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Intermediates in the Formation of the Chlorophyll Isocyclic Ring[1]

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ABSTRACT

Cell-free, organelle-free synthesis of Mg-2,4-divinylpheoporphyrin a_5 (MgDVP) from Mg-protoporphyrin IX monomethyl ester (Mg-Proto Me) has been described (Wong and Castelfranco 1984 Plant Physiol 75: 658–661). This system consists of plastid membrane and stromal fractions and requires O_2, NAD(P)H and *S*-adenosylmethionine (SAM). The synthetic 6-methyl-β-ketopropionate analog of Mg-Proto Me was converted to MgDVP by the same catalytic system in the presence of O_2 and NADPH. SAM was not required. A compound (X) displaying the kinetic behavior of an intermediate was isolated from reaction mixtures with Mg-Proto Me as the substrate, but not with the 6-methyl-β-ketopropionate analog as the substrate. X was identified as the 6-methyl-β-hydroxypropionate analog of Mg-Proto Me by conversion to the dimethyl ester with CH_2N_2 and comparison with authentic 6-β-hydroxydimethyl ester. X was converted to MgDVP by the same catalytic system in the presence of O_2 and NADPH. We conclude that the conversion of Mg-Proto Me to MgDVP proceeds through the 6-β-hydroxy and the 6-β-ketopropionate esters in agreement with earlier suggestions.

The formation of the Chl isocyclic ring requires modification of the 6-methylpropionate side-chain of Mg-Proto Me.[2] Granick (10) first suggested that this transformation involves the β-oxidation of the 6-methylpropionate side-chain to a methyl-β-ketopropionate group. The active methylene carbon atom, situated between the carboxylate ester and the β-carbonyl, then becomes bonded to the γ-bridge carbon in an oxidizing cyclization reaction, during which two hydrogens are lost (Fig. 1; for further clarification of the chemical structures of postulated intermediates, see [16, Fig. 1]). Ellsworth and Aronoff (5, 6) described mutants of *Chlorella* that accumulated Mg-porphyrins containing methyl acrylate, methyl-β-hydroxypropionate, and methyl-β-ketopropionate side-chains. These correspond to the intermediates in the conversion of the 6-methylpropionate side-chain to the isocyclic ring as postulated by Granick. Chereskin *et al.* (3) proposed a mechanism in which molecular O_2 is directly involved in the reaction, possibly in the hydroxylation of the 6-methylpropionate side-chain to the β-hydroxypropionate ester.

Recently, we have prepared an organelle-free enzyme system from developing chloroplasts which is active in converting Mg-Proto Me to MgDVP in the presence of O_2 and NADPH (15). In the present study we have shown that the synthetic 6-methyl-β-ketopropionate analog can also be converted to the cyclized product by our complex multi-enzyme preparation. A preliminary account of this work has been presented (17). We also report the isolation of an intermediate in the enzymic cyclization reaction of Mg-Proto Me which has been identified as the 6-methyl-β-hydroxypropionate analog of Mg-Proto Me (Mg-HP Me).

MATERIALS AND METHODS

Materials. Hepes, Tes, NADPH (tetrasodium salt, type III enzymically reduced), SAM (chloride salt), cysteine-HCl, EDTA, sorbitol, and Proto Me_2 were obtained from Sigma. BSA (fatty acid poor) was obtained from Calbiochem. $MgCl_2$ was obtained from Baker. DMSO (reagent grade) was purchased from Matheson, Coleman, and Bell. Percoll was obtained from Pharmacia. Acetone, Hexanes, and diethyl ether (for anesthesia) were obtained from Mallinckrodt. Glass-distilled methanol and acetone (for HPLC) were purchased from Burdick and Jackson. PIC-Reagent A was obtained from Waters Associates and diluted according to the direction of the manufacturer.

Methods. *Plant Tissue.* Cucumber seeds (*Cucumis sativus* L. cv Beit Alpha) were germinated in the dark at room temperature for 5 to 6 d (12). Etiolated seedlings were exposed to white light (60–80 $\mu E\ m^{-2}\ s^{-1}$ PAR at 28–30°C) for 20 h.

Plastid Isolation and Preparation of Reconstituted Cyclase System. Isolation of developing cucumber chloroplasts by Percoll centrifugation and preparation of reconstituted cyclase system were as described by Wong and Castelfranco (15).

Incubation Conditions. Routine incubations were carried out in 1 ml of buffer containing 10 mM Hepes, 20 mM Tes, 1 mM $MgCl_2$, and 1 mM EDTA (pH 7.7). Substrates and cofactors appropriate to the reaction along with 5.8 to 9.6 mg plastid protein were included. Incubations were carried out in the dark to prevent phototransformation or photodestruction of newly synthesized MgDVP. All incubations were carried out at 30°C for 1 h and terminated by freezing at −15°C.

Preparation of Substrates. The biosynthesis of Mg-Proto Me was according to Chereskin *et al.* (3). Purified Mg-Proto Me was dissolved in 100% DMSO to a final concentration of 500 μM. The synthesis of DV Mg-KP Me was according to Goff (9). MV Mg-KP Me was synthesized by published methods (4). These esters have, respectively, emission maxima at 605 to 606 and 608, and excitation maxima at 425 and 420 (in diethyl ether at

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[2] Abbreviations: Mg-Proto Me, Mg protoporphyrin IX monomethyl ester; DV, 2,4-divinyl; MV, 2-vinyl,4-ethyl; MgDVP, Mg-2,4-divinyl pheoporphyrin a_5; Mg-Proto, Mg-protoporphyrin IX; Mg-Proto Me_2, Mg-protoporphyrin IX dimethyl ester; Mg-A Me, acrylate derivative of Mg-Proto Me (see Fig. 1 for structure); Mg-HP Me, β-hydroxy derivative of Mg-Proto Me (see Fig. 1 for structure); Mg-HP Me_2, 7-methylpropionate ester of Mg-HP Me; Mg-KP Me, β-keto derivative of Mg-Proto Me (see Fig. 1 for structure); Mg-KP Me_2, 7-methylpropionate ester of Mg-KP Me; Proto Me_2, protoporphyrin IX dimethyl ester; Pheobide, pheophorbide *a*; SAM, *S*-adenosylmethionine.

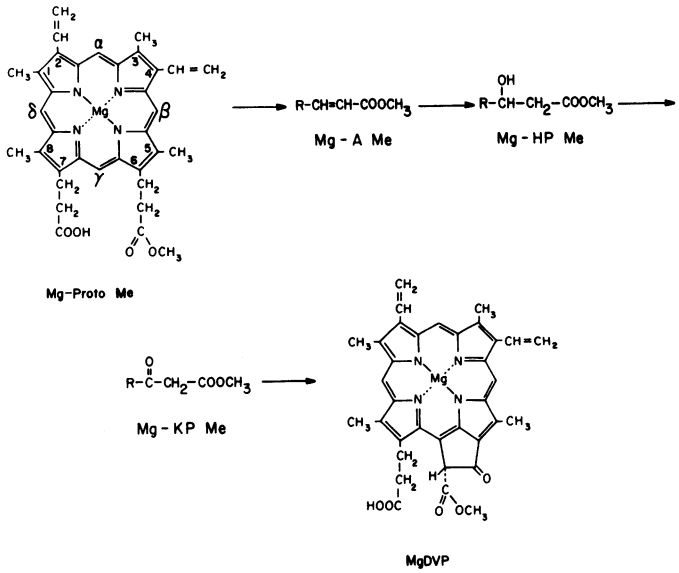

FIG. 1. Mechanism of conversion of Mg-Proto Me to MgDVP according to Granick (10).

297K). Concentration of these substrates were determined assuming molar emission coefficients equal to that of Mg-Proto Me.

Porphyrin Standards. Mg-Proto Me_2 standard was prepared from Proto-Me_2 by refluxing with $Mg(C10_4)_2$ in anhydrous pyridine (8). Mg-Proto Standard was prepared from Mg-Proto Me_2 as described by Chereskin and Castelfranco (2). Chlide standard was prepared just as Pchlide (2), except that etiolated cucumber cotyledons were exposed to white light (60–80 $\mu E\ m^{-2}\ s^{-1}$ PAR) for 5 min prior to harvest. Mg-HP Me_2 was prepared by $NaBH_4$ reduction of the DV Mg-KP Me_2 (D. A. Goff and K. M. Smith, unpublished data).

MgDVP Extraction and Determination. MgDVP formed during incubation was extracted into diethyl ether by a previously described procedure (1) and was determined using a Perkin-Elmer MPF 44-A fluorescence spectrophotometer as described by Chereskin *et al.* (3). To analyze reaction products by reversed-phase HPLC, diethyl ether extracts of incubation mixtures were washed with 50 mM phosphate buffer (pH 6.8) and H_2O. Dissolved H_2O in the ether was removed by freezing at 15°C overnight prior to injection.

HPLC Equipment. A Waters Associates model 6000A solvent delivery system was used. This system was equipped with a Rheodyne model 7125 injector, a Rheodyne solvent filter, a Beckman Ultrasphere-ODS 5 μm, 250 × 4.6 mm i.d., reversed-phase column, a Waters Associates model 440 absorbance detector set at 405 nm, and a Sargent model DSRG recorder. Solvent systems employed are described in figure legends. All solvents were filtered through Rainin Nylon-66, 0.45 μm pore size filter prior to use on HPLC.

RESULTS

Reactivity of Synthetic 6-β-Keto Analogs of Mg-Proto Me. MgDVP can be formed from Mg-Proto Me in a reconstituted cyclase system containing plastid membranes and a stromal protein fraction, O_2, and NADPH (15). Mg-Proto can also be used as substrate for MgDVP formation provided SAM is added, indicating that SAM-Mg-Proto methyl transferase (EC 2.1.1.11) is also present in this preparation. In the present study, synthetic 6-β-keto-propionate analogs of Mg-Proto Me (DV and MV Mg-KP Me) were tested (Tables I and II). Table I, experiment 1, shows that DV Mg-KP Me was converted to MgDVP by the cyclase system. If either enzymic component, membrane pellet, or stromal supernatant, was omitted from the incubation mixture, no activity was observed. O_2 and NADPH were both required for MgDVP synthesis. In the absence of either cofactor, no MgDVP was formed from DV Mg-KP Me (Table I, experiment 2).

Table I. *Requirements for the Conversion of DV Mg-KP Me to MgDVP by the Oxidative Cyclase System*

Component concentrations were: Mg-Proto Me and Mg-KP Me, 10 μM; NADPH, 5 mM; SAM, 1 mM. Experiment 1 contained 3.7 mg supernatant protein and 2.2 mg pellet protein per sample. Experiment 2 contained 5.8 mg plastid protein per sample, and experiment 3 9.1 mg plastid protein per sample. Other incubation conditions were as described in "Materials and Methods". In Tables I to III, entries represent the mean and the range of duplicates.

Incubation Conditions	MgDVP Formed
	$pmol \cdot mg^{-1} protein \cdot h^{-1}$
Experiment 1	
Mg-Proto Me	224
DV Mg-KP Me	169 ± 1
DV Mg-KP Me, minus pellet	Trace
DV Mg-KP Me, minus supernatant	Trace
Experiment 2	
No substrate added	Trace
Mg-Proto Me	217
DV Mg-KP Me	178 ± 5
DV Mg-KP Me, minus O_2	Trace
DV Mg-KP Me, minus NADPH	Trace
Experiment 3	
Mg-Proto Me	320 ± 14
Mg-Proto Me, minus SAM	171 ± 4
DV Mg-KP Me	169 ± 9
DV Mg-KP Me, minus SAM	169 ± 7

Table II. *Conversion of MV Mg-KP Me to Pchlide by the Cyclase System*

All samples contained 1 mM SAM, 5 mM NADPH, 10 μM MV Mg-KP Me, 4.0 mg supernatant protein, and 5.9 mg pellet protein, unless specified otherwise. Other incubation conditions were as described in "Materials and Methods".

Incubation Conditions	Pchlide Formed
	$pmol \cdot mg^{-1} protein \cdot h^{-1}$
MV Mg-KP Me	173 ± 7
MV Mg-KP Me, minus pellet	Trace
MV Mg-KP Me, minus supernatant	Trace

The stimulatory effect of SAM in the synthesis of MgDVP from Mg-Proto Me was previously explained as SAM counteracting the esterase activity found in our enzyme preparation by re-esterifying any Mg-Proto that was formed from Mg-Proto Me during incubation (3). This stimulatory effect of SAM was not observed when DV Mg-KP Me was used as substrate. DV Mg-KP Me was converted to MgDVP equally well, regardless of the presence of SAM in the reaction mixture (Table I, experiment 3).

Since Mg-Proto Me and MgDVP are DV compounds, it is logical to assume that the putative intermediates are also DV. However, MV Mg-KP Me was also active as substrate for the cyclization reaction. Table II shows that MV Mg-KP Me was converted to the corresponding cyclized product by the cyclase system. Low temperature fluorescence indicated that this cyclized product was Pchlide, the MV analog of MgDVP (excitation max at 450 nm in diethyl ether, 2-methylbutane, ethanol, 2:2:1 by volume, at 77°K [3]).

Isolation of the 6-β-Hydroxy Analog of Mg-Proto Me from

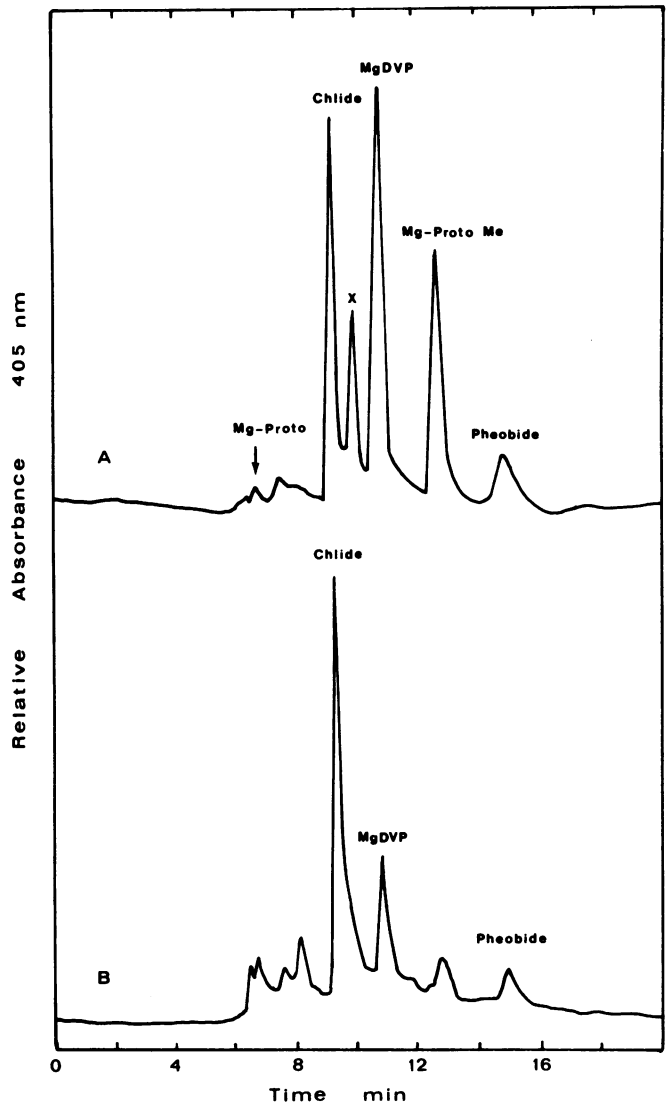

FIG. 2. Accumulation of X in incubation mixtures. All incubations contained 5 mM NADPH, 1 mM SAM, 5.8 mg plastid proteins, and 10 μM Mg-Proto Me (A) or 10 μM DV Mg-KP Me (B). Other incubation conditions were as described in "Materials and Methods". Column was developed at a flow rate of 1 ml/min with 70% methanol:30% 5 mM PIC Reagent A in H_2O. After 3 min, the solvent was changed to 70% methanol:30% H_2O, and the pigments were eluted in 20 min (7). Peaks were separated on HPLC, collected, and identified by comparison with standards.

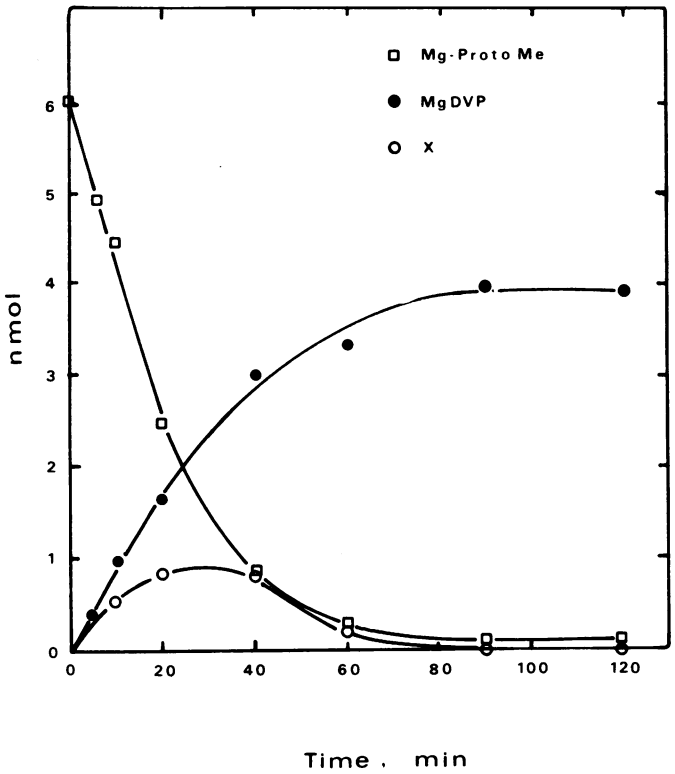

FIG. 3. Time-course study of the formation of X and MgDVP. All incubations contained 5 mM NADPH, 1 mM SAM, 10 μM Mg-Proto Me, and 9.6 mg plastid protein. Other conditions were as described in "Materials and Methods". At the end of each incubation, Mg-porphyrins were extracted into diethyl ether and separated on HPLC as described in Figure 2 legend. Mg-Proto Me, X, and MgDVP were then quantitized spectrofluorimetrically with reference to standards.

Table III. *Conversion of Compound X to MgDVP by the Cyclase System*

All samples contained 1 mM SAM, 5 mM NADPH, and 8.1 mg plastid protein. Two μM compound X and 2 μM Mg-Proto Me were included where indicated. Other incubation conditions were as described in "Materials and Methods".

Substrates	MgDVP Formed	Per Cent Conversion
X	966 ± 55	48
Mg-Proto Me	655 ± 4	33

Cyclization Reaction Mixtures. A compound (X) accumulated in the reaction mixture with Mg-Proto Me as substrate (Fig. 2A), but not in the reaction mixture with DV Mg-KP Me as substrate (Fig. 2B). X eluted between Mg-Proto and Mg-Proto Me on the reversed-phase column, suggesting that X is intermediate in polarity between these two compounds. X was collected from HPLC. Diethyl ether and H_2O were added to the 70% methanol eluate to cause a phase separation. The upper phase was withdrawn and washed with H_2O to remove any dissolved methanol. The excitation and emission maxima in ether at room temperature were identical to those of Mg-Proto Me; namely, excitation, 420 nm; emission, 594 nm. These spectral characteristics were not changed by treatment with CH_2N_2 (Table IV).

A study was carried out to determine whether the time course of accumulation of X was consistent with its possible role as a reaction intermediate. During a 2-h incubation period, X first accumulated and then disappeared while the final product, MgDVP, gradually built up until it leveled off (Fig. 3). This behavior suggested an intermediate-product relationship. Isolated, purified X was readily converted to MgDVP by the cyclase system (Table III). Both the polarity (between Mg-Proto and Mg-Proto Me) and spectrofluorimetric properties (identical to Mg-Proto Me) of X are consistent with the Mg-HP Me structure.

Identification of X was carried out by comparing the methyl derivative of X (methylated with CH_2N_2) with authentic Mg-HP Me_2. Methylated X had identical spectrofluorimetric properties as the authentic Mg-HP Me_2 (Table IV). These values matched those of Mg-Proto Me. These two compounds also showed identical retention times on reversed-phase HPLC in different solvent systems (Table V). Mg was removed from these two compounds by treatment with 2 N HCl, and the two free porphyrins were compared. Spectrofluorimetric properties and retention time of the Mg-free methylated X were identical to those

Table IV. *Spectrofluorimetric Properties of Derivatized X, Mg-HP Me_2, Mg-Proto Me_2, and Proto Me_2 in Diethyl Ether at 297 K*

X was methylated with CH_2N_2. Excess CH_2N_2 was added to 0.5 ml diethyl ether solution of X at 0°C. After 5 min, CH_2N_2 was removed under a stream of N_2. The methylated X residue was redissolved in diethyl ether. Acidification was carried out by adding two drops of 2 N HCl to 0.5 ml of metalloporphyrin solution in diethyl ether. After shaking, the diethyl ether was removed in a stream of N_2. Half ml diethyl ether was then added back and mixed with the aqueous phase. After phase separation, the diethyl ether containing the Mg-free pigments was withdrawn.

Compounds	Excitation Maximum	Emission Maximum
	nm	
Methylated X	420	594
Acidified methylated X	407	633
Mg-HP Me_2	420	594
Acidified Mg-HP Me_2	407	633
Mg-Proto Me_2	420	594
Proto Me_2	407	633

Table V. *Retention Times of Derivatized X and Mg-HP Me_2 on Reversed-Phase HPLC in Different Solvent Systems*

Column was developed at a flow rate of 1 ml/min with the mentioned solvents. Sample size: 2 to 10 μl.

	Methanol			Acetone	
Compounds	100%	98%	96%	80%	75%
Methylated X	3.5–3.6	4.1	4.9	3.8	6.2
Acidified methylated X	6.5				
Mg-HP Me_2	3.5–3.6	4.1	4.9	3.8	6.2
Acidified Mg-HP Me_2	6.5				

of the metal-free derivative of Mg-HP Me_2 (Tables IV and V). Both excitation and emission maxima matched Proto Me_2.

DISCUSSION

Rebeiz and *et al.* (13, 14) reported the accumulation of "longer wavelength metalloporphyrins" in cucumber cotyledons which were suggested to be intermediates between Mg-Proto Me and Pchlide. These longer wavelength metalloporphyrins were not characterized; however, the spectral characteristics reported by these authors are not inconsistent with the fluorimetric propeties of synthetic MV and DV Mg-KP Me.

The compound X, accumulated in reaction mixtures with Mg-Proto Me as the substrate, was identified as Mg-HP Me by comparison of its methylated derivative with authentic Mg-HP Me_2. Spectrofluorimetric and HPLC results both support the Mg-HP Me structure. While the synthetic Mg-HP Me_2 was used as a standard in this study, the corresponding monoester, Mg-6-hydroxypropionate methyl ester-7-propionic acid (Mg-HP Me), has not yet been synthesized. Therefore, direct biochemical studies starting with synthetic Mg-HP Me as substrate have not yet been carried out.

Both DV and MV Mg-KP Me were converted to their corresponding cyclized products, namely, MgDVP (DV Pchlide) and Pchlide (MV Pchlide) (Tables I and II). Therefore, this cyclase system seems to have no specificity toward the 4-position of the macrocycle. The substrate specificity of the cyclase system is further discussed in another paper (16).

According to proposed mechanisms (2, 3, 10), Mg-KP Me would be the immediate precursor of MgDVP. It was interesting to find that the requirements for its conversion to MgDVP were almost identical to the requirement for the conversion of Mg-Proto Me to MgDVP. The two enzymic components (plastid membrane pellet and stromal supernatant), O_2, and NADPH were all needed for activity (Table I). At this point, we do not know how O_2 and NADPH are involved in the conversion of Mg-KP Me to MgDVP. The only difference observed between reactions with the two substrates, Mg-Proto Me and Mg-KP Me, is related to the effect of added SAM. SAM had no effect on the conversion of Mg-KP Me to MgDVP. This may be explained by the hypothesis that either the methyl esterase (3) or the methyl transferase (EC 2.1.1.11) is active toward Mg-Proto Me but not toward Mg-KP Me. It is also known that β-keto esters are hydrolyzed less readily than the corresponding simple esters (11), and furthermore, any hydrolysis of the 6-β-ketopropionate ester produces the 6-β-ketopropionic acid, which is readily decarboxylated to the 6-acetyl compound (9). The latter is no longer a substrate for the enzymic cyclization reaction (Y.-S. Wong and D. A. Goff, unpublished data).

In conclusion, this study agrees with the proposed mechanisms in which the formation of the isocyclic ring from Mg-Proto Me involves β-hydroxy and β-keto intermediates. We do not know yet how the 6-β-hydroxypropioante ester is formed from the initial 6-propionate ester. Perhaps this reaction proceeds through an acrylate intermediate followed by the hydration of the α-β double bond as suggested by Granick (10) and Ellsworth and Aronoff (5, 6). Or perhaps the mechanism involves a direct attack on the β-carbon, by some reduced O_2 species produced by the interaction of NADPH and O_2 as in mixed function oxidase type reactions (3, 15).

The elucidation of the three specific biochemical steps in this sequence, namely, the formation of the β-hydroxypropionate ester from Mg-Proto Me, the oxidation of the β-hydroxypropionate ester to the corresponding β-ketopropionate ester, and the cyclization of the latter to mgDVP, will constitute the object of future research.

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