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Effects of Iron and Oxygen on Chlorophyll Biosynthesis[1]

II. OBSERVATIONS ON THE BIOSYNTHETIC PATHWAY IN ISOLATED ETIOCHLOROPLASTS

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ABSTRACT

The conversion of L-glutamate to δ-aminolevulinate, in preparations of cucumber etiochloroplasts incubated *in vitro*, was inhibited by protoheme IX and Mg-protoporphyrin IX. Mg-protoporphyrin IX was destroyed in the presence of air and plastids; this breakdown was accelerated by *S*-adenosyl methionine. Mg-protoporphyrin IX was also converted to protochlorophyllide *in vitro*. This conversion exhibited an absolute requirement for atmospheric oxygen and was strongly stimulated by *S*-adenosyl methionine and by darkness.

Based on these results, and on the results of the preceding paper (Spiller, Castelfranco, Castelfranco 1981 Plant Physiol 68: 107–111), a comprehensive hypothesis for the role of O_2 and Fe in chlorophyll biosynthesis is formulated.

It is widely accepted that either Fe or O_2 deficiency results in chlorosis in higher plants (19). There is also an abundance of experimental data on the effects of manipulation of these two nutrients on Chl levels (19). However, as yet there is no unified explanation for these many and diverse observations.

O_2 is required in higher plant tissues for the accumulation of ALA[3] (2). It is probable that this *in vivo* O_2 requirement reflects the need for molecular O_2 in aerobic respiration, which is necessary to generate ATP in the common test plants (*e.g.* cucumber, bean, barley). This O_2 requirement, however, persists in isolated plastids incubated in the presence of added ATP. Anaerobiosis caused a 30% inhibition in the conversion of [^{14}C]glutamate to [^{14}C]ALA in cell-free plastid preparations from greening cucumber cotyledons (23) and 58% inhibition in plastids from greening barley (14). The reason for this O_2 requirement in isolated plastids supplied with ATP is not known. There is also an inhibition by anaerobiosis of the conversion of ALA to Proto by isolated cucumber etiochloroplasts (5). This O_2 requirement appears to be at the coproporphyrinogen oxidase level. Anaerobiosis does not interfere with Pchlide photoreduction but prevents the subsequent transformation of the newly formed Chlide to Chl *a* (21). In particular, the reduction of geranylgeranyl Chlide is blocked by anaerobiosis (17).

Several workers have shown that iron deficiency induced either by growing plants on Fe-deficient media (19) or by chelating the endogenous Fe with reagents such as α,α′-DP, *o*-phenanthroline and 8-hydroxyquinoline (7, 20) results in the accumulation of intermediates in the Chl biosynthetic pathway. Another effect of Fe deficiency might be to decrease Heme formation. Heme synthesis has been shown to occur in both etioplasts and mitochondria of barley (15), in which tissue Heme and Chl share a common ALA pool (4). It is probable that there is some plastidic control over the relative rates of Heme and Chl synthesis from common precursors. Therefore, interference with Heme synthesis by artificially lowering the activity of Fe^{2+} ion should alter the levels of the intermediates of the Chl branch.

This paper reports on the effects of Fe deficiency induced by the chelating agent, α,α′-DP, and of anaerobiosis on Chl biosynthesis in cell-free plastids isolated from cucumber cotyledons. Three segments of the Chl biosynthetic pathway were studied: (a) the conversion of Glu to ALA; (b) the conversion of ALA to Proto and Mg-Proto; and (3) the conversion of Mg-Proto to Pchlide.

MATERIALS AND METHODS

Plant Tissue. Cucumber seeds (*Cucumis sativus* L., cv. Beit Alpha MR 46811-6180 or Alpha Green 46714-5224) were germinated in the dark at room temperature for 5 to 6 days as described previously (11). The seedlings were exposed to white light (40–50 $\mu E\ m^{-2}s^{-1}$ PAR, General Electric F40WW mainlighter warm white fluorescent bulbs). For the assays using Glu or ALA as starting material, the light exposure lasted 4 h. For assays using Mg-Proto as starting material, greening time was increased to 20 h.

Isolation of Etiochloroplasts. Etiochloroplasts were isolated from greening cucumber cotyledons by previously published procedures. They were used for experiments requiring four different assays: (a) Glu conversion to Proto and Mg-Proto (5, 6); (b) ALA conversion to Proto and Mg-Proto (6); (c) Glu conversion to ALA (23); (d) Mg-Proto conversion to Pchlide. For the assay of porphyrin accumulation from Glu or ALA, plastids were isolated by the method of Weinstein and Castelfranco (22). Plastids for the other assays were obtained by the procedure of Pardo *et al.* (16). The conversion of Glu to ALA utilized P_2 of that method, while the conversion of Mg-Proto to Pchlide utilized P_3. The plastid pellets were resuspended in solution A (0.5 M sucrose, 1 mM $MgCl_2$, 1 mM EDTA, 20 mM Tes, 10 mM Hepes [pH 7.7]) with appropriate cofactors and cosubstrates.

Incubation Conditions. All samples were incubated with substrates and cofactors appropriate to the particular reaction and 1.4 to 9.9 mg plastid protein in 1 ml solution A. All incubations were terminated by freezing at −15 °C. Other treatments are specified in the figure and table legends.

Preparation of Mg-Proto Substrate. Mg-protoporphyrin dimethyl ester was hydrolyzed to the diacid in 1.5 M KOH in 80% methanol overnight at room temperature. Half volume H_2O and one volume diethyl ether were added. The upper phase containing

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[3] Abbreviations: ALA, δ-aminolevulinic acid; Proto, protoporphyrin IX; α,α′-DP, α,α′-dipyridyl; Heme, Fe-protoporphyrin IX; Mg-Proto, Mg-protoporphyrin IX; Mg-Proto Me_2, Mg-protoporphyrin IX dimethyl ester; Mg-Proto(Me), a mixture of Mg-Proto and Mg-Proto Me of unknown proportion; Proto Me_2, protoporphyrin IX dimethyl ester; SAM, *S*-adenosyl methionine; Mg-Proto Me, Mg-protoporphyrin IX monomethyl ester.

the unreacted Mg-Proto Me_2 was discarded. Then, ½ volume saturated NaCl and 1/16 volume 0.25 M monosodium maleate were added. The pH was adjusted to 6.8 and the sample was extracted repeatedly with diethyl ether. The ether was washed with water and the dissolved water was removed by freezing. The ether was then evaporated under reduced pressure to a volume of 5 ml. The concentration was determined by the *A* at 419 nm using an extinction coefficient of 308 mM^{-1} (13). The Mg-Proto was then extracted quantitatively into equivolume 10 mM KOH.

Pigment Extraction and Assay Procedure. Porphyrins formed during the *in vitro* assays were extracted into diethyl ether by a previously described procedure (6). Porphyrins and metalloporphyrins in the diethyl ether extracts were assayed using a Perkin-Elmer PMF 44-A fluorescence spectrophotometer. The results in Table I were obtained using an excitation wavelength of 410 nm with a 20 nm slit width. The emission peak heights for Mg-Proto and Proto were measured respectively at 594 and 634 nm with a 4-nm slit width. For all other experiments, the excitation wavelength was 420 nm with a 20-nm slit width. Emission peak heights for Mg-Proto(Me) and Pchlide were measured with a 2-nm slit width. The position of the peak maximum for Pchlide was at 628 to 629 nm. Uncorrected spectra were obtained with a scan speed of 60 nm/min. Concentrations were determined by reference to standard Mg-Proto Me_2, Proto Me_2, and Pchlide measured under identical conditions.

Spectrofluorimetric Standards. The standards were synthetic Mg-Proto Me_2 (a gift of Dr. Kevin Smith), Proto Me_2 (Sigma), and Pchlide prepared from cucumber cotyledons by a modification of the procedure of Castelfranco *et al.* (6) for extraction of other Mg-porphyrins. Cotyledons were picked under green safe light from 5-day-old dark grown cucumbers and ground with mortar and pestle in acetone: 1 M NH_4OH (9:1), using 5 ml of solvent for each gram of tissue. The brei was centrifuged at 43,500*g* for 10 min. The pellets were washed in ammoniacal acetone and the supernatants combined. The supernatants (200 ml) were then washed 4 times with 150 ml hexanes. To the lower phase, an equal volume of saturated NaCl and 1/60 volume of 0.25 M monosodium maleate were added, and the pH adjusted to 6.8 with HCl. The aqueous solution was then extracted with equal volumes of diethyl ether until most of the fluorescence was transferred to the upper phase. The diethyl ether was then washed with ⅓ volume of 50 mM K-phosphate (pH 6.8), and twice with ⅓ volume distilled H_2O. It was then dried by freezing out the water at −15 °C and concentrated by evaporation under reduced pressure. The Pchlide concentration in the diethyl ether was determined spectrophotometrically using the equations of Anderson and Boardman (1). This Pchlide preparation was used to prepare standard curves for the quantitation of Pchlide synthesized in our incubations.

Other Procedures. [^{14}C]ALA formed *in vitro* from L-[U-^{14}C]glutamate was isolated and quantitated by the procedure of Weinstein and Castelfranco (23).

Protein was determined by the biuret method using BSA as standard.

RESULTS

The effects of the iron chelating agent, α,α′-DP, on some partial reactions of the Chl biosynthetic pathway are shown in Tables I and II. Addition of α,α′-DP had no effect on the accumulation of Proto and Mg-Proto(Me), if the substrate was ALA (Table I, experiment 2), but strongly stimulated this accumulation if the substrate was Glu (Table I, experiment 1).

The inhibition of ALA synthesis in the presence of Heme, Mg-Proto, and Proto is shown in Figure 1, and the effect of α,α′-DP on the conversion of Glu to ALA, in the presence of Heme, Mg-Proto, and Proto is given in Table II. Experiment 1 shows that α,α′-DP alone stimulated the formation of ALA from Glu. Heme was clearly an inhibitor of ALA formation from Glu and α,α′-DP had no effect on this inhibition. Proto also inhibited ALA formation, but the inhibition was completely reversed by α,α′-DP. The most likely explanation for this observation is that Proto did not inhibit ALA formation directly, but was converted to Heme in our plastid preparation which then inhibited ALA synthesis. The formation of Heme from exogenous Proto was prevented by α,α′-DP, and therefore, no inhibition of ALA formation was observed. Experiment 2 shows that the inhibition by added Mg-Proto was partially reversed by inclusion of α,α′-DP. This result suggests the presence of an inhibition by endogenous Heme formed during the incubation.

The conversion of Mg-Proto to Pchlide by an etiochloroplast preparation from cucumber cotyledons required air and SAM. Figure 2 shows the disappearance of Mg-Proto and the accumulation of Pchlide as a function of time in the presence of SAM, in air and in N_2. The incubations were carried out in the dark to prevent the photoreduction of newly synthesized Pchlide to Chlide. In air, the accumulation of Pchlide leveled off after 40

Table I. *Effect of α,α′-DP on Porphyrin Accumulation from Glu or ALA*

Plastids were incubated in 1 ml solution A (pH 7.7), containing 4 μmol GSH, 0.6 μmol NAD, and 2 mg BSA in low light (3–5 μE m^{-2} s^{-1} PAR); 0.2 μmol α,α′-DP was included where indicated. In experiment 1, 8.0 mg plastid protein, 80 nmol ALA, and 10 μmol ATP were added. In experiment 2, 5.6 mg plastid protein, 5 μmol Glu, and 1.5 μmol ATP were added. The mean and range for two samples are given.

	Proto	Mg-Proto
	pmol mg^{-1} h^{-1}	
Experiment 1		
+ Glu	30.0 ± 6.7	6.14 ± 0.17
+ Glu + α,α′-DP	56.6 ± 2.3	29.6 ± 0.5
Experiment 2		
+ ALA	30.0 ± 1.0	15.8 ± 0.8
+ ALA + α,α′-DP	30.1 ± 0.3	15.3 ± 0.2

Table II. *Effect of α,α′-DP, Heme, Mg-Proto, and Proto on the Conversion of Glu to Ala*

Plastids were incubated in 1 ml solution A (pH 7.7) containing 1 μmol L-[U-^{14}C]Glu (0.5 μCi/μmol), 1.5 μmol ATP, 0.6 μmol NAD, 4 μmol GSH, 2 mg BSA, and 2 μmol levulinic acid; 0.2 μmol α,α′-DP was included where indicated. Proto and Heme concentrations in experiment 1 were 10 μM. Plastid protein was 8.5 mg in experiment 1, and 9.9 mg in experiment 2. All incubations were for 1 h at 37°C under low light (3–5 μE m^{-2} s^{-1} PAR). Adapted from reference (3) by permission of the publisher. The mean and range for two samples are given in experiment 1 and in succeeding tables.

	Rate of ALA Formation
	pmol h^{-1}
Experiment 1	
Control	1,324 ± 0.5
α,α′-DP	2,141 ± 151
Proto	771 ± 53
Proto + α,α′-DP	1,653 ± 25
Heme	258 ± 9
Heme + α,α′-DP	287 ± 21
Experiment 2	
Control	1,161
1.4 M Mg-Proto	628
1.4 M Mg-Proto + α,α′-DP	873
2.8 M Mg-Proto	430
2.8 M Mg-Proto + α,α′-DP	847
4.6 M Mg-Proto	442
4.6 M Mg-Proto; α,α′-DP	812

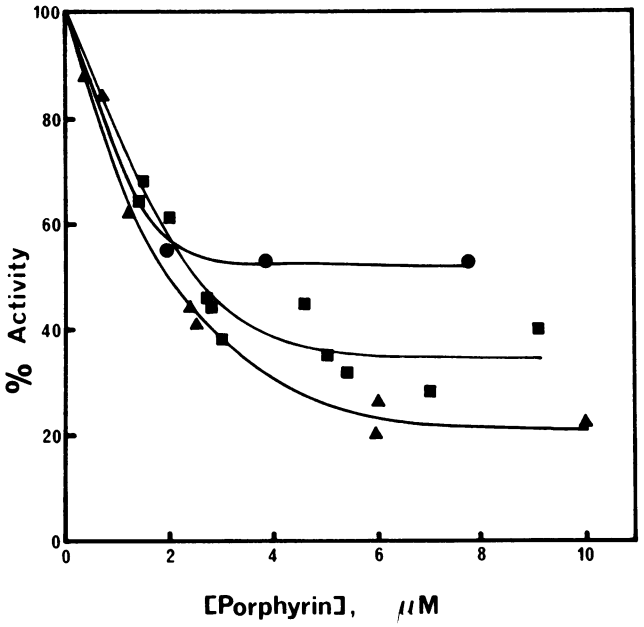

FIG. 1. Inhibition of ALA synthesis by Proto, Mg-Proto, and Heme. Plastids were incubated in 1 ml solution A (pH 7.7), containing 1 μmol L-[U-^{14}C]Glu (0.5 μCi/μmol) 1.5 μmol ATP, 0.6 μmol NAD, 4 μmol GSH, 2 mg BSA, 2 μmol levulinic acid, and 5.6 to 9.9 mg plastid protein. The inhibition of ALA formation by Heme (▲), Mg-Proto (■), and Proto (●), was studied in 2, 3, and 1 experiments, respectively. All incubations were for 1 h at 37°C.

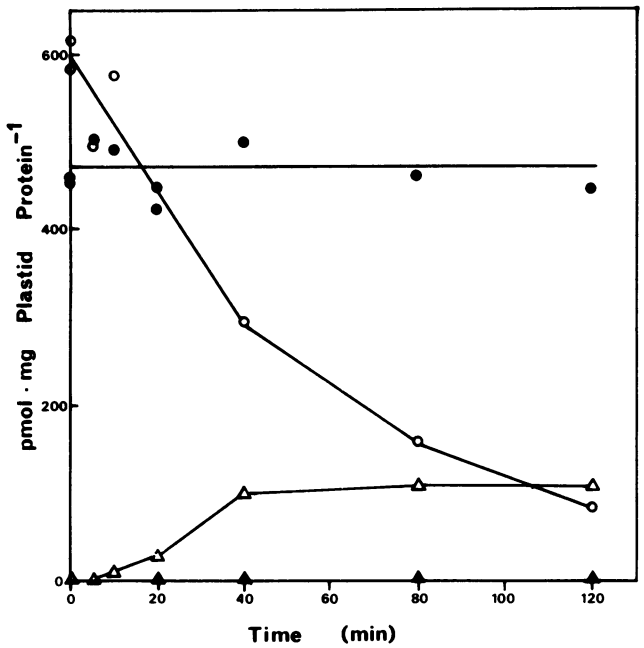

FIG. 2. Mg-Proto (●, ○) disappearance, and Pchlide (▲, △) formation in air (○, △) and nitrogen (●, ▲). Plastids were incubated in 1 ml solution A (pH 7.7), containing 1 nmol Mg-Proto, 1 μmol SAM, 4 μmol GSH, and 2 mg BSA. Anaerobic samples were flushed with N_2 for 5 min prior to incubation. Reaction vessels were incubated for the times indicated at 30°C wrapped in foil. Plastid protein was 3.8 mg/flask for the aerobic samples and 3.6 mg/flask for the anaerobic samples.

min at 30 °C; Mg-Proto disappearance, however, continued for the duration of the experiment. Neither Mg-Proto disappearance nor Pchlide accumulation occurred in N_2 atmosphere.

Mg-protoporphyrin IX disappearance was also demonstrated (although at a slower rate) in the absence of added SAM (Fig. 3). Increasing amounts of plastids increased the rate of Mg-Proto disappearance, both in the presence and in the absence of added SAM. Pchlide formation was stimulated by added SAM and by increased amounts of plastids.

Pchlide formation from Mg-Proto in the presence of SAM and plastids also exhibited light-dark effects (Table III). Dark incubation enhanced Pchlide accumulation in the presence and the absence of added SAM. The combined effects of SAM plus dark were more than additive.

In the presence of SAM, plastids and light (line 2), there was a large drop in Mg-Proto that was not accounted for by the Pchlide accumulated; but in the dark control (line 4), the drop in Mg-Proto appeared to be balanced stoichiometrically by the formation of Pchlide.

In a separate experiment, the effect of room lighting on Mg-Proto in the absence of plastids was investigated (data not shown). Even at this low light intensity (3–5 μE $m^{-2}s^{-1}$ PAR) there was 26% photodecomposition of Mg-Proto at 30 °C in 1 h. Added SAM had no effect on Mg-Proto in the absence of plastids. Strong

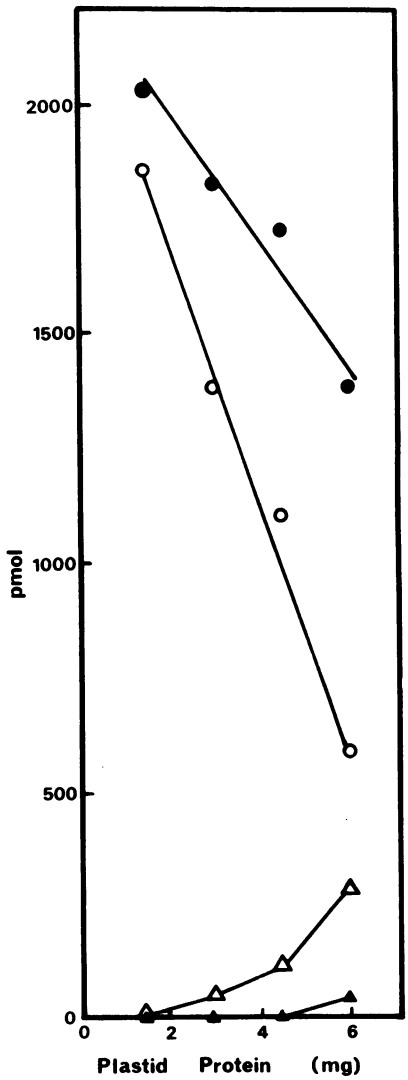

FIG. 3. Mg-Proto (●, ○) disappearance, and Pchlide (▲, △) formation with increasing plastid protein in the presence (○, △) or absence (●, ▲) of SAM. Plastids were incubated in 1 ml of solution A (pH 7.7), containing 1.2 nmol Mg-Proto, 4 μmol GSH, and 2 mg BSA; 1 μmol SAM was included where indicated. All reaction vessels were incubated for 1 h at 30°C, wrapped in foil.

Table III. *Effect of Light and Dark on the Conversion of Mg-Proto to Pchlide in the Presence and Absence of SAM*

Plastids were incubated in 1 ml of solution A (pH 7.7), containing 1.2 nmol Mg-Proto, 4 μmol GSH, 2 mg BSA, and 5.5 mg plastid protein. One μmol SAM was included, where indicated. Dark samples were wrapped in foil; light samples were exposed to 3 to 5 μE m^{-2} s^{-1} PAR. All samples were incubated 1 h at 30°C.

Incubation Conditions	Mg-Proto(Me)	Pchlide
	pmol	
− SAM, light	881 ± 125	0.0
+ SAM, light	98.2 ± 0.5	86.9 ± 3.9
− SAM, dark	1,078 ± 67	271 ± 2
+ SAM, dark	488 ± 37	609 ± 19

Table IV. *Effect of SAM or ATP plus Met on the Conversion of Mg-Proto to Pchlide*

Plastids (5.3 mg protein) were incubated in 1 ml solution A (pH 7.7), containing 1 nmol Mg-Proto, 4 μmol GSH, and 2 mg BSA; 1 μmol SAM, or 5 μmol ATP and/or 2 μmol Met were included where indicated. Reaction vessels were incubated 1 h at 30°C, wrapped in foil.

Incubation Conditions	Pchlide
	pmol mg^{-1} h^{-1}
+ ATP	25.0 ± 0.9
+ Met	20.2 ± 0.5
+ ATP, + Met	43.2 ± 0.0
+ SAM	33.6 ± 2.1

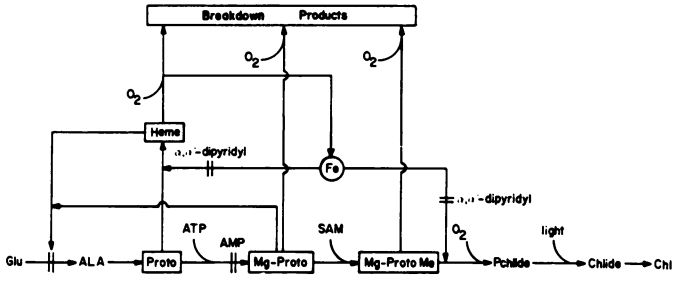

FIG. 4. Role of O_2 and Fe in Chl biosynthesis.

light (90 μE $m^{-2}s^{-1}$ PAR, 20 w F20T12-CW Cool White, Westinghouse) caused almost complete disappearance of Mg-Proto in the absence of plastids, at 30 °C in 1 h (data not shown).

The addition of α,α′-DP in the presence of SAM caused a partial inhibition of the conversion of Mg-Proto to Pchlide, which never exceeded 30% under our experimental conditions (data not shown). Attempts to stimulate Pchlide formation by addition of $FeSO_4$ to the reaction flasks were unsuccessful.

Table IV shows that the effect of added SAM on the conversion of Mg-Proto to Pchlide could be duplicated by the addition of ATP plus Met. L-Methionine plus ATP supported Pchlide formation from Mg-Proto better than either Met or ATP alone.

DISCUSSION

Figure 4 gives a schematic representation of Chl biosynthesis emphasizing the points where this process is affected by O_2 and Fe. According to this scheme, the stimulation of Proto and Mg-proto synthesis from Glu by α,α′-DP: (Table I) occurs because the step between Glu and ALA is inhibited by Heme (Fig. 1, Table II), and Heme synthesis in the plastids is prevented by Fe sequestering agents such as α,α′-DP. Mg-protoporphyrin IX and, possibly, Mg-Proto Me are also effective feedback inhibitors of ALA synthesis (Table II). The concentration range in which these metabolites are effective *in vitro* leads us to postulate that Heme

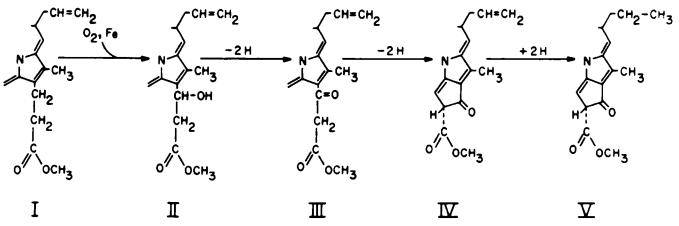

FIG. 5. Conversion of Mg-Proto Me to Pchlide. I, Mg-Proto Me; II, hydroxy-derivative of Mg-Proto Me; III, keto-derivative of Mg-Proto Me; IV, Mg-2, 4-divinyl pheoporphyrin A_5; V, Pchlide.

and Mg-protoporphyrins are likewise feedback regulators of tetrapyrrole synthesis *in vivo*.

Heme and Mg-protoporphyrins are also sensitive to oxidative degradation. Mg-porphyrins are destroyed by cell-free plastid preparations in the presence of O_2 (Fig. 2) and an O_2-dependent Heme destruction has been observed in plant tissue homogenates (Jones and Hendry, personal communication). Therefore, we think that molecular O_2 is involved in the oxidative breakdown of the primary feedback inhibitors that control ALA synthesis. It has been pointed out that O_2 stimulates the conversion of [^{14}C]Glu to [^{14}C]ALA in etiochloroplasts, even in the presence of added ATP (14, 23). This stimulation may be due to the aerobic breakdown of minute quantities of Heme and Mg-Proto that are present in the plastids and partially inhibit the activity of the ALA-synthesizing system.

The role of O_2 in the conversion of Mg-Proto to Pchlide is more striking. This conversion is absolutely dependent on atmospheric O_2 (Fig. 2). An Fe-containing oxygenase has been proposed to catalyze this conversion (7, 12). However, we were unable to demonstrate a requirement for Fe within the time frame of our own *in vitro* experiments. We did see a consistent partial inhibition of Pchlide formation by α,α′-DP, but no stimulation by added Fe. Probably, Fe is needed for the synthesis of an Fe-containing protein, but once this enzyme is formed, there is only a very slight dissociation of the metal ion. Therefore, the addition of Fe salts and addition of Fe-chelators do not cause dramatic changes in short-term experiments with isolated plastids. Vlcek and Gassman (20), in long-term experiments (6–12 h), have shown that α,α′-DP prevents the conversion of Mg-Proto Me to Pchlide in detached bean leaves.

The role of this Fe-containing enzyme system might be to hydroxylate the β-carbon of the C-6 methyl propionate sidechain (*i.e.* the methylene group, α to the macrocycle) (Fig. 5). This transformation could be accomplished either by a mixed function oxidase, or by dehydrogenation followed by hydration of the resulting double bond as proposed by Ellsworth and Aronoff (8, 9). The Fe requirement could be explained in either case. A mixed function oxidase could be a complex Fe-protein, while a dehydrogenase would produce a reduced cosubstrate (probably, a flavin) which might be reoxidized by molecular O_2, and Fe could be involved in this reoxidation step. Subsequently, the hydroxyl group could be oxidized to a keto group followed by the formation of the cyclopentanone ring as proposed by Granick (10).

In our incubations, we have failed to detect any emission peaks between 600 and 615 nm, which have been ascribed to the biosynthetic intermediates between Mg-Proto Me and Mg-2,4-divinyl pheoporphyrin A_5 (18). Possibly, the steps after the initial hydroxylation are very fast under our own experimental conditions, and thus these intermediates do not accumulate.

The availability of SAM appears to be important in controlling the methylation of Mg-Proto and, therefore, the synthesis of Chl in developing plastids. The enzyme Met adenosyl-transferase (EC 2.5.1.6) seems to be present in the intact plastids because ATP plus Met can be as effective in stimulating the conversion of Mg-Proto to Pchlide as added SAM (Table IV).

The *in vitro* conversion of Mg-Proto to Pchlide increased if the reaction was carried out in the dark (Table III). This increase in Pchlide recovery could result from protection of substrate (Mg-Proto), intermediate (Mg-Proto Me), or product (Pchlide) from photochemical breakdown. It could also reflect the light requirement for Pchlide conversion to Chlide.

These experiments support the proposal that an Fe-dependent oxygenase is involved in the conversion of Mg-Proto Me to Pchlide. The findings form a picture of the roles of Fe and O_2 in Chl biosynthesis which can be tested further by *in vivo* and *in vitro* experiments.

Acknowledgments—We are obliged to Dr. Kevin Smith for his generous gift of Mg-Proto Me_2. The cucumber seed was a gift of Moran Seeds, Inc., Box 3091, Modesto, CA. Mr. T. P. Fuesler helped by drawing the figures and proof reading the manuscript. We are indebted to Elsevier/North Holland for permission to reproduce Table II from reference (3).

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