The Biosynthesis of δ-Aminolevulinic Acid in Higher Plants

I. ACCUMULATION OF δ-AMINOLEVULINIC ACID IN GREENING PLANT TISSUES

Received for publication August 2, 1973 and in revised form October 5, 1973

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ABSTRACT
δ-Aminolevulinic acid dehydrase activity in cucumber (Cucumis sativus L. var. Alpha green) cotyledons did not change as the tissue was allowed to green for 24 hours. δ-Aminolevulinic acid accumulated in greening cucumber cotyledons, and barley (Hordeum sativum L. var. Numar) and bean (Phaseolus vulgaris L. var. Red Kidney) leaves incubated in the presence of levulinic acid, a specific competitive inhibitor of δ-aminolevulinic acid dehydrase. The rate of δ-aminolevulinic acid accumulation in levulinic acid-treated cucumber cotyledons paralleled the rate of chlorophyll accumulation in the controls, and the quantity of δ-aminolevulinic acid accumulated compensated for the decrease in chlorophyll accumulation. When levulinic acid-treated cucumber cotyledons were returned to darkness, δ-aminolevulinic acid accumulation ceased.

δ-Aminolevulinic acid accumulation showed an absolute requirement for oxygen and was inhibited drastically by cyanide and azide, and to a lesser extent by arsenite and malonate. 2,4-Dinitrophenol, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea, sodium fluoroacetate, and hydroxylamine hydrochloride showed no effect under the conditions tested. Freezing and thawing of the tissue completely prevented the accumulation of δ-aminolevulinic acid.

The findings of this investigation are consistent with the hypothesis that δ-aminolevulinic acid is a chlorophyll precursor in higher plants, and that chlorophyll biosynthesis is regulated at the level of the formation of δ-aminolevulinic acid.

The biosynthesis of Chl is thought to follow the same route as that of heme from ALA, the first biosynthetic intermediate unique to the tetrapyrrrole pathway, to protoporphyrin IX, the immediate precursor of heme and Mg porphyrins (7). Insertion of the Mg into the protoporphyrin nucleus and modification of the side chains lead to protochlorophyllide, which is then photoreduced to chlorophyllide in greening plants, although in some algae and gymnosperms, light is not necessary for this reduction. Chlorophyllide is subsequently phyltylated to yield Chl.

The primary regulation of the Chl biosynthetic pathway is thought to occur before ALA formation because: (a) no intermediates other than protochlorophyllide accumulate when greening is inhibited by placing plants in the dark; and (b) exogenous ALA is converted to protochlorophyllide in plants kept in the dark, and a portion of this ALA-induced protochlorophyllide can be converted to Chl during subsequent incubation of the plant in the light (16, 20).

In animal tissues, bacteria, and yeast, ALA is formed by the condensation of succinyl CoA and glycine, catalyzed by the pyridoxal phosphate-requiring enzyme succinyl CoA-glycine succinyl transferase (ALA synthetase) yielding α-amino, β-keto adipic acid, which is immediately decarboxylated to ALA and CO₂ (derived from the carboxyl carbon of glycine) (11). In photosynthetic bacteria and in some heme-forming systems, ALA synthetase is a site of regulation of the pathway, being subject to feedback inhibition and induction (5, 12).

The evidence for the presence of ALA synthetase in greening plant tissue is very weak. Although the enzyme would be expected to be very active during the rapid phase of Chl synthesis in greening etiolated tissues, it has not been detected in extracts from such material. Where ALA synthetase has been reported in plant sources, the evidence for its existence has been fragmentary, and the relation of the enzyme to Chl synthesis remains undetermined (14, 17, 24, 25).

In this study we have concerned ourselves with the following questions: (a) whether ALA is indeed synthesized by greening plant tissues; (b) if so, whether this ALA is the precursor of the tetrapyrrrole moiety of Chl; (c) whether the formation of ALA is the rate-limiting step in the biosynthetic pathway leading to Chl in greening plant tissues.

In these experiments we have used the ALA-dehydrase inhibitor levulinic acid to induce the accumulation of ALA in greening plant tissues. This technique was originated by Beale (2, 3) to demonstrate the biosynthesis of ALA in the unicellular alga Chlorella and was subsequently used for the same purpose in bacteria (10), Euglena (18), corn, and bean leaves (9). In a companion paper, we describe an inquiry into the in vivo source of this ALA (4).

MATERIALS AND METHODS

Reagents. Levulinic acid and Ehrlich’s reagent (p-dimethylaminobenzaldehyde) were purchased from Sigma Chemical Co., dimethylsulfoxide from J. T. Baker, ethylacetocetate and acetyl acetone (2,4-pentanedione) from Matheson Coleman and Bell, and Botran (2,6-dichloro-4-nitroaniline) from Duco Products Co., division of Upjohn Co., Kalamazoo, Mich. DCMU, obtained from E. I. duPont de Nemours and Co., was recrystallized from aqueous acetone.

Plant Material. Cucumber seeds (Cucumis sativus L. var. Alpha green), a gift of the Niagara Chemical Division, FMC Corporation, Modesto, Calif. were germinated in complete
darkness for 6 days at 25 C (8). Barley seeds (Hordeum sativum L. var. Numar) were germinated for 7 days in the dark at 25 to 26 C in vermiculite watered with Hoagland solution and beans (Phaseolus vulgaris L. var. Red Kidney) were soaked for 30 min in a saturated Botran solution and germinated for 10 days under the same conditions as barley.

**Incubation Conditions.** Cucumber cotyledons, barley, or bean primary leaves were incubated at 25 to 28 C in water containing various concentrations of levulinic acid and other components. The cucumber cotyledons were excised with about 5 mm of hypocotyl hook; 2.0 ml of bathing solution was used per 50 cotyledon pairs. Barley tissue was cut into 10-mm segments and floated on bathing solution contained in a Petri dish 9.0 cm in diameter. Primary bean leaves were excised, separated, and floated in Petri dishes as above. Illumination, when required, was 240 to 260 ft-c from a cool-white fluorescent source.

**Determination of ALA.** ALA was determined by the method of Mauzerall and Granick (13). The ALA was reacted with either acetylacetone at pH 4.6 or with ethylacetoacetate at pH 6.8, and the resulting pyrroles were detected by reaction with modified Ehrlich reagent containing 2 mM HClO4, (13). The absorbance of the pink color was determined at 552.5 nm in a Zeiss PMQ II spectrophotometer.

**ALA Dehydrase Assay.** The enzyme ALA dehydrase (E.C. 4.3.1.24) was assayed by a modification of the method of Burnham and Lascelles (5). Ten grams of cotyledons were ground in a mortar and pestle at 0 C in 20 ml of 0.33 M sorbitol containing 40 mM HEPES, pH 7.8, 5 mM MgCl2 and 0.1% sodium ascorbate, filtered through Miracloth, then incubated at 37 C in the presence of ALA. The reaction was stopped by the addition of 5% trichloroacetic acid, and porphobilinogen was determined by the method of Mauzerall and Granick (13).

**Pigment Extraction and Determinations.** Pigments were extracted by grinding the tissue in 90% acetone, containing 10 mM NH4OH and saturated with MgCO3. Grinding was performed with a Polytron homogenizer. The brei was centrifuged for 10 min at 27,000g, and the pellet was extracted again with the same solvent. Both supernatants were combined and cleared by centrifugation at 35,000g for 2 hr.

**Spectrophotometric Measurements.** Protoporphyr(ide) and chlorophyll(ide) content were determined from the absorbances at 626, 645, and 663 nm, using the absorption coefficients of Anderson and Boardman (1), converted to molar values. Correction for light scattering was accomplished by measurement of light absorption at 700 nm, and subtracting an appropriate fraction from the measured absorption at the other wavelengths, according to a scattering curve derived by measuring apparent light absorption, at all four wavelengths, of a dilute suspension of milk. The scattering corrections apply only to this particular spectrophotometer (Zeiss Model PMQ II). The actual equations employed are as follows (in nmol of pigment per ml of acetone extract): chlorophyll a = 14.19 664 - 2.97 665 + 0.33 666 - 12.68 667; chlorophyll b = -4.66 664 + 26.03 665 - 3.59 666 + 20.87 667; protoporphyr(ide) = -4.49 664 + 7.58 665 + 33.22 666 + 27.01 667.

**Use of Dimethylsulfoxide in Conjunction with Levulinic Acid.** In all experiments with intact cucumber cotyledons using levulinic acid plus other compounds, 10% dimethylsulfoxide was added to all incubation mixtures including control mixtures containing levulinic acid only. This was done in order to overcome possible permeability barriers associated with the water-repellent surface of the cotyledons (6). It was determined that the presence of 10% dimethylsulfoxide had no effect on ALA accumulation in the presence of levulinic acid. Dimethylsulfoxide was omitted in the experiments with barley and bean tissue.

**RESULTS**

**ALA Dehydrase from Cucumber Cotyledons.** Six-day-old etiolated cucumber cotyledons were found to maintain the same level of ALA dehydrase activity as the cotyledons were allowed to green for 24 hr at 28 C. The Km for ALA was approximately 0.75 mM under the incubation conditions described above. In the presence of 5 mM ALA initial concentration, the reaction rate was constant for at least 60 min.

**ALA Accumulation in Cucumber Cotyledons Treated with Levulinic Acid**

**Concentration Effects of Levulinic Acid.** Six-day-old etiolated cucumber cotyledons were incubated at 28 C in the light. After 4.5 hr, samples were treated with various concentrations of levulinic acid and allowed to incubate for 2 more hours, then the tissue was homogenized, and the accumulated ALA was determined (Table I). Because 100 mM levulinic acid gave the highest yields of ALA, it was chosen as the standard concentration in subsequent experiments with cucumber cotyledons.

**Correlation of Rates of ALA and Chl Synthesis.** Etiolated cucumber cotyledons were incubated in the light, and samples were taken for periodic Chl determination. Other samples were withdrawn at regular intervals and incubated with 100 mM levulinic acid for 1 hr in the light, after which the accumulated ALA was determined. The rates of Chl synthesis and ALA accumulation are plotted (Fig. 1). A close relationship between shapes of the resulting curves is observed over the first 8 hr of the greening process.

**Effect of Returning Tissue to Darkness.** Etiolated cucumber cotyledons were illuminated for 4.5 hr, then were treated with 100 mM levulinic acid. After 1 additional hr in the light, some of the samples were placed in the dark at 25 C. Samples were taken at regular intervals and the accumulated ALA was determined (Fig. 2). ALA accumulation stopped within 1 hr after the tissue was returned to the dark.

**Stoichiometry of ALA and Chl Formation.** Etiolated cucumber cotyledons were illuminated for 4.5 hr, then some samples were treated with 100 mM levulinic acid and others were left untreated. After 2 additional hr, both Chl and accumulated ALA were measured in all samples. One hundred mM levulinic acid was found to inhibit Chl synthesis by 50% under these conditions, but the total ALA synthesized (moles ALA accumulated plus 8 times moles of Chl formed) in the levulinic acid-treated tissue approximated that of the controls (Table II).

**Effect of Metabolic Inhibitors and Anaerobiosis.** Etiolated cucumber cotyledons were illuminated for 4.5 hr, then treated with 100 mM levulinic acid and one of the inhibitors listed in Table III, and allowed to incubate for 2 more hours, after which the ALA was determined and compared to a sample treated with levulinic acid only. In the N2-treated sample, N2 was allowed to flush a covered Petri dish containing the cotyledons. This sample was compared to a similar one flushed with air. Anaerobiosis inhibited ALA accumulation almost completely, while in the presence of the inhibitors, malonate and arsenite showed a moderate but appreciable inhibition, whereas the other inhibitors had a relatively small effect or none at all (Table III). The inhibitions caused by anaerobiosis or KCN could not be reversed by the addition of 100 mM α-ketoglutarate, 100 mM glycine, and 10 mM NAD. Furthermore, the inhibition caused...
by anaerobiosis was not reversed by 100 mm glycine, 100 mm succinic acid, and 5 mm ATP.

Effect of Freezing and Thawing. Etiolated cucumber cotyledons were illuminated for 4 hr, then rapidly frozen by placing the cotyledons in a single layer on a Petri dish and floating the dish on a Dry Ice-ethanol bath for 15 min. They were then thawed by floating the dish in a 28 C water bath. ALA accumulation was next measured after 2 hr of illumination at 28 C in the presence of 100 mm levulinic acid. The freeze-thaw treatment completely inhibited ALA accumulation. The ability to accumulate ALA could not be restored by addition of 50 mm potassium phosphate buffer, pH 7.0, 50 mm α-ketoglutarate or 50 mm glutamic acid with or without buffer, or 50 mm glycine and 50 mm succinic acid in the same buffer.

RESPONSE OF OTHER PLANT TISSUES TO LEVULINIC ACID

Barley. Seven-day-old etiolated barley seedlings were illuminated intact for 4 hr at 30 C, then the primary leaves were cut into 1-cm segments and allowed to float in Petri dishes containing 2.5 ml of solution of levulinic acid. After 3 additional hr of incubation, the leaf segments were homogenized in 5% HClO4, and ALA was determined. Twenty mm levulinic acid was found to cause the higher ALA accumulation of the two concentrations tested (Table IV).

Chl synthesis was inhibited by both concentrations of levulinic acid; but in the presence of 100 mm levulinic acid a net loss of Chl from the tissue occurred. At either concentration levulinic acid caused a marked inhibition of the total ALA (moles ALA plus 8 times moles Chl) accumulated; while with cucumber cotyledons the ALA and the Chl were essentially additive even with 100 mm levulinic acid (compare Tables II and IV).

Beans. Ten-day-old etiolated bean seedlings were illuminated for 5 hr at 28 C, then the primary leaves were excised, separated, and placed in Petri dishes, along with 2.5 ml of levulinic acid solution. After 3 more hr of illumination, the tissue was homogenized and ALA was determined. ALA accumulation increased with increasing levulinic acid concentration up to 20 mm, and then reached a plateau (Table V).

![Diagram](https://example.com/diagram.png)

Fig. 1. Correlation of Chl formation and ALA accumulation in greening cucumber cotyledons. Six-day-old etiolated cucumber cotyledons were illuminated with 240 ft-c of fluorescent light at 28 C. Periodic samples were taken for Chl determinations. Other samples were taken, incubated with 100 mm levulinic acid and 10% dimethylsulfoxide for 1 hr under the same light and temperature conditions as above, and then accumulated ALA was determined. Amounts of ALA accumulated/hr-g tissue in treated samples (ALA) and Chl formed/hr-g tissue in untreated samples (CHL) are both plotted against hours since beginning of illumination.

Table I. ALA Accumulation in Greening Cucumber Cotyledons Treated with Levulinic Acid

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<th>Levulinic Acid Conc (mm)</th>
<th>ALA Accumulated in 2 Hr (mmoles/g fresh wt)</th>
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Table II. Effect of Dimethylsulfoxide on ALA Accumulation

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Table III. Effect of Glutarate on ALA Accumulation

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from succinyl CoA and glycine has been found to be the site of regulation of the pathway. An exception to this general finding may exist in Neurospora, where it has been reported that the second enzyme in the pathway, ALA dehydrase, is the site of regulation (15).

Table III. Inhibition of Levulinic Acid-induced ALA Accumulation in Cucumber Cotyledons by Various Substances

Etiolated cotyledons were preilluminated for 4.5 hr, then illumination was continued for 2 hr in the presence of 10% dimethylsulfoxide, 100 mM levulinic acid, and the inhibitor, applied by wetting the cotyledons (N2-treated samples were flushed with a constant stream of the gas).

Table IV. ALA and Chl Accumulation in Levulinic Acid-treated Barley Leaves

Seven-day-old etiolated barley leaves were preilluminated for 4 hr, then the leaves were cut into 10-mm sections and floated on the indicated solutions during continued illumination for 3 more hr.

Table V. ALA Accumulation in Levulinic Acid-treated Bean Leaves

Ten-day-old etiolated bean seedlings were preilluminated for 5 hr, then primary leaves were excised and bathed with the indicated solution under continued illumination for 3 more hr.

DISCUSSION

The regulation of a biochemical pathway is generally considered to be exerted most effectively at the enzymatic step which produces the first compound unique to that pathway. Chl and heme are thought to share a common biosynthetic pathway from ALA to protoporphyrin IX (7). In studies on heme synthesis in mammalian (12) and bacterial (22) systems, and of bacteriochlorophyll synthesis (5, 23), the formation of ALA
The formation of ALA is generally thought to be the limiting step in the pathway leading to protochlorophyllide in etiolated higher plant tissue. This conclusion is based primarily on experiments wherein ALA fed to the etiolated plant tissues results in higher protochlorophyllide content than in control tissues. At least some of this ALA-protochlorophyllide can be photoconverted to chlorophyll(ide) when the etiolated tissue is illuminated (16, 20).

Presumably, the enzymes involved in the pathway from ALA to protochlorophyllide are present in the etiolated tissue in nonlimiting quantities, but it is not presently known whether in general these enzymes are constitutive or if they are induced during the course of greening. Steer and Gibbs (21) were able to demonstrate changes in ALA dehydrase activity in greening bean leaves, but these changes were not sufficiently large to indicate a regulatory function for this enzyme. Our finding that ALA dehydrase activity is maintained at a constant level as etiolated cucumber cotyledons are allowed to become green, supports the hypothesis that this enzyme is not involved in the regulation of the Chl pathway.

Although the enzyme ALA synthetase (succinyl CoA-glycine succinyl transferase) has been reported to occur in crude plant extracts, there have been either preliminary accounts or abstracts not followed by more comprehensive confirmatory studies (14, 24) or they are subject to the following criticisms: (a) lack of correlation of enzyme activity with changing rates of Chl synthesis; (b) lack of the crucial test for succinyl transferase activity which is the incorporation into ALA of the methylene carbon of glycine but exclusion of the carboxyl carbon; (c) lack of adequate precautions against microbial contamination during the preparation or incubation of the extracts (17, 25).

It has recently become possible to measure ALA formation in vivo by inhibiting ALA dehydrase with the competitive inhibitor levulinic acid and measuring the accumulation of ALA as it is formed (2, 3). A potential disadvantage of this method is the possibility of other effects of levulinic acid on cell metabolism, and therefore of secondary effects on ALA accumulation. An awareness of this possibility leads us to seek converging lines of evidence from experiments with different plant tissues and experimental conditions.

We have shown here that the time course of ALA accumulation in greening cucumber cotyledons treated with levulinic acid corresponds to the time course of Chl formation in control tissue (Fig. 1), confirming similar findings in Chlorella (2) and corn (9). Light is required for ALA accumulation in levulinic acid-treated cucumber cotyledons (Fig. 2) as it is in other higher plants (9) and in Euglena (18). In all these cases there is a corresponding light requirement for Chl accumulation in control tissues.

In growing Chlorella cultures, it was shown that the total quantity of ALA synthesized, that is, the moles of accumulated ALA plus 8 times the moles of Chl formed (since 8 molecules of ALA are required to form 1 of Chl), was the same in levulinic acid-treated cultures as in untreated controls, even when Chl synthesis was inhibited 50%. This finding provided strong evidence that the ALA which accumulated in the presence of levulinic acid was indeed destined for Chl synthesis and that the levulinic acid was without side-effect, serving only to divert ALA from the Chl pathway. This stoichiometry has now been demonstrated in cucumber cotyledons (Table II). However, in barley leaves, this could not be shown. Levulinic acid, at a concentration sufficient to cause accumulation of free ALA, inhibited the formation of total ALA (Table IV). It is interesting that in long term incubations of bean and corn leaves employed by Harel and Klein (9), levulinic acid treatment caused a stimulation of total ALA accumulation. We have not investigated such possibly compensatory ALA synthesis occurring in long term incubations.

An aerobic environment appears to be absolutely required for the accumulation of ALA in levulinic acid-treated cucumber cotyledons (Table III). Although both cyanide and azide were relatively potent inhibitors (72 and 66%, respectively, at 3 mM), 1 mM 2,4-dinitrophenol inhibited ALA accumulation by only 25%. These results suggest that some metal-containing terminal oxidase, not necessarily cytochrome oxidase, is involved in ALA synthesis, but that oxidative phosphorylation per se may not be. The preliminary results obtained with the citric acid cycle inhibitors (i.e. the moderate inhibition of ALA production observed in the presence of malonate and arsenite and the lack of inhibition with fluoracetate) (Table III) are difficult to explain. The negligible inhibition obtained with DCMU on ALA accumulation (Table III) suggests that photosynthetic O₂ evolution (and noncyclic photophosphorylation) is not involved in ALA synthesis. This result is not surprising, since photosynthesis is probably not yet operative at these early stages of chloroplast development and DCMU was reported not to inhibit Chl formation in greening Euglena (19), although, in a more recent report (18), DCMU caused a partial inhibition of ALA accumulation in the presence of levulinic acid in Euglena. However, it should be noted that in the experiments summarized in Table III no attempt was made to evaluate the extent of penetration of each inhibitor into the tissue.

Freezing and thawing completely destroys the ability of cotyledon tissue to accumulate ALA in the presence of levulinic acid. This finding argues for the involvement of membrane bound systems in the formation of ALA.

In conclusion, we have shown that ALA accumulates in etiolated cucumber cotyledons, barley, and bean leaves treated with levulinic acid, and we have extended some of the results which have been previously obtained in Chlorella (2), corn, bean (9), and Euglena (18). By using a number of different criteria, we have shown that the ALA which accumulates under these conditions is destined for Chl biosynthesis.

These studies have provided us with the tools to approach a very basic question: namely, "what are the immediate precursors of the Chl-precursor ALA?" A preliminary answer to this question, implicating organic molecules other than glycine and succinyl CoA, has been obtained and forms the object of our next report (4).

Acknowledgment—We thank L. Ginslin for performing the chlorophyll determinations.

LITERATURE CITED