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Accumulation of Photodynamic Tetrapyrroles Induced by Acifluorfen-Methyl

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ABSTRACT

Treatment with acifluorfen-methyl (AFM), methyl 5-(2-chloro-4-[trifluoromethyl] phenoxy)-2-nitrobenzoate, inhibited protochlorophyllide synthesis in dark-held, δ-amino levulinic acid-fed, excised cotyledons of cucumber (*Cucumis sativus* L.). Protochlorophyllide and protoporphyrin IX levels in AFM-treated cotyledons were inversely related and dependent on AFM concentration; as the herbicide dose increased, protoporphyrin IX levels also increased with a concomitant loss of protochlorophyllide. Significant protoporphyrin IX accumulation was induced by concentrations of AFM from the linear region of the membrane disruption dose response curve. The pattern of precursor accumulation seen in HPLC chromatograms from extracts of AFM-treated tissue indicate that Mg insertion into the tetrapyrrole ring is inhibited, suggesting interference with Mg-chelatase. An inhibitor of δ-amino levulinic acid synthesis, gabaculine (3-amino-2,3-dihydrobenzoic acid), completely blocked the membrane disruption activity of AFM in illuminated cotyledons. Protoporphyrin IX accumulating in AFM-treated tissues may serve as the primary photosensitizer for initiating lipid peroxidation.

As a consequence of the rapid induction of lipid peroxidation, illumination of plant tissues treated with *p*-nitro DPE[1] herbicides results in the disruption of a wide range of biological constituents and functions (18, 26). This broadly destructive activity by the DPE herbicides has made it difficult to monitor specific interactions between these compounds and light-dependent *in vivo* physiological processes. These effects lead to one of the most readily visible manifestations of the treatment of whole plants with DPE herbicides: the bleaching of the foliar tissue as a consequence of pigment photooxidation.

In an earlier study (15) greening was induced in AFM-treated cucumber cotyledons without triggering a loss in plasmalemma membrane integrity under a regime of low intensity, intermittent illumination. Yet, even under these conditions, we observed significant reductions in the levels of Chl accumulated in AFM-treated tissue. These results suggested the possibility that, in addition to the pigment bleaching in DPE-treated plant tissue which occurs under continuous illumination, AFM might also directly inhibit pigment biosynthesis in a manner not related to photooxidation.

We have since found further evidence that the DPE herbicides directly inhibit Chl synthesis prior to the formation of Pchl(ide). Rather than being only a secondary effect, the physiological consequences of the site of inhibition are such that this interaction with the Chl biosynthetic pathway may be directly related to the light-activated, membrane-disrupting activity of the DPE herbicides.

[1] Abbreviations: DPE, diphenyl ether; AFM, acifluorfen-methyl; ALA, δ-amino levulinic acid; Pchl(ide), a mixture of Pchlide and Pchlide ester; proto IX, protoporphyrin IX; Mg-proto IX, Mg-protoporphyrin IX; Mg-proto IX Me, Mg-protoporphyrin IX monomethyl ester; LA, levulinic acid: LSD (0.05), least significant difference at the 5% probability level.

MATERIALS AND METHODS

Plant Material. Cucumber (*Cucumis sativus* L. cultivar 'Wisconsin SMR 18') was grown, and cotyledons were harvested and prepared as previously described (15).

Efflux Experiments. Loss of membrane integrity was determined as a function of radiolabeled 3-*O*-methyl-D-[U-^{14}C]glucose efflux, a modification of the procedure of Orr *et al.* (26) as described by Halling and Peters (15). Continuous (600 $\mu E \cdot m^{-2} \cdot s^{-1}$ PAR) and intermittent (25 $\mu E \cdot m^{-2} \cdot s^{-1}$ PAR) illumination was supplied as in the earlier report (15). Herbicide treatments and incubation conditions are specified in the figure captions.

Spectrophotometric Pigment Analyses. Cotyledons were treated as in the efflux experiments except for the omission of the radiolabeled sugar additions. Treatment sets of five intact cotyledons were extracted in 5 ml of methanol for 48 h in the dark at 4°C following intermittent illumination. Total Chl was determined using the extinction coefficients of MacKinney (22), and total carotenes were measured as described previously (15). Porphyrin synthesis was induced in dark-incubated, etiolated cotyledons by treatment with 5 mM ALA. Pchl(ide) levels in methanolic extracts were estimated from the absorbance at 629 nm, utilizing the extinction coefficient of Koski and Smith (19).

Tetrapyrrole Extraction. The extraction of tetrapyrroles from ALA-fed, dark-incubated, etiolated cotyledons and their separation from carotenes and lipids was accomplished according to the method of Duggan and Gassman (8). Briefly, treatment sets of 50 cotyledons were homogenized at room temperature in 10 ml of basic acetone and centrifuged to remove debris. The pellet was then extracted with 5 ml of basic acetone and was centrifuged; afterward, the supernate was combined with the first acetone extract to yield fraction I (the pooled aqueous acetone extracts) and a nonpigmented pellet. Fraction I was washed with an equal volume of petroleum ether to remove carotenes and lipids. The petroleum ether fraction was back-extracted with basic acetone, and this extract was added to fraction I. The pH of fraction I was brought to 6.8 with the addition of 0.4 ml of saturated NaCl and the dropwise addition of 0.5 M KH_2PO_4. Fraction I was then extracted twice with 3 ml volumes of diethyl ether. The combined ether extracts were subsequently evaporated to dryness under vacuum, then solvated in 5 ml of methanol. The extraction procedure was conducted entirely under green safelights.

HPLC Separations. The methanol extracts were analyzed on a Varian Vista 5500 HPLC utilizing a 5 μm, 250 × 4.5 mm, IBM-C_{18} reverse phase column. Fifty μl of pigment extract was injected onto the column with the aid of a Varian 8085 autosam-

pler. Separation of porphyrin carboxylic acids was done according to the method of Bonkovsky *et al.* (3), which eliminates the need for sample esterification. Briefly, tetrapyrroles were eluted using a 0.1 M ammonium phosphate-methanol mobile phase employing a 15 min gradient from 61 to 100% methanol at a flow rate of 1.5 ml/min. Porphyrins were detected and identified with a HP1040A HPLC photodiode array detection system set at 400 nm and used subsequently to scan detected peaks from 350 to 600 nm. Peak areas were integrated by a Hewlett-Packard Series 4400X System consisting of a HP-9816 desktop computer equipped with Nelson XTRA Chrom Software interfaced to the detector through a Nelson Analytical 760 A/D intelligent interface.

Experimental Design. All experiments were set out as completely randomized blocks and analyzed by a simple one-way ANOVA made on combined experiments. Each experiment was conducted two to three times, and all treatment values presented in figures are the means from all replicates combined across experiments.

Chemicals. AFM and Mg-proto IX were made in the synthesis labs at the FMC Princeton Research and Development facility. Mg-proto IX was synthesized according to the method of Fuhrhop and Smith (13). Proto IX was purchased from Porphyrin Products (Logan, UT). Baker Analyzed Reagent HPLC grade methanol and ammonium phosphate monobasic was purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). Other reagents were purchased from Aldrich or Sigma.

RESULTS AND DISCUSSION

Total Chl accumulation in intermittently illuminated cotyledons was inhibited by AFM as a linear dose response with an I_{50} of 2.5 μM (Fig. 1). Previously, we had determined that 5 μM AFM had no effect on plasmalemma integrity under this same illumination regime (15). Therefore, it appeared that Chl accumulation was highly sensitive to AFM and could be affected by the herbicide independently of extensive lipid peroxidation. When intermittently illuminated cotyledons, treated in the same manner as in the Chl synthesis experiments, were subsequently placed under continuous illumination, significant membrane disruption, measured as efflux of radiolabeled 3-OMG, occurred. The dose response for efflux was similar to Chl synthesis inhibition; 50% of the total radioactivity loaded in the cotyledons was lost at 3 μM AFM (Fig. 2).

A shared primary event would be one explanation for the similarity of the two response curves. Under these experimental conditions, it was not possible to distinguish between pigment photodestruction and direct inhibition of pigment synthesis. It was possible that even under the low intensity, intermittent illumination, that the herbicide, or an herbicide-pigment complex, was serving as a photosensitizer causing oxidation of Chl and/or its precursors. This same photosensitizing function under stronger, sustained illumination might then lead to lipid peroxidation.

Pigment synthesis can be induced in dark-held cucumber cotyledons by the addition of exogenous ALA; the cotyledons will then readily transform the ALA all the way to Pchl(ide) without illumination (6, 8). AFM interfered with dark, ALA-induced pigment synthesis (apparent I_{50} = 10 μM), indicating a direct effect on the synthetic pathway independent of photooxidation (Fig. 3). Spectral scans of extracts taken from cotyledons treated in this manner showed that the 440 nm peak was dramatically reduced in the 3 μM AFM treatments relative to the controls, suggesting a reduction in Pchl(ide) levels (Fig. 4). Maxima in the scans from AFM treatments were detected at 400, 538, and 576 nm, characteristic of proto IX (8). If proto IX were present at sufficiently high levels, this could have interfered with pigment quantitation, so that some of the apparent reduction in Pchl(ide) indicated in Figure 3 may have been due to proto IX accumulation. To better determine the effect of AFM on the levels of specific pigments, the extracts were analyzed chromatographically.

Using the technique of Bonkovsky *et al.* (3), it proved possible

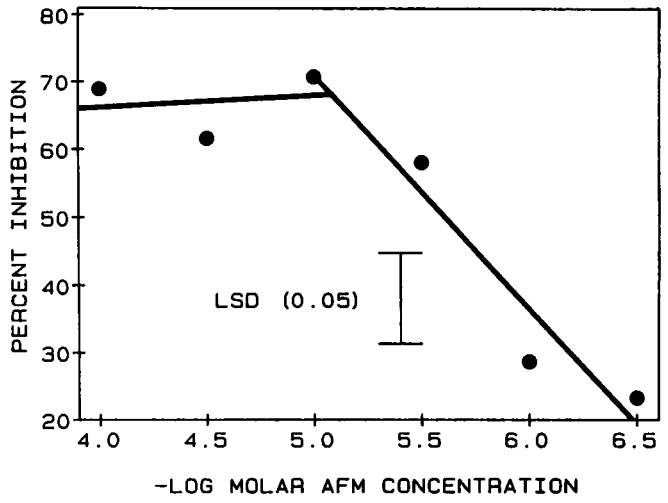

FIG. 1. Inhibition of greening in excised cotyledons, under intermittent illumination, as a function of AFM concentration. Under green safelights, excised cotyledons were placed on media treated with various concentrations of AFM, then held in darkness for 6 h. Treatments were then exposed to intermittent illumination (1 min of light in 10 min cycles) for a period of 10 h. Replicated treatment sets of five cotyledons were then sampled and extracted for spectrophotometric pigment analysis. The level of total Chl was determined as described by MacKinney (22). The average control value was 2.2 μg Chl·cotyledon^{-1}.

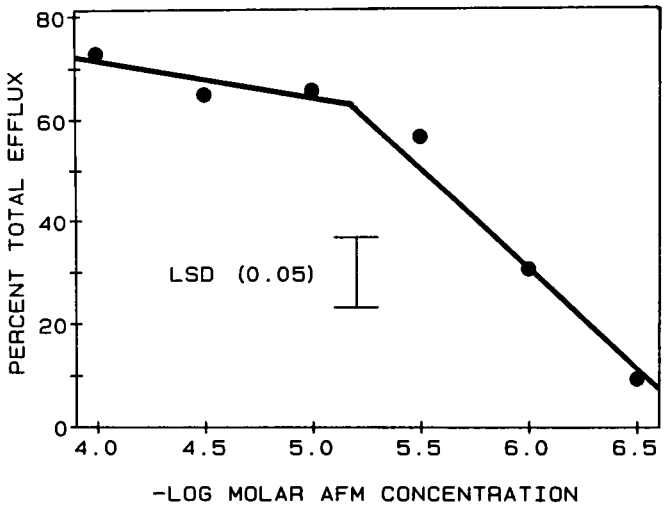

FIG. 2. Membrane damage induced in excised cotyledons as a function of AFM concentration. After loading with 3-*O*-methyl-D-[U-^{14}C] glucose, excised cotyledons were washed under green safelights, placed on media containing various concentrations of AFM, then held in darkness for 6 h. Treatments were then exposed to intermittent illumination (1 min of light in 10 min cycles) for a period of 10 h. Light-activated membrane disruption was induced by placing the treatments under a continuous, high intensity illumination source (600 μE·m^{-2}·s^{-1}; PAR) for 3 h. The medium was then recovered from replicated treatment sets of five cotyledons and sampled for effluxed radioactivity. Leakage induced by the herbicide treatments is expressed as a percent of the total uptake of radioactivity; therefore, increasing injury is expressed as an increase in the percentage value. Average control value was 2% efflux.

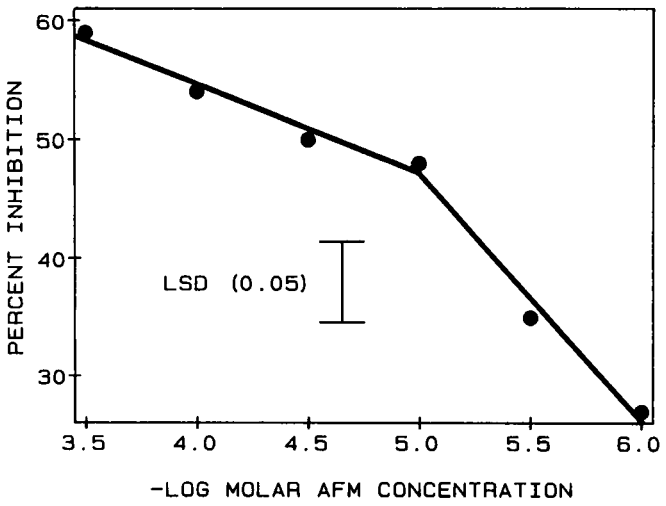

FIG. 3. Inhibition of greening in ALA-fed, dark-held, excised cotyledons, as a function of AFM concentration. Under green safelights, excised cotyledons were placed on media treated with various concentrations of AFM and dark-incubated for 6 h; 5 mM ALA was then added to all treatments, which were held for an additional 16 h in darkness. Replicated treatment sets of five cotyledons were sampled and extracted for spectrophotometric pigment analysis. The level of Pchl(ide) was estimated from the absorbance at 629 nm, utilizing the extinction coefficient of Koski and Smith (19). The estimates of Pchl(ide) levels were not corrected for possible interference by proto IX. The average control value was 4 μg Pchl(ide)·cotyledon^{-1}.

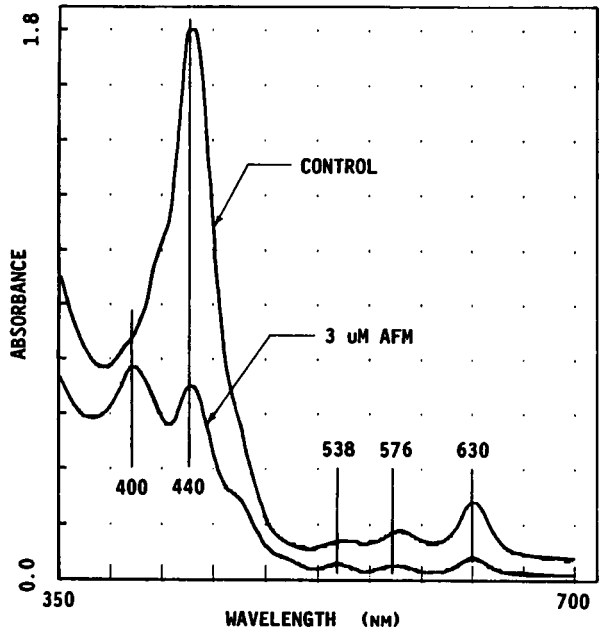

FIG. 4. Visible spectra of pigment extracts from control *versus* 3 μM AFM-treated cotyledons. Under green safelights, excised cotyledons were placed on AFM-treated media and dark-incubated for 6 h, 5 mM ALA was added, and treatments were then held for an additional 16 h in darkness. Replicated treatment sets of five cotyledons were sampled and extracted for spectrophotometric pigment analysis.

to satisfactorily separate the Chl precursors proto IX and Mg-proto IX by HPLC (Fig. 5). The acidity of the initial gradient conditions used here, pH 4.2, is similar to that cited by Castelfranco *et al.* (5) for HPLC separation of Chl precursors. These conditions afforded better development and separation of peaks than more alkaline initial gradients; however, we found that Mg-

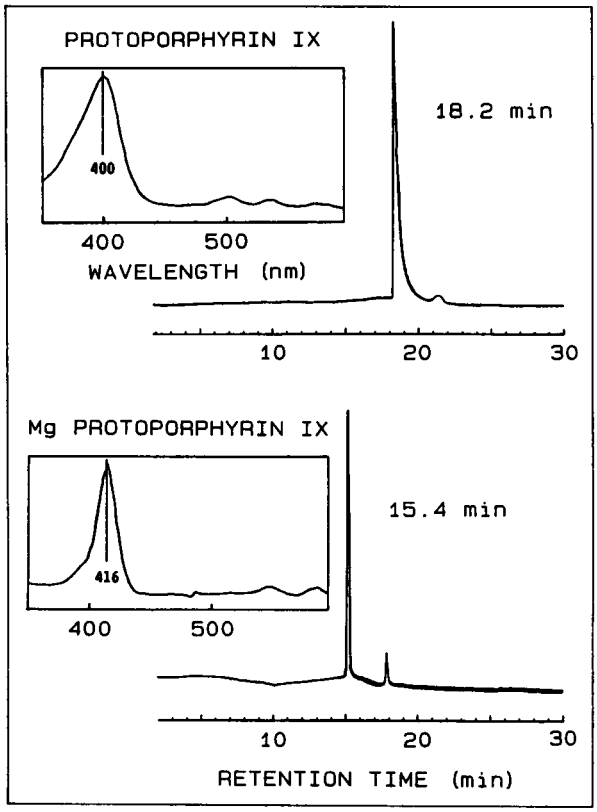

FIG. 5. Analysis by HPLC of porphyrin standards. Authentic proto IX and Mg-proto IX were solubilized in 100% methanol and chromatographed on a C_{18} reversed-phase column according to Bonkovsky *et al.* (3). Major peaks absorbing at 400 nm are designated by retention time in minutes. Insets show absorption spectra of the major peaks as measured by an on-line photodiode-array, visible wavelength detector (Hewlett-Packard 1040A).

proto IX was unstable at pH 4.2, losing the metal cation to degrade back to proto IX. With the gradient changing from 61 to 100% methanol in 15 min, degradation of authentic Mg-proto IX to proto IX on the column was observed (note trailing peak in Fig. 5) leading to losses usually of less than 15%. With a more alkaline initial gradient, pH > 6, there was no indication of any Mg-proto IX degradation, but cotyledon extracts showed no discernible differences in Mg-proto IX or proto IX levels when separations by both sets of gradient conditions were compared.

Spectra taken of each of the peaks of the chromatographed standards show maxima at values in agreement with Gough (14), 416 nm for Mg-proto IX and 400 nm for proto IX (Fig. 5, insets). Extracts from dark-incubated controls that had been fed ALA yielded two major peaks (Fig. 6A). The smaller showed the same retention time and spectra as authentic proto IX; the largest peak had an absorption maximum at 437 to 440 nm suggesting that it was Pchlide, the usual accumulation product in cucumber cotyledons treated in this manner (6, 8). The iron chelator, 2,2-dipyridyl, stimulates porphyrin and Mg-porphyrin formation by inhibiting protohaem synthesis (7, 8, 30). High concentrations of 2,2-dipyridyl inhibit the conversion of Mg-proto IX to Pchl(ide) *in vivo* leading to an accumulation of Mg-proto IX and Mg-proto IX Me (8). HPLC chromatograms of cotyledons treated with 10 mM 2,2-dipyridyl showed the appearance of a peak with the same retention time as authentic Mg-proto IX (Fig. 6A). The 15.4 min peak from the 2,2-dipyridyl extract had an absorbance spectrum identical to Mg-proto IX (data not shown).

Treatment of cotyledons with AFM simultaneously reduced

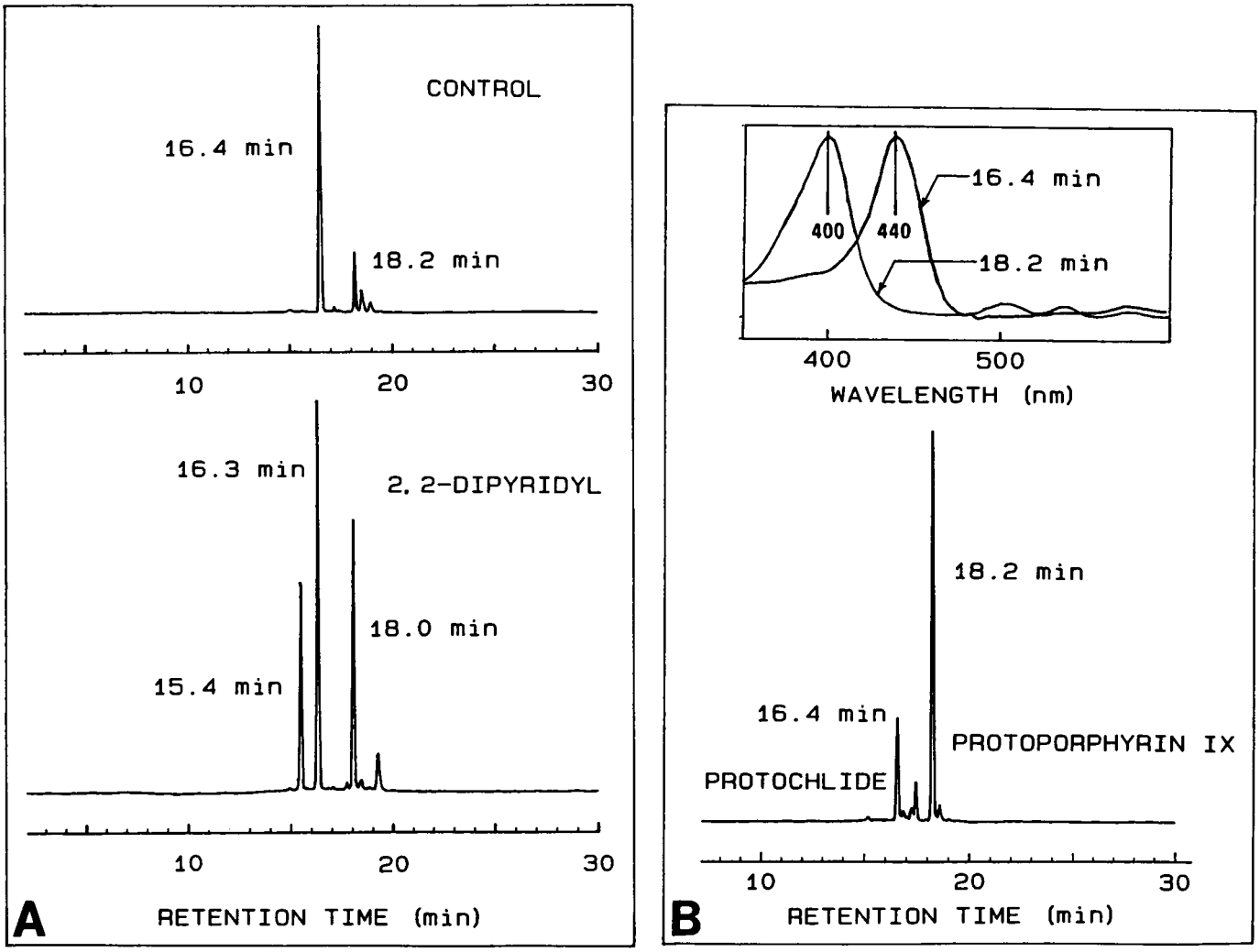

FIG. 6. A, Pigment extracts from untreated and 2,2-dipyridyl treated cotyledons separated by HPLC. Under green safelights, excised cotyledons were placed on media treated with AFM or 10 mM 2,2-dipyridyl and were dark-incubated for 6 h; 5 mM ALA was then added to all treatments, which were held for an additional 16 h in darkness. Treatment sets of 50 cotyledons were extracted; the porphyrins were evaporated to dryness from ether, then taken up in methanol and chromatographed on a reversed-phase C_{18} column according to Bonkovsky *et al.* (3). Major peaks absorbing at 400 nm are designated by retention time in minutes. B, Pigment extracts from AFM-treated cotyledons separated by HPLC. Under green safelights, excised cotyledons were placed on media treated with 300 μM AFM and were dark-incubated for 6 h; 5 mM ALA was then added to all treatments, which were held for an additional 16 h in darkness. Treatment sets of 50 cotyledons were extracted; the porphyrins were evaporated to dryness from ether, then taken up in methanol and chromatographed on a reversed-phase C_{18} column as according to Bonkovsky *et al.* (3). Major peaks absorbing at 400 nm are designated by retention time in minutes. The inset shows absorption spectra of the major peaks from the AFM treatment as measured by an on-line photodiode-array, visible wavelength detector (Hewlett-Packard 1040A).

levels of the 16.4 min peak and stimulated levels of the 18.2 min peak relative to controls. Absorbance maxima of the 16.4 and 18.2 min peaks were at 400 and 440 nm, respectively; concentrations of AFM up to 300 μM were investigated, but only these two major peaks were observed; there was no indication of a major peak that would correspond to Mg-proto IX in AFM treatments (Fig. 6B). Additions of authentic proto IX to AFM-treated extracts caused an increase in a single peak with retention time of 18.2 min, and overlays of spectral scans of the 18.2 min peak from AFM treatments showed an exact match with spectra of authentic proto IX (data not shown). We interpret the chromatograms from AFM treatments as indicating that proto IX is accumulating in AFM-treated cotyledons due at least in part to an inhibition of its conversion to Pchl(ide). Thus it appears that AFM affects *in vivo* tetrapyrrole synthesis in the dark in a manner that differs significantly from iron chelators such as 2,2-dipyridyl.

When ALA is administered, the amounts of putative Pchlide and proto IX in AFM-treated cotyledons were inversely related and dependent on AFM concentration; as the herbicide dose increased, proto IX levels also increased with a concomitant loss of apparent Pchlide (Fig. 7). Significant proto IX accumulation was induced by concentrations of AFM from the linear region of the membrane disruption dose response curve, from 1 to 3 μM (*cf.* Figs. 7 and 2). As indicated in Figure 7, the percentage increase in proto IX accumulation was much greater than the reduction of Pchlide. These HPLC peaks contained only nonesterified Pchlide; therefore, there may have been a greater reduction in an acidic/esterified Pchlide pool than is reflected in the reductions shown here. However, the study by McCarthy *et al.* (25) indicates that Pchlide ester should have comprised only a small proportion of the total Pchlide, and that inhibition of a synthetic step immediately following proto IX would be expected to affect levels of both forms of Pchlide equally. A more probable explanation for the seeming disparity between the relative effect

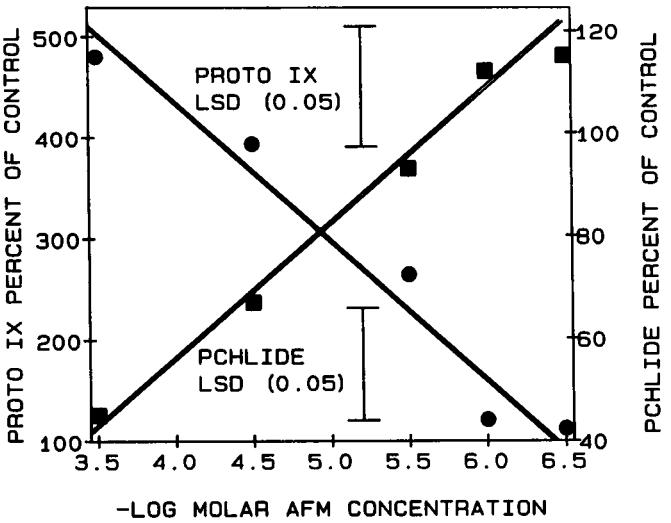

FIG. 7. Proto IX accumulation and Pchlide reduction as a function of AFM concentration. Under green safelights, excised cotyledons were placed on media treated with various concentrations of AFM and were dark-incubated for 6 h; 5 mM ALA was then added to all treatments which were held for an additional 16 h in darkness. Treatment sets of 50 cotyledons were extracted; the porphyrins were evaporated to dryness from ether extracts, then taken up in methanol and chromatographed on a reversed-phase C_{18} column as according to Bonkovsky *et al.* (3). Levels of proto IX (●——●) and Pchlide (■—■) in treatments, relative to the controls, were determined on the basis of the area under the relevant peaks measured at 400 nm by an on-line photodiode-array, visible wavelength detector (Hewlett-Packard 1040A). Peak area was integrated by a Hewlett-Packard 9816 microcomputer connected with the detector through a Nelson Analytical 760 series A/D interface.

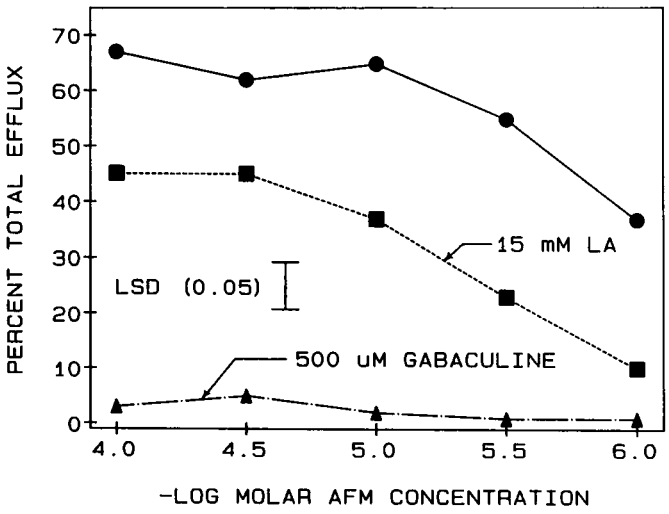

FIG. 8. Effect of inhibitors of porphyrin precursor formation on the membrane-disrupting activity of AFM. After being loaded with 3-*O*-methyl-D-[U-^{14}C]glucose, excised cotyledons were washed under green safelights, placed on media treated with AFM, then held in darkness for 6 h. Inhibitors, 500 μM gabaculine (▲-·-·-▲) or 15 mM LA (■– – –■), were added to selected replicates, and all treatments, including AFM alone (●——●), were incubated in the dark for a further 10 h. Treatments were then exposed to intermittent illumination (1 min of light in 10 min cycles) for a period of 4 h. Light-activated membrane disruption was induced by placing the treatments under a continuous, high intensity illumination source (600 $\mu E \cdot m^{-2} \cdot s^{-1}$; PAR) for 3 h. Treatment medium was then recovered and sampled for effluxed radioactivity. Controls and 500 μM gabaculine alone showed identical efflux levels of 2%; 15 mM LA alone induced 4% leakage.

of AFM on Pchlide and proto IX may be related to uneven distribution of AFM throughout the intact cotyledons. In the illuminated efflux and pigment synthesis experiments, it appeared that tissue in the center of the cotyledons was less affected by AFM than that at the periphery so that synthesis of Pchlide may have been less inhibited in cells in the center, while outer cells were more greatly affected and so accumulated high levels of proto IX. Extending the duration of AFM pretreatments to 6 h did not seem to eliminate the apparent concentration gradient in the treated tissues.

Although the chromatograms of AFM-treated extracts show an accumulation of proto IX and a reduction in a peak probably associated with Pchlide, there is no indication of an accumulation of Mg-protoporphyrins. This suggests that the effect of AFM on ALA-induced Pchlide synthesis in dark-incubated cotyledons is the inhibition of the insertion of Mg into the tetrapyrrole ring. The enzyme responsible for this synthetically difficult reaction, Mg-chelatase (5, 6) may be the primary site of action of this herbicide. Accumulation of proto IX, a known, potent photosensitizer and O_2 radical generator could then initiate the lipid peroxidation reactions associated with AFM treatments (4, 6, 28). Studies by Fuesler *et al.* (12) attempting to locate the chelatase have suggested that it is at or near the surface of the plastid envelope. This correlates with a sequential ultrastructural study by Kenyon *et al.* (18) examining intercellular damage caused by acifluorfen, which indicated that the first membrane system affected in treated leaves was the plastid envelope.

If the accumulation of photodynamic tetrapyrroles induced by DPE herbicides initiates the lipid peroxidation reactions, then inhibition of ALA synthesis should reduce membrane disruption in AFM-treated tissue by preventing the formation of the endogenous photosensitizers. Porphyrin synthesis can be inhibited with LA which reduces synthesis of porphobilinogen by competing with ALA for the enzyme ALA dehydrase (1, 2, 5, 8). Alternatively, the activity of glutamate 1-semialdehyde aminotransferase can be blocked with gabaculine, thus inhibiting plastid ALA synthesis (17). Figure 8 shows that inhibition of tetrapyrrole production by LA significantly reduced the level of membrane disruption caused by AFM in cucumber cotyledons; the effectiveness of LA as an inhibitor of Chl synthesis was probably limited by the ability of the charged molecule to penetrate intercellularly. More dramatically, 500 μM gabaculine completely abolished AFM activity; even at 100 μM AFM, the level of efflux from gabaculine-protected cotyledons was not significantly different from untreated controls. The tissue in this experiment was not treated until after the cotyledons had been harvested, thus carotene levels were not greatly affected by the gabaculine treatments. Cotyledons treated with 500 μM gabaculine showed a 15% reduction in total carotenes after the intermittent light incubation, but after transfer to the continuous high intensity illumination, the rate of accumulation of total carotenes was the same in controls and gabaculine treatments.

Respiration and protein synthesis inhibitors have been reported as reducing AFM activity in excised cucumber cotyledons, but gabaculine appears to be the most effective antagonist to AFM activity yet reported in pigmented cotyledons (9, 15). This result argues that a photoactive excitation complex is not formed *in vivo* between AFM and carotenes as has been suggested (15, 26). Significant reduction of the membrane disrupting activity of DPE herbicides by photosynthetic electron transport inhibitors have been reported for both higher plant and algal systems (20, 23). It is interesting then to note that there have been reports of specific effects of phenyl ureas and triazines on porphyrin metabolism (10, 29). More generally, inhibition of photosynthesis would reduce ATP and NADPH levels in chloroplasts which in turn would depress ALA synthesis (16).

Effective weed control with the diphenyl ethers requires treatment of very young plants shortly after the seedlings have emerged (21). Accumulation of photodynamic tetrapyrroles in the rapidly growing and expanding leaves of such plants, as a result of DPE-induced Chl synthesis inhibition, could be a major contributor to the phytotoxic action of these herbicides. The gabaculine experiments suggest that tetrapyrrole accumulation may be the only important mechanism for AFM-induced membrane disruption in cucumber cotyledons. However, if the nitrofen-susceptible, yellow rice mutants reported by Matsunaka (24) do not synthesize plastid tetrapyrroles, then our hypothesis could not account for the activity of all DPE herbicides in all systems.

Etioplasts that retain the chelatase function have been described in detail (5, 8, 11, 27). Therefore, it should be possible with such preparations to begin to investigate the effect of AFM on *in vitro* Chl synthesis and to more precisely describe the site and type of inhibition. Rebeiz *et al.* (28) have examined the potential of endogenously accumulated porphyrins as herbicides. But experiments are also needed to determine what intercellular concentration of protoporphyrin IX is required to initiate significant lipid peroxidation, and how those concentrations relate to the levels accumulated in the presence of AFM.

Acknowledgments—The authors are obliged to Dr John W. Lyga for the synthesis and isolation of Mg-protoporphyrin IX.

Note added in revision: Accumulation of tetrapyrroles in AFM-treated soybean cells and cucumber hypocotyls was recently reported by M. Matringe and R. Scalla at the 1987 British Crop Protection Conference.

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