Plant Physiol. (1974) 53, 206-215

Induction of Porphyrin Synthesis in Etiolated Bean Leaves by Chelators of Iron[1,2]

Received for publication August 14, 1973 and in revised form October 9, 1973

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ABSTRACT

Primary leaves of 7- to 9-day-old etiolated seedlings of *Phaseolus vulgaris* L. var. Red Kidney infiltrated in darkness with aqueous solutions of α,α'-dipyridyl, *o*-phenanthroline, pyridine-2-aldoxime, pyridine-2-aldehyde, 8-hydroxyquinoline, or picolinic acid synthesize large amounts of magnesium protoporphyrin monomethyl ester and lesser amounts of magnesium protoporphyrin, protoporphyrin, and protochlorophyllide. Pigment formation proceeds in a linear manner for up to 21 hours after vacuum infiltration with 10 mM α,α'-dipyridyl. Etiolated tissues of *Zea mays* L., *Cucumis sativus* L., and *Pisum sativum* L. respond in the same way to dipyridyl treatment. Compounds active in eliciting this response are aromatic heterocyclic nitrogenous bases which also act as bidentate chelators and form extremely stable complexes with iron; other metal ion chelators, such as ethylenediaminetetraacetic acid, salicylaldoxime, and sodium diethyldithiocarbamate, do not elicit any pigment synthesis. The ferrous, ferric, cobaltous, and zinc chelates of α,α'-dipyridyl are similarly ineffective. If levulinic acid is supplied to etiolated bean leaves together with α,α'-dipyridyl, porphyrin production is inhibited and δ-aminolevulinic acid accumulates in the tissue. Synthesis of porphyrins proceeds in the presence of 450 micrograms per milliliter chloramphenicol or 50 micrograms per milliliter cycloheximide with only partial diminution. We propose that heme or an iron-protein complex blocks the action of the enzyme(s) governing the synthesis of δ-aminolevulinic acid in etiolated leaves in the dark and that iron chelators antagonize this inhibition, leading to the biosynthesis of δ-aminolevulinic acid and porphyrins.

When higher plants are germinated in darkness, their leaves fail to produce any Chl. Instead, these leaves synthesize a small amount of the Chl precursor, pchld.[3] Upon illumination as brief as 1 msec, pchld is transformed to chld (5). Within 10 to 30 min following the illumination, chld is esterified to Chl and more pchld is synthesized (5).

The amount of pchld in a dark-grown bean leaf is only about 0.3% of the amount of Chl which the leaf can accumulate if placed under continuous illumination. If, however, ALA is administered to the leaf in darkness, the organ will synthesize large amounts of pchld (16, 22). Thus, it appears that one effect of illumination is to stimulate the synthesis of ALA (22) either by activation or synthesis of an enzyme which can produce this compound (15, 44). Since essentially no other intermediates in the Chl biosynthetic pathway accumulate in darkness in the presence of ALA, it would appear that the other enzymes in this pathway are present and active at levels which do not limit Chl synthesis and that the synthesis of ALA is the rate-limiting step in Chl biosynthesis. The synthesis of ALA has been shown to be the rate-limiting step in bchl biosynthesis in photosynthetic bacteria (37) and in heme biosynthesis in animal tissues (23, 38).

Granick (20, 22) reported that treatment of dark-grown barley leaves with α,α'-dipyridyl in the presence of ALA causes the synthesis of several porphyrin intermediates in addition to the synthesis of additional pchld. Among the intermediates are proto, Mg proto, and Mg proto ME. Of these three porphyrins, proto is considered to be an intermediate in both heme and Chl synthesis, while the magnesium porphyrins are specific to the Chl pathway (21). Granick (22) also reported that dipyridyl increased the utilization of exogenous ALA from 20 to 40%. He argued that since dipyridyl or pyridine-2-aldoxime seemed to increase the total porphyrin yield from ALA, iron was probably not directly involved in the enzymes of the Chl biosynthetic pathway (21).

The studies reported here indicate that the effect of dipyridyl on porphyrin synthesis in etiolated leaves appears to be at least 2-fold: (*a*) on the conversion of Mg proto ME to Chl and (*b*) on the biosynthesis of ALA. We have found that dipyridyl and related iron chelators stimulate the biosynthesis of these porphyrins in the absence of exogenous ALA, which suggests that one effect is to mimic the administration of ALA.

MATERIALS AND METHODS

Growth and Manipulation of Plant Material. Seeds of *Phaseolus vulgaris* L. var. Red Kidney, *Zea mays* L., *Cucumis sativus* L. var. Chicago Pickling, and *Pisum sativum* L. var. Alderman were germinated in moistened vermiculite in total darkness as previously described (13). All manipulations of

[1] This research was supported in part by National Science Foundation Grant GB 35627 and by a grant from the University of Illinois, Chicago Circle Research Board. Part of these studies were presented at the annual meeting of the Midwest Section, American Society of Plant Physiologists, August 1973, Lafayette, Ind.

[2] These studies form part of a thesis to be submitted by J. D. to the University of Illinois at Chicago Circle in partial fulfillment of requirements for the M.S. degree.

[3] Abbreviations: ALA: δ-aminolevulinic acid; bchl: bacteriochlorophyll; chld: chlorophyllide; copro: coproporphyrin III; coprogen: coproporphyrinogen III; Mg proto: magnesium protoporphyrin IX; Mg proto ME: magnesium protoporphyrin IX monomethyl ester; pchld: protochlorophyllide; pchl: protochlorophyll; proto: protoporphyrin IX; Zn proto DME: zinc protoporphyrin IX dimethyl ester

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plant material were performed under a dim green safelight (13). Seven- to 9-day-old primary bean leaves were excised from petioles and vacuum infiltrated in test solutions. The leaves were then transferred to Petri dishes containing filter paper moistened with the appropriate test solution and incubated in darkness for the indicated period. Excised 8-day-old maize leaves, cucumber cotyledons, and pea epicotyls were treated similarly.

***In Vivo* Spectrophotometry.** Absorption spectra of intact leaves were recorded with a Hitachi Perkin-Elmer Model 356 spectrophotometer as previously described (13). The slit was set to 0.5 nm (0.125 mm).

Leaf Irradiation. Leaves were irradiated by discharging a xenon arc 3 cm from the leaf surface (13). This flash was of sufficient intensity to photoconvert all of the phototransformable pchld to chld but caused essentially no change in the other porphyrins.

Extraction and Determination of Porphyrins. Leaf samples were homogenized in 40 parts of acetone-0.1 N NH_3 (9:1, v/v) in a Waring Blendor according to the procedure of Granick (20) as modified by Rebeiz *et al.* (47). The homogenate was vacuum-filtered through an asbestos-coated sintered glass funnel, and the residue was resuspended in another equal volume of the extraction solution and refiltered. The two filtrates were combined and the residue, which was nonfluorescent, was discarded. The combined filtrate was extracted with an equal volume of petroleum ether (b.p. range: 39–58 C) to remove most of the carotenoids, lipids, and traces of pchl and Chl. The petroleum ether was extracted with one-fifth its volume of acetone-0.1 N NH_3 (9:1). The ammoniacal acetone extracts were combined, and the petroleum ether phase was discarded.

The solution containing the porphyrins was overlayed with one-sixth its volume of peroxide-free diethyl ether, followed by the addition of one-seventeenth its volume of saturated aqueous NaCl plus one-seventieth its volume of 0.5 M NaH_2PO_4 to lower the pH to about 7. The porphyrins were then quantitatively transferred to the ether phase. The nonfluorescent aqueous phase was discarded, and the ethereal solution was extracted with 2 volumes of a saturated aqueous solution of $MgCO_3$.

All operations were performed under dim illumination (less than 1 ft-c). The pigments were at no time exposed to higher light intensities except during brief examinations with a long wavelength UV lamp (Wood's). Similarly, at no time were the solutions evaporated to dryness.

The ether extract (hereafter termed the "crude ether extract") was examined immediately in a Coleman-Hitachi 124 spectrophotometer (slit width: 0.5 nm). Concentration of the extract was accomplished by evaporation in a stream of N_2 at 35 to 40 C. The solution was made anhydrous with crystalline NaCl (12). Absorption spectra were recorded with the aid of a Coleman 165 recorder. Interfaced between the spectrophotometer and recorder was a Coleman scale expansion accessory. Estimation of Mg proto ME + Mg proto was based upon the equation given by Rebeiz *et al.* (48). Estimation of pchld in ether was performed using the extinction coefficient given for pchl by Boardman (5) at 624 nm. The extinction of Mg proto (ME) at 628 nm is less than 1% of the extinction of pchld (18) and was, therefore, ignored in the estimation of pchld in the crude ether extract. The level of proto was found to be so low as not to interfere with the estimation of the other pigments.

Chromatography. The pigments in the crude ether extract were separated by chromatography on thin layers of silica gel (Silplate-22, Brinkmann Instruments, Cantiague, N. Y.) in benzene-ethyl acetate-ethanol (4:1:1) (47). The silica was heated for 30 min at 110 C prior to use, and the solvents were anhydrous. The pigments were chromatographed at 22 C in total darkness, examined briefly under a Wood's lamp, and the individual separated pigments were scraped into acetone-methanol (4:1) before the chromatogram had dried out. The silica was removed by centrifugation, and the solutions were immediately examined for their absorbance properties with either the 356 or 124 spectrophotometers.

Crystalline standards of copro and proto methyl esters were hydrolyzed in 3 N HCl for 12 hr. This yielded a mixture of partial esters as chromatographic standards (20). After hydrolysis, the solution was neutralized with saturated sodium acetate, and the porphyrins were extracted into ether. These ether solutions were then dried and concentrated, as previously described, before application to chromatograms. Zn proto DME was prepared by the method of Ellsworth and Lawrence (11).

Determination of ALA and ALA Synthetase. ALA was estimated by a modification of the methods of Urata and Granick (55) and Mauzerall and Granick (42). One g of leaf tissue was ground in a mortar with 7 ml of 5% trichloroacetic acid. The suspension was clarified by centrifugation and the supernatant applied to a column (0.7 × 6 cm) of Dowex-50×8 (200–400 mesh) in the H^+ form. The column was then washed with 10 ml of distilled H_2O followed by 7 ml of 0.5 M sodium acetate. An additional 5 ml of 0.5 M sodium acetate were applied to elute the column, and the eluate was brought to pH 4.6 with 1.1 ml of 1 N HCl. One-tenth ml of acetyl acetone was added to the solution which was then placed in a glass-stoppered volumetric flask and immersed in a boiling H_2O bath for 10 min. After cooling, the pH was adjusted to 8.5 with 0.85 ml of 2 N KOH, and the solution was applied to a column (0.7 × 2.0 cm) of Dowex-1×8 (200–400 mesh) in the acetate form. The column was washed with 6 ml of H_2O followed by 4 ml of 0.1 N acetic acid. The ALA-pyrrole was eluted with 5 ml of methanol-glacial acetic acid (2:1, v/v). One and one-half ml of the eluate were combined with 1.5 ml of modified Ehrlich's reagent, and the absorbance at 552 nm was determined 15 min later using $E_{mM}^{1\,cm} = 53.0$.

The identity of the pyrrole was confirmed by chromatography with authentic ALA-pyrrole on thin layers of cellulose in l-butanol-1 N NH_3 (1:1, v/v). The pyrroles were visualized by spraying with Ehrlich's reagent.

To assay for ALA synthetase, primary leaves of 8-day-old etiolated bean seedlings were vacuum-infiltrated in 1 mM α,α'-dipyridyl, incubated for 18 hr in darkness, washed with distilled H_2O, and ground in a mortar and pestle with 3 volumes of 0.1 M sodium phosphate buffer, pH 7.0, containing 0.35 M NaCl (46) plus a small amount of acid-washed sand. All operations were performed at 0 to 4 C under dim light (1 ft-c) (46). The homogenate was centrifuged at 200*g* for 5 min, and the supernatant served as the source of the enzyme. ALA synthetase was assayed according to the method of Burnham (9).

Chemicals. The following were obtained from Sigma Chemical, St. Louis, Mo.: diethylenetriamine pentaacetic acid, nitrilotriacetic acid, salicylic acid, bathophenanthroline sodium sulfonate, bathocuproine sodium sulfonate, levulinic acid (grade I), neocuproine, thiosemicarbazide, sodium diethyldithiocarbamate, thioglycolic acid, 2,3-dimercaptopropanol, picolinic acid, dipicolinic acid, pyridine-2-aldehyde, pyridine-2-aldoxime, 8-hydroxyquinoline hemisulfate, pyroglutamic acid, pyridoxine HCl, pyridoxal HCl, nicotinic acid, nicotinamide, pyridine-4-aldoxime, imidazole, proto IX dimethyl ester (cryst.), copro III tetramethyl ester, and ALA·HCl.

The following were obtained from Eastman Organic Chemicals, Rochester, N. Y.: salicylaldehyde (pract.), salicylaldox-

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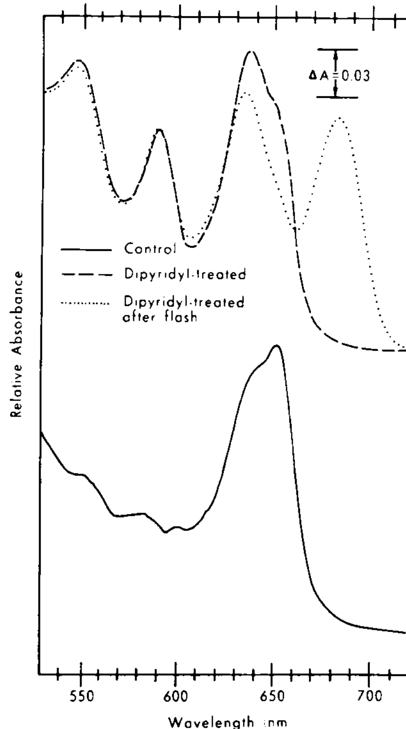

FIG. 1. *In vivo* absorption spectra of 8-day-old etiolated bean leaves treated with 10 mM α,α'-dipyridyl for 17 hr.

ime, acetyl acetone, *o*-aminophenol (pract.), and 8-aminoquinoline. The following were obtained from Fisher Chemical, Fairlawn, N. J.: α,α'-dipyridyl, *o*-phenanthroline, EDTA, dithiooxamide, and dimethylglyoxime. Quinoline-2-carboxylic acid and quinoline-8-carboxylic acid were obtained from K and K Laboratories, Plainview, N. Y. All solvents and inorganic compounds were reagent-grade. Chloramphenicol sodium succinate was a gift of Parke Davis and Company, Detroit, Mich. Cycloheximide (Acti-Dione) was a gift of the Upjohn Company, Kalamazoo, Mich.

RESULTS

Effect of α,α'-Dipyridyl on the *in Vivo* Absorption of Etiolated Bean Leaves. Eight-day-old etiolated Red Kidney bean leaves were incubated with 10 mM α,α'-dipyridyl in the dark for 16 hr at 22 to 24 C, and the *in vivo* absorption spectrum was determined (Fig. 1). These leaves differ conspicuously from untreated leaves in having absorption peaks at 552 and 590 nm. In addition, the absorption at 633 nm is higher than that at 650 nm, indicating a greater proportion of $pchld_{633}$, the species of pchld which is not directly phototransformable to chld (13, 14, 16, 22), as compared to phototransformable $pchld_{650}$, the pchld species associated with the pchld holochrome (13, 14, 16, 22). Five min after irradiation of either control (not shown) or treated leaves with a 1-msec light flash, the absorption at 650 nm has diminished, and the absorption peak at 683 nm associated with chld has appeared. However, the 552 and 590 nm peaks are essentially unchanged in the treated leaves (Fig. 1). These additional peaks are probably due to a metalloporphyrin such as Mg proto.

CHARACTERIZATION OF THE CRUDE ETHER EXTRACT OF DIPYRIDYL-TREATED LEAVES

Absorption Studies and Time Course of the Response. The absorption spectra of crude ether extracts of etiolated bean leaves treated with 10 mM α,α'-dipyridyl for various periods of time in darkness are shown in Figure 2. Within 3 hr after vacuum infiltration with dipyridyl, absorption peaks at 552 and 590 nm appear. These peaks increase in intensity with time up to at least 21 hr. An absorption band (Soret) at 418 nm increases in parallel to the 552 and 590 nm bands (not shown). In addition, the pchld band at 624 nm increases during the infiltration period. By the 21st hr, some proto is starting to appear as evidenced by the absorption peak at 505 nm. The position and ratios of these peaks which appear in response to infiltration with dipyridyl are very similar to those of Mg proto (ME) in ether (1, 11, 17–20, 28, 47). Zn proto DME was found to have absorption peaks at 345, 544, and 581 nm in ether with a Soret at 414 nm (Table I).

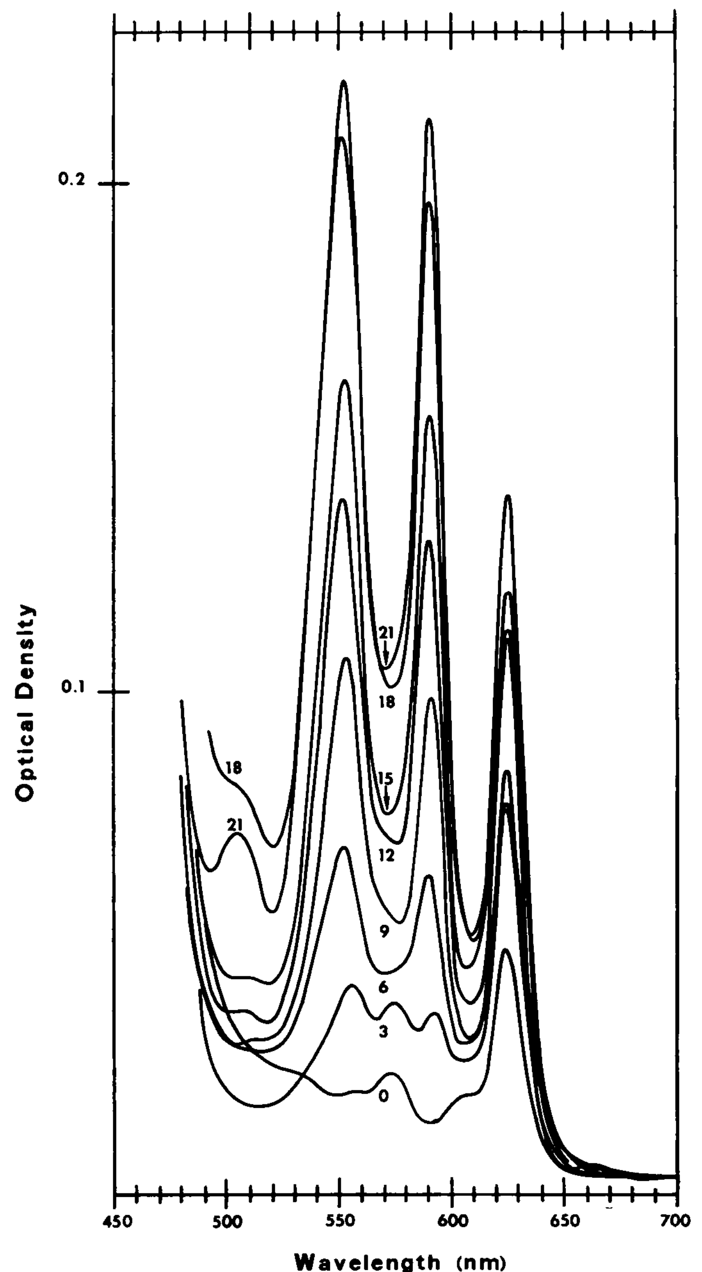

FIG. 2. Absorption spectra of ether extracts of 8-day-old etiolated bean leaves treated with 10 mM α,α'-dipyridyl for various periods of time (hr) in darkness. Each extract was prepared from 0.5 g of tissue as described under "Materials and Methods."

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Table I. *Absorption Maxima and Relative Peak Height of Several Porphyrins in Organic Solvents*

Porphyrin	Solvent	Peak Maxima						Reference
		nm						
Mg proto	Ether		418		553	587		18
	80% acetone				552 (1.03)[1]	590 (1.00)		18
	0.02 N KOH-50% ethanol	340 (1.85)	418 (17.8)		551 (1.1)	589 (1.0)		19
Mg proto ME	Ether				553 (1.0)	591 (1.0)		20
	Ether		419 (17.1)	510 (0.20)	553.5 (1)	591 (1)		28
	Ether		420		553	591		47
	Ether		419 (5.95)		551 (0.96)	589 (1.00)		17
	Ether		418		553	592		18
	0.02 N KOH-50% ethanol	340 (1.21)	418 (15.5)	510 (0.15)	551 (1.07)	589 (1.00)		10
	80% acetone-20% methanol		416.5 (15)	515 (0.33)	552 (1.06)	589 (1.00)		—[2]
Mg proto DME	Ether	340 (1.13)	419 (16.9)	510 (0.13)	551 (1.00)	589 (1.00)		19
	Ether		418		551	590		1
	Ether		419 (20)		551 (1.0)	589 (1.0)		11
	0.02 N KOH-50% ethanol	342 (1.66)	418 (15.6)	510 (0.20)	551 (1.08)	590 (1.00)		10, 19
Zn proto DME	Ether		414		543	582		1
	Ether		414 (16)		543 (0.9)	582 (1.0)		11
	Ether		414		544	581		—[3]
	80% acetone-20% methanol	345	416		545	583		—[3]
Proto	Ether	405	501	535	577		632	18
	80% acetone	403	503	538	575		630	18
	80% acetone-20% methanol	403	503	537	574		630	—[4]
Proto ME	Ether	405	504	537	576		632	18
Proto DME (Sigma)	Ether	404 (24.0)	503 (2.25)	536 (1.81)	576 (1.01)	605 (0.23)	633 (1.00)	12
	80% acetone-20% methanol	402	503	536	575		630	—[5]
Proto, dication	2.5% sodium dodecyl sulfate	412		557	602			12
	2.7 M HCl	408 (45.3)		554 (2.34)	598 (1.00)			12
	2.7 M HCl	409		556	600			18
Proto ME, dication	80% acetone-20% methanol-0.1% HCl	413		559	602			—[2]
Proto DME, dication (Sigma)	80% acetone-20% methanol-0.1% HCl	413		559	602			—[5]
Pchld	Ether	432 (8.16)	535 (0.18)	571 (0.37)	623 (1.00)			32
	Ether	432	530	567	622			18
	Ether	432 (7.6)	535 (0.23)	574 (0.35)	624 (1.00)			—[6]
	80% acetone	434	538	575	628			18
	80% acetone-20% methanol	432 (6.24)	535 (0.23)	573 (0.37)	625 (1.00)			—[6]

[1] Numbers in parentheses indicate relative height.
[2] Component II, R_F = 0.70, these studies (see "Results" for details).
[3] These studies.
[4] Component IV, R_F = 0.11, these studies (see "Results" for details).
[5] Chromatographed in benzene-ethyl acetate-ethanol (4:1:1) (R_F = 0.96) and eluted into acetone-methanol (4:1).
[6] Component I, R_F = 0.54, these studies (see "Results" for details).

The change in Mg proto (ME) and in pchld in both dipyridyl-treated as well as H_2O-treated leaves, as a function of time, is shown in Figure 3. Synthesis of these porphyrins is linear over the entire 21-hr incubation period, and there is no apparent lag or induction period. These results indicate that dipyridyl stimulates the synthesis of magnesium porphyrins in bean leaves.

Chromatographic Separation and Pigment Characterization. The crude ether extract obtained from both control and dipyridyl-treated etiolated bean leaves was chromatographed on silica gel. The result is shown in Figure 4. Extracts of control leaves contain essentially one porphyrin, a green spot exhibiting red fluorescence under a Wood's lamp, with R_F = 0.54. Extracts of dipyridyl-treated leaves contain at least four porphyrins which were eluted and characterized. A fifth pigment, with R_F = 0.35, was found in trace amounts and not further characterized. One of the four porphyrins resembled the single porphyrin found in control extracts. This porphyrin, also green with red fluorescence, with R_F = 0.54, appeared to be a major component in the extract. However, a reddish brown spot exhibiting orange fluorescence, with R_F = 0.70, was also a major component. The other two porphyrin spots were estimated to be minor components. One of these resembled the major component with R_F = 0.70 in its spectral properties but ran with R_F = 0.17. The other was a faint brown spot, fluorescing red, with R_F = 0.11.

Component I: R_F = 0.54. Upon elution into acetone-methanol (4:1), the absorption spectrum of this porphyrin was determined and is shown in Figure 5. The absorption spectrum of the porphyrin at this R_F from both treated and untreated leaves resembles the absorption spectrum of pchld (32). Absorption peaks were recorded at 431.5, 535, 573, and 625 nm (Table I).

Component II: R_F = 0.70. The absorption spectrum of this porphyrin in acetone-methanol is shown in Figure 6. Absorption peaks are evident at 416.5, 552, and 589 nm (Table I). This spectrum is similar to the absorption spectrum of

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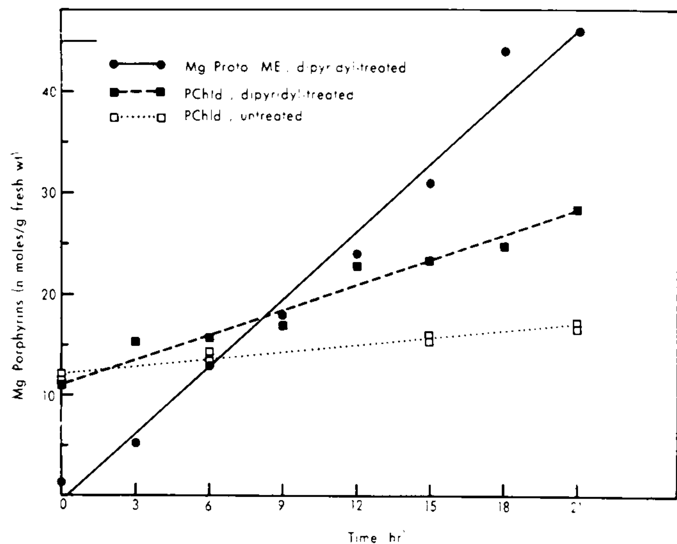

FIG. 3. Pchld and Mg proto (ME) synthesis in 8-day-old etiolated bean leaves in darkness after vacuum infiltration with 10 mM α,α'-dipyridyl or H_2O. The lines were drawn by the method of least squares; correlation coefficients for each were greater than 0.98.

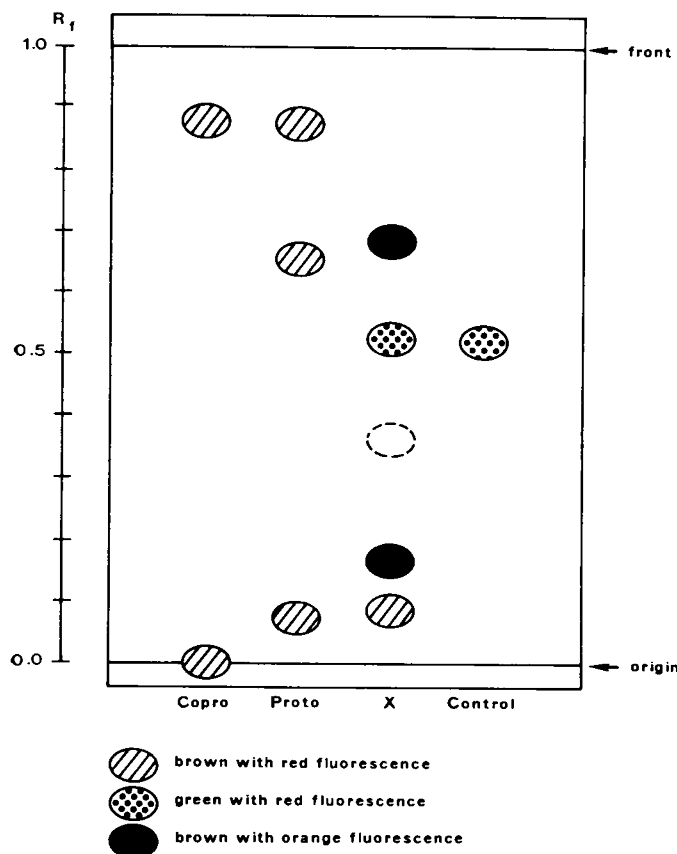

FIG. 4. Diagram of a chromatogram of copro, copro tetramethyl ester, proto, proto ME, proto DME, and the porphyrins in ether extracts of dipyridyl-treated (×) and control leaves. Separation on silica gel at 22 C in benzene-ethyl acetate-ethanol (4:1:1).

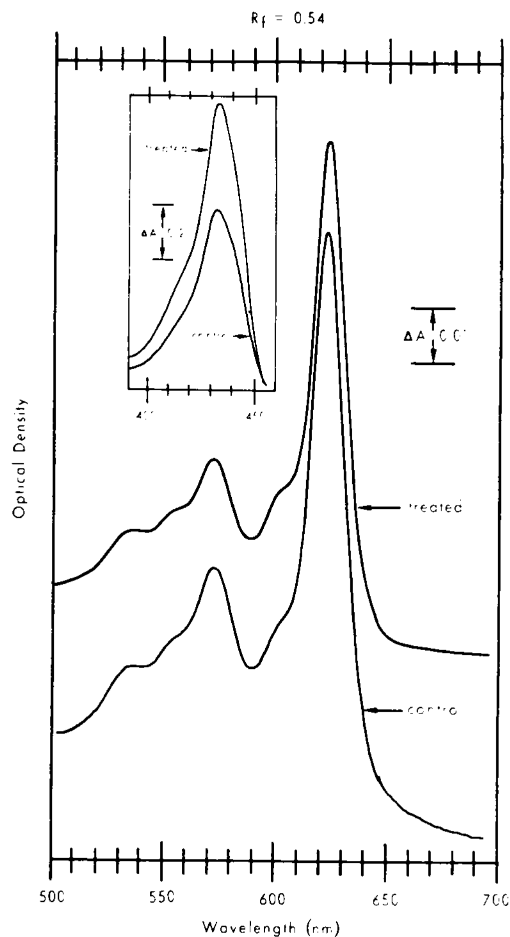

FIG. 5. Absorption spectra of the porphyrins at R_F = 0.54 eluted from the chromatogram in Fig. 4 into acetone-methanol (4:1).

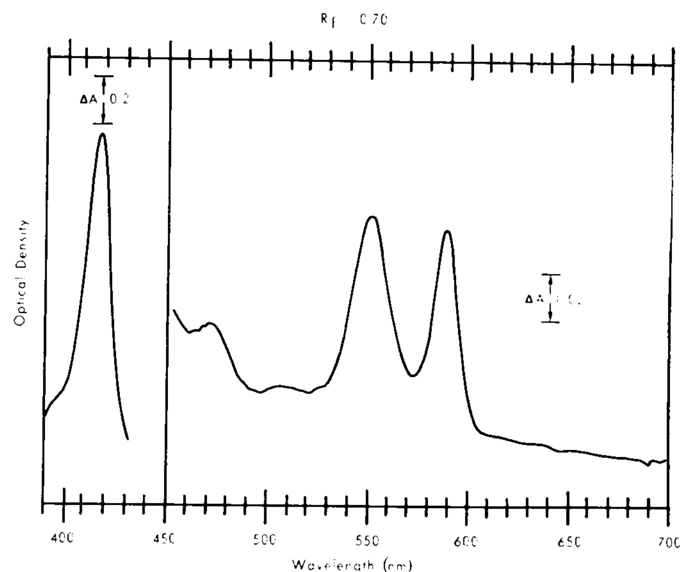

FIG. 6. Absorption spectrum of the porphyrin at R_F = 0.70 eluted from the chromatogram in Fig. 4 into acetone-methanol (4:1).

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Mg proto (ME) (1, 10, 11, 17–20, 28, 47). An additional absorption peak at 471 nm is present in the eluate containing component II. A spectrally similar contaminant was also present in purified extracts of Mg proto derived from a *Chlorella* mutant and was thought to be a carotenoid (19). A yellow nonfluorescent spot, probably a xanthophyll (*cf.* Fig. 1, ref. 47), was noted on the chromatogram (Fig. 4); its R_F was slightly greater than that of component II. When the fluorescence activation spectrum of this component was recorded, no excitation band was observed at 471 nm (J. Duggan and M. Gassman, unpublished data), confirming the supposition that the absorption peak at this wavelength is attributable to a nonfluorescent contaminant such as a carotenoid.

The high R_F (0.70) of the component in the benzene-ethyl acetate-ethanol solvent system suggests that it is probably Mg proto ME rather than Mg proto (47). The solubility properties of this component further suggest that it has only one free carboxyl group. This porphyrin could not be transferred immediately to 2 N HCl or to 0.1 N NH_3; it could, however, be transferred to 0.1 N NH_3-methanol (3:1) (20).

By comparison, Zn proto DME was found to have absorption bands at 345, 416, 545, and 583 nm in this solvent (Table I).

Component III: R_F = 0.17. The spectral properties of this component are identical to those of component II, R_F = 0.70 (Fig. 6). Since component II has a relatively low R_F, its identity is probably Mg proto.

Component IV: R_F = 0.11. The absorption spectrum of this component in acetone-methanol is shown in Figure 7. This spectrum is identical to the absorption spectrum of crystalline proto. By comparison with the chromatographically separated proto, proto ME, and proto DME (Fig. 4), we conclude that this component is proto and that both carboxyl groups are not esterified.

Effect of Acid on Components II and III. Acid treatment followed by neutralization of these components converted them to compounds which are spectrally identical to authentic proto.

Effect of Various Concentrations of Dipyridyl on the Induction of Porphyrin Synthesis. Eight-day-old etiolated bean leaves were infiltrated with various concentrations of α,α'-dipyridyl for 16 hr in darkness, the pigments extracted into ether, and the magnesium porphyrin content determined spectrophotometrically on the crude ether extracts. The results (Fig. 8) show that Mg proto (ME) content is a function of the concentration of inducer. Pchld content, however, exhibits a different response: concentrations of dipyridyl up to 1 mM induce pchld synthesis. Concentrations greater than this inhibit further pchld synthesis. Thus, it appears that dipyridyl has two effects upon this tissue: (*a*) induction of total magnesium

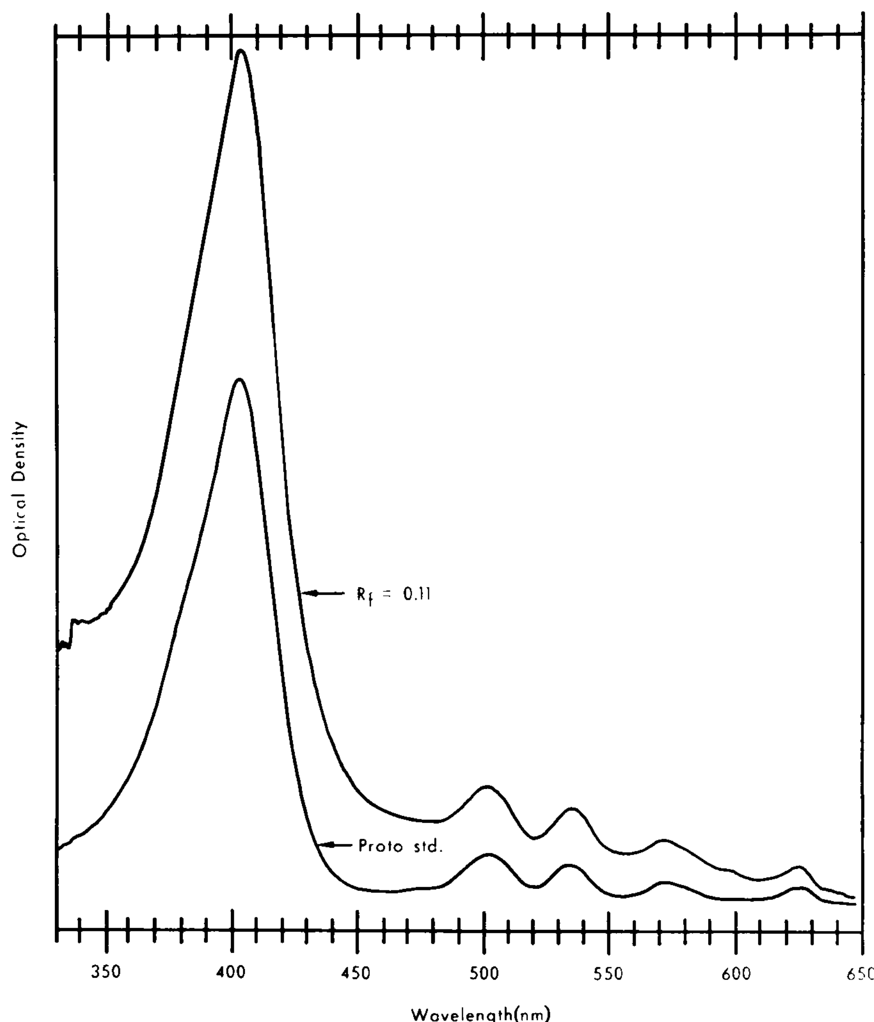

FIG. 7. Absorption spectra of the porphyrin at R_F = 0.11 eluted from the chromatogram in Fig. 4 into acetone-methanol (4:1) and of crystalline proto in the same solvent.

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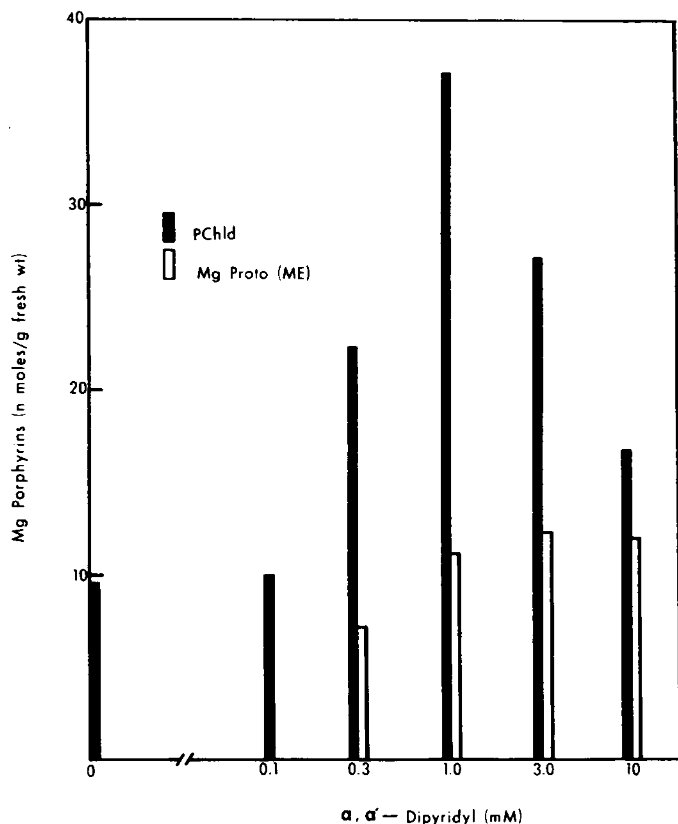

FIG. 8. Porphyrinogenic response of 8-day-old etiolated bean leaves to various concentrations of α,α′-dipyridyl. The tissue was vacuum infiltrated with the various solutions and incubated in darkness for 16 hr. The pigments were determined spectrophotometrically in ether extracts of the tissue.

porphyrin synthesis and (*b*) inhibition of the conversion of Mg proto ME to pchld.

Compounds Active in Inducing Porphyrin Synthesis in Etiolated Bean Leaves. Table II presents the compounds which are effective in inducing the synthesis of Mg proto ME, Mg proto, proto, and pchld in etiolated leaves during a 16-hr incubation in darkness. Dipyridyl was also found to induce these same porphyrins in 8-day-old etiolated cucumber cotyledons, 8-day-old etiolated maize leaves, and 8-day-old pea epicotyls. All of the compounds in Table II are aromatic heterocyclic nitrogenous bases and are bidentate chelators; all form extremely stable complexes with iron (6, 25). One compound which did not elicit the porphyrinogenic response was bathophenanthroline sodium sulfonate. However, the large size of this molecule and its charged group may preclude its entering the leaves and reaching the site of synthesis, presumably in the etioplast. Even the most effective inducers, *i.e.* α,α′-dipyridyl and *o*-phenanthroline, showed evidence of restricted entrance and mobility (*e.g.* patches of fluorescence on the leaf at the lower concentrations). Toxic symptoms began to appear at concentrations ranging from 10 to 20 mM, depending upon the compound examined.

The following compounds were ineffective in inducing the synthesis of porphyrins, at the concentrations indicated: EDTA, 0.1 to 10 mM; diethylenetriamine pentaacetic acid, 1 to 10 mM; nitrilotriacetic acid, 0.1 to 10 mM; salicylaldoxime, 0.1 to 10 mM; salicylaldehyde, 0.1 to 10 mM; salicylic acid, 0.1 to 10 mM; bathophenanthroline sodium sulfonate, 1 to 10 mM (in 1% dimethyl sulfoxide); bathocuproine sodium sulfonate, 1 to 10 mM; neocuproine, 5 mM; sodium diethyldithiocarbamate, 0.1 to 10 mM; thiosemicarbazide, 10 mM; thioglycolic acid, 10 mM; 2,3-dimercaptopropanol (BAL), 10 mM; dithiooxamide, H_2O-saturated solution; dimethylglyoxime, H_2O-saturated solution; *o*-aminophenol, 10 mM; 8-aminoquinoline, 5 mM; dipicolinic acid, 1 to 5 mM; α,α′-dipyridyl, 10 mM, + $FeCl_2$, 10 mM; α,α′-dipyridyl, 5 mM, + $ZnCl_2$, 5 mM; imidazole, 10 mM; nicotinic acid, 10 mM; nicotinamide, 10 mM; pyridoxine HCl, 10 mM; pyridoxal HCl, 10 mM; pyroglutamic acid, 10 mM; quinoline-2-carboxylic acid, 0.5 to 10 mM; quinoline-8-carboxylic acid, 0.5 to 1 mM.

Effect of Protein Synthesis Inhibitors on Porphyrin Synthesis Induced by Dipyridyl. Eight-day-old etiolated bean leaves were infiltrated with 450 μg/ml chloramphenicol and 50 μg/ml of cycloheximide, separately and in combination, in the presence of 10 mM α,α′-dipyridyl for 16 hr in darkness. The magnesium porphyrins were then extracted into ether and the content of Mg proto (ME) and pchld determined spectrophotometrically. The results are shown in Table III. Chloramphenicol alone has no significant effect upon the synthesis of either type of porphyrin. However, cycloheximide alone inhibits the synthesis of Mg proto (ME) by 42%. In combination with chloroamphenicol, cycloheximide inhibits the synthesis of Mg proto (ME) by 52% and pchld by 42%.

Determination of ALA Levels in Dipyridyl-treated Levulinic Acid-treated Leaves. Since the rate of synthesis of ALA is be-

Table II. *Compounds Which Induce Porphyrin Synthesis in Etiolated Bean Leaves in Darkness*

Compound	Effective Concn[1]
	mM
α,α′-Dipyridyl	0.5–10
o-Phenanthroline	0.5–10
8-Hydroxyquinoline	0.5-5[2]
Pyridine-2-aldoxime	10
Pyridine-2-aldehyde	10
Picolinic acid	10
α,α′-Dipyridyl:$MgCl_2$ (1:1)	10
α,α′-Dipyridyl:EDTA (1:1)	5

[1] A positive response was determined by spectral examination of a crude ether extract prepared from leaves treated with each compound. The identity of the porphyrins was confirmed in each case by chromatography; they were found to be qualitatively the same for each inducer.

[2] Concentrations above 5 mM were toxic.

Table III. *Effect of Chloramphenicol and Cycloheximide upon the Dipyridyl-induced Synthesis of Mg Proto (ME) and Pchld in Etiolated Bean Leaves*

The porphyrins were determined spectrophotometrically in crude ether extracts; SE of the mean of three determinations.

Treatment	*Mg Proto (ME)*	*Pchld*
	nmoles/g fr wt	
Control	0.0	14.9 ± 0.98
10 mM dipyridyl	27.0 ± 6.3	26.0 ± 0.40
10 mM dipyridyl + 450 μg/ml chloramphenicol	22.7 ± 1.4	24.6 ± 0.40
10 mM dipyridyl + 50 μg/ml cycloheximide	15.7 ± 0.7	26.5 ± 1.3
10 mM dipyridyl + 450 μg/ml chloramphenicol + 50 μg/ml cycloheximide	15.7 ± 1.0	20.7 ± 0.52

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lieved to be very low in etiolated plant leaves in the dark and the level of ALA is considered to limit the rate of pchld (and porphyrin) synthesis (15, 16, 22, 44), it appeared that the iron chelators might induce porphyrin synthesis by increasing ALA synthesis. To determine whether dipyridyl-induced porphyrin production involved ALA synthesis, the specific inhibitor of ALA utilization, levulinic acid (26, 45), was applied to the leaves in the presence or absence of dipyridyl. This compound has been shown to cause ALA accumulation (2–4, 24, 26, 49) and inhibition of Chl synthesis (2, 3, 24, 49) in a number of organisms. The concentration of levulinic acid employed, 15 mM, was found to be optimal with respect to both ALA accumulation and inhibition of dipyridyl-stimulated porphyrin synthesis in bean leaves. Lower concentrations did not appreciably affect the dipyridyl-induced response and higher concentrations were toxic to the leaves over prolonged periods.

The results, shown in Table IV, demonstrate that levulinic acid does indeed inhibit porphyrin synthesis stimulated by dipyridyl over a 10-hr incubation period. In addition, there is a large increase in the ALA content of the leaves. Furthermore, ALA levels in dipyridyl-treated leaves are higher than those in untreated leaves, both in the presence and absence of levulinic acid (Table IV). This supports the hypothesis that dipyridyl stimulates the biosynthesis of ALA. However, no ALA synthetase activity was detected in homogenates of dipyridyl-treated or untreated leaves.

DISCUSSION

α,α'-Dipyridyl, *o*-phenanthroline, pyridine-2-aldoxime, pyridine-2-aldehyde, picolinic acid, and 8-hydroxyquinoline belong to a class of compounds which form extremely stable complexes with iron (6, 25). These chelators are unusual in that their iron complexes are more stable than their copper, zinc, cobalt, or nickel complexes (6); *i.e.* they do not follow the Mellor-Malley series (43) for metal-chelator-complex stability to which compounds such as EDTA, salicylaldoxime, and sodium diethyldithiocarbamate belong. These compounds are aromatic heterocyclic nitrogenous bases which act as bidentate chelators. It has been proposed that the reason that these compounds form such stable complexes with iron is that the iron-chelator-complex exists in the form of five-membered aromatic rings capable of assuming several resonance structures (Fig. 9) (6).

Table IV. *Effect of Levulinic Acid upon ALA Synthesis and Dipyridyl-induced Porphyrin Synthesis in Etiolated Bean Leaves*

Primary leaves of 8-day-old etiolated seedlings were infiltrated in darkness with or without 15 mM levulinic acid, in the presence or absence of 5 mM α,α'-dipyridyl for 10 hr. The porphyrins were determined spectrophotometrically in crude ether extracts. ALA was extracted from the tissue in 5% trichloroacetic acid. The number of determinations is given in parentheses. See "Materials and Methods" for details.

Treatment	Pchld (3)	Mg proto (ME) (3)	ALA (3)
	nmoles/g fresh wt		
control	14.8 ± 0.5[1]		2.5 ± 0.1
5 mM dipyridyl	24.1 ± 0.2	16.9 ± 0.5	6.6 ± 0.3
15 mM levulinic acid	12.6 ± 0.2		25.4 ± 0.2
5 mM dipyridyl + 15 mM levulinic acid	18.9 ± 0.6	5.7 ± 0.2	54.9 ± 1.9

[1] SE of the mean.

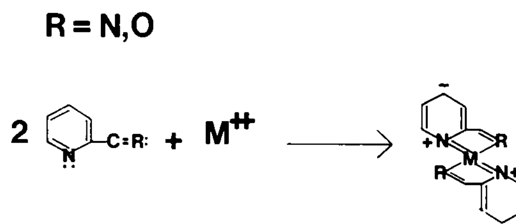

FIG. 9. Proposed model for iron-complex formation by aromatic heterocyclic nitrogenous bases acting as bidentate chelators. After Brandt *et al.* (6).

The stimulation of porphyrin synthesis by these iron chelators may result from the inactivation of an iron-protein which might inhibit the enzyme(s) governing ALA synthesis in etiolated leaves in darkness. The inactivation of the iron-protein-inhibitor would be accomplished by the removal of iron. Since other metal chelators do not evoke this response, we conclude that the stability of the iron-protein-inhibitor complex *in vivo* is quite high.

Another explanation for the stimulation of ALA synthesis by iron chelators is that there is an inactivation of an iron-protein repressor of the enzyme(s) governing ALA synthesis. However, the effect of dipyridyl was found to be relatively insensitive to 450 μg/ml chloramphenicol or 50 μg/ml cycloheximide (Table III). The 42% inhibition of pchld synthesis by cycloheximide plus chloramphenicol may reflect the relative instability of the enzyme(s) controlling the synthesis of ALA or its precursors; *i.e.* approximately half of the level of these enzymes may turnover in 16 hr in the dark. The enzyme(s) controlling the synthesis of ALA was estimated to have an apparent turnover time of 10 (52) to 90 (44) min in irradiated barley leaves and 30 min in *Chlorella* (2, 3). The longer apparent turnover time reported here may be due to the uncoupling of the enzyme(s) from a possible iron-protein inhibitor. In addition, the ALA synthetic enzyme may be unstable under irradiation. ALA synthetase in potato peels is reported to be inactivated *in vitro* by light (46). Other enzymes of the porphyrin biosynthetic pathway may also turnover during this 16-hr dark period. This may also contribute to the diminution in total porphyrin yield in the presence of protein synthesis inhibitors. In any event, these results suggest that the hypothetical iron-protein is more likely to be an inhibitor of the ALA synthetic enzyme(s) than a repressor.

Iron plays an important role in both heme and Chl synthesis. The ferrous ion is incorporated into proto to form heme (33). However, iron-deficient bacteria excrete large amounts of copro, a porphyrin which precedes proto (as the porphyrinogen) in the biosynthetic pathway (10, 34–36, 54). This observation is unusual because: (*a*) proto, rather than copro, might be expected to accumulate if iron were lacking for heme synthesis and (*b*) more copro is synthesized by iron-deficient cells than can be accounted for by heme synthesis in iron-sufficient cells (34, 36). Thus, various investigators have concluded that iron plays a catalytic role in the conversion of copro to proto in bacteria in addition to its stoichiometric role in heme synthesis (36, 54). The discrepancy between copro excreted by iron-deficient cells and heme synthesized by normal cells has been explained by Lascelles (37). Since her studies have shown that ALA synthetase, the rate-limiting enzyme in bchl and heme synthesis in the photosynthetic bacterium *Rhodopseudomonas* spheroides (37), is inhibited and repressed by heme (37), she reasons that the level of this enzyme escalates when heme levels in the cell drop due to iron-deficiency (37). The inability of the cell to sustain heme levels which can

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effectively corepress and/or inactivate ALA synthetase leads to the synthesis of large amounts of copro.

The following observations support the catalytic role of iron in the conversion of copro to proto: (*a*) Iron chelators such as *o*-phenanthroline and α,α'-dipyridyl inhibit cell extracts which can catalyze the conversion of coprogen to proto (50, 53); (*b*) addition of iron to suspensions of photosynthetic bacteria (10, 31, 36) or to tobacco leaf discs (27), both of which are iron-deficient, lowers their capacity to form copro from exogenous substrates and increases the production of bchl by the bacteria or proto by the leaf tissue. (*c*) a mutant of tomato exists which, if cultured in a medium which renders iron unavailable to the plant, will form copro from exogenous substrates but, if iron is made available to the plant by changing the culture medium, the mutant produces a normal phenotype and will synthesize considerably more proto and Chl from substrates while producing less copro (39). It is interesting to note that etiolated bean leaves do not excrete any copro in the presence of iron chelators (Fig. 4). Thus, if the bean enzyme which converts coprogen to proto contains iron, then these chelators are apparently unable to remove the iron and inactivate this enzyme.

Jones (29, 30) has studied the response of *Rhodopseudomonas spheroides* to culture in the presence of 8-hydroxyquinoline. He reports that this compound inhibits bchl synthesis and causes the excretion of a variety of porphyrins, the majority of which are believed to play a role in the biosynthesis of Chl from Mg proto. The effect was subsequently ascribed to the presence of hydroxyquinoline plus Cu^{2+} (31). Iron levels did not appear to be involved in this process but did affect the excretion of copro and Mg proto ME. Thus, it would appear that, with respect to the role of iron in Chl synthesis, plants are similar in one respect to bacteria: both require iron in their culture medium to convert coprogen to proto. On the other hand, plants differ from bacteria in their response to 8-hydroxyquinoline: plants excrete Chl intermediates "early" in the biosynthetic pathway from magnesium porphyrins to Chl while bacteria excrete "late" intermediates.

An alternate explanation for the stimulation of porphyrin production by iron chelators is that heme synthesis might be blocked, thereby diverting the porphyrin precursors into the magnesium porphyrin pathway. This proposal would require sufficient heme synthesis in untreated leaves to account for the additional magnesium porphyrin and phorbin synthesis in treated leaves. However, preliminary experiments indicate that heme synthetic levels in untreated leaves are less than 1% of magnesium porphyrin and phorbin synthetic levels in treated leaves. Thus, such an explanation is unlikely.

If heme or a hemoprotein is responsible for inhibition or repression or both of ALA synthesis in higher plants, as in the case of photosynthetic bacteria, then a turnover of such a heme compound in the absence of heme synthesis (*i.e.* in the presence of dipyridyl) could account for the stimulation of ALA and porphyrin synthesis. Determination of heme content in the leaves before and after incubation with dipyridyl has shown that heme levels in treated tissue are significantly lower than in untreated tissue (J. Duggan and M. Gassman, unpublished data).

Strand *et al.* (51) have recently reported the induction of ALA synthetase in cultured avian hepatocytes by α,α'-dipyridyl and bathophenanthroline. Since these compounds interfere with heme synthesis, the results were interpreted as providing further evidence that heme represses ALA synthetase. However, their results do not exclude the possibility that dipyridyl acts directly on ALA synthetase or an inhibitor thereof.

Since the level of ALA appears to limit Chl synthesis in green plants (15, 16, 21, 22, 24, 44), and iron chelators cause the synthesis of magnesium porphyrins in darkness in etiolated bean leaves, it is suggested that iron chelators may stimulate the synthesis of ALA. The inhibition of dipyridyl-induced magnesium porphyrin synthesis by levulinic acid (Table IV) partially supports this hypothesis. Levulinic acid is a specific inhibitor of ALA dehydrase (45), the enzyme converting ALA into porphobilinogen. Administration of this compound to the growth medium of *Chlorella* (2, 3), *Euglena* (49), corn or bean leaves (24), or cucumber cotyledons (4) causes an inhibition of Chl synthesis and a build-up of ALA. Since the results reported here suggest that the dipyridyl-stimulated synthesis of porphyrins is only partially diminished by inhibitors of protein synthesis, it would appear that these chelators cause the activation of the enzyme(s) which synthesize ALA.

The application of dipyridyl to etiolated bean leaves in darkness results in an elevated level of ALA as compared to untreated leaves (Table IV). Treatment of leaves with levulinic acid results in an inhibition of pchld synthesis and an increase in ALA levels. Dipyridyl-stimulated porphyrin production is inhibited by levulinic acid and the ALA content is further elevated. In contrast to the situation in algae (2), the molar reduction in porphyrin levels by levulinic acid in bean leaves results in an inhibition of pchld synthesis and in increase in ALA levels (24).

There have been several reports dealing with the participation of iron in the biosynthesis of ALA. Brown (7) and Vogel *et al.* (56) presented evidence that Fe^{2+} is involved in ALA synthesis in erythrocytes, and Marsh *et al.* (40, 41) reported that iron-deficient cowpea leaves were unable to synthesize ALA. On the other hand, Fe^{2+} has been reported to inhibit ALA synthetase in *R. spheroides* (8). Perhaps, an iron-protein inactivates ALA synthetase in dark-grown bean leaves and light alleviates this inactivation. Iron chelators might destroy this inactivator by removing the iron.

The stimulation of porphyrin synthesis by iron chelators provides a new and powerful tool for investigating the control of ALA and porphyrin synthesis in plant tissues. Despite their stimulatory effect upon ALA synthesis, these compounds have deleterious effects associated with their action; *viz.* the inhibition of the conversion of Mg proto ME to pchld (Fig. 8). This, of course, suggests that the enzyme which acts on Mg proto ME may contain iron in a state similar to that of the complex which inhibits ALA synthetase. Jones (28) has observed Mg proto ME excretion into the growth medium of iron-deficient *R. spheroides* and has proposed that iron may be necessary for the conversion of Mg proto ME to Chl as well as in earlier steps. Studies are in progress to assess the role of light in relation to this response.

Acknowledgments—The authors thank L. Schurig for able technical assistance during these studies. We are indebted to S. Granick and C. Rebeiz for their helpful criticisms of the manuscript.

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