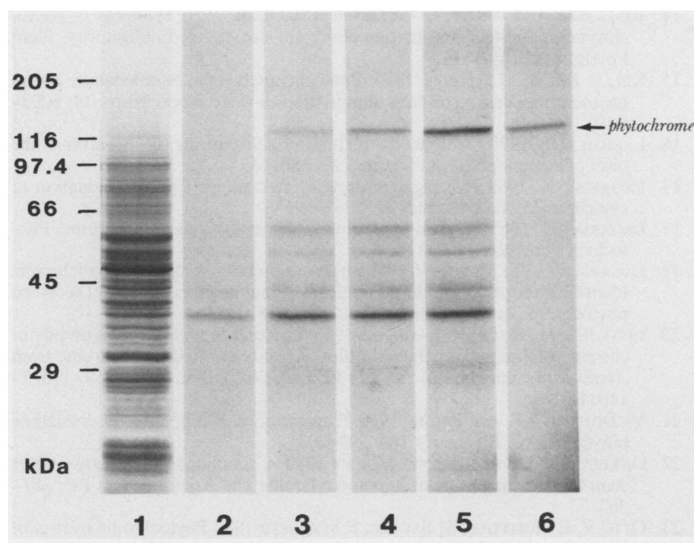
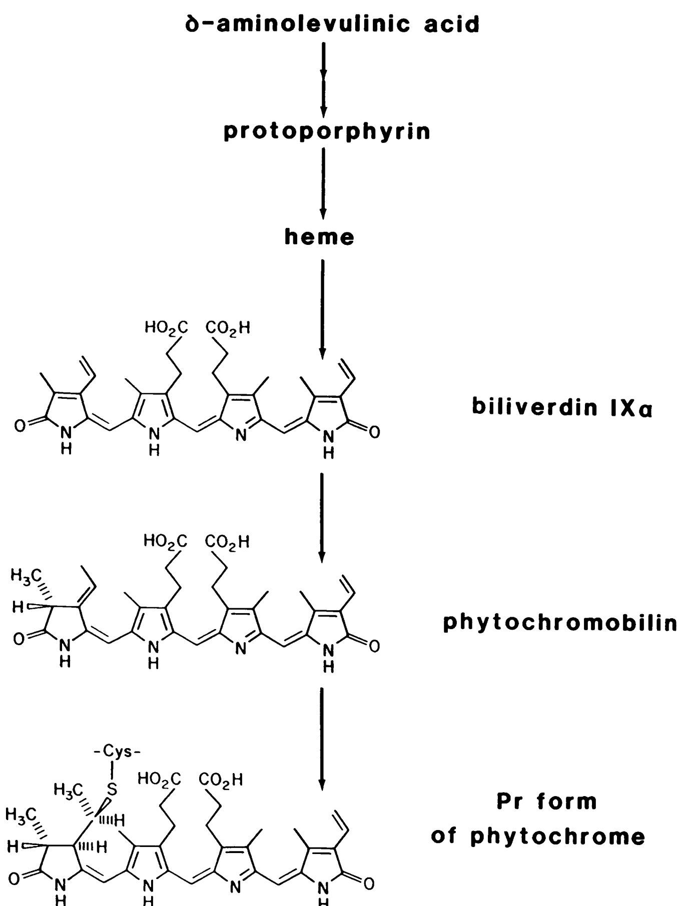



FIG. 4. Time course of incorporation of [4-$^{14}$C]ALA into soluble proteins of gabaculine-grown *Avena* seedling explants. Shoot tips from etiolated gabaculine-grown seedlings were incubated in darkness at 25°C in 15 mM Hepes buffer (pH 7.4) containing [4-$^{14}$C]ALA, as described under "Materials and Methods." Soluble extracts were prepared at indicated times up to 5 h, and the proteins were resolved on a 7.5 to 15% (T) gradient SDS polyacrylamide gel. Lane 1 is a representative Coomassie blue stained gel. Lanes 2 to 5 are 25 d exposure fluorographs of *Avena* extracts following 1, 2, 3, and 5 h incubations with radiolabeled ALA, respectively. Lane 6 is a 25 d fluorograph of an immunoprecipitate from an extract after 5 h incubation with [4-$^{14}$C]ALA. The monospecific rabbit antiserum towards phytochrome used for immunoprecipitation is described in Figure 2. Total protein loads are 150 $\mu$g/lane.




SCHEME I. Proposed biosynthetic pathway of the phytochrome chromophore.

ALA metabolites into the protein moiety. ALA is catabolized by plant tissue, and radiolabel derived from it can appear in animo acid pools (7, 10). In addition, ALA conversion to porphobilinogen, which can also be catabolized to amino acids (8), provides yet another possible pathway for nonspecific radiolabeling of the phytochrome protein. However, the relatively few proteins labeled during the 5 h incubation period with [4-$^{14}$C]ALA (Fig. 4) argue against such labeling. Indirect incorporation would require an exceedingly rapid turnover or new synthesis of the six labeled proteins. In addition, evidence that the predominant pathway of ALA catabolism in etiolated barley seedlings results in the loss of C4 as $CO_2$ (7) suggests that such a salvage pathway for ALA would not yield as efficient radiolabeling as we observe in these studies. While it is possible that the observed radiolabeling pattern represents a stress response towards wounding (*i.e.* seedling excision) and/or ALA or gabaculine treatment, the observation that phytochrome is such a 'stress protein' has no literature precedent. Based on the above arguments, we conclude that the observed labeling of phytochrome and the five smaller proteins is due to specific incorporation of [4-$^{14}$C]ALA into covalently bound heme, Chl or bilatriene prosthetic groups. To directly address the question of chromophore- or protein-specific incorporation of radiolabel from [4-$^{14}$C]ALA or any other labeled potential phytochromobilin precursor such as biliverdin, efficient methods must be developed to cleave the thioether linkage between the chromophore and apoprotein.

The question of whether newly synthesized chromophore is attached to preformed apoprotein or to newly synthesized apoprotein during the 5 h recovery period in the presence of ALA or biliverdin remains an important unanswered question. Our immunochemical results indicate that there is only a slight increase (if any) in the level of total extractable phytochrome protein in seedlings incubated with ALA or biliverdin. This increase in immunochemically detectable phytochrome was significantly less than the increase detected spectrophotometrically. These results support the hypothesis of a posttranslational attachment of chromophore to preformed apoprotein.

The lack of availability of radiolabeled biliverdin has prevented experiments similar to those performed with [4-$^{14}$C]ALA, however, the kinetics of spectrally detectable phytochrome recovery in seedlings administered biliverdin strongly support its intermediacy in the synthesis of the phytochrome chromophore. Biliverdin has been shown to be an intermediate in the biosynthesis of the prosthetic group(s) of the light harvesting accessory chromoprotein phycocyanin from the red alga *Cyanidium caldarium* (1, 4). Thus, the two structurally related bilatriene prosthetic groups likely share a common biosynthetic pathway which involves the formation of free linear tetrapyrrole precursors rather than the intermediacy of a heme protein precursor as proposed earlier (17). It is also worthy of note that the biliverdin used in the present studies is impure, the natural IX$\alpha$ isomer being but a minor component (21). While it is reasonable to infer that the IX$\alpha$ isomer is responsible for the recovery of spectrally active phytochrome in *Avena* seedlings, the role of the other bilin isomers in phytochrome biosynthesis needs to be addressed. Perhaps these contaminants may be responsible for the observed toxicity of biliverdin towards protein levels in developing seedlings.

In conclusion, these studies support the biosynthetic pathway for the phytochrome chromophore illustrated in Scheme I. The data presented here also imply that the biosynthesis of the phytochrome chromophore and apoprotein are not tightly coupled in etiolated *Avena* seedlings. Thus, the potential for inde-

**pendent regulation of phytochrome levels through modulation of the pathway of chromophore biosynthesis exists and may prove to be an important means to mediate phytochrome levels in plants.**

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## LITERATURE CITED

1. Beale SI, J Cornejo 1983 Biosynthesis of phycocyanobilin from exogenous labeled biliverdin in *Cyanidium caldarium*. Arch Biochem Biophys 227: 279-286
2. Bonner BA 1967 Incorporation of delta-aminolevulinic acid into the chromophore of phytochrome. Plant Physiol 42: S-11
3. Briggs WR, HW Siegelman 1965 Distribution of phytochrome in etiolated seedlings. Plant Physiol 40: 934-941
4. Brown SB, JA Holroyd, DI Vernon 1984 Biosynthesis of phycobiliproteins. Incorporation of biliverdin into phycocyanin of the red alga *Cyanidium caldarium*. Biochem J 219: 905-909
5. Colbert JT, HP Hershey, PH Quail 1983 Autoregulatory control of translatable phytochrome mRNA levels. Proc Natl Acad Sci USA 80: 2248-2252
6. Colbert JT, HP Hershey, PH Quail 1985 Phytochrome regulation of phytochrome mRNA abundance. Plant Mol Biol 5: 91-101
7. Duggan JX, E Meller, ML Gassman 1982 Catabolism of 5-aminolevulinic acid to $CO_2$ by etiolated barley leaves. Plant Physiol 69: 19-22
8. Duggan JW, E Meller, ML Gassman 1982 Catabolism of porphobilinogen by etiolated barley leaves. Plant Physiol 69: 602-608
9. Gardner G, HL Gorton 1985 Inhibition of phytochrome synthesis by gabaculine. Plant Physiol 77: 540-543
10. Gassman ML, JX Duggan, LC Stillman, LM Vlcek, PA Castelfranco, B Wezelman 1978 Oxidation of chlorophyll precursors and its regulation to the control of greening. *In* G Akoyunoglou, JH Argyroudi-Akoyunoglou, eds, Chloroplast Development. Elsevier/North Holland, Amsterdam, pp 167-181
11. Hawkes R, E Niday, J Gordon 1982 A dot-immunoblotting assay for monoclonal and other antibodies. Anal Biochem 119: 142-147
12. Houghten RA, CH Li 1983 Reduction of sulfoxides in peptides and proteins. Methods Enzymol 91: 549-559
13. Johnson DA, JW Gautsch, JR Sportsman, JH Elder 1984 Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Anal Techn 1: 3-8
14. Jones AM, CD Allen, G Gardner, PH Quail 1986 Synthesis of phytochrome apoprotein and chromophore are not coupled obligatorily. Plant Physiol 81: 1014-1016
15. Kelly JM, JC Lagarias 1985 Photochemistry of 124 kilodalton *Avena* phytochrome under constant illumination *in vitro*. Biochemistry 24: 6003-6010
16. Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage $T_4$. Nature 227: 680-685
17. Lagarias JC 1982 Bile pigment-protein interactions. Coupled oxidation of cytochrome *c*. Biochemistry 21: 5962-5967
18. Lagarias JC 1985 Progress in the molecular analysis of phytochrome. Photochem Photobiol 42: 811-820
19. Lagarias JC, FM Mercurio 1985 Structure-function studies on phytochrome. Identification of light-induced conformational changes in 124 kDa *Avena* phytochrome *in vitro*. J Biol Chem 260: 2415-2423
20. Litts JC, JM Kelly, JC Lagarias 1983 Structure-function studies on phytochrome. Preliminary characterization of highly purified phytochrome from *Avena sativa* enriched in the 124 kDa species. J Biol Chem 258: 11025-11031
21. McDonagh AF, LA Palma 1980 Preparation and properties of crystalline biliverdin IX$\alpha$. Biochem J 189: 193-208
22. Oakley BR, DR Kirsh, NR Morris 1980 A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Anal Biochem 105: 361-363
23. Otto V, E Mosinger, M Sauter, E Schaefer 1983 Phytochrome control of its own synthesis in *Sorghum vulgare* and *Avena sativa*. Photochem Photobiol 38: 693-700
24. Otto V, E Schaefer, A Nagatani, KT Yamamoto, M Furuya 1984 Phytochrome control of its own synthesis in *Pisum sativum*. Plant Cell Physiol 25: 1579-1584
25. Rando RR, FW Bangerter 1976 The irreversible inhibition of mouse brain $\gamma$-aminobutyric acid (GABA)-$\alpha$-ketoglutaric acid transaminase by gabaculine. J Am Chem Soc 98: 6762-6764
26. Read SM, DH Northcote 1981 Minimization of variation in the response to different proteins of the Coomassie blue G dye-binding assay for protein. Anal Biochem 116: 53-64
27. Shropshire W Jr, H Mohr 1983 Photomorphogenesis. Encyclopedia of Plant Physiology, New Series Vol 16A and 16B. Springer-Verlag, New York
28. Skinner MK, MD Griswold 1983 Fluorographic detection of radioactivity in polyacrylamide gels with 2,5-diphenyloxazole in acetic acid and its comparison with existing procedures. Biochem J 209: 281-284
29. Towbin H, T Staehelin, J Gordon 1979 Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76: 4350-4354