

Porphyrin Accumulation and Export by Isolated Barley (*Hordeum vulgare*) Plastids¹

Effect of Diphenyl Ether Herbicides

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We have investigated the formation of porphyrin intermediates by isolated barley (*Hordeum vulgare*) plastids incubated for 40 min with the porphyrin precursor 5-aminolevulinic acid and in the presence and absence of a diphenylether herbicide that blocks protoporphyrinogen oxidase, the enzyme in chlorophyll and heme synthesis that oxidizes protoporphyrinogen IX to protoporphyrin IX. In the absence of herbicide, about 50% of the protoporphyrin IX formed was found in the extraplastidic medium, which was separated from intact plastids by centrifugation at the end of the incubation period. In contrast, uroporphyrinogen, an earlier intermediate, and magnesium protoporphyrin IX, a later intermediate, were located mainly within the plastid. When the incubation was carried out in the presence of a herbicide that inhibits protoporphyrinogen oxidase, protoporphyrin IX formation by the plastids was completely abolished, but large amounts of protoporphyrinogen accumulated in the extraplastidic medium. To detect extraplastidic protoporphyrinogen, it was necessary to first oxidize it to protoporphyrin IX with the use of a herbicide-resistant protoporphyrinogen oxidase enzyme present in *Escherichia coli* membranes. Protoporphyrinogen is not detected by some commonly used methods for porphyrin analysis unless it is first oxidized to protoporphyrin IX. Protoporphyrin IX and protoporphyrinogen found outside the plastid did not arise from plastid lysis, because the percentage of plastid lysis, measured with a stromal marker enzyme, was far less than the percentage of these porphyrins in the extraplastidic fraction. These findings suggest that of the tetrapyrrolic intermediates synthesized by the plastids, protoporphyrinogen and protoporphyrin IX, are the most likely to be exported from the plastid to the cytoplasm. These results help explain the extraplastidic accumulation of protoporphyrin IX in plants treated with photobleaching herbicides. In addition, these findings suggest that plastids may export protoporphyrinogen or protoporphyrin IX for mitochondrial heme synthesis.

There has been increased interest in the mechanism of plant Chl and heme synthesis due to recent studies showing that the light-dependent action of photobleaching diphenylether herbicides involves the accumulation of protoporphyrin IX, an intermediate in the heme and Chl synthetic pathways. This photoactive porphyrin can generate membrane-damag-

ing radicals upon exposure to light and oxygen (Matringe and Scalla, 1987; Kouji et al., 1988; Lydon and Duke, 1988; Matringe and Scalla, 1988a, 1988b; Sandmann and Böger, 1988; Witkowski and Halling, 1988; Becerril and Duke, 1989a, 1989b; Bowyer et al., 1989; Duke et al., 1989; Kouji et al., 1989; Lehnen et al., 1990). These herbicides inhibit the plastid-associated enzyme protoporphyrinogen oxidase, which converts protoporphyrinogen to protoporphyrin IX (Matringe et al., 1989a, 1989b; Witkowski and Halling, 1989; Jacobs et al., 1990; Versano et al., 1990; Camadro et al., 1991). Herbicide inhibition of this enzyme should lead to accumulation of its substrate, protoporphyrinogen, within the plastid, although this accumulation has not been directly demonstrated. Furthermore, the mechanism and site of oxidation of this accumulated protoporphyrinogen to protoporphyrin IX in the presence of herbicide have not been elucidated. Several recent observations suggest that, in the presence of herbicide, protoporphyrinogen is oxidized to protoporphyrin IX at sites within the cell but external to the chloroplast. To examine this possibility further, in the present study we attempt to determine whether plastids can export a portion of the protoporphyrinogen synthesized within the plastid.

The demonstration of protoporphyrinogen export by plastids would also have implications for the compartmentalization of the biosynthetic pathway for heme within the plant cell and, in particular, for the role of the mitochondrion in heme synthesis (Beale and Weinstein, 1990; Thomas and Weinstein, 1990). In the animal cell, ALA synthase, coproporphyrinogen oxidase, protoporphyrinogen oxidase, and ferrochelatase are in the mitochondria, whereas ALA dehydratase, PBG deaminase, and uroporphyrinogen III decarboxylase are in the cytoplasm. The chloroplast, however, contains all the enzymes necessary for heme and Chl synthesis from glutamate, the earliest precursor, and recent studies have indicated that the plant cell contains no ALA dehydratase or PBG deaminase in the cytoplasm or the mitochondria (Smith, 1988). This observation suggests that the chloroplast may synthesize and transport intermediates to the plant mitochondrion for heme synthesis. Because the plant mitochondrion contains the last two heme-synthetic

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Abbreviations: AFM, acifluorfen methyl; ALA, 5-aminolevulinic acid; PBG, porphobilinogen; PCA, perchloric acid.

enzymes, protoporphyrinogen oxidase (Jacobs et al., 1982a) and ferrochelatase (Jones, 1968; Porra and Lascelles, 1968; Little and Jones, 1976), the demonstration that the chloroplast can export protoporphyrinogen and/or protoporphyrin IX would lend considerable support to the proposal that the mitochondrion is dependent on the plastid for a supply of precursors of heme biosynthesis.

MATERIALS AND METHODS

Materials

Protoporphyrin IX, Mg-protoporphyrin IX, coproporphyrin III, and uroporphyrin III were purchased from Porphyrin Products (Logan, UT). All chemicals, including Percoll, were purchased from Sigma. Barley seed (*Hordeum vulgare* L. var Birka) and AFM (99%) were obtained as previously described (Jacobs et al., 1991).

Preparation of Plastids

Six-day-old, dark-grown barley plants were allowed to green in daylight for 4 h. The shoots (50 g) were excised and homogenized (Waring blender, 2 × 5 s) in 150 mL of isolation buffer (0.5 M Suc, 0.05 M Tris [pH 7.5], 1 mM DTT, 1 mM EDTA, 1 mM MgCl₂, 0.2% BSA). The brei was filtered through four layers of Miracloth and centrifuged at 150g for 1 min. The supernatant was centrifuged at 2000g for 2 min and the pellet resuspended in 2.5 mL of isolation buffer without BSA. To isolate intact plastids, 5 mL of crude plastids were layered over a step gradient of 25 mL of 40% Percoll and 10 mL of 80% Percoll and centrifuged at 6000g for 15 min. All centrifugations were carried out in a Sorval HB-40 swinging bucket rotor. The diffuse layer of intact plastids was removed with a Pasteur pipette. The Percoll was removed by dilution of the plastid fraction with isolation medium without BSA and centrifugation at 3000g for 5 min. The plastids were resuspended in a minimum volume of isolation medium without BSA. All fractionation steps were carried out at a temperature of 4°C.

Determination of Plastid Intactness

Plastid intactness before addition to the porphyrin synthesis assay was determined by measurement of the latency of the stromal enzyme 6-phosphogluconate dehydrogenase (Journet and Douce, 1985). Plastids were added to 1.0 mL of reaction mixture consisting of 300 mM Suc, 50 mM Tris (pH 7.9), 5 mM MgCl₂, 0.5 mM NADP. The reaction was started by the addition of 2 mM gluconate-6-P. NADP reduction was measured at 340 nm. The plastids were then lysed to release the stromal enzyme by addition of 0.01 mL of 2.5% Triton X-100. Intactness of the plastids was evaluated by comparison of the rates of NADP reduction before and after plastid lysis by the detergent.

To determine the extent of plastid lysis during the 40-min incubation period, plastids were incubated in the assay buffer without DTT, NADP, and ALA. At the beginning of the assay and after 40 min, the plastids and extraplastidic medium were separated by centrifugation as described below. The sedimented plastids were lysed by resuspension in a volume

of distilled water equal to the original assay volume. The 6-phosphogluconate dehydrogenase activities of the two fractions were compared to evaluate the extent of release of the stromal enzyme into the medium during incubation.

Measurement of Porphyrin-Synthesizing Activity

Porphyrin synthesis by intact plastids was measured by a modification of the procedure of Pardo et al. (1980). Plastids were incubated in 0.5 mL of reaction mixture, consisting of 500 mM Suc, 4 mM ALA, 1 mM MgCl₂, 1 mM EDTA, .025 mM Tris (pH 7.9), 1.5 mM ATP, 4 mM DTT, 0.6 mM NADP, and 2 mg of BSA at room temperature and in darkness. After 40 min of incubation at room temperature on a platform shaker, the reaction mixture was centrifuged at 3000g for 5 min to separate the plastids from the supernatant, and 0.5 mL of 1 M PCA:methanol (1:1, v/v) was added to each fraction. These fractions were then stored overnight under refrigeration. After centrifugation to remove the precipitated protein, the concentrations of coproporphyrin, uroporphyrin, and protoporphyrin IX present in the plastids or the supernatant were quantitated in PCA:methanol on a Perkin-Elmer 650 10S spectrofluorometer by the method of Grandchamp et al. (1980). The identity of the porphyrins in several assays was confirmed by HPLC (Bonkovsky et al., 1986) and by TLC of the free porphyrins (Henderson, 1989) to verify the spectrofluorometric assay. These fluorescence assays determine porphyrins and not porphyrinogens; most porphyrinogens, however, are converted to their corresponding porphyrins after several hours in acidic solvents under aerobic conditions.

In acidic solvents, Mg-protoporphyrin IX is demetalated and is measured as protoporphyrin IX. Therefore, where indicated, Mg-protoporphyrin IX was determined by extraction with acetone-NH₄OH on a separate aliquot of the reaction mixture (Castelfranco et al., 1979). The Mg-protoporphyrin IX extracted had the same fluorescence excitation and emission spectra in acetone as authentic Mg-protoporphyrin IX. In experiments in which Mg-protoporphyrin IX and free protoporphyrin IX were determined separately, the Mg-protoporphyrin IX concentration, analyzed in the basic solvent, was subtracted from the total protoporphyrin IX content, analyzed in the acidic solvent, to determine the concentration of free protoporphyrin IX. The herbicide AFM was added as previously described (Jacobs et al., 1991) to some assays at a concentration of 10 μM to inhibit protoporphyrinogen oxidase.

Detection of Accumulated Protoporphyrinogen by Oxidation with *Escherichia coli* Enzyme

In some experiments, *Escherichia coli* membranes were added to enzymically oxidize protoporphyrinogen to protoporphyrin IX. The membranes were prepared by growing *E. coli* (strain ATCC 25922) in nutrient broth for 10 h at 37°C with vigorous aeration. The cells were harvested by centrifugation and disrupted by sonication (Branson; model W185D) (twenty 30-s treatments with intermittent cooling on ice). After unbroken cells were removed by centrifugation (12,000g for 20 min), the membranes were sedimented at 144,000g for 2 h, washed once in 0.05 M Tris buffer (pH 7.5),

and resuspended in this buffer to give a final protein concentration of approximately 15 mg mL⁻¹. The *E. coli* membranes had no coproporphyrinogen oxidase activity (assayed as in Jacobs et al., 1991) and were unable to convert ALA to coproporphyrinogen or protoporphyrinogen in the assay described above. In some experiments, *E. coli* enzyme was added to plastids at the beginning of the incubation period with ALA, and porphyrins were analyzed by solvent extraction after the incubation. In other experiments, the *E. coli* enzyme was added to the supernatant fraction following centrifugation of the plastids at the end of the incubation with ALA (see "Results"). To follow the oxidation of protoporphyrinogen in these supernatants, the *E. coli* enzyme was added, and the supernatant was scanned (excitation 410 nm, emission from 580–680 nm) with a Perkin-Elmer 650-10 S spectrofluorometer (excitation slit, 2 nm; emission slit, 5 nm). Protoporphyrin IX was detected by its emission peak at 633 nm, which was readily differentiated from the emission peaks of uroporphyrin and coproporphyrin at 620 and 615 nm, respectively. Protoporphyrin IX stock dissolved in 1.25% Triton X-100 was added to the assay buffer to calibrate the concentration of protoporphyrin IX formed.

Determination of Protoporphyrinogen Recovery after Addition to Plastid Extract

Protoporphyrinogen was prepared by reduction of protoporphyrin IX with sodium amalgam (Jacobs and Jacobs, 1982b) and neutralized by dilution with an equal volume of 0.5 M Tris (pH 7.5), containing DTT (50 mM) (Jacobs et al., 1990). Broken plastids (prepared by freeze-thawing gradient-purified plastids) were incubated in an assay mixture consisting of 0.05 M Tris (pH 7.5), 5 mM DTT, 1 mM EDTA, and protoporphyrinogen in a total volume of 0.250 mL. *E. coli* membranes were added at either the beginning of the assay or after a 40-min incubation period. After further incubation for 30 to 40 min to allow all protoporphyrinogen present to be oxidized by the *E. coli* membranes, PCA-methanol was added to analyze the porphyrin. The concentration of protoporphyrin IX present was determined spectrophotometrically in PCA-methanol as described above.

RESULTS

Extraplastidic Accumulation of Porphyrins Synthesized from Excess ALA by Intact Barley Plastids

We determined the partitioning of the various porphyrin intermediates synthesized from ALA between the intact plastid and the extraplastidic medium, after separation by centrifugation. It was necessary to use ALA as a substrate, because heme and Chl synthesis from precursors before ALA is normally well regulated, producing no accumulation of tetrapyrrole intermediates. In the experiments shown in Table I, 15 to 25% of the uroporphyrin and 5 to 15% of the Mg-protoporphyrin IX were found in the supernatant fraction. However, 50% of the protoporphyrin IX was found outside the plastids, suggesting that this porphyrin is preferentially exported. Three additional experiments in which three different plastid preparations were used reproduced the finding that uroporphyrin was mainly within the plastid, whereas the protoporphyrin IX was mainly extraplastidic (Fig. 1). In these latter experiments, the protoporphyrin IX fraction includes both protoporphyrin IX and a lesser amount of Mg-protoporphyrin IX, because analysis was by the PCA-methanol method, which removes the metal from Mg-protoporphyrin IX. However, this would not modify our conclusion, because Mg-protoporphyrin IX was located only within the plastid (see Table I).

In all experiments, visual observation of porphyrin fluorescence immediately following addition of PCA-methanol indicated that a significant portion of the porphyrins were still present as porphyrinogens, because fluorescence increased greatly after overnight storage to aerobically oxidize porphyrinogens to porphyrins.

Effect of a Photobleaching Herbicide and Protoporphyrinogen Oxidase from *E. coli* on Porphyrin Accumulation by Barley Plastids

When the assay was conducted in the presence of the photobleaching herbicide AFM, no protoporphyrin IX was recovered from either the plastid or the extraplastidic fraction, but the amounts of uroporphyrin and coproporphyrin found were unaffected (Table II). This result was unexpected, because it apparently contradicts the finding that, in intact plants, photobleaching herbicides markedly increase proto-

Table I. Distribution between the plastids and the supernatant of porphyrins synthesized from ALA

Gradient-purified plastids were prepared, tested for intactness, and incubated with ALA as described (see "Materials and Methods" for all procedures). After an incubation period of 40 min, the assay mixture was separated by centrifugation into plastids and supernatant fractions. Coproporphyrin (copro), uroporphyrin (uro), and protoporphyrin IX (proto) were determined by extraction with PCA-methanol. Mg-protoporphyrin IX (Mg proto) was determined by extraction with acetone-NH₄OH. Plastid latency was 75% in experiment 1 and 71% in experiment 2. The protein concentration was 0.1 mg/assay in experiment 1 and 0.2 mg/assay in experiment 2. The data represent the means and ranges of duplicate assays.

Experiment	Inside Plastid				Outside Plastid			
	copro	uro	proto	Mg proto	copro	uro	proto	Mg proto
	<i>pmol of porphyrin/mg of protein</i>							
1	79 ± 15	306 ± 62	804 ± 69	164 ± 2	27 ± 3	103 ± 8	726 ± 15	10 ± 0
2	156 ± 34	1161 ± 195	873 ± 136	263 ± 2	136 ± 10	224 ± 0	871 ± 22	46 ± 7

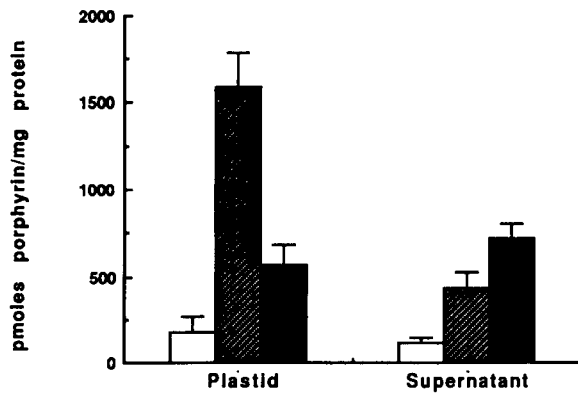


Figure 1. Distribution of coproporphyrin, uroporphyrin, and protoporphyrin IX between the plastid and the supernatant. Intact plastids were incubated with ALA for 40 min and fractionated as described in "Materials and Methods." Coproporphyrin, uroporphyrin, and protoporphyrin IX were determined by extraction with PCA-methanol (see "Materials and Methods"). In this procedure, Mg-protoporphyrin IX is measured as protoporphyrin IX. Data represent means and ranges of duplicate assays of three experiments with three separate plastid preparations. □, Coproporphyrin; ▨, uroporphyrin; ■, protoporphyrin IX.

protoporphyrin IX accumulation. However, this finding agrees with that of other investigators who have also reported that diphenyl ether herbicides markedly decrease protoporphyrin IX accumulation from ALA by isolated plastids (Mito et al., 1991; Nandihalli et al., 1991).

To investigate this further, we included *E. coli* membranes in the assay mixture with the herbicide. *E. coli* membranes have an active herbicide-resistant protoporphyrinogen oxidase that oxidizes protoporphyrinogen to protoporphyrin IX in the presence of AFM (Jacobs et al., 1990). Under these conditions, we found that the supernatant fraction contained

more protoporphyrin IX than was found when no herbicide was present (Table II). Thus, the *E. coli* membranes oxidized the protoporphyrinogen that had accumulated in the assay mixture and permitted its detection as protoporphyrin IX by fluorescence spectroscopy after addition of the PCA-methanol solvent. This was the first direct demonstration that diphenyl ether herbicides caused the formation and accumulation of protoporphyrinogen by isolated plastids, which then transported much of the tetrapyrrole into the extraplastidic medium. In addition, these findings indicate that in the presence of ALA and diphenyl ether herbicide, uro-, copro-, and protoporphyrinogens accumulate but are not oxidized in the assay mixture at neutral pH. Upon the addition of the PCA-methanol solvent, uro- and coproporphyrinogens are readily oxidized to their respective porphyrins during overnight storage. However, protoporphyrinogen is not oxidized to protoporphyrin by this solvent treatment and is, therefore, not detected.

To confirm the extraplastidic accumulation of protoporphyrinogen, plastids were separated from the supernatant fractions by centrifugation after incubation for 40 min with ALA in the presence or absence of AFM. The supernatant fractions were scanned directly (without addition of any solvent) in a scanning spectrofluorometer (see "Materials and Methods"). A peak with an excitation maximum at 410 nm and an emission maximum at 633 nm, compatible with those of protoporphyrin IX, was readily apparent in the supernatant fraction containing no AFM. No porphyrin fluorescence was observed in the supernatant containing AFM. *E. coli* membranes were added to both supernatants to oxidize any protoporphyrinogen to protoporphyrin IX. Periodic scanning of the supernatants showed an increase in the protoporphyrin IX peak of the supernatant containing AFM, until, after 20 min, the protoporphyrin IX peaks of both supernatants were the same (Fig. 2). Emission peaks for coproporphyrin (615 nm) or uroporphyrin (620 nm) were not seen during this

Table II. Effect of AFM and *E. coli* membranes on the accumulation of protoporphyrin IX in plastids and supernatant

Plastids were incubated (see "Materials and Methods") with ALA for 40 or 55 min (experiments 1 and 2, respectively). The assay mixture was separated by centrifugation into plastid and supernatant fractions. Coproporphyrin (copro), uroporphyrin (uro), and protoporphyrin IX (proto) were determined by extraction with PCA-methanol (see "Materials and Methods"). In this procedure, Mg-protoporphyrin IX was measured as protoporphyrin IX. Where indicated, AFM (10 μM) was added to inhibit protoporphyrinogen oxidase and/or *E. coli* membranes (0.75 mg of protein/assay) were added to oxidize the protoporphyrinogen present in the supernatant. Unincubated plastids contained less than 1 pmol of porphyrin mg^{-1} of protein (data not shown). Experiments 1 and 2 contained 0.24 and 0.19 mg of plastid protein/assay, respectively. The data are means and ranges of duplicate assays.

	Inside Plastid			Outside Plastid		
	copro	uro	proto	copro	uro	proto
	<i>pmol of porphyrin mg⁻¹ of protein</i>					
Experiment 1						
No addition	146 ± 12	1325 ± 42	442 ± 50	121 ± 12	458 ± 17	604 ± 62
AFM	150 ± 12	1325 ± 200	<1	154 ± 12	433 ± 50	<1
AFM + <i>E. coli</i> membranes	162 ± 4	558 ± 208	<1	217 ± 75	683 ± 92	1375 ± 42
Experiment 2						
No addition	63 ± 63	1842 ± 176	326 ± 100	79 ± 5	594 ± 17	173 ± 26
AFM	126 ± 5	2105 ± 142	<1	153 ± 15	689 ± 16	<1
AFM + <i>E. coli</i> membranes	210 ± 5	1210 ± 200	189 ± 16	284 ± 47	900 ± 84	1400 ± 126

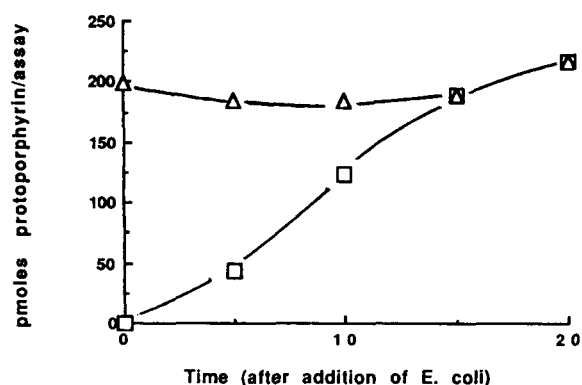


Figure 2. Demonstration of protoporphyrinogen accumulation in the supernatant of AFM-inhibited plastids by direct oxidation with *E. coli* membranes. Plastids were separated from the supernatant fractions by centrifugation after incubation for 40 min with ALA in the presence or absence of AFM. *E. coli* membranes were added to both supernatants to oxidize any protoporphyrinogen to protoporphyrin IX. Periodic scanning of the supernatants showed an increase in the protoporphyrin IX peak of the supernatant containing AFM. Δ, No herbicide; □, AFM (10 μM).

period, confirming that these porphyrins remained as porphyrinogens in the supernatant and were aerobically oxidized to porphyrin only after the addition of PCA-methanol (see Table II). These results verify that significant amounts of protoporphyrinogen can be exported from intact plastids in the presence photobleaching herbicides.

Evaluation of the Role of Plastid Lysis in Extraplasmidic Porphyrin Accumulation

The above observations raise the question of whether the protoporphyrin(ogen) present in the supernatant represents porphyrin synthesized within the plastid and exported from intact plastids or porphyrin released by lysis of plastids during the incubation with ALA. We determined the degree of plastid intactness both before and after the 40-min incubation period by measuring the stromal marker enzyme 6-phosphogluconate dehydrogenase, which is inaccessible to exogenous substrate in the intact plastid but is fully accessible after lysis of plastids (see "Materials and Methods"). The degree of intactness was determined in each experiment and was between 70 and 90% at the beginning of the assay period. In three experiments, we found that the plastid intactness decreased on average by a further 11% during the 40-min incubation period. These findings indicate that only 11% of the porphyrin present in the supernatant could be due to porphyrin synthesized within the plastid during the 40-min incubation with ALA and released by plastids that lysed during the incubation period. Although 11% lysis can account for the Mg-protoporphyrin IX found in the supernatant and a significant portion of the uroporphyrin found in the supernatant (Table I), plastid lysis cannot account for the 50% of the protoporphyrin(ogen) found in the extraplasmidic medium. Therefore, this protoporphyrin(ogen) was synthesized within the plastid and exported from intact plastids.

To exclude the possibility that the protoporphyrin(ogen) in

the supernatant was synthesized by enzymes released into the extraplasmidic fluid from the small percentage of lysed plastids present in the assay mixture, plastids were incubated for 40 min in the usual assay mixture (without ALA). The intact plastids were removed by centrifugation, and the supernatant was incubated with ALA for an additional 40 min. Under these conditions only small amounts of coproporphyrin (4.5 ± 1.5 pmol/mg⁻¹ of plastid protein) and uroporphyrin (36.5 ± 1.5 pmol/mg⁻¹ of plastid protein) were synthesized from ALA. No protoporphyrin IX was found, even in the presence of *E. coli* membranes, which would have oxidized any protoporphyrinogen present. Thus, extraplasmidic enzymes released from lysed plastids under these conditions do not synthesize protoporphyrinogen, confirming that the extraplasmidic protoporphyrin(ogen) was synthesized within the plastid and then exported.

Stability and Recovery of Protoporphyrinogen in Plastid Extracts and in the Solvent Extraction Procedure Used for Porphyrin Analysis

The question of protoporphyrinogen stability and recovery is important for understanding the experiments reported above. To examine protoporphyrinogen stability, we determined the recovery of added protoporphyrinogen after incubation with plastid preparations in the presence of AFM to prevent enzymic protoporphyrinogen oxidation. In all cases, the reactions were stopped by the addition of PCA-methanol to precipitate the protein, followed by overnight storage (see "Materials and Methods"), which is often utilized to chemically oxidize porphyrinogens to porphyrins. The results showed that, under our assay conditions and in the presence of broken plastids and herbicide, the maximum recovery of protoporphyrinogen (measured as protoporphyrin IX) was obtained when *E. coli* protoporphyrinogen oxidase was added at the beginning of the incubation period (Table III, line 1).

Table III. Recovery of protoporphyrinogen from plastid extracts treated with AFM

Protoporphyrinogen was incubated for 40 min under standard conditions but without ALA (see "Materials and Methods") with plastids broken by freezing and thawing. AFM (10 μM) was added to inhibit plastid protoporphyrinogen oxidase. *E. coli* membranes (0.37 mg of protein/assay) were added to oxidize the protoporphyrinogen where indicated, either at the beginning or end of the 40-min incubation period, and the incubation was continued for an additional 40 min. The assay mixture was extracted with PCA-methanol and the protoporphyrin IX concentration determined spectrophotometrically. The plastid protein concentration was 0.5 mg/assay. The data represent the means and ranges of duplicate determinations.

Treatment	Protoporphyrin IX Recovered
	μM
Plastids + AFM + <i>E. coli</i> membranes added at $t = 0$	0.66 ± 0.16
Plastids + AFM + <i>E. coli</i> membranes added at $t = 40$ min	0.59 ± 0.03
Plastids + AFM (no <i>E. coli</i> membranes)	0.05 ± 0

If the incubation with protoporphyrinogen was allowed to proceed for 40 min, there was still excellent recovery of protoporphyrinogen when *E. coli* enzyme was added after the incubation period but before the PCA-methanol (Table III, line 2). This result indicates that protoporphyrinogen remains stable for at least 40 min under these assay conditions in the presence of plastids and herbicide. However, if the *E. coli* enzyme was not added to the assay mixture, less than 10% of the protoporphyrinogen was recovered by the PCA-methanol procedure (Table III, line 3). This result showed that, if protoporphyrinogen was not converted to protoporphyrin IX before addition of the extracting solvent, it was mostly lost during the PCA-methanol solvent procedure utilized in these assays.

We also compared the enzymic oxidation of protoporphyrinogen by *E. coli* to the chemical oxidation of protoporphyrinogen by iodine (Porra and Falk, 1964). Under conditions identical with those shown in Table III, line 2, the recovery of protoporphyrinogen as protoporphyrin IX in the presence of AFM and plastids was compared after oxidation of the added protoporphyrinogen by *E. coli* protoporphyrinogen oxidase or a small excess of iodine solution followed by decolorization with Cys according to the procedure described by Porra and Falk (1961, 1964). The enzymic oxidation gave a 2-fold better recovery of protoporphyrinogen as protoporphyrin IX than did the chemical oxidation. We conclude that, although iodine is an effective oxidant for protoporphyrinogen under some conditions (Porra and Falk, 1964), it is less effective than the *E. coli* enzyme under our assay conditions. There could be several explanations for this, but they are beyond the scope of this investigation.

DISCUSSION

It is reported in this study that significant amounts of the heme and Chl precursors protoporphyrin IX and/or protoporphyrinogen were found outside of intact plastids incubated with excess ALA. Three lines of evidence indicated that this accumulation of protoporphyrin(ogen) was due to a specific export process from intact plastids rather than leakage from lysed plastids. First, the relatively small percentage of lysis during the incubation (measured with a stromal marker enzyme) was far less than the percentage of protoporphyrin(ogen) found external to the plastid in the supernatant fraction. Second, uroporphyrin, an earlier precursor, and Mg-protoporphyrin IX, a later precursor, were found largely within the plastid. Finally, the low level of porphyrin biosynthetic enzymes released from lysed plastids and present in the extraplastidic fluid was shown to be unable to convert ALA to protoporphyrin(ogen), excluding the possibility that enzymes released from lysed plastids synthesized the protoporphyrin(ogen) found in the extraplastidic fluid. Thus, all of these observations collectively indicate that, of the heme and Chl precursors that were synthesized by and accumulated within the plastid, only protoporphyrin IX and protoporphyrinogen appeared to be specifically exported.

When combined with other findings, our results show how photobleaching herbicides may cause accumulation of phototoxic protoporphyrin IX within the plant cell: the protoporphyrinogen that accumulates within the plastid when proto-

porphyrinogen oxidase is inhibited by the herbicide can be exported and oxidized to protoporphyrin IX outside of the plastid through the mediation of an herbicide-resistant protoporphyrinogen-oxidizing activity that we recently demonstrated in membranes such as the plasma membrane (Jacobs et al., 1991). Although extraplastidic formation of protoporphyrin IX from protoporphyrinogen could theoretically occur spontaneously by autooxidation, several observations are more compatible with an active herbicide-resistant mechanism associated with extraplastidic membranes for this oxidation. For instance, we found in this study that, when isolated plastids synthesized porphyrins in the presence of herbicide, the accumulated protoporphyrinogen was not spontaneously oxidized to protoporphyrin IX in the absence of a source of herbicide-resistant protoporphyrinogen oxidase activity. In the intact leaf, the latter can be provided by extraplastidic membranes. In addition, an extraplastidic mechanism of protoporphyrinogen oxidation also offers the best explanation for the following three observations: protoporphyrin IX appears rapidly in intact leaves after herbicide treatment in the dark (Becerril and Duke, 1989a, 1989b; Matsumoto and Duke, 1990); protoporphyrin IX accumulates mostly outside of the plastids in the cytoplasm or at the plasma membrane (Lehnen et al., 1990); protoporphyrin IX accumulates rather than being converted by plastid enzymes to later Chl intermediates.

Our finding that protoporphyrin(ogen) accumulates outside the plastid suggests that protoporphyrin(ogen) may flow from the chloroplast to the mitochondrion for the synthesis of mitochondrial heme. The plant mitochondrion contains protoporphyrinogen oxidase and ferrochelatase, the two enzymes necessary for the conversion of protoporphyrinogen to protoporphyrin IX and protoporphyrin IX to heme (Jones, 1968; Porra and Lascelles, 1968; Little and Jones, 1976; Jacobs et al., 1982a). This is consistent with the demonstration that two enzymes earlier in the pathway, ALA dehydratase and PBG deaminase, are primarily plastidic and are not detected either in the cytoplasm, as in animal cells, or in mitochondria (Smith, 1988).

Interesting questions arise from these findings. How is protoporphyrin IX or protoporphyrinogen released from the plastid? Is there an active export mechanism or a simple diffusion through the outer membrane? The location of the early enzymes of Chl synthesis within the chloroplast may provide some insight. Protoporphyrinogen oxidase has recently been shown to be present on the envelope of the chloroplast (Matringe et al., 1992). Because protoporphyrinogen is converted to protoporphyrin IX at the envelope, both intermediates may be exported when physiological conditions are such that they become present in excess. It is interesting that protoporphyrin IX is the least water soluble of the early porphyrin intermediates and may be best able to diffuse through the lipid membrane. Alternatively, protoporphyrinogen may be actively transported into the cytoplasm and into the mitochondrion where it is oxidized by mitochondrial protoporphyrinogen oxidase and converted to heme by ferrochelatase.

These observations concerning the effect of AFM on protoporphyrin IX synthesis clarify some confusing aspects of herbicide action. Most important, our findings conclusively

demonstrate for the first time that this herbicide causes the accumulation of significant quantities of protoporphyrinogen in cell-free preparations of plant tissues. In addition, our results explain the observation that diphenyl ether herbicides enhance protoporphyrin IX accumulation in intact plants but inhibit protoporphyrin IX accumulation by isolated plastids. We found that this herbicide caused protoporphyrinogen to accumulate in plastid suspensions but that this protoporphyrinogen was not oxidized to protoporphyrin after the addition of the PCA-methanol solvent used for porphyrin analysis in our cell-free preparations.

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