

Inhibition of Phytochrome Synthesis by Gabaculine

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ABSTRACT

Gabaculine (5-amino-1,3-cyclohexadienylcarboxylic acid), a transaminase inhibitor, also inhibits chlorophyll formation in plants, and the effect of this compound can be counteracted by 5-aminolevulinic acid (ALA) (Flint, personal communication, 1984). Since it is probable that ALA also serves as a precursor to phytochrome, the effects of gabaculine on phytochrome synthesis in developing etiolated seedlings were examined using *in vivo* spectrophotometry. Preemergence treatment with gabaculine was found to inhibit initial phytochrome synthesis in peas (*Pisum sativum* L.), corn (*Zea mays* L.), and oats (*Avena sativa* L.). In general, reduction in phytochrome correlated with reduction in chlorophyll. However, the extent of inhibition of phytochrome synthesis was not as great as that of chlorophyll synthesis, perhaps due to preexisting phytochrome in the seed. Foliar treatment of etiolated pea seedlings prior to light-induced destruction of phytochrome inhibited subsequent phytochrome resynthesis in the dark. These results suggest that both initial synthesis and resynthesis of phytochrome require *de novo* synthesis of chromophore as well as apoprotein.

Growth and development of an etiolated seedling is accompanied by an increase in the level of photoreversible phytochrome, the chromoprotein that is the photoreceptor for many responses in plant photomorphogenesis. This apparent synthesis of phytochrome is paralleled by *de novo* synthesis of the phytochrome apoprotein, as shown by deuterium-density labeling techniques (19). While substantive data do exist on the synthesis (and degradation) of the phytochrome protein, there is virtually no information on the biosynthesis of the phytochrome chromophore. Spectral evidence has suggested that the chromophore is a linear tetrapyrrole (23), although only recently has the structure of the red-absorbing form of the pigment been rigorously elucidated (16). The exact structure of the Pfr chromophore still remains unclear. A preliminary report (4) indicated that radioactivity from [¹⁴C]ALA², a common precursor of porphyrins and bile pigments, was incorporated into photoreversible phytochrome; however, no technical details of methods or results were ever published.

It has been suggested (2) that the synthesis of ALA may be compartmentalized in plants, the ALA in mitochondria (and the cytoplasm?) coming from the condensation of glycine and succinyl CoA (the principal pathway in animals and bacteria) and the ALA in plastids from the intact carbon skeleton of glutamate or α -ketoglutarate (the C-5 pathway). The intermediate steps in the formation of ALA from glutamate are not clearly understood.

One suggestion (13) is that glutamate, via glutamate-1-P, is reduced to glutamate-1-semialdehyde, which undergoes an isomerization involving transamination to form ALA. Alternatively, glutamate may be converted to α -ketoglutarate, reduced to 4,5-dioxovalerate, and transaminated in the presence of an amino group donor such as L-alanine to form ALA (3, 14).

Gabaculine (5-amino-1,3-cyclohexadienylcarboxylic acid) is a natural product isolated from a culture filtrate of *Streptomyces toyocaensis* that has been shown to be an irreversible inhibitor of the mammalian enzyme γ -aminobutyric acid- α -ketoglutaric acid transaminase (GABA-T) (15, 21). Recently, D. H. Flint (personal communication, 1984; manuscript in preparation) has shown that gabaculine is a potent inhibitor of Chl formation in plants, and his observation that the effect of gabaculine was counteracted by ALA indicated that the gabaculine-induced block occurred on the pathway of ALA synthesis.

The availability of gabaculine as an inhibitor of ALA formation and its apparent specificity for the C-5 pathway based on mechanistic reasons (Flint, personal communication, 1984) raised similar questions for other ALA-derived chromophores in plants. Specifically, in the case of phytochrome, was the phytochrome chromophore derived from the same pathway as Chl, and, if the C-5 pathway was confined to plastids, what would the results say as to the subcellular localization of phytochrome chromophore synthesis? Thus, we decided to examine the effect of gabaculine on phytochrome synthesis in developing etiolated seedlings.

Levels of phytochrome in a developing seedling are not constant. As an etiolated seedling begins to grow, phytochrome levels increase (8). Levels of phytochrome also change in a light-grown plant during the daily light/dark cycle. When the seedling is exposed to red light, phytochrome is transformed to the Pfr form which is proteolytically degraded (7, 18). Under continuous light, phytochrome levels reach a steady state when the rate of degradation equals the rate of synthesis (20, 22). The rate of phytochrome synthesis appears to be unaffected by light. When the plant is returned to darkness, phytochrome again begins to accumulate (20).

In this report we address three questions. (a) Does gabaculine inhibit initial phytochrome synthesis in germinating pea seedlings? (b) Is phytochrome resynthesis after destruction affected in the same way as initial synthesis? (c) Do other species, in addition to peas, respond in the same fashion?

MATERIALS AND METHODS

Peas—Initial Phytochrome Synthesis upon Germination. Approximately 50 pea seeds (*Pisum sativum* L., cv Alaska; W. Atlee Burpee) were placed in a 14-cm Petri dish on cellulose packing material (Kim-Pak, Kimberly-Clark) moistened with 30 ml water or 30 ml of 0.6 mM gabaculine. Two dishes were prepared for each treatment. The seeds were placed in the dark at 27°C. After 3 d, an additional 8 ml of the appropriate solution was added to each dish to compensate for loss by evaporation. After 5 d, 30

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² Abbreviation: ALA, 5-aminolevulinic acid.

ml of solution was added to each dish, and one dish from each treatment was transferred to continuous cool white fluorescent light.

At the end of 7 d, the plumules of the plants that had been transferred to the light were excised and extracted in acetone, and their Chl content was determined by the procedure of Arnon (1). The epicotyl hooks of the seedlings that had been kept in the dark were excised, the plumules were discarded, and the phytochrome content of the hook tissue was measured *in vivo* using a dual wavelength spectrophotometer as described previously (11). The experiments shown here were duplicated with similar results, and several similar experiments with slightly differing protocols were also carried out.

Peas—Phytochrome Resynthesis after Destruction. *Preemergence Application.* In control experiments, pea seeds were soaked in 85 ml Hutner's medium (9) for 3 h, planted in Jiffy Plus Potting Mix (Jiffy Products of America, West Chicago, IL) in 40 × 80 mm crystallizing dishes, and grown in the dark for 7 day at 27°C and 80% RH. For gabaculine treatment, the compound was dissolved in the Hutner's medium and, after seed soaking, the treatment solution was combined with the potting mix prior to planting.

Phytochrome destruction and resynthesis were achieved by the protocol of Clarkson and Hillman (5). Intact plants were treated with a succession of 15-min exposures to red light separated by 105-min periods of darkness. After the fourth red light period, plants were returned to darkness. At various time intervals, 10-mm epicotyl sections were excised 1 mm below the hook for phytochrome measurement as described above. Each data point was determined on duplicate 0.2-g tissue samples, and the means of the values are presented. Each experiment was replicated at least three times with qualitatively similar results.

Foliar Application. Untreated etiolated pea plants were grown in crystallizing dishes as described. One h prior to the first red light period, each dish of intact plants was sprayed with 2.0 ml of gabaculine in surfactant (0.5% [w/v] Tween 20 plus 0.5% Span 80). A Kontes TLC sprayer was used for application. Red light treatments and phytochrome measurements were carried out as indicated above.

Corn—Initial Phytochrome Synthesis. Fifteen corn seeds (*Zea mays* L., cv WF9 × Bear 38) were imbibed in 85 ml of treatment solution with varying concentrations of gabaculine for 3 h and planted in Jiffy Plus Potting Mix combined with the same treatment solution. Plants were grown in the dark for 3 to 4 d, and coleoptiles, including primary leaf tissue, were harvested for *in vivo* phytochrome measurements as described above. At the time of measurement, a replicate set of samples was placed under cool white fluorescent light for 24 h, and then Chl content was determined.

Oats—Initial Phytochrome Synthesis. Five g of husked oat seeds (*Avena sativa* L., cv Victory, USDA CI2020) was imbibed in a 100 × 15 mm plastic Petri dish in 20 ml of 1% agar containing 1 mM gabaculine. After 4 d growth in the dark at 27°C, phytochrome was measured in excised coleoptiles (includ-

ing primary leaves) as above. For each treatment, duplicate 0.25-g samples were prepared, each containing about 16 shoots. At the time of phytochrome measurement, a parallel set of plants was placed under cool white fluorescent lights for 24 h before Chl extraction and measurement.

Chemicals and Light Sources. D,L-Gabaculine was purchased from Calbiochem-Behring Corp. and used in a water solution. Red light was obtained from a Leitz Prado slide projector through a Corion 660 nm interference filter (10).

RESULTS

Inhibition of Phytochrome Synthesis upon Germination—Peas. The initial experiments were carried out with germinating pea seedlings. That gabaculine got into the plants and was active is shown by its effect on Chl formation (Table I). Although there was some detectable Chl in the treated plants, its amount was less than 1% of the control value. This observation confirms, for pea plumules, the report of Flint (personal communication, 1984) that gabaculine inhibits Chl biosynthesis in barley leaves.

The effect of gabaculine on phytochrome synthesis is shown in Table II. Whether expressed on a per plant or a fresh weight basis, 0.6 mM gabaculine inhibited the formation of 80 to 90% of the photoreversible phytochrome.

Inhibition of Phytochrome Resynthesis after Destruction. The experiment in Table II indicates that the Chl biosynthesis inhibitor gabaculine is capable of inhibiting the increase in phytochrome content that occurs upon germination and development of an etiolated seedling. Net phytochrome synthesis also occurs when a plant is returned to the dark following light-induced destruction. The experiment in Figure 1 was carried out to determine whether this phytochrome 'resynthesis' is also subject to inhibition by gabaculine. As observed by Clarkson and Hillman (5), in pea seedlings the absolute level of phytochrome must fall below a certain critical level before resynthesis occurs. Reduction to this level was achieved by three successive cycles of 15 min red light/105 min dark. This sequence of irradiations caused significant destruction and, after the plants were returned to the dark, resynthesis increased the measurable photoreversible phytochrome to 70% of the initial dark level (Fig. 1, —). Gabaculine at 1 mM completely inhibited phytochrome resynthesis as well as the initial phytochrome synthesis in the dark (Fig. 1, ·····).

Because of the mode of application of the compound in the experiments just described, it is not possible to separate the effect on initial synthesis from that on resynthesis. (Perhaps the initial inhibition of ALA synthesis resulted in a phytotoxic 'shock' that secondarily affected phytochrome resynthesis.) Therefore, etiolated pea plants containing normal levels of phytochrome were sprayed with gabaculine 1 h prior to the red light treatments leading to destruction and subsequent resynthesis. Under these conditions, gabaculine clearly inhibits phytochrome synthesis (Fig. 2). Furthermore, the inhibition appears to be concentration-dependent. After 48 h in the dark, the plants sprayed with 1 mM gabaculine had 55% of the phytochrome of the control whereas

Table I. *Inhibition of Chl Formation in Pea Seedlings by Gabaculine*

Alaska pea seeds were imbibed and germinated in water containing gabaculine, grown in the dark for 5 d, and then transferred to continuous white light for 48 h before extraction of Chl. Each treatment contained 37 seedlings.

Treatment	Total Chl	Fresh Wt	Chl/Plumule	Chl/g Fresh Wt
	mg	g	μg	mg
Water	8.74	0.42	236	20.8
Gabaculine, 0.6 mM	0.0239	0.205	0.65	0.117
% of control			0.3%	0.6%

Table II. Inhibition of Phytochrome Synthesis in Etiolated Pea Seedlings by Gabaculine

Alaska pea seeds were imbibed and germinated in water containing gabaculine and grown in the dark for 7 d. Phytochrome content was then measured *in vivo* with a dual wavelength spectrophotometer. The control sample contained 22 epicotyl hooks, and the gabaculine-treated sample contained 30 hooks.

Treatment	Total Phytochrome [$\Delta(\Delta A)$]	Fresh Wt g	Phytochrome/Hook	Phytochrome/g Fresh Wt
Water	0.273	0.28	0.012	0.975
Gabaculine, 0.6 mM	0.053	0.54	0.002	0.098
% of control			17%	10%

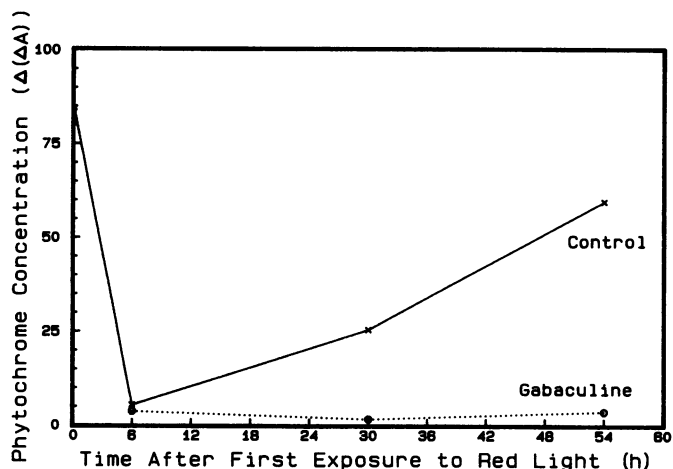


FIG. 1. Inhibition of phytochrome resynthesis in peas—soil treatment with gabaculine. Pea seeds were imbibed and germinated in Hutner's medium (—) or medium containing 1 mM gabaculine (.....), and grown in potting mix for 7 d in the dark. Destruction and resynthesis of phytochrome were induced by a series of four 15-min red light treatments separated by 105 min of darkness. Phytochrome concentration was determined by *in vivo* dual wavelength spectrophotometry at 660 and 730 nm on 0.2-g samples taken just prior to, and 6, 30, and 54 h after the initial red light treatment. Phytochrome concentration is given as $\Delta(\Delta A)$ per g fresh weight of tissue. The control sample after 48 h of resynthesis had an activity of 5.96 ± 0.36 (SE) $\times 10^{-2}$ $\Delta(\Delta A)$ /g fresh weight.

the plants treated with 3 mM gabaculine had only 14% of the control levels.

Inhibition of Phytochrome Synthesis in Corn Seedlings. Since gabaculine showed broad-spectrum herbicidal activity with symptoms of lack of Chl, it would be expected that this compound could also inhibit phytochrome synthesis in a broad range of species. The compound was tested in etiolated corn seedlings at 0.3, 1.0, and 3.0 mM, and inhibited both phytochrome and Chl formation (Fig. 3). As was observed in the case of pea seedlings (Tables I and II), the degree of inhibition of Chl biosynthesis in corn coleoptiles was greater than the degree of inhibition of phytochrome formation.

Inhibition of Phytochrome Synthesis in Oat Seedlings. The effect of gabaculine in etiolated oat seedlings is similar to that in corn. At 1 mM gabaculine, the phytochrome content of treated oat shoots was 55% of the control (0.90 $\Delta(\Delta A)$ /g fresh weight compared with 0.161 $\Delta(\Delta A)$ /g fresh weight). As in the case of the other species, the degree of inhibition of Chl biosynthesis (to 20% of the control) was greater than that of phytochrome.

DISCUSSION

The fundamental importance of these experiments is that both initial synthesis and resynthesis of phytochrome require *de novo*

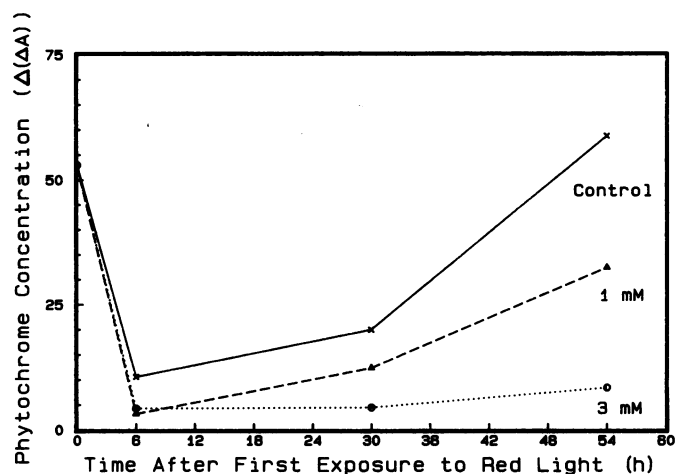


FIG. 2. Inhibition of phytochrome resynthesis in peas—foliar treatment with gabaculine. Untreated etiolated pea plants were grown and irradiated as described for Figure 1. One h prior to the first red light treatment, plants were sprayed with 2.0 ml surfactant (—), surfactant containing 1 mM gabaculine (---), or surfactant containing 3 mM gabaculine (.....). Samples were taken for *in vivo* phytochrome measurement as in Figure 1.

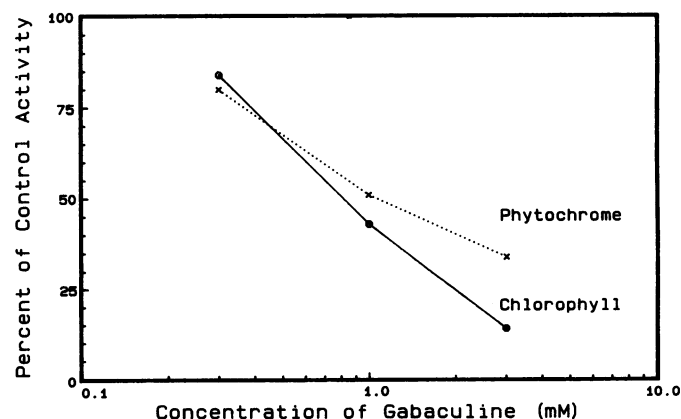


FIG. 3. Inhibition of initial phytochrome synthesis in corn seedlings. Seeds were imbibed and germinated in Hutner's medium containing 0.3, 1.0, or 3.0 mM gabaculine and grown in the dark for 3 to 4 d. Phytochrome content (.....) was assayed in duplicate 0.25-g samples of shoots by *in vivo* spectrophotometry, and replicate samples were transferred to white light for 24 h before Chl extraction and measurement (—). Values are given as percentage of control. The untreated control tissue had a phytochrome concentration of 1.63 ± 0.46 (SE) $\times 10^{-2}$ $\Delta(\Delta A)$ /0.25 g fresh weight.

synthesis of chromophore as well as apoprotein, and this synthesis of chromophore is dependent on the synthesis of ALA. From the point of view of biosynthetic regulation, these inhibitors of ALA biosynthesis provide a valuable probe for examining the relationship between the phytochrome chromophore and its apoprotein. One obvious question that can be addressed is what happens to apoprotein synthesis under the conditions of Figure 2? This is especially interesting in view of the recent finding that phytochrome controls the level of its own messenger RNA (6).

The results indicate that the bulk of the phytochrome chromophore synthesized in etiolated peas, corn, and oats shares a common pathway with Chl. However, there is a quantitative discrepancy between the effects in Tables I and II. In the presence of gabaculine, less than 1% of the control level of Chl was synthesized, whereas 10 to 20% of the phytochrome was still measurable. There are at least three explanations for the presence of this amount of phytochrome. The first, and most likely, is that the 10 to 20% represents phytochrome chromophore that already existed in the dry seed prior to imbibition. Many seeds have measurable levels of phytochrome, and many seeds show phytochrome control of germination (see 24 for review.) Second, it is possible that phytochrome synthesis more effectively competes for available ALA than does Chl synthesis. The third possibility is that a small portion of the phytochrome is made by the classical ALA synthetase from glycine and succinyl CoA. This could be examined by measuring incorporation of radioactivity into phytochrome from glycine specifically labeled in the C-2 position. (The carboxyl carbon of glycine is lost as CO₂ in the synthetase reaction.) This experiment would also be a definitive test as to whether gabaculine is specific for the C-5 pathway.

Given the literature cited above, these data imply that the bulk of the newly synthesized phytochrome chromophore is manufactured in the plastid. This conclusion is consistent with the observations of Grombein *et al.* (12) that under conditions following phytochrome destruction in the light, reappearance of photoreversibility in the dark occurred primarily in the subcellular fraction enriched for plastids and not in the mitochondrial fraction.

The general argument appears valid that a transaminase inhibitor of ALA synthesis also inhibits both initial synthesis and resynthesis of phytochrome in a range of species. In the course of this work, other classes of Chl inhibitors were also examined for their effects on phytochrome content. Levulinic acid and 4,6-dioxoheptanoic acid (2, 17), inhibitors of ALA dehydratase, often inhibited Chl formation in our systems at high concentrations, and occasionally this response was associated with an apparent inhibition of phytochrome biosynthesis. But the results with these compounds were extremely variable, and clear cut inhibitory effects were accompanied by relatively high phytotoxicity. In contrast, gabaculine inhibited phytochrome formation quite reproducibly at concentrations that caused few other morphological abnormalities than lack of Chl.

The activity of gabaculine allows us to address a central question in the control of growth and development by phytochrome: are phytochrome responses controlled by the absolute level of the pigment (or Pfr) or are they controlled solely by the photostationary equilibrium? In other words, what are the phys-

iological consequences of the inhibition of phytochrome synthesis? Since in the natural environment plants are exposed to white light containing roughly equivalent proportions of red and far-red, there is a constant balance between destruction and resynthesis, and the experiments here suggest that this resynthesis requires new chromophore synthesis. Experiments are now underway to determine the effect of gabaculine on physiological responses to phytochrome photoconversion.

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LITERATURE CITED

1. ARNON DJ 1949 Copper enzymes in isolated chloroplasts, polyphenoloxidase in *Beta vulgaris* L. *Plant Physiol* 24: 1-15
2. BEALE SI 1978 δ -Aminolevulinic acid in plants: its biosynthesis, regulation, and role in plastid development. *Annu Rev Plant Physiol* 29: 95-120
3. BEALE SI, SP GOUGH, S GRANICK 1975 Biosynthesis of δ -aminolevulinic acid from the intact carbon skeleton of glutamic acid in greening barley. *Proc Natl Acad Sci USA* 72: 2719-2723
4. BONNER BA 1967 Incorporation of delta-aminolevulinic acid into the chromophore of phytochrome. *Plant Physiol* 42: S 11
5. CLARKSON DT, WS HILLMAN 1967 Apparent phytochrome synthesis in *Pisum* tissue. *Nature* 213: 468-470
6. COLBERT JT, HP HERSHEY, PH QUAIL 1983 Autoregulatory control of translatable phytochrome mRNA levels. *Proc Natl Acad Sci USA* 80: 2248-2252
7. COLEMAN RA, LH PRATT 1974 Phytochrome: immunocytochemical assay of synthesis and destruction. *Planta* 119: 221-231
8. FRANKLAND B 1972 Biosynthesis and dark transformations of phytochrome. In K Mitrakos, W Shropshire Jr, eds, *Phytochrome*. Academic Press, New York, pp 195-225
9. FURUYA M, WS HILLMAN 1964 Observations on spectrophotometrically assayable phytochrome *in vivo* in etiolated *Pisum* seedlings. *Planta* 63: 31-42
10. GARDNER G 1983 The effect of growth retardants on phytochrome-induced lettuce seed germination. *J Plant Growth Regul* 2: 159-163
11. GARDNER G, MA GRACEFFO 1982 The use of a computerized spectroradiometer to predict phytochrome photoequilibria under polychromatic irradiation. *Photochem Photobiol* 16: 349-354
12. GROMBEIN S, W RÜDIGER, R HAMPP 1978 Destruction and possible *de novo* synthesis of phytochrome in subcellular fractions of laminae from *Avena sativa* L. *Planta* 141: 273-277
13. KANNANGARA CG, SP GOUGH 1978 Biosynthesis of δ -aminolevulinic acid in greening barley leaves: glutamate 1-semialdehyde aminotransferase. *Carlsberg Res Commun* 43: 185-194
14. KLEIN O, D DORNEMANN, H SENER 1980 Two biosynthetic pathways to 5-aminolevulinic acid in algae. *Int J Biochem* 12: 725-728
15. KOBAYASHI K, S MIYAZAWA, A TERAHARA, H MISHIMA, H KURIHARA 1976 Gabaculine: γ -aminobutyrate aminotransferase inhibitor of microbial origin. *Tetrahedron Lett* 7: 537-540
16. LAGARIAS JC, H RAPOPORT 1980 Chromopeptides from phytochrome. The structure and linkage of the Pr form of the phytochrome chromophore. *J Am Chem Soc* 102: 4821-4828
17. MELLER E, ML GASSMAN 1981 Studies with 4,6-dioxoheptanoic acid on etiolated and greening barley leaves. *Plant Physiol* 67: 1065-1068
18. PRATT LH, GH KIDD, RA COLEMAN 1974 An immunochemical characterization of the phytochrome destruction reaction. *Biochim Biophys Acta* 365: 93-107
19. QUAIL PH, E SCHÄFER, D MARMÉ 1973 *De Novo* synthesis of phytochrome in pumpkin hooks. *Plant Physiol* 52: 124-127
20. QUAIL PH, E SCHÄFER, D MARMÉ 1973 Turnover of phytochrome in pumpkin cotyledons. *Plant Physiol* 52: 128-131
21. RANDO RR 1977 Mechanism of the irreversible inhibition of γ -aminobutyric acid- α -ketoglutaric acid transaminase by the neurotoxin gabaculine. *Biochemistry* 16: 4604-4610
22. SCHÄFER E, B MARCHAL, D MARMÉ 1972 *In vivo* measurements of the phytochrome photostationary state in far red light. *Photochem Photobiol* 15: 457-464
23. SIEGELMAN HW, BC TURNER, SB HENDRICKS 1966 The chromophore of phytochrome. *Plant Physiol* 41: 1289-1292
24. SMITH H 1975 *Phytochrome and Photomorphogenesis*. McGraw-Hill, London