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In Vitro Synthesis of the Chlorophyll Isocyclic Ring[1]

TRANSFORMATION OF MAGNESIUM-PROTOPORPHYRIN IX AND MAGNESIUM-PROTOPORPHYRIN IX MONOMETHYL ESTER INTO MAGNESIUM-2,4-DIVINYL PHEOPORPHYRIN a_5

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ABSTRACT

Developing chloroplasts of *Cucumis sativus* L., cv Beit Alpha which were incubated with either Mg-protoporphyrin IX or Mg-protoporphyrin IX monomethyl ester in darkness produced a partially phototransformable protochlorophyllide species that was tentatively identified as Mg-2,4-divinyl pheoporphyrin a_5. *S*-Adenosylmethionine stimulated Mg-2,4-divinyl pheoporphyrin a_5 formation irrespective of the starting material used. In the case of Mg-protoporphyrin IX monomethyl ester, this stimulation was attributed to the need to remethylate substrate that had been hydrolyzed by an endogenous methylesterase which converts part of the added Mg-protoporphyrin IX monomethyl ester to Mg-protoporphyrin IX.

NADP and NADPH stimulated the conversion of Mg-protoporphyrin IX to Mg-2,4-divinyl pheoporphyrin a_5. The conversion required oxygen and was half saturated at 50 micromolar dissolved O_2. The conversion was insensitive to inhibitors of iron-sulfur and heme-containing proteins, to Cu chelators, H_2O_2, and peroxide scavengers. However, the conversion was extremely sensitive to phenazine methosulfate, methylene blue, and methyl viologen.

A decrease of the plastids' ability to convert Mg-protoporphyrin IX to Mg-2,4-divinyl pheoporphyrin a_5 after lysis in 0.1 molar NaCl suggested a requirement for plastid integrity.

The conversion of Mg-Proto Me[3] to Pchlide requires modification of the tetrapyrrole side chains at positions 4 and 6. The methyl propionate side chain at position 6 gives rise to the isocyclic ring. Granick (11) first suggested that this transformation involves the β-oxidation of the methyl propionate side chain to a 3-ketopropionate group. The active methylene carbon atom, situated between the carboxylate ester and the β-carbonyl, would then become bonded to the γ-bridge in an oxidizing cyclization reaction, during which two hydrogens are lost. This cyclization sequence would result in MgDVP which is converted to Pchlide by the hydrogenation of the vinyl group at position 4.

MgDVP was found in a tan mutant of *Rhodopseudomonas spheroides* (28), in wildtype *R. spheroides* treated with 8-hydroxyquinoline (16), and in the inner seed coat of *Cucurbita pepo* (18). Certain photosynthetic bacteria excrete MgDVP into the medium (24, 30). Conclusive evidence for the structure of the pigment isolated from 8-hydroxyquinoline-treated cultures was obtained by Jones (17).

Griffiths and Jones (13) tested the MgDVP accumulated by a mutant of *R. spheroides* and showed that it was photoconverted by a barley etioplast preparation that was also able to convert added Pchlide.

Recently, Belanger and Rebeiz (1, 2) have shown that MgDVP is present and undergoes phototransformation in a number of plant species. Cell-free systems have been described (7, 21, 22) that are able to convert porphyrin precursors to Pchlide. However, in none of these studies have Pchlide and MgDVP been resolved.

Ellsworth and Hervish (7) observed the conversion of ^{3}H-labeled Mg-Proto to Pchlide by crude homogenates of etiolated wheat seedlings. Required cofactors included SAM, CoA, ATP, and Pi. Their results were expressed in terms of specific radioactivity and do not permit the calculation of the actual yield of Pchlide formed.

Mattheis and Rebeiz (21) observed the conversion of Mg-Proto Me to Pchlide by a preparation of developing chloroplasts extracted from greening cucumber cotyledons. CoA, methanol, ATP, NAD, and Pi were added to the incubation. The percent conversions reported were high (51% on the basis of added Mg-Proto Me), but the total *in vitro* synthesis of Pchlide was low (approximately 10 pmol of Pchlide mg^{-1} plastid protein h^{-1}).

In a recent study (4), we reported on the cell-free conversion of Mg-Proto to Pchlide, dependent on O_2 and SAM. In the present study we have optimized the system, analyzed the cofactor requirement and the effect of metabolic inhibitors. We have reexamined the product and shown that it probably consists of MgDVP, rather than Pchlide.

MATERIALS AND METHODS

Materials. NADP (monosodium salt), NADPH (tetrasodium salt, type III enzymically reduced), SAM (chloride salt), cysteine HCl, EDTA, DCPIP (Na salt), FMN, PMS, β-carotene (type III, from carrots), GSH, ferredoxin (type III from spinach), and DIECA (sodium salt) were obtained from Sigma.

Hepes, Tes, BSA (fatty acid poor), and L-amino acid oxidase (*Crotalus adamanteus* venom) were obtained from Calbiochem.

KCN, H_2O_2 (solution 30%), sucrose, NaCl, $Mg(ClO_4)_2$, and diethyl ether (for anesthesia) were obtained from Mallinckrodt.

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[3] Abbreviations: Mg-proto Me, Mg-protoporphyrin IX monomethyl ester; MgDVP, Mg-2,4-divinyl pheoporphyrin a_5; Mg-proto, Mg-protoporphyrin IX; SAM, *S*-adenosylmethionine; DCPIP, dichlorophenolindophenol; FMN, flavin mononucleotide; PMS, phenazine methosulfate; DIECA, diethyldithiocarbamic acid; DMSO, dimethylsulfoxide; MB, methylene blue; DSPD, *N,N*-disalicylidene-1,3-propanediamine; MV, methyl viologen; DHA, dehydroascorbic acid; sulfo-DSPD, disulfodisalicylidene-1,3-propanediamine; Mg-proto Me_2, Mg-protoporphyrin IX dimethyl ester; Proto Me_2, protoporphyrin IX dimethyl ester; Mg-Proto (Me), a mixture of Mg-Proto and Mg-Proto Me in unknown proportions; Chl(ide), a mixture of Chl and Chlide in unknown proportions; EPA, diethyl ether, 2-methylbutane, absolute ethanol (2:2:1 by volume).

NaN_3 (practical), glucose, DMSO (reagent grade), and MB were obtained from Matheson, Coleman, and Bell.

Glucose oxidase and catalase were obtained from Worthington Biochemical Corp., Freehold, N.J.

$MgCl_2$ and $K_3Fe(CN)_6$ (Baker analyzed) were obtained from Baker Chemical Co.

L-Leucine was obtained from Fisher Scientific Co. DSPD was obtained from Ames Laboratory, Milford, CT. Glutathione peroxidase (from bovine red blood cells) was from Toyoba Co., Tokyo. MV (paraquat dichloride 99.2%) was from Ortho, Chevron Chemical Co., Richmond, CA. Silica gel H (according to Stahl) was from Merck AG., Darmstadt, Germany. Polyethylene was from PolySciences, Inc., Warrington, PA. GSSG was from C. F. Boehringer and Soehne, GmBH Mannheim, Germany. DHA was from K and K Laboratories, 121 Express St., Plainview, NY. Sulfo-DSPD was a gift of A. Trebst.

Methods.

Plant Tissue. Cucumber seeds (*Cucumis sativus* L., cv Beit Alpha), a gift of Moran Seed Co. Inc., Modesto, CA, were germinated in the dark at room temperature for 5 to 6 d as described previously (14). The seedlings were exposed to white light (40 to 50 $\mu E\ m^{-2}s^{-1}$ PAR, General Electric F20T12 WW warm white fluorescent bulbs) for 20 h.

Isolation of Plastids. Greening cucumber cotyledons (50–60*g*) were used as the source of a preparation containing intact plastids (23). In some experiments, plastids received a NaCl wash instead of the usual wash through 35 ml of buffered 0.6 M sucrose. The crude plastid pellet (P_2) was resuspended in 5 ml per pellet of 0.2% BSA and 5 mM cysteine in solution A (0.5 M sucrose, 1 mM $MgCl_2$, 1 mM EDTA, 20 mM Tes, 10 mM Hepes, pH 7.7). Each pellet suspension was further diluted with 35 ml of either 0.35 M, 0.2 M, or 0.1 M NaCl containing 1 mM $MgCl_2$, 1 mM EDTA, 20 mM Tes, 10 mM Hepes (pH 7.7). After 5 min at 4°C, the suspensions were centrifuged at 500*g* for 15 min. The pellets, prepared by either method, were resuspended in solution A with the appropriate cofactors and substrates.

Routine Incubation Conditions. All samples were incubated with substrates and cofactors appropriate to the reaction along with 5.2 to 10.1 mg plastid protein in 1 ml of solution A. All samples were depleted of endogenous MgDVP by a 5-min light treatment (see Table I for details). Incubations were performed in darkness to prevent phototransformation or photodestruction of newly formed MgDVP. All incubations were done at 30°C for 1 h and terminated by freezing at −15°C. Other treatments are specified in the figure and table legends.

Air-N_2 and air-CO mixtures were prepared by means of a Dial-A-Gas precision dilution system from Calibrated Instruments, Inc. Plastids, suspended in solution A containing 0.6 mM NADP and 0.6 mM NADPH, were added to the main compartment of the reaction vessel and given a 5-min light treatment (40 to 50 $\mu E\ m^{-2}\ s^{-1}$ PAR). Mg-Proto (3 nmol) and SAM (1 μmol) were added to the side arm. The gases were flushed through the reaction vessels for 5 min, after which the plastids were mixed with the substrates in the side arm. Subsequent incubation was carried out as described above.

Substrates for the Enzyme Reaction. Mg-Proto Me_2 was prepared from Proto Me_2 by refluxing with anhydrous $Mg(ClO_4)_2$ in anhydrous pyridine (10). Mg-Proto was prepared from synthetic Mg-proto Me_2 as described by Chereskin and Castelfranco (4) with the modification that the Mg-Proto in the final diethyl ether solution was transferred quantitatively to an equal volume of 10 mM KOH.

The synthesis of Mg-Proto Me was modified from Granick (12). Thirty 5-d-old etiolated cotyledon pairs were incubated with 2.5 ml of 10 mM K-phosphate buffer (pH 6.5) containing 50 μmol ALA, and 20 μmol α,α'-dipyridyl in 0.05 ml methanol. Incubation was carried out in the dark at 30°C for 18 to 20 h. The product was purified by HPLC (9). The Mg-Proto Me peaks from several chromatographic separations were pooled. The Mg-Proto Me was transferred to diethyl ether and the methanol was removed by repeated washing with H_2O. The ether was dried by storing it overnight at −15 to −18°C and decanting it from the ice crystals. The ether was evaporated under reduced pressure and the residue containing Mg-Proto Me was redissolved in 20% DMSO. The porphyrin product migrated as a single band on silica gel H thin layers.

Pigment Extraction and Assay Procedure. Porphyrins formed during the incubations were extracted into diethyl ether by a

Table I. *Effect of Light on MgDVP Formation*

All incubations included 4 mM GSH and 0.2% BSA; 5 μM Mg-Proto and 1 mM SAM were included where indicated. Plastid protein was 6.9 mg in experiment 1 and 6.8 mg in experiment 2. Light exposures were at 30 ± 1°C under white light (40 to 50 $\mu E\ m^{-2}\ s^{-1}$ PAR, General Electric F20T12WW warm light fluorescent bulbs). Other conditions are described in "Materials and Methods." In this table, and Tables III to V, the tabulated values represent the mean and the range of two samples.

	MgDVP
	pmol mg^{-1} protein h^{-1}
Experiment 1	
Zero time	96.2 ± 3.3
5 min light, no incubation	13.2 ± 0.6
5 min light, 1 h incubation	12.6 ± 1.8
5 min light, 1 h incubation with Mg-Proto and SAM	51.6 ± 0.5
Experiment 2	
5 min light, 1 h incubation with Mg-Proto and SAM	56.6 ± 1.5
5 min light, 1 h incubation with Mg-Proto and SAM, 1 min light	31.8 ± 2.5

Table II. *Corrected Fluorescence Spectra of Standard Pchlide, Standard MgDVP, Endogenous MgDVP, and Products from Incubations with Mg-Proto or Mg-Proto Me*

Standard Pchlide and MgDVP were extracted from dark-grown cucumber cotyledons and separated on polyethylene thin layers. Endogenous MgDVP was extracted from plastids isolated from cucumber cotyledons greened for 20 h. The pigment product in the two bottom rows was formed during *in vitro* incubation of isolated plastids, previously exposed to 5 min of light to phototransform the endogenous MgDVP. Incubations included 1 mM SAM, 0.6 mM NADP, 0.6 mM NADPH, and 10 μM Mg-Proto or Mg-Proto Me, as indicated, and other conditions described in "Materials and Methods." All samples were purified by TLC and eluted with ether. Corrected excitation and emission spectra were determined as indicated in "Materials and Methods."

	Ether (298 K)		EPA (77 K)	
	Excitation maximum	Emission maximum	Excitation maximum	Emission maximum
	nm			
Standard Pchlide	433–434	625–626	450	632
Standard MgDVP	440	627	456	633
Endogenous MgDVP	440	627	456	633
Product: Mg-Proto incubation	440	627	456	633
Product: Mg-Proto Me incubation	440	627	456	633

previously described procedure (3). Porphyrins and metalloporphyrins in the diethyl ether extracts were assayed using a Perkin-Elmer MPF 44-A fluorescence spectrophotometer. Mg-Proto(Me) was measured with an excitation of 420 nm and a slit width of 20 nm. MgDVP was measured with an excitation of 440 nm and a slit width of 20 nm. The emission maxima for Mg-Proto(Me) and MgDVP were 594 and 627 to 629 nm, respectively, measured with a 2-nm slit width. Uncorrected spectra were obtained with a scan speed of 60 nm/min using a 430-nm cut-off filter. Concentrations were determined by reference to standard Mg-Proto Me_2 and Pchlide measured under identical conditions.

Spectrofluorimetric Standards. The standards were synthetic Mg-Proto Me_2 and Pchlide prepared from cucumber cotyledons by the method of Chereskin and Castelfranco (4). This product is really a mixture of Pchlide and MgDVP (2). Our measurements of MgDVP were based on the assumption that the molar emission coefficients of both components are essentially equal. However, if this were not so, we would merely introduce into our measurements a proportionality error that is consistent throughout all our data.

Thin Layer Chromatography. Pchlide preparations were separated into monovinyl and divinyl Pchlide (MgDVP) by TLC on polyethylene (2). The plates were developed in acetone/dodecane (99:1, v/v) in the dark at 4°C. Two fluorescent bands were separated. They were reported as monovinyl and divinyl Pchlide by Belanger and Rebeiz (2). The two bands were scraped and eluted into diethyl ether. Biosynthetic Mg-Proto Me was analyzed by chromatography on thin layers of silica gel H, developed with toluene:ethyl acetate:ethanol:dodecane (80:40:20:1) at room temperature in the dark. The plates were viewed under UV light.

Corrected Spectra. For the spectrofluorimetric identification of MgDVP, corrected spectra were taken at room temperature in diethyl ether and at 77 K in EPA, using a DCSU-2 accessory to the Perkin Elmer MPF-44A Fluorescence Spectrophotometer. Monovinyl and divinyl Pchlide (MgDVP) separated by chromatography on polyethylene thin layers were used as standards. Excitation and emission spectra were determined with a 430-nm cut-off filter. For the spectra at 77 K, the diethyl ether extracts were evaporated to dryness under N_2 and the residues were redissolved in EPA.

RESULTS

This paper examines further the characteristics of the plastid-catalyzed conversion of Mg-Proto to MgDVP. The product, measured by the height of the emission at 628 to 629 nm, has been identified as MgDVP; however, it is the same product that was previously referred to as 'Pchlide,' *i.e.* Mg-2-vinyl, 4-ethyl-pheoporphyrin a_5 (4). MgDVP is present to the extent of 800 pmol g^{-1} fresh weight in whole cotyledonary tissue after 20 h exposure to light. The effect of light on both endogenous MgDVP and MgDVP synthesized *in vitro* in isolated plastids was investigated (Table I). Endogenous MgDVP in the isolated plastids used in these experiments was approximately 100 pmol/mg plastid protein; however, after a 5-min light treatment, it decreased to 10% to 15% of the original value. Incubation (1 h, 30°C) did not restore the MgDVP level unless the substrates, Mg-Proto and SAM (4), were added. The newly formed MgDVP was also partly light sensitive. A 1-min light treatment decreased the newly formed MgDVP by approximately 50% (Table I). A 5-min treatment did not reduce the level further.

Pchlide (R_F, approximately 0.78) and MgDVP (R_F, approximately 0.62) were separated by TLC. Although the R_F values were quite variable from one chromatography to the next, the relationship between the two bands remained quite constant. The spots were scraped, eluted with diethyl ether, and the eluates were examined spectrofluorimetrically at room temperature in diethyl ether and at 77 K in EPA (Table II). The spectrum of the slow-moving component was red shifted with respect to that of the fast-moving component. In the emission, the difference was 1 or 2 nm. In the excitation, the difference was 6 or 7 nm. Our results are therefore comparable to those of Belanger and Rebeiz (2), even though our low-temperature spectra were not taken in the same solvent. In agreement with these workers, we choose to call the fast-moving spot, Pchlide (or monovinyl Pchlide), and the slow-moving spot, MgDVP (or divinyl Pchlide).

The endogenous Pchlide component present in our etiochloroplast preparation was routinely transformed by exposure to light before incubation with test materials, in order to decrease our zero-time blank (Table I). Analysis of this pigment showed that it was chromatographically and spectrofluorimetrically similar to the slow-moving, red-shifted Pchlide component of etiolated cucumber tissue and was therefore identified as MgDVP (2) (Table II). The product formed during the incubation, using either Mg-

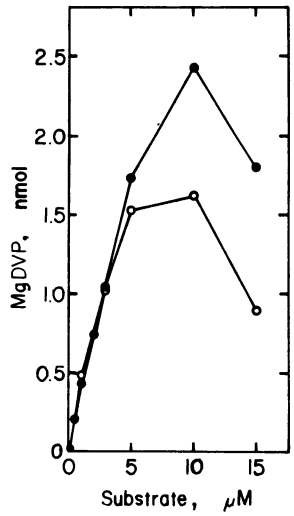

FIG. 1. Requirement for the Mg-porphyrin substrates: Mg Proto and Mg-Proto Me. Incubation included 1 mM SAM, 0.6 mM NADP, and 0.6 mM NADPH. For dependence of MgDVP formation on exogenous Mg-Proto (●–●–●), incubation contained 6.7 mg plastid protein. For dependence of MgDVP formation on exogenous Mg-Proto Me (O–O–O) incubations contained 5.2 mg plastid protein. Other conditions were as described in "Materials and Methods."

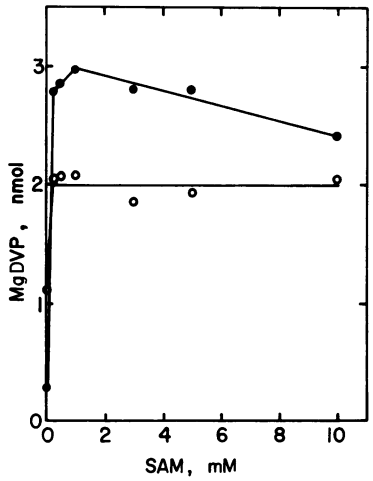

FIG. 2. Requirement for SAM in presence of either Mg-Proto or Mg-Proto Me. Incubations included 0.6 mM NADP, 0.6 mM NADPH, and either 10 μM Mg-Proto or 10 μM Mg-Proto Me, as indicated. For Mg-Proto (●–●–●) curve, incubation contained 7.3 mg plastid protein; for Mg-Proto Me curve (O–O–O), incubations contained 7.2 mg plastid protein. Other conditions were as described in "Materials and Methods."

Table III. *Comparison of Mg-Proto and Mg-Proto Me Substrates for the Formation of MgDVP*

All samples contained 10 μl DMSO, 24.3 μl of 10 mM KOH, 0.6 μmol NADP, 0.6 μmol NADPH, and 6.9 mg plastid protein. The substrate was either 10 μM Mg-Proto or 10 μM Mg-Proto Me; 1 mM SAM was added as indicated. In this and subsequent tables, the plastids were given a 5-min light treatment before the *in vitro* incubation to deplete the endogenous MgDVP. Other conditions as described in "Materials and Methods."

Substrates	MgDVP
	pmol
Mg-Proto	455 ± 20
Mg-Proto + SAM	3708 ± 90
Mg-Proto Me	1728 ± 1
Mg-Proto Me + SAM	3213 ± 16

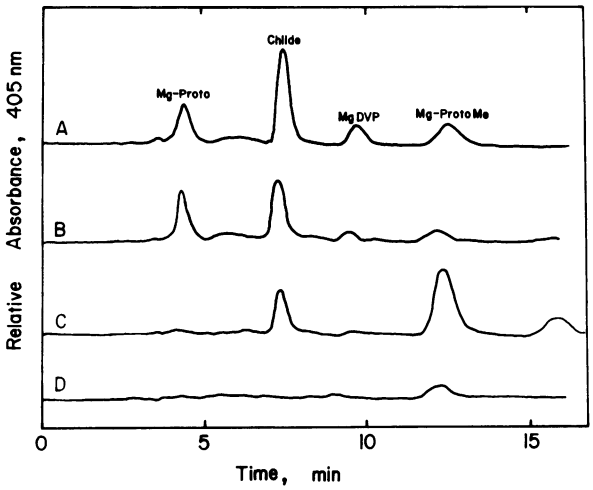

FIG. 3. Demethylation of Mg-Proto Me during incubation. All incubations included 0.6 mM NADP, 0.6 mM NADPH, and 5 μm Mg-Proto Me. Incubations A to C contained 7.5 mg plastid protein. Peaks were separated by HPLC and identified by comparison with authentic standards (9). A, Intact plastids; B, sonicated plastids (sonication was for 3 × 5 s at three-fourths maximum intensity using a BIOSONIC Sonicator); C, boiled plastids (100°C for 5 min); D, no plastids. Other conditions were as described in "Materials and Methods."

Table IV. *Effect of Triphosphopyridine Nucleotides on MgDVP Formation*

Incubations included 0.2% BSA, 6.4 mg plastid protein, 4 mM GSH, 1 mM SAM, and 3 μM Mg-Proto. NADP and NADPH were 0.6 mM. Other conditions were as described in "Materials and Methods."

Additions	MgDVP
	pmol
None	372 ± 12
NADP	477 ± 38
NADPH	548 ± 9
NADP + NADPH	622 ± 20

Proto or Mg-Proto Me as the substrate, was likewise shown to consist of MgDVP by chromatographic and spectrophotometric criteria (Table II).

Requirements for MgDVP Formation. The requirements for O_2 and SAM have already been reported (4). Concentration curves for the two substrates Mg-Proto and Mg-Proto Me are shown in Figure 1. The latter substrate was tested in 2% DMSO. In a preliminary experiment it was found that this solvent is not inhibitory at this concentration (data not shown). For both substrates the saturating concentration is approximately 10 μM; higher concentrations appeared to be inhibitory.

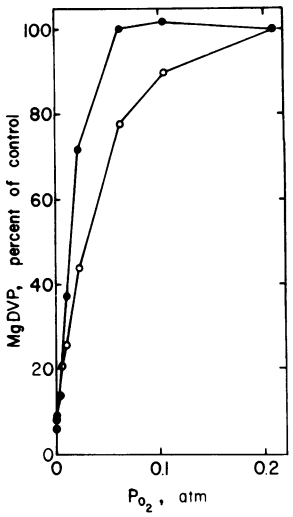

FIG. 4. Dependence of conversion of Mg-Proto to MgDVP on O_2 in N_2 and CO. Data for each curve were obtained from two experiments. Incubation vessels contained 3 μM Mg-Proto, 1 mM SAM, 0.6 mM NADP, 0.6 mM NADPH, and 5.7 to 8.1 mg plastid protein. Incubations carried out in 100% air were used as controls and curves were normalized accordingly. Other conditions were as described in "Materials and Methods." Symbols O——O, air-N_2 mixtures; ●——●, air-CO mixtures.

Table V. *Effect of NaCl Washes on the Ability of Plastids to Form MgDVP*

Plastids were isolated and washed with sucrose or NaCl solutions as described in "Materials and Methods." Incubations included 3 μM Mg-Proto, 1 mM SAM, 0.6 mM NADP, 0.6 mM NADPH, and 5.6 to 8.6 mg plastid protein; other conditions were as described in "Materials and Methods."

Treatment	MgDVP	
	pmol	*pmol mg^{-1} protein h^{-1}*
0.6 M sucrose	11150 ± 15	188 ± 1
0.35 M NaCl	1124 ± 54	202 ± 10
0.2 M NaCl	1078 ± 8	126 ± 1
0.1 M NaCl	378 ± 1	50.2 ± 0.1

The dependence of MgDVP formation on SAM concentration, using either Mg-Proto or Mg-Proto Me as substrate is shown in Figure 2. It can be seen that in both cases the system is saturated by 1 mM SAM, the concentration used in our routine assay. With Mg-Proto there is an almost total requirement for SAM; with Mg-Proto Me, there is still a significant stimulation by SAM.

Figures 1 and 2 suggest that added Mg-Proto Me may be slightly less effective in MgDVP formation than added Mg-Proto. Table III confirmed the stimulation by SAM with both substrates and indicated that, in the presence of saturating SAM concentration, Mg-Proto is approximately 15% more effective than Mg-Proto Me in the formation of MgDVP. It was not obvious why SAM should stimulate the formation of MgDVP if Mg-Proto Me is used as the substrate (Fig. 2 and Table III). This point was investigated by a separate experiment, in which the plastids were incubated with Mg-Proto Me in the absence of SAM, and after the incubation the pigments were extracted and separated by HPLC (9). During the incubation, Mg-Proto Me is converted to Mg-Proto as well as to MgDVP (Fig. 3). This hydrolytic reaction is enzymic, since it does not take place when the plastids are omitted or boiled. Added SAM would enhance the formation of MgDVP by remethylating any Mg-Proto that is formed during the course of the incubation.

The two cofactors NADP and NADPH are added routinely to our incubation at a concentration of 0.6 mM each. The stimulation

due to these two cofactors is somewhat variable and sometimes, but not always, there is an additive effect (Table IV). NAD and NADH were much less effective than NADP and NADPH (data not shown).

The dependence of MgDVP formation on O_2 concentration is shown in Figure 4. Air was mixed with N_2 to give the partial pressures of O_2 indicated on the abscissa. MgDVP formation was half-saturated at a partial pressure of approximately 0.04 atm (50 μM dissolved O_2).

CoA was reported to stimulate the incorporation of ^{14}C-labeled ALA (25) and ^{3}H-labeled Mg-Proto (7) into Pchlide. When this cofactor was tested in our system, no stimulation of Mg-Proto conversion to MgDVP was observed (data not shown).

Organelle Integrity. The plastid preparation (P_3), which is isolated in buffered 0.6 M sucrose, does not lose activity on washing with 0.35 M NaCl. The system's response to decreasing NaCl concentration in the plastid wash is shown in Table V. Activity remained high at 0.35 and 0.2 M NaCl. However, MgDVP formation decreased dramatically if the plastids were washed with 0.1 M NaCl.

A parallel electron microscopy study (data not shown) verified the intactness of P_3. The plastids used in the present study contained prolamellar bodies and stroma lamellae as well as developing grana stacks. Retention of stroma indicated intactness. By these criteria, intactness decreased with decreasing NaCl concentration. This loss of intactness was not reflected in a parallel loss of enzymic activity in the 0.35 and 0.2 M NaCl-washed plastids. However, the 0.1 M NaCl-washed plastids, which were nearly 100% broken, had lost most of their enzymic activity (Table V).

Inhibitor Studies. Our previous work (4) indicated the requirement of Fe in MgDVP formation. The possibility that a hemoprotein could be involved in the oxidative cyclization of the methyl propionate side chain was tested by the addition of CO, KCN, and NaN_3. These three nucleophiles form ligands with the central Fe atom of many hemoproteins excluding the access of substrates to the oxidation-reduction site. Air in CO appeared to be more effective in bringing about MgDVP formation than air in N_2 (Fig. 4). Presumably, this apparent stimulation by CO was due to the inhibition of some competing side reaction. At any rate, CO was not inhibitory. Because of equipment limitations, Figure 4 had to be compiled from four separate experiments. In a single experiment, plastids were incubated with 3 μM Mg-Proto and 1 mM SAM under the following gas mixtures: 50% air in N_2, 50% air in CO, 10% air in N_2, 10% air in CO. The extent of MgDVP formation was dependent on the concentration of air, but not on the kind of diluent gas (N_2 or CO) (data not shown). KCN was essentially noninhibitory (10% inhibition at 1 mM); NaN_3 was slightly inhibitory (45% inhibition at 1 mM) (data not shown).

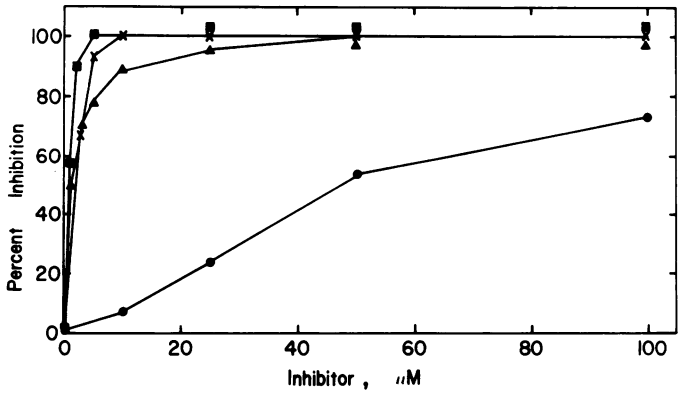

FIG. 5. Inhibition of MgDVP formation by MB, PMS, MV, and FMN. Four curves are result of four different experiments. All incubations included 10 μM Mg-Proto, 0.6 mM NADP, 0.6 mM NADPH, 1 mM SAM, and 5.7 to 7.2 mg plastid protein. Other conditions were as described in "Materials and Methods." Symbols: ■——■, MB inhibition curve; ×——×, PMS inhibition curve; ▲——▲, MV inhibition curve; ●——●, FMN inhibition curve.

Table VI. *Effect of Some Known Metabolic Inhibitors on MgDVP Formation*

Table VI was compiled from seven different experiments. In all cases, incubations included 3 μM Mg-Proto, 1 mM SAM, 0.6 mM NADP, 0.6 mM NADPH, and 5.5 to 10.1 mg plastid protein. Other conditions were as described in "Materials and Methods." DSPD was added in DMSO such that the final concentration of DMSO was 1.2%. Glucose oxidase was suspended in H_2O and centrifuged at 39,000*g* for 5 min; the supernatant was added to the incubation. β-Carotene was added in 20% ethanol such that the final ethanol concentration was 0.8%. All test materials were compared to appropriate controls. A unit of GSH peroxidase is defined as the amount of enzyme required to oxidize 1 nmol NADPH ml^{-1} min^{-1}.

Test Materials Added	MgDVP
	% of control
1 mM DIECA	81
10 mM DIECA	<5
1 mM DSPD	51
1 mM sulfo-DSPD	108
5 mM glucose + 1 mg glucose oxidase	39
5 mM L-leucine + 0.5 mg L-amino acid oxidase	63
0.1 mM H_2O_2	95
0.5 mg catalase	99
4 mM GSH + 5 units GSH peroxidase	94
32 μM β-carotene	58

Sodium diethyldithiocarbamate (DIECA) is a Cu-chelating agent that has proven useful in the detection of Cu-containing enzymes. Although it has affinity for other divalent metals, it chelates Cu most strongly and gives 90% to 100% inhibition at 1 mM concentration for such Cu-containing proteins as ascorbic acid oxidase, polyphenol oxidase, and amine oxidase (15, 20). However, in our system DIECA inhibited less than 20% at 1 mM concentration (Table VI). The result does not support the involvement of a Cu-containing oxidase in the conversion of Mg-Proto to MgDVP.

The possible involvement of a third class of metalloproteins, Fe,S-proteins, in the conversion of Mg-Proto to MgDVP was examined. Addition of 0.2 mg of soluble spinach ferredoxin to our usual developing plastid material gave no stimulation; if the plastids were made more permeable either by a brief osmotic shock or by a wash with 0.35 M NaCl, slight stimulation (14–21%) by added spinach ferredoxin was observed (data not shown). If the plastids were washed vigorously to remove the endogenous ferredoxin, the activity was irreversibly lost. A known inhibitor of ferredoxin-dependent reactions in photosynthesis, DSPD (29), inhibited the formation of MgDVP by 50% at 1 mM (Table VI). However, in photosynthetic systems this compound was shown to inhibit noncyclic electron transport at sites other than ferredoxin (19). The Na sulfonate analog of DSPD, which was reported to be more specific for the ferredoxin site (19), gave no inhibition at 1 mM concentration (Table VI).

Inhibitions ranging between 37% and 61% were observed with H_2O_2-generating systems (glucose-glucose oxidase and L-leucine-L-amino acid oxidase). However, no effect was observed with 0.1 mM H_2O_2 or with 0.5 mg of catalase (Table VI).

The involvement of a peroxy-intermediate was tested by adding GSH and GSH peroxidase (27). This mixture had no effect on the formation of MgDVP (Table VI). Finally, the involvement of singlet oxygen was tested by the addition of β-carotene (8), which was found to produce a 42% inhibition at 32 μM concentration.

Three redox materials were found to be powerful inhibitors of MgDVP formation: PMS, MV, and MB; with all of these 50% inhibition occurred at 2 μM or less (Fig. 5). FMN was less

inhibitory, with 50% inhibition occurring at approximately 50 μM (Fig. 5). Of the other redox materials tested, DCPIP was inhibitory at 100 μM concentration (84% inhibition), but not at 20 μM; whereas $K_3Fe(CN)_6$, DHA, and GSSG did not inhibit at 100 μM.

DISCUSSION

In spite of the fact that our experimental tissue was exposed to continuous light for 20 h, it contained considerable levels of a phototransformable Pchlide component (Table I) reputed to be MgDVP. The identification of our product as MgDVP (Table II) must still be considered as tentative, although the product seems to correspond to the pigment identified as "divinyl protochlorophyllide" (MgDVP) by Belanger and Rebeiz (2). A more rigorous identification is needed and we are currently investigating the use of different spectroscopic techniques for this purpose. The relatively high steady-state concentration of MgDVP found in greening tissue suggests that the resynthesis of MgDVP must be fairly rapid with respect to both the reduction of the vinyl side chain in the 4 position and the photoreduction of the double bond in the 7 to 8 position.

The *in vitro* synthesis of MgDVP from Mg-Proto or Mg-Proto Me required SAM and O_2, in agreement with a previous report (4), and NADP(H). With the *in vitro* system saturated with respect to both SAM and Mg-Proto(Me), a 30% conversion of the porphyrin substrate to MgDVP was observed (Figs. 1 and 2, Table III). The stimulation of the reaction by SAM, even when the substrate was Mg-Proto Me, was explained by the observation of a hydrolytic side reaction that removed the methyl group from the carboxylate ester (Fig. 3).

The nature of the oxidizing reactions converting the methyl propionate side chain to the isocyclic ring has not been elucidated, but some aspects of this process are becoming clear: (a) Molecular O_2 is definitely required (Fig. 4 and [4]). (b) A hemoprotein terminal oxidase similar to P_{450} does not seem to be involved because little or no inhibition was observed with CO (Fig. 4), KCN, and NaN_3. Inhibitor studies likewise did not yield any strong leads as to the involvement of Cu-proteins, Fe,S-proteins, singlet O_2, or oxygen radicals (Table VI). (c) There is a striking inhibition by artificial electron aceptors, of which the reduced form is autooxidizable in air. In the case of the three strongest inhibitors, MB, PMS, and MV (Fig. 5), the uncharged reduction products could easily penetrate through the membranes to a lipophilic inhibition site.

In the absence of added plastids, 10 μM PMS caused a 96% decrease in added NADPH in a 60-min incubation, whereas 10 μM MB and 50 μM MV caused no detectable decrease in the added NADPH. In the presence of plastids a loss of the added NADPH was observed; this loss was increased enormously by 10 μM PMS, but only slightly by 10 μM MB or 50 μM MV. Thus, it seems possible that NADPH depletion is a factor in the inhibition of the reaction by PMS. On the other hand, the slight effect of NADPH oxidation by MB and MV does not appear sufficient to account for the observed inhibition of MgDVP formation. Perhaps FMN is a weaker inhibitor in this system (Fig. 5) because of the more polar character of reduced FMN, which restricts its diffusion into the membrane, and DCPIP is a weaker inhibitor because of its relatively high oxidizing potential ($E_0' = 0.22$ at pH 7.0) (26).

Granick (11) proposed that the formation of the isocyclic ring is preceded by the β-oxidation of the 6-methyl propionate side chain to the 6-ketoderivative of Mg-Proto Me. Ellsworth and Aronoff (5, 6) isolated from *Chorella* mutants a number of compounds that they identified as the proposed β-oxidation intermediates of both divinyl and vinyl-ethyl series, namely, as the acrylate, β-hydroxypropionate and β-ketopropionate derivatives of Mg-Proto Me and of dihydro Mg-Proto Me [4-ethyl(4-desvinyl)Mg-Proto Me]. The dihydro series would result if the reduction of the 4-vinyl side chain preceded the oxidation of the 6-methyl propionate side chain, in which case, the cyclization product would not be MgDVP, but Pchlide.

Our results do not support a conventional β-oxidation, analogous to the β-oxidation of long-chain fatty acyl-CoA derivatives, because such a process would be stimulated rather than inhibited by MB, PMS, and MV since these compounds act as electron carriers between reduced pyridine and flavin nucleotides and O_2. Moreover, in a conventional β-oxidation sequence, O_2 can be replaced by other terminal electron acceptors, for example by DCPIP in the presence of PMS. All attempts to replace O_2 by other acceptors in our system have given negative results.

At this point, we would favor a mechanism in which O_2 is directly involved in the reaction, possibly in the hydroxylation of the side chain to yield the β-hydroxypropionate intermediate. That Ellsworth and Aronoff (5, 6) isolated the acrylate derivative cannot be used as an argument against this type of mechanism because the dehydration of the β-hydroxypropionate ester would be greatly facilitated by the presence of the aromatic ring system on one side of the hydroxyl function, and of the carboxylate ester on the other side. Therefore, the acrylate derivative of Mg-Proto Me could be formed from the β-hydroxy derivative either *in situ* or in the course of the isolation.

In conclusion, an experimental system is now available that permits the *in vitro* synthesis of the isocyclic ring at rates of approximately 300 pmol mg^{-1} plastid protein h^{-1}. The formation of the cyclized product, MgDVP, seems to be relatively uncomplicated by side reactions. This system is suitable to test the effect of added substrates, cofactors, and inhibitors on the overall process. We think that it will be of great usefulness in analyzing the mechanism of this portion of the Chl biosynthetic pathway.

Note Added in Proof. The product of the *in vitro* incubation was identical with authentic MgDVP extracted from freeze-dried cells of *Rhodopseudomonas spheroides* mutant V3 (a gift of Dr. O. T. G. Jones) as shown by: chromatography on polyethylene thin layers, (b) emission and excitation spectra at 298 and 77 K. Moreover, NMR spectroscopy of the isolated *in vitro* product revealed three meso hydrogens and two vinyl side chains, supporting the assignment of the MgDVP structure (B. M. Chereskin, P. A. Castelfranco, and J. Dallas, unpublished data).

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