LIGHT DEPENDENT FORMATION OF δ-AMINOLEVULINIC ACID IN ETIOLATED LEAVES OF HIGHER PLANTS

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SUMMARY

Levulinic acid inhibited the synthesis of chlorophyll and caused accumulation of δ-aminolevulinic acid in greening etiolated leaves of corn and bean seedlings. This is the first direct evidence for the synthesis of δ-aminolevulinic acid in higher plants. No stoichiometric relationship was found between the inhibion of cholorophyll synthesis and δ-aminolevulinic acid accumulation. The formation of δ-aminolevulinic acid was light dependent and the rate of its formation increased after an initial lag period of 2-4 hours.

Aminolevulinic acid synthetase participates in the synthesis of chlorophyll in photosynthetic bacteria by catalyzing the formation of ALA from succinyl-CoA and glycine [1]. It has been suggested that in algae and higher plants also, ALA is an intermediate in the biosynthesis of chlorophyll [2,3].

The production of ALA in algae has only recently been demonstrated directly. Beale [4] showed that light grown *Chlorella* cells excrete ALA in the presence of LA, a competitive inhibitor of ALA dehydratase [5]. A similar situation exists in *Euglena*, where ALA accumulates in greening cells in the presence of LA [6]. So far, no direct evidence is available for the synthesis of ALA in higher plants as part of the biosynthetic pathway leading to chlorophyll. In this communication we shall show that in higher plants LA inhibits chlorophyll synthesis as in *Chlorella* and *Euglena*, and that ALA accumulates under these conditions.

Abbreviations: ALA — δ-aminolevulinic acid; LA — levulinic acid.

MATERIALS AND METHODS

Corn (*Zea mays*, var. Neve Yaar 170) and bean (*Phaseolus vulgaris*, var. bulgarian) seeds were germinated and grown in vermiculite in the dark at 22°. The primary leaves of 9-10 day old bean seedlings were collected under a green safe-light and floated on the appropriate solutions at pH 6.0. After 2 hours in the dark the leaves were illuminated with 80 ft.c. of white fluorescent light or remained in the dark. Leaves from 8-9 day old corn seedlings were treated similarly, but stood with their cut bases in small flasks in front of a fan to accelerate uptake of solutions. After various periods the leaves were ground in ice-cold water with a mortar and pestle, cold CCl_3COOH was added to a final concentration of 4% and the extract centrifuged at 10,000 *g* for 15 min. The supernatant was brought to pH 4.6 with 1 M Na acetate and ALA determined according to Mauzerall and Granick [7].

For chromatography, ALA from the leaf extracts or commercially obtained (Sigma), was condensed with acetylacetone, extracted with ethylacetate and concentrated according to Irving and Elliot [8]. The ALA-pyrrole (2-methyl-3-acetyl-4-propionic acid pyrrole) thus obtained was chromatographed on paper [9] and on silica gel plates (M. Voelm, Germany) [8]. Chlorophyll was extracted from leaves with 80% acetone and determined spectrophotometrically. Levulinic acid (grade I, Sigma) was purified as described by Beale [4].

RESULTS AND DISCUSSION

Levulinic acid inhibited chlorophyll synthesis and caused accumulation of ALA when added to etiolated corn and bean leaves exposed to light (Table 1). In the dark only traces of ALA accumulated. In the presence of sucrose, known to enhance chlorophyll synthesis in the bean [10], more ALA accumulated

TABLE 1. The effect of levulinic acid (LA) on the formation of δ-aminolevulinic acid (ALA) in greening leaves of corn and bean seedlings

Treatment	Corn		Bean	
	ALA µmole/g	Chlorophyll µg/g	ALA µmole/g	Chlorophyll µg/g
Water, light	0.04 ± 0.03	318 ± 35	0.44 ± 0.17	60 ± 11
LA, light	7.62 ± 0.22	184 ± 19	5.40 ± 1.00	32 ± 7
Sucrose, light	0.08 ± 0.05	239 ± 25	1.08 ± 0.20	187 ± 22
Sucrose + LA, light	4.66 ± 0.55	163 ± 24	8.78 ± 1.50	76 ± 12
Water, dark	0.13 ± 0.03	14* ± 3	0.25 ± 0.24	16* ± 1
LA, dark	0.15 ± 0.04	7 ± 2	0.50 ± 0.50	
Sucrose, dark	0.12 ± 0.05	20 ± 4	0.00	
Sucrose + LA, dark	0.36 ± 0.01	9 ± 3	0.00	

*Protochlorophyll(ide)

Duration of experiments: corn — 22 hours; bean — 18 hours. Concentration of levulinic acid: 20 mM for corn; 15 mM for bean. Sucrose concentration 0.2 M. Results are given per g fresh weight as mean and standard error of 4-6 experiments.

than in its absence. In corn, sucrose slightly inhibited chlorophyll synthesis and less ALA was formed when the leaves were treated with LA and sucrose as compared with leaves which were given LA alone.

Figure 1 shows the effect of various concentrations of LA on the formation of chlorophyll and ALA. Increasing the concentration of LA up-to 50 mM caused an increase in ALA accumulation and a decrease in chlorophyll synthesis.

The ALA in the leaf extracts was identified by thin layer and paper chromatography. The Rf values for the pyrrole — 0.5 on thin layer and 0.2 on paper chromatograms — were identical with those obtained for the pyrrole prepared from commercial ALA and corresponded to values reported in the literature [8,9]. Aminoketones can interfere with the colorimetric determination of ALA [7]. However, no indications were found for the presence of aminoketones in the leaf extracts. This was established by ion-exchange chromatography [9],

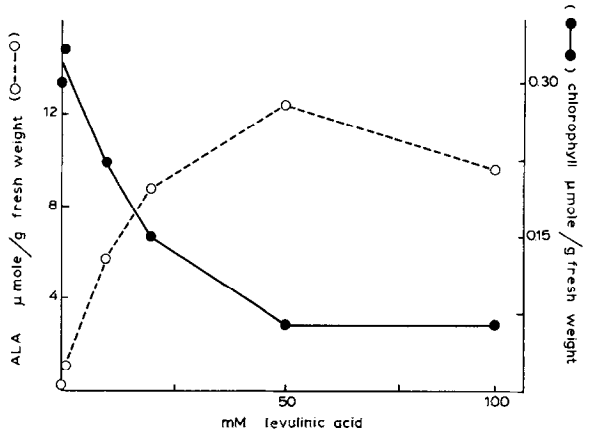

Fig. 1. The effect of various concentrations of levulinic acid on the formation of δ-aminolevulinic acid and chlorophyll in greening corn leaves. Etiolated 8 day old leaves were treated with levulinic acid for 2 hours in the dark and a following period of 22 hours in light.

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thin layer and paper chromatography and by the modified colorimetric method of Granick [11]. Evidently, the product formed with the Ehrlich reagent was due to the presence of ALA-pyrrole.

Small amounts of ALA were also detected in the LA containing media on which the leaves were floated. The ALA in the media amounted to 10-15% of that found in the extracts. Beale [4] reported that in *Chlorella* the amount of ALA excreted exactly matched the difference in amounts of chlorophyll synthesized in the presence and absence of LA. Under the conditions prevailing during our experiments this stoichiometric relationship does not occur in either bean or corn. The amount of ALA found in the extracts of both bean and corn leaves was always 5-10 times higher than that expected from the difference in chlorophyll between treated and untreated leaves, taking into account that 8 molecules of ALA are required for the formation of a tetrapyrrole ring (Table 1, Fig. 1).

The time course of ALA accumulation in corn leaves treated with LA is similar to that of chlorophyll accumulation in untreated leaves (Fig. 2).

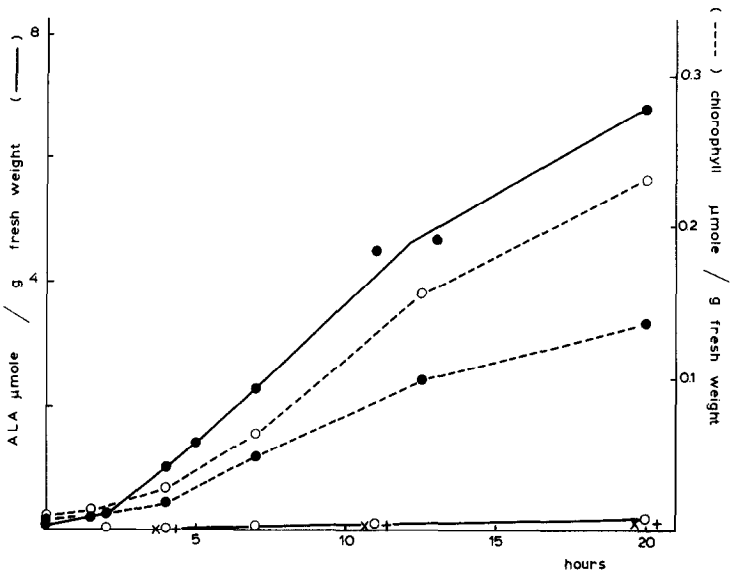

Fig. 2. The effect of levulinic acid on the accumulation of ALA and chlorophyll in greening corn leaves.
o light, water
× dark, water
• light, 20 mM levulinic acid
+ dark, 20 mM levulinic acid

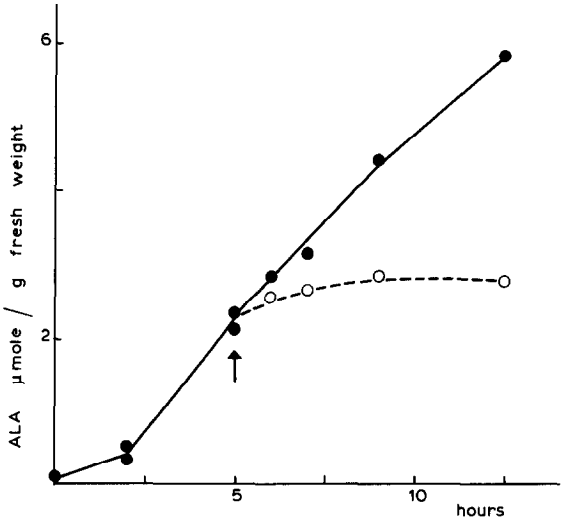

Fig. 3. The accumulation of ALA in corn leaves in the dark after illumination for 5 hours.
o control: leaves treated with 20 mM levulinic acid in continuous light.
• same treatment but leaves were returned to darkness after 5 hours in light.

TABLE 2. The effect of succinate and glycine on the accumulation of ALA in greening corn leaves in the presence of 20 mM levulinic acid.

Treatment	μmole ALA/ g fresh weight* 5 hrs light	13 hrs light	22 hrs light
Water	0.00	0.03	0.03 ± 0.02
levulinic acid (LA)	1.36	4.51	7.35 ± 0.23
0.05 M glycine	0.04	0.02	0.00
0.05 M glycine + LA	1.72	4.95	11.05 ± 0.98
0.05 M succinate	0.00	0.00	0.00
0.05 M succinate + LA	1.88	6.10	10.94 ± 0.48

*Mean and standard error of 3 experiments

When leaves were returned to the dark after 5 hours exposure to light, ALA accumulation ceased within 2-3 hours (Fig. 3). This resembles the cessation of protochlorophyll(ide) synthesis upon transfer of illuminated leaves to darkness and indicates that ALA accumulation too requires continuous light.

ALA synthetase has yet to be detected in tissues of higher plants and to our knowledge the data reported here comprise the first direct observation of ALA synthesis in such tissues. The finding that higher plant tissues produce ALA is in itself no proof that this process is catalyzed by ALA synthetase. This, however, is suggested by the enhancement of ALA accumulation in corn leaves by succinate and glycine (Table 2).

The direct involvement of ALA in the synthesis of chlorophyll in higher plants has been deduced from observations that exogenous ALA increases the formation of protochlorophyll(ide) in the dark [2,12] and accelerates chlorophyll production in the light [13,14]. The finding that ALA accumulation is light dependent and related to a decrease in chlorophyll synthesis supports this suggestion. The parallel effect of sucrose on chlorophyll synthesis and on ALA formation also indicates that these two processes are related. The

lack of stoichiometry between ALA formation and inhibition of chlorophyll synthesis in the presence of LA may be due to the release of the ALA producing system from a tightly coupled control mechanism. It may also be related to the low intensity of light used in our experiments to avoid bleaching since chlorophyll accumulation and the formation of ALA might have different light requirements.

It has been suggested that the formation of ALA plays a central role in the metabolic control of chlorophyll biosynthesis [2,3]. The demonstration of a light dependent formation of ALA in higher plants makes it possible to approach more directly problems concerning the control mechanisms of chlorophyll synthesis.

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