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

III. Evidence for Functional Heterogeneity of the ALA Pool

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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 protoclorophyllide (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-dioxohexapetonic 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 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 plastidic ALA pool.
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-carboxethoxy-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, Heps, 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; MgCl2·6H2O was from Mallinckrodt. Ethyl acetocetate and acetylacetone were from Aldrich; SDA (Dupnonol) 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 ethylacetocetate 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 acetocetate pyrrole [2-methyl-3-carboxethoxy-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 IC50 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](https://www.plantphysiol.org)
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 14C-2-glycine, unlabeled succinate, other pertinent cofactors, and GAB. The extracted ALA did not incorporate significant radioactivity from 14C-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 1C).

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.

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

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;"

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>pmol Pchlide x 8 mg protein</th>
<th>Inhibition by LVA %</th>
</tr>
</thead>
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<tr>
<td>Zero-time</td>
<td>48</td>
<td>100</td>
</tr>
<tr>
<td>No inhibitor</td>
<td>1668 ± 24</td>
<td>100</td>
</tr>
<tr>
<td>10 mM LVA</td>
<td>163 ± 4</td>
<td>9.8</td>
</tr>
<tr>
<td>1 mM GAB</td>
<td>1564 ± 26</td>
<td>100</td>
</tr>
<tr>
<td>1 mM GAB, 10 mM LVA</td>
<td>149 ± 5</td>
<td>9.5</td>
</tr>
</tbody>
</table>

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;"

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pmol Pchlide x 8 mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero-time</td>
<td>32</td>
</tr>
<tr>
<td>6 mM Glu</td>
<td>1384 ± 32</td>
</tr>
<tr>
<td>6 mM Glu, 50 μM ALA</td>
<td>2208 ± 24</td>
</tr>
</tbody>
</table>

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).
isolated chloroplasts

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\textsuperscript{-1}; in the DOHA treatment it was 4774 pmol ALA-mg protein\textsuperscript{-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 I 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 \textit{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-
been definitively wanted Pchlide. persisted the destinations, different regulatory mechanisms, regulation The plant (17, Figure (17)).

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 $\mu$M; 2, Fe-chelatase, $K_m$ (protoporphyrin IX) = 0.2 $\mu$M. Scheme B illustrates our ‘two-ALA-pool’ hypothesis.

Protoporphyrin 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 protoporphyrin by the preferential inhibition of ALAp over ALAHe. 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 ALAp is under phytochrome control, whereas the other ALA pool (ALAHe) is regulated by a protoporphyrin 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 protoporphyrin turnover persisted after Chl had reached its maximum level, the ALA formed during the night and needed to make protoporphyrin 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. Eich 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 ALApH, which is more susceptible to AHA than the ALApH that gives rise to Chl. Kannanagara and Gough (20) observed that green 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 ALApH 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 ALApH 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 protoporphyrin 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.

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