Plant Physiol. (1990) 92, 172–178
0032-0889/90/92/172/07/$01.00/0

Received for publication June 6, 1989
and in revised form August 29, 1989

Regulation of 5-Aminolevulinic Acid (ALA) Synthesis in Developing Chloroplasts[1]

III. Evidence for Functional Heterogeneity of the ALA Pool

Laiqiang Huang[2] and Paul A. Castelfranco*

Department of Botany, University of California, Davis, California 95616

ABSTRACT

Gabaculine and 4-amino-5-hexynoic acid (AHA) up to 3.0 millimolar concentration strongly inhibited 5-aminolevulinic acid (ALA) synthesis in developing cucumber (*Cucumis sativus* L. var Beit Alpha) chloroplasts, while they hardly affected protochlorophyllide (Pchlide) synthesis. Exogenous protoheme up to 1.0 micromolar had a similar effect. Exogenous glutathione also exhibited a strong inhibitory effect on ALA synthesis *in organello* but hardly inhibited Pchlide synthesis. Pchlide synthesis *in organello* was highly sensitive to inhibition by levulinic acid, both in the presence and in the absence of gabaculine, indicating that the Pchlide was indeed formed from precursor(s) before the ALA dehydratase step. The synthesis of Pchlide in the presence of saturating concentrations of glutamate was stimulated by exogenous ALA, confirming that Pchlide synthesis was limited at the formation of ALA. The gabaculine inhibition of ALA accumulation occurred whether levulinic acid or 4,6-dioxohepatonic acid was used in the ALA assay system. ALA overproduction was also observed in the absence of added glutamate and was noticeable after 10-minute incubation. These observations suggest that although Pchlide synthesis *in organello* is limited by ALA formation, it does not utilize all the ALA that is made in the *in organello* assay system. Gabaculine, AHA, and probably also protoheme, inhibit preferentially the formation of that portion of ALA that is not destined for Pchlide. A model proposing a heterogenous ALA pool is described.

ALA[3] is the universal precursor for the synthesis of Chl and other tetrapyrroles. Since it was discovered that in higher plants ALA is synthesized from the intact carbon skeleton of Glu (2, 3), the C-5 pathway has been extended to algae, cyanobacteria, and many other bacteria (4). It is now generally believed that in plants and most algae ALA destined for all cellular tetrapyrroles is synthesized via the C-5 pathway in the plastid (4). Studies with particulate-free extracts from various organisms have revealed that transformation of Glu to ALA requires at least a tRNA and three enzymes, *i.e.* glutamyl-tRNA synthetase, glutamyl-tRNA, hydrogenase, and GSA aminotransferase (4, 14, 22).

Gabaculine, an inhibitor of certain ω-aminotransferases, has been reported to inhibit tetrapyrrole synthesis in plants and algae by preventing the synthesis of ALA (7, 9, 11, 21, 27). It has been shown to block ALA synthesis by inhibiting GSA aminotransferase, which catalyzes the conversion of GSA to ALA (12, 21, 24). AHA, another ω-aminotransferase inhibitor, has been reported to inhibit Chl and phytochrome chromophore synthesis in plants, more strongly than GAB (8). Exogenous protoheme has long been known as a potent inhibitor of ALA synthesis in various biological systems (4, 5). Of the three enzyme activities required for ALA formation from Glu, protoheme exerts its effect on the dehydrogenase (13) and probably also the ligase (4). The physiological role of endogenous heme in regulating ALA synthesis and the tetrapyrrole pathway is yet to be elucidated.

Despite the recent rapid advance in the study of the individual enzymatic steps, there is still a wide gap in our comprehension of the regulation of ALA synthesis and of the ways in which ALA is utilized for the synthesis of all cellular pyrroles.

In our studies of Chl biosynthesis, we have used a preparation of intact, developing chloroplasts isolated from greening cucumber cotyledons that is competent to carry out both Pchlide and ALA synthesis from Glu (15, 16). With this biological system, we have previously observed that Pchlide synthesis from Glu is controlled at ALA formation (17). Yet, more ALA is produced than is needed to account for Pchlide synthesis; this 'ALA-overproduction' is not the consequence of a feedback inhibition of ALA synthesis by Pchlide (17). A large portion, but not all, of the ALA-synthesizing capacity assayed *in organello* was dependent upon light and dark treatments of the seedlings prior to chloroplast isolation (17), and this light regulation was shown to be a low fluence phytochrome response (19). Therefore, it seemed that part of the ALA, including the ALA that is related to Pchlide synthesis, was under phytochrome control, while the rest was not phytochrome-regulated and was not involved in Pchlide synthesis; this raised the suspicion of a basic heterogeneity in the plastidic ALA pool.

In the present study, we have investigated the effects of GAB, AHA, protoheme, and GSH on both ALA and Pchlide synthesis in the isolated chloroplasts and have obtained additional evidence for the functional heterogeneity of the plas-

[1] Supported by National Science Foundation Grant DMB 8415321.

[2] Present address: Carnegie Institution of Washington, Department of Plant Biology, Stanford, CA 94305

[3] Abbreviations: ALA,5-aminolevulinic acid≥A, 4-amino-5-hexynoic acid; DMABA, *p*-dimethylaminobenzaldehyde; DOHA, 4,6-dioxoheptanoic acid; GAB, gabaculine, or 3-amino-2,3-dihydrobenzoic acid; GSA, glutamate-1-semialdehyde; [H], reducing power consisting of 4 mM NADPH and 4 mM glucose-6-phosphate; LVA, levulinic acid; SAM, *S*-adenosyl-L-methionine.

tidic ALA pool. A preliminary account of this work has been presented elsewhere (18).

MATERIALS AND METHODS

Materials

Cucumber (*Cucumis sativus* L. var Beit Alpha) seed was from Harris Moran Seeds, Salinas, CA 93901.

Synthetic ALA pyrroles (2-methyl-3-carbethoxy-4-[3-propionic acid]pyrrole and 2-methyl-3-acetyl-4-[3-propionic acid]pyrrole) and GSA pyrrole (2-methyl-3-acetyl-5-[3-propionic acid]pyrrole) were kindly provided by Drs. Kevin M. Smith and Paul Liddell, Department of Chemistry, University of California, Davis, CA 95616.

The following were purchased from Sigma: ATP, NADPH, glucose-6-phosphate, Glu, GSH, LVA, SAM, ALA, DMABA, cysteine, EDTA, Hepes, Tes, sorbitol, Percoll, and Coomassie brilliant blue G.

Bovine serum albumen and DOHA were obtained from Calbiochem. GAB was from Fluka AG, Switzerland. AHA was from Merrell Dow Research Institute Pharmaceuticals Inc., Cincinnati, OH 45215. Protoheme IX was from Porphyrin Products, Logan, UT 84321; $MgCl_2 \cdot 6H_2O$ was from Mallinckrodt. Ethyl acetoacetate and acetylacetone were from Aldrich; SDA (Duponol) was from Dupont, and Cation Exchange Resin (AG 50W-X8, 100–200 mesh, hydrogen form) was from Bio-Rad Laboratories. Acetone, Hexanes, Diethyl ether (for anesthesia), and DMSO were from Fisher Scientific.

Methods

Plant Material, Chloroplasts Isolation, and Incubation Conditions

Cucumber seeds were germinated in complete darkness for 6 d, and the seedlings were illuminated continuously for 20 h as reported previously (10). Chloroplasts were isolated (10, 17) and briefly illuminated to remove the small amount of Pchlide present in the isolated plastids; they were then incubated in the dark as described previously, using 3 to 5 mg of plastid protein per mL of incubation (17).

ALA Assay

ALA was extracted, purified, and determined spectrophotometrically (27). The identity of the product accumulated in the biological incubation mixtures was confirmed by coupling with ethylacetoacetate or acetylacetone and treating the reaction mixtures with Ehrlich's reagent. The colored products obtained had visible absorption spectra identical to the products made from standard ALA. The two ALA pyrroles made by total synthesis, the ethyl acetoacetate pyrrole [2-methyl-3-carbethoxy-4(3-propionic acid)pyrrole], and the acetylacetone pyrrole [2-methyl-3-acetyl-4-(3-propionic acid)pyrrole] also gave spectra that agreed with those obtained from the biological reaction mixtures. The salient features of these spectra were a sharp maximum at 552 to 553 nm and a shoulder at 520 to 525 nm (23, 25). Chemically synthesized GSA-acetylacetone pyrrole [2-methyl-3-acetyl-5-(3-propionic acid)-pyrrole] at concentrations one to three times as high as the concentrations of our biological ALA samples had no significant absorption in the 500 to 600 nm region in the presence of Ehrlich reagent. Therefore, GSA, if present, could not be detected in our ALA quantitation.

Other Analytical Methods

Protein determinations, ALA synthesis assays, and Pchlide extraction and quantitation were carried out as described recently (17).

RESULTS

The *in organello* accumulation of ALA was severely inhibited by GAB; inhibition was striking up to 200 μM, with an I_{50} of approximately 120 μM, but appeared to be saturated between 1 and 3 mM; at 3 mM GAB, ALA accumulation was still about 28% of the control (Fig. 1). In contrast, Pchlide accumulation was essentially unaffected by GAB up to 1 mM (Fig. 1) and slightly affected by 3 mM GAB (Fig. 1). By 500 μM GAB or higher, the amount of ALA accumulated had become essentially equivalent to that of Pchlide (Fig. 1). Thus, GAB eliminated 'ALA-overproduction.' AHA had about the same effect as GAB on the *in organello* ALA and Pchlide accumulations (Fig. 2).

The effect of exogenous protoheme on ALA and Pchlide accumulation was similar to that of GAB and AHA, although the differential inhibition was less striking (Fig. 3). Protoheme gave 50% inhibition at approximately 0.5 μM. The inhibition of Pchlide accumulation as a function of protoheme concentration displayed a biphasic course (Fig. 3). When protoheme was below 1.0 μM, ALA accumulation was inhibited much more than Pchlide accumulation, resulting in the convergence of the two curves (Fig. 3). At 1.0 μM protoheme, the amount of ALA accumulated was not much greater than that of Pchlide expressed in 'ALA-equivalents' (Fig.3). Further ex-

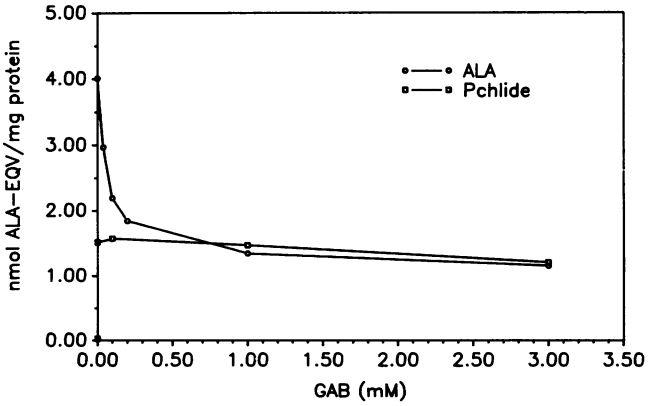

Figure 1. Effect of GAB on ALA and Pchlide synthesis in isolated chloroplasts. Chloroplasts isolated from greening cotyledons were incubated in the dark for 1 h with 6 mM Glu, 3 mM ATP, 4 mM [H], 1 mM SAM, and other additions indicated in 'Materials and Methods.' Ten mM LVA was added only to the incubations for ALA accumulation. In Figures 1 to 6 and all the tables, the values for Pchlide were converted into ALA equivalents (ALA-EQV) by multiplying by 8. The unincubated zero-time controls for both ALA and Pchlide are shown by the two nearly overlapping points on the Y-axis nearest the origin.

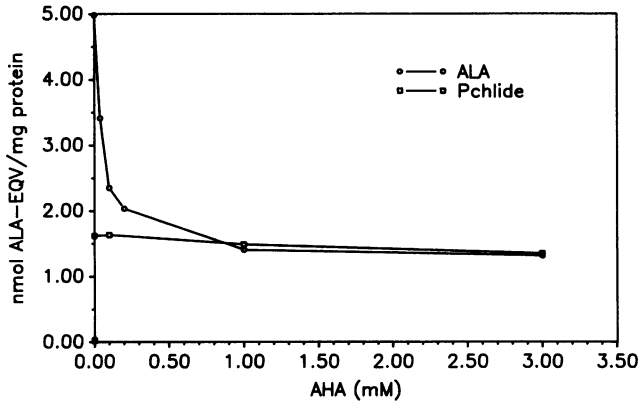

Figure 2. Effect of AHA on ALA and Pchlide synthesis in isolated chloroplasts. All the experimental conditions were as in Figure 1. The unincubated zero-time controls are shown on the Y-axis as in Figure 1.

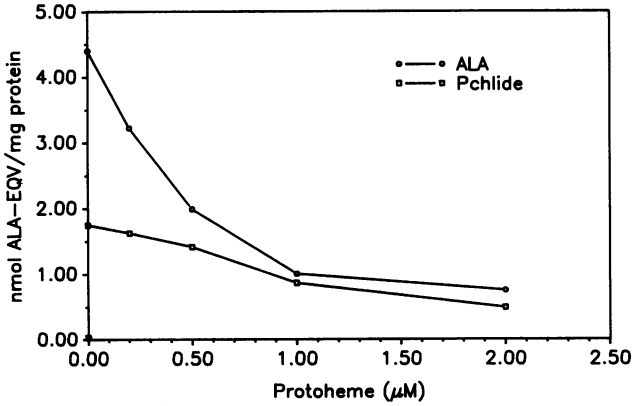

Figure 3. Effect of protoheme on ALA and Pchlide synthesis in isolated chloroplasts. Protoheme was supplied in DMSO and all samples contained 2.85% (v/v) DMSO. Other experimental details were as in Figure 1. The unincubated zero-time controls are shown on the Y-axis as in Figure 1.

periments revealed that Pchlide formation from exogenous ALA was also inhibited by protoheme at concentrations greater than 0.5 μM (data not shown). In contrast to GAB and AHA, protoheme at higher concentrations completely inhibited both ALA and Pchlide accumulation from added Glu (data not shown).

The effect of the three inhibitors, GAB, AHA, and protoheme, on ALA overproduction is highlighted in Table I. Table IA emphasizes that ALA overproduction in the presence of 6 mM Glu was eliminated by 1 mM GAB or 1 mM AHA. In the absence of the exogenous substrate (Table IB), ALA and Pchlide synthesis *in organello* was expectedly lower (15–17), but the effect of the two inhibitors (GAB and AHA) on ALA overproduction was qualitatively the same. Table IC shows that exogenous protoheme reduced, but did not completely eliminate, ALA overproduction.

In light of the recent report that GSH potentiates protoheme inhibition of ALA synthesis by *Chlorella* extracts (28), we tested the effect of GSH on ALA and Pchlide synthesis in isolated chloroplasts. GSH strongly inhibited ALA synthesis but had little effect on Pchlide synthesis (Fig. 4).

The effect of LVA on Pchlide accumulation with or without GAB is presented in Table II. In both cases, 10 mM LVA inhibited Pchlide accumulation by 90%. One mM GAB only slightly inhibited the accumulation of Pchlide, regardless of the presence of LVA (Table II). Table III shows that under conditions where Glu-dependent Pchlide synthesis is fully saturated (15, 17), addition of ALA further increased Pchlide formation.

In light of the report that LVA, but not DOHA, increases the sensitivity of GSA aminotransferase to GAB (12), it was crucial to determine if *in organello* ALA accumulation in the presence of DOHA, was also inhibited by GAB. Figure 5 shows the inhibition of ALA accumulation in the presence of either 1.5 mM DOHA or 10 mM LVA as a function of GAB concentration. The concentrations of these two ALA dehydratase inhibitors were suggested by previous studies (16, 17, 25). As GAB concentration increased, ALA accumulation in the presence of LVA or DOHA were inhibited to about the same extent. In both cases, the inhibition appeared to be saturated at 1 to 3 mM GAB.

The possibility that ALA overproduction is somehow dependent on the presence of exogenous Glu was tested in a kinetic experiment without added substrate, but otherwise identical to one reported previously in which 6 mM Glu was included (17; Fig. 1). The results of this experiment (Fig. 6) indicate that ALA overproduction is not a function of the exogenous substrate and, moreover, show that it is observed after a 10 min incubation and is therefore not dependent on the longer incubation time (60 min) used routinely in this study.

To test the possibility that the ALA that was accumulated in the presence of GAB might be synthesized via the glycine pathway (26), the isolated chloroplasts were incubated with ^{14}C-2-glycine, unlabeled succinate, other pertinent cofactors, and GAB. The extracted ALA did not incorporate significant radioactivity from ^{14}C-2-glycine (data not shown), indicating that involvement of the glycine pathway is not likely.

DISCUSSION

The present study on the effects of GAB, AHA, and protoheme on ALA and Pchlide synthesis *in organello* has shown that ALA formation can be dramatically inhibited without appreciably decreasing Pchlide synthesis. ALA formation was sharply decreased in the presence of low concentrations of these three inhibitors (Figs. 1–3). At higher concentrations, GAB or AHA had very little additional effect (Figs. 1 and 2), whereas protoheme continued to inhibit ALA synthesis (Fig. 3). On the other hand, Pchlide synthesis was essentially unaffected by GAB or AHA (Figs. 1 and 2; Table 1A and B) and was moderately inhibited by protoheme (Fig. 3; Table 1C). The ALA-overproduction phenomenon, which was examined in a previous study (17), could be eliminated by 1.0 mM GAB or AHA (Table I, A and B) and greatly reduced by 1.0 μM protoheme (Table IC).

ALA synthesis was also sharply inhibited by GSH (Fig. 4), which was recently shown to enhance the protoheme inhibition of ALA synthesis by soluble *Chlorella* extracts but to

Table I. *Effect of GAB, AHA, and Protoheme on 'ALA-overproduction' in Isolated Chloroplasts*

Chloroplasts isolated from greening cotyledons were incubated in the dark for 1 h with 3 mM ATP, 4 mM [H], 1 mM SAM, and other additions indicated in "Materials and Methods;" 10 mM LVA was included only in the incubations for ALA accumulation. In parts A and C, 6 mM Glu was added; experiment B contained no exogenous substrate. In part C, protoheme was supplied in DMSO and all samples contained 2.85% (v/v) DMSO.

Inhibitor	ALA	% Control	Pchlide	% Control	ALA/Pchlide Ratio
	pmol ALA Eqv·mg protein^{-1}				
A.					
Zero-time	29		31		
Control	4730	100	1570	100	3.01
1 mM GAB	1466	31.0	1516	96.6	0.97
1 mM AHA	1323	28.0	1492	95.0	0.89
B.					
Zero-time	8		6		
Control	2080	100	749	100	2.78
1 mM GAB	643	30.9	729	97.3	0.88
1 mM AHA	609	29.3	699	93.3	0.87
C.					
Zero-time	27		17		
Control	4243	100	1676	100	2.53
Protoheme					
0.2 μM	3011	71.0	1554	92.7	1.94
0.5 μM	1888	44.5	1339	79.9	1.41
1.0 μM	934	22.0	798	47.6	1.17

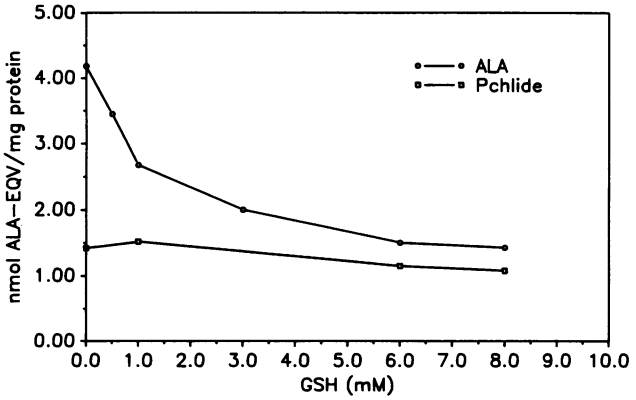

Figure 4. Effect of GSH on ALA and Pchlide synthesis in isolated chloroplasts. All experimental conditions were as in Figure 1.

Table II. *Effect of LVA on Pchlide Synthesis in the Presence and Absence of GAB*

Isolated chloroplasts were incubated in the dark for 1 h with 6 mM Glu, 3 mM ATP, 4 mM [H], 1 mM SAM, and other additions indicated in "Materials and Methods."

Inhibitor	$\frac{\text{pmol Pchlide} \times 8}{\text{mg protein}}$	Inhibition by LVA % control
Zero-time	48	
No inhibitor	1668 ± 24	100
10 mM LVA	163 ± 4	9.8
1 mM GAB	1564 ± 26	100
1 mM GAB, 10 mM LVA	149 ± 5	9.5

Table III. *Stimulation of Pchlide Synthesis by Exogenous ALA in the Presence of Saturating Glu*

Isolated chloroplasts were incubated in the dark for 1 h with 3 mM ATP, 4 mM [H], 1 mM SAM, and other additions indicated in "Materials and Methods."

Substrate	$\frac{\text{pmol Pchlide} \times 8}{\text{mg protein}}$
Zero-time	32
6 mM Glu	1384 ± 32
6 mM Glu, 50 μM ALA	2208 ± 24

have no inhibitory effect in the absence of protoheme (28). We interpret our finding as a potentiation of the inhibition by the endogenous protoheme present in the isolated plastids. Like GAB and AHA, GSH had only a slight effect on Pchlide synthesis.

Two possible explanations were considered for the observation that, as shown in Figures 1 to 4, Pchlide was inhibited much less than ALA. First, is Pchlide being synthesized from preexisting intermediates in the porphyrin pathway? Table II shows that this is unlikely, since Pchlide synthesis was equally sensitive to LVA both in the presence and absence of GAB. Furthermore, we have repeatedly seen that the isolated chloroplasts prior to incubation contained essentially no ALA or Pchlide (Figs. 1–3; Tables I and II, the zero-times) or other intermediates between ALA and Pchlide that are spectrofluorimetrically detectable (17). Second, is Pchlide synthesis in these plastids saturated by the ALA that is made from the exogenous Glu? Again, this seems improbable, since the *in organello* synthesis of Pchlide in the presence of saturating Glu could be stimulated by the addition of ALA (Table III).

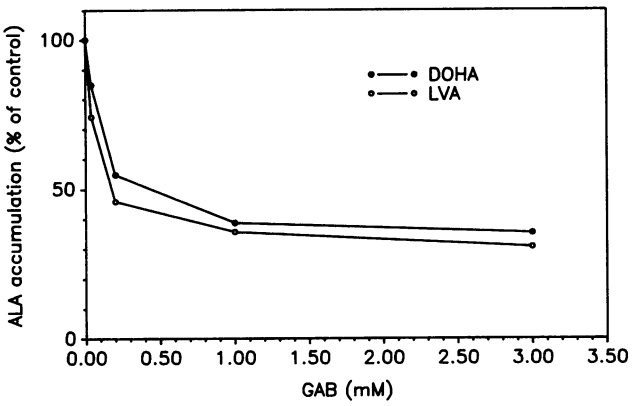

Figure 5. Effect of replacing LVA by DOHA on GAB inhibition of ALA accumulation. Isolated chloroplasts were incubated for 1 h in the dark with 6 mM Glu, 3 mM ATP, 4 mM [H], other additions indicated in 'Materials and Methods,' plus either 10 mM LVA or 1.5 mM DOHA. At GAB = 0, activity in the LVA treatment was 4171 pmol ALA·mg protein^{-1}; in the DOHA treatment it was 4774 pmol ALA·mg protein^{-1}.

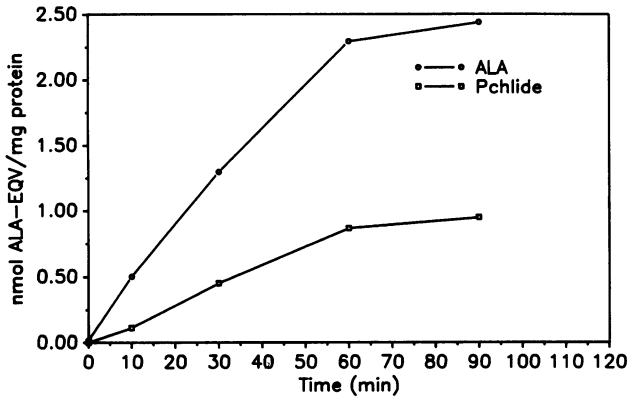

Figure 6. Synthesis of ALA and Pchlide in isolated chloroplasts in the absence of added Glu. Chloroplasts were incubated in the dark with 3 mM ATP, 4 mM [H], 1 mM SAM and other additions indicated in 'Materials and Methods.' Ten mM LVA was added only to the incubations for ALA accumulation (top curve).

Moreover, previous results have also established that Pchlide synthesis is limited by the availability of ALA (5; 17, Fig. 2).

The strong inhibition of ALA synthesis by GAB (Fig. 1; Table I) might be explained by a recent report that certain keto compounds such as LVA (but not DOHA) can predispose GSA aminotransferase to GAB inhibition (12). However, in Figure 5 we showed that substitution of LVA with DOHA made little difference in the inhibition of ALA synthesis by GAB. It should be noted that Avissar and Beale (1) also failed to confirm this potentiation of GAB inhibition by certain keto compounds.

The residual accumulation of ALA in the presence of high concentrations of GAB or AHA (Figs. 1 and 2; Table I) could conceivably be explained on the basis of accumulation of GSA (21) followed by a nonenzymatic transamination to ALA (12; also, J. D. Weinstein, personal communication). We have not attempted to see whether GSA accumulation, or nonenzymatic transamination of GSA occurred in our system. On the other hand, exogenous protoheme, which inhibits ALA synthesis before, rather than after GSA (4, 13), and therefore would not cause the accumulation of GSA, behaved similarly to GAB and AHA in our system. Endogenous protoheme could not be tested directly, but in the light of recent observations of Weinstein *et al.* (28), it is logical to regard the observed GSH inhibition of ALA synthesis (Fig. 4) as due to the endogenous protoheme. These findings suggest that the differential effect on ALA and Pchlide synthesis is independent of GSA accumulation or nonenzymatic ALA synthesis.

Two experimental conditions used in this study might be considered to be fairly unusual: first, the high concentration of exogenous substrate (6 mM Glu); second, the long incubation time (60 min) used in these experiments with isolated chloroplasts. It is clear, however, that neither of these conditions is responsible for ALA overproduction (*cf.* Table IA with Table IB; *cf.* Fig. 6 with Fig. 1 of ref. 17; note that in both of these figures ALA overproduction is already strikingly apparent after 10 min incubation). Therefore, neither the high concentration of exogenous substrate nor the long incubation can explain the inhibition of ALA synthesis by the various exogenous compounds tested in this study, which leads to the elimination or marked reduction of ALA overproduction.

We should try to understand these results and the results of the two previous papers in this series (17, 19) in terms of a unifying hypothesis for the regulation of tetrapyrrole synthesis in developing chloroplasts.

A linear reaction sequence from Glu to Pchlide will not explain ALA-overproduction unless Pchlide inhibits its own synthesis by a feedback mechanism at the level of ALA formation. However, in our developing chloroplast system no such feedback mechanism was observed (17). On the other hand, branched metabolic sequences can readily explain ALA-overproduction because, in that case, both the ALA that is headed toward Pchlide and the ALA that is headed elsewhere would accumulate in the presence of an ALA dehydratase inhibitor. This alternate destination for ALA is most probably the Fe-branch of the tetrapyrrole pathway. Net heme accumulation in greening seedlings is low, but labeling studies have suggested that heme turns over at a rapid rate (6).

Two branched metabolic sequences are shown in Figure 7. The scheme shown in Figure 7A is partly a summary of the conventional wisdom on this subject in greening cells (see recent review articles, including refs. 4 and 5). Figure 7A is not meant to exclude the possibility of other branching points in addition to protoporphyrin. Figure 7B differs in that the branching point between the Mg and the Fe tetrapyrroles occurs not at protoporphyrin, but much earlier, at the synthesis of ALA itself.

It is difficult to explain the data we have obtained on the synthesis of Pchlide in isolated chloroplasts (this paper plus refs. 17 and 19) on the basis of scheme 7A. On the one hand, the enhancement of Pchlide synthesis by exogenous ALA (Table III; ref. 17, Table I) and the inhibition of Pchlide synthesis by added LVA (Table II; ref. 17, Fig. 2) suggest that ALA synthesis is rate determining. On the other hand, the results presented in this paper with known inhibitors of ALA synthesis *in vitro*, GAB, AHA, GSH, and protoheme (Figs. 1–5; Table I) suggest that ALA synthesis must be fast relative to its conversion to Pchlide. The branching point at proto-

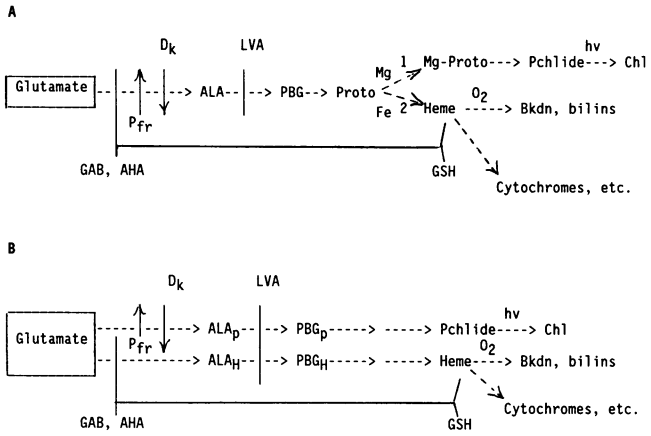

Figure 7. Two hypothetical schemes summarizing biosynthesis and regulatory events relating ALA, Pchlide, and other members of the pyrrole pathway. Scheme A is adapted from Castelfranco and Beale (5, Fig. 3). Arabic numerals 1 and 2 indicate the two protoporphyrin IX chelatases: 1, Mg-chelatase, K_m (protoporphyrin IX) = 3.5 μM; 2, Fe-chelatase, K_m (protoporphyrin IX) = 0.2 μM. Scheme B illustrates our 'two-ALA-pool' hypothesis.

porphyrin does not help us out of this dilemma if we assume that the ratio between the affinities of Mg and Fe chelatases remains constant. The protoporphyrin branching point could solve this problem for us only if the addition of GAB or AHA were to favor Mg chelation relative to Fe chelation, thus compensating for the drop of ALA synthesis. This assumption, however, seems rather unlikely. For these reasons, we deem that the scheme in Figure 7A is not consistent with our observations.

On the other hand, the scheme in Figure 7B can easily explain the effect of GAB, AHA, GSH, and protoheme by the preferential inhibition of ALA_H over ALA_P. The scheme in Figure 7B would also explain the effects of dark and dark-light pretreatments of the seedlings prior to chloroplast isolation (17, 19) by postulating that the synthesis of ALA_P is under phytochrome control, whereas the other ALA pool (ALA_H) is regulated by a protoheme feedback mechanism which is potentiated by GSH.

One could visualize certain physiological advantages to a developing plant cell of having two targeted ALA pools under different regulation mechanisms. For example, if protoheme turnover persisted after Chl had reached its maximum level, the ALA formed during the night and needed to make protoheme could not be diverted to 'free' Pchlide, which could be photoactive and therefore toxic during the following light period. The scheme in Figure 7A, on the other hand, does not offer any protection against the accumulation of unwanted Pchlide.

In conclusion, our currently favored working hypothesis (Fig. 7B) postulates two distinct pools of ALA with different metabolic destinations, different regulatory mechanisms, and different degrees of susceptibility to the ALA synthesis inhibitors used in this study. While we favor scheme B, we cannot claim that scheme B has been proven or that scheme A has been definitively rejected.

Our hypothesis can also shed light on other results that have not yet received a satisfactory explanation. Elich and Lagarias (8) reported that *Avena* seedlings germinated in AHA were impaired with respect to both phytochrome and Chl synthesis, but phytochrome accumulation was inhibited to a much greater extent. We would speculate that the phytochrome chromophore is derived from ALA_H, which is more susceptible to AHA than the ALA_P that gives rise to Chl. Kannangara and Gough (20) observed that greening barley seedlings, returned to darkness after 2 h of light, stopped accumulating Chl. However, the soluble ALA-synthesizing enzymes did not decrease compared with the control kept in continuous light. From the standpoint of our hypothesis (Fig. 7B), we might suggest that the soluble, stromal, ALA-synthesizing enzymes from barley are those responsible for the synthesis of ALA_H and that their activity, therefore, is not correlated with Chl accumulation.

Finally, our hypothesis requires that there is little or no crossover from the branch that begins at ALA_H to the Pchlide and Chl pathway. As a corollary of this requirement, we would expect that the enzymes between ALA and protoporphyrin are duplicated and occur in two sequences that are functionally separate from each other. The functional separation of ALA synthesis and ALA utilization *in organello* could conceivably be brought about by cytological or enzymological differences within each plastid. On the other hand, this heterogeneity could reside in different populations of plastids, since our suspension was isolated from whole cucumber cotyledons, containing a number of cell types, and also since each cell type could conceivably contain more than one type of plastids.

Measurement of protoheme synthesis and turnover in isolated plastid suspensions and determination of the form and nature of the postulated functional heterogeneity of ALA shall be the tasks of further research.

ACKNOWLEDGMENTS

We thank Harris Moran Seeds of Salinas, CA, for its gift of cucumber seed and the Merrell Dow Research Institute of Cincinnati, OH, for its gift of AHA. We are indebted to Drs. Kevin M. Smith and Paul Liddell for the synthetic ALA and GSA pyrroles and to Dr. Caroline Walker for reading this manuscript and making many useful suggestions.

LITERATURE CITED

1. **Avissar YJ, Beale SI** (1989) Biosynthesis of tetrapyrrole pigment precursors. Pyridoxal requirement of the aminotransferase step in the formation of δ-aminolevulinate from glutamate in extracts of *Chlorella vulgaris*. Plant Physiol **89:** 852–859
2. **Beale SI, Castelfranco PA** (1973) ^{14}C incorporation from exogenous compounds into δ-aminolevulinic acid by greening cucumber cotyledons. Biochem Biophys Res Commun **52:** 143–149
3. **Beale SI, Castelfranco PA** (1974) The biosynthesis of δ-aminolevulinic acid in higher plants. II. Formation of ^{14}C-δ-aminolevulinic acid from labeled precursors in greening plant tissues. Plant Physiol **53:** 297–303
4. **Beale SI, Weinstein JD** (1989) Tetrapyrrole metabolism in photosynthetic organisms. *In* HA Dailey, ed, Biosynthesis of Heme and Chlorophylls. McGraw Hill, New York, pp 287–391
5. **Castelfranco PA, Beale SI** (1983) Chlorophyll biosynthesis: recent advances and areas of current interest. Annu Rev Plant Physiol **34:** 241–278

6. **Castelfranco PA, Jones OTG** (1975) Protoheme turnover and chlorophyll synthesis in greening barley tissue. Plant Physiol **55:** 485–490
7. **Elich TD, Lagarias JC** (1987) Phytochrome chromophore biosynthesis. Both 5-aminolevulinic acid and biliverdin overcome inhibition by gabaculine in etiolated *Avena sativa L.* seedlings. Plant Physiol **84:** 304–310
8. **Elich TD, Lagarias JC** (1988) 4-Amino-5-hexynoic acid—a potent inhibitor of tetrapyrrole biosynthesis in plants. Plant Physiol **88:** 747–751
9. **Flint DH** (1984) Gabaculine inhibits δ-aminolevulinic acid synthesis in chloroplasts (abstract No. 965). Plant Physiol 75: S-170
10. **Fuesler TP, Castelfranco PA, Wong Y-S** (1984) Formation of Mg-containing chlorophyll precursors from protoporphyrin IX, δ-aminolevulinic acid, and glutamate in isolated, photosynthetically competent, developing chloroplasts. Plant Physiol **74:** 928–933
11. **Hill CM, Pearson SA, Smith AJ, Rogers LJ** (1985) Inhibition of chlorophyll synthesis in *Hordeum vulgare* by 3-amino-2,3-dihydrobenzoic acid (gabaculine). Biosci Rep **5:** 775–781
12. **Hoober JK, Kahn A, Ash DE, Gough SP, Kannangara CG** (1988) Biosynthesis of δ-aminolevulinate in greening barley leaves. IX. Structure of the substrate, mode of gabaculine inhibition, and the catalytic mechanism of glutamate 1-semialdehyde aminotransferase. Carlsberg Res Commun **53:** 11–25
13. **Huang D-D, Wang W-Y** (1986) Chlorophyll synthesis in *Chlamydomonas* starts with the formation of glutamyl-tRNA. J Biol Chem **261:** 13451–13455
14. **Huang D-D, Wang W-Y, Gough SP, Kannangara CG** (1984) δ-Aminolevulinic acid-synthesizing enzymes need an RNA moiety for activity. Science **225:** 1482–1484
15. **Huang L, Castelfranco PA** (1986) Regeneration of magnesium-2,4-divinyl pheoporphyrin a_5 (divinyl protochlorophyllide) in isolated developing chloroplasts. Plant Physiol **82:** 285–288
16. **Huang L, Castelfranco PA** (1988) A re-examination of 5-aminolevulinic acid synthesis by isolated, intact, developing chloroplasts: the O_2 requirement in the light. Plant Sci **54:** 185–192
17. **Huang L, Castelfranco PA** (1989) Regulation of 5-aminolevulinic acid synthesis in developing chloroplasts. I. Effect of light/dark treatments *in vivo* and *in organello.* Plant Physiol **90:** 996–1002
18. **Huang L, Castelfranco PA** (1989) Effect of three inhibitors on ALA and Pchlide synthesis in isolated chloroplasts (abstract No. 298). Plant Physiol **89:** S-50
19. **Huang L, Bonner BA, Castelfranco PA** (1989) Regulation of 5-aminolevulinic acid synthesis (ALA) in developing chloroplasts. II. Regulation of ALA-synthesizing capacity by phytochrome. Plant Physiol **90:** 1003–1008
20. **Kannangara CG, Gough SP** (1979) Biosynthesis of δ-aminolevulinate in greening barley leaves. II. Induction of enzyme synthesis by light. Carlsberg Res Commun **44:** 11–20
21. **Kannangara CG, Schouboe A** (1985) Biosynthesis of δ-aminolevulinate in greening barley leaves. VII. Glutamate 1-semialdehyde accumulation in gabaculine treated leaves. Carlsberg Res Commun **50:** 179–191
22. **Kannangara CG, Gough SP, Bruyant P, Hoober JK, Kahn A, von Wettstein D** (1988) $tRNA^{Glu}$ as a cofactor in δ-aminolevulinate biosynthesis: steps that regulate chlorophyll synthesis. Trends Biochem Sci **13:** 139–143
23. **Mauzerall D, Granick S** (1956) The occurrence and determination of δ-aminolevulinic acid and porphobilinogen in urine. J Biol Chem **219:** 435–446
24. **Mayer SM, Beale SI, Weinstein JD** (1987) Enzymatic conversion of glutamate to δ-aminolevulinic acid in soluble extracts of *Euglena gracilis.* J Biol Chem **262:** 12541–12549
25. **Meller E, Gassman ML** (1981) The effects of levulinic acid and 4,6-dioxoheptanoic acid on the metabolism of etiolated and greening barley leaves. Plant Physiol **67:** 728–732
26. **Shemin D, Russell CS** (1983) δ-Aminolevulinic acid, its role in the biosynthesis of porphyrins and purines. J Am Chem Soc **75:** 4873–4874
27. **Weinstein JD, Beale SI** (1985) Enzymatic conversion of glutamate to δ-aminolevulinate in soluble extracts of the unicellular green alga *Chlorella vulgaris.* Arch Biochem Biophys **237:** 454–464
28. **Weinstein JD, Howell R, Leverette R, Brignola P** (1989) Heme inhibition of δ-aminolevulinic acid (ALA) synthesis is facilitated by glutathione in cell-free extracts from *Chlorella* (abstract No. 443). Plant Physiol **89:** S-74