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Studies with 4,6-Dioxoheptanoic Acid on Etiolated and Greening Barley Leaves[1]

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ABSTRACT

4,6-Dioxoheptanoic acid (DA), an inhibitor of 5-aminolevulinic acid (ALA) dehydratase (EC 4.3.1.24), causes ALA to accumulate at the expense of chlorophyll when applied to greening leaves of *Hordeum vulgare* L. var. Larker. Preincubating etiolated leaves with DA in darkness eliminates the lag phase in ALA accumulation during a subsequent exposure to illumination. More than 50% of the DA taken up during a 2-hour incubation disappeared during a subsequent 4-hour incubation. These results suggest that barley leaves can metabolize DA, and the products of this metabolism may enhance the capacity for ALA synthesis.

4,6-Dioxoheptanoic acid is an effective inhibitor of ALA[3] dehydratase from animal (5, 12) and plant (14) systems. When applied to etiolated barley leaves exposed to light, it inhibits the conversion of ALA to porphobilinogen, thereby causing ALA to accumulate at the expense of Chl (14). LA is also an inhibitor of this enzyme and has been used extensively as a tool to study the physiology and biochemistry of ALA biosynthesis in higher plants and algae (2, 8).

In a comparative study of the effects of LA and DA on etiolated and greening barley leaves, we found (14) that DA was a more effective inhibitor of several cellular processes, including Chl synthesis, than was LA. Duggan *et al.* (3, 4) have reported that LA is extensively metabolized by etiolated and greening barley leaves. In this paper, we report a number of studies with DA, some of which indicate that DA is also metabolized. We also report that DA and, to a lesser extent, LA shorten the lag phase in ALA accumulation by etiolated leaves subjected to irradiation.

MATERIALS AND METHODS

Growth and Manipulation of Plant Material. Barley (*Hordeum vulgare L.* var. Larker) seeds, obtained from Field Seed Farm, Byron, MN, were germinated in vermiculite in darkness at 22 C. All manipulations of plant material were carried out under a dim green safelight (6). The apical 5-cm portion of 7-day-old leaves was excised in the dark and divided into 1-cm segments. One-g lots were placed into 125-ml Erlenmeyer flasks containing 2.5 to 10 μmol DA or 25 μmol LA in 1.0 ml of 50 mM K-phosphate (pH 5.0) and incubated up to 6 h in the dark or in the light.

Irradiations. Irradiations were carried out at 25 C for the indicated time under Sylvania Cool White High-Output fluorescent lamps, supplemented by incandescent lamps, at an irradiance of 3.2×10^4 ergs/cm^2·s.

Determination of ALA Accumulation *in vivo*. ALA, which accumulated in irradiated barley leaves treated with DA or LA, was determined as described previously (9).

Chl Extraction and Determination. Chl was extracted into 90% acetone and determined as previously described (14).

Measurement of DA Uptake. At the conclusion of an experiment, 50 ml of water was added to each incubation flask, and the leaf tissue was removed and washed with an excess of water. The washed leaves were gently blotted, then frozen in liquid N_2 and homogenized with 10 ml of 5% TCA in a chilled mortar. The suspension was centrifuged at 18,000*g* for 15 min, and the supernatant was assayed for DA content.

Determination of DA. DA was measured by a modification of the method given by Ebert *et al.* (5). To 1 ml of supernatant was added 1 ml of 2 M Na-acetate buffer (pH 4.6) containing 10 μmol of ALA. The mixture was heated in a boiling water bath for 20 min to produce a DA-ALA pyrrole. After cooling, an equal volume of modified Ehrlich's reagent was added, and the absorbance of the resulting pink-colored complex was assayed in a Coleman Perkin-Elmer model 124 spectrophotometer equipped with a model 165 recorder. The optical density of this complex was found to follow the Beer-Lambert law for concentrations of DA up to 0.5 mM. The levels of DA that were measured in the tissue were found not to interfere with the determination of ALA.

Purification and Thin-Layer Chromatography of the Pyrroles. The pyrroles produced by reacting ALA with either acetylacetone or DA were purified according to the method of Irving and Elliot (10). Both pyrroles were chromatographed on thin layers of cellulose (E. Merck) using 1-propanol: 1-butanol: 5% NH_4OH (1:2:1) and on thin layers of silica gel 60 (E. Merck), using 1-butanol: 10 N NH_4OH: H_2O (49:1:50).

Determination of DA Metabolism in Barley Leaves. Twenty g of 7-day-old etiolated barley-leaf segments were placed into a 14-cm Petri dish containing 20 ml of 10 mM DA in 50 mM K-phosphate (pH 5.0) and incubated for 2 h at 22 C in the dark. The leaf segments were then washed with a large excess of water, gently blotted dry, and 1-g samples were placed into 5-cm Petri dishes containing 1 ml of water. Incubations were continued for up to 4 h in darkness, and samples were periodically removed for DA determination (see above). The amount of DA that leached out into the incubation solution was also determined.

Chemicals. ALA and LA were purchased from Sigma Chemical Co.; DA, from Calbiochem-Behring Corp., San Diego, CA; *p*-dimethylaminobenzaldehyde and acetylacetone, from Fisher Scientific, Fair Lawn, NJ. Inorganic chemicals were reagent grade.

RESULTS

Effect of DA and LA on ALA Accumulation in Irradiated Leaves. DA and LA inhibit ALA dehydratase and cause ALA to

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[3] Abbreviations: ALA, 5-aminolevulinic acid; DA, 4,6-dioxoheptanoic acid; LA, levulinic acid.

accumulate at the expense of Chl in irradiated barley leaves (14). The kinetics of ALA accumulation in DA- and LA-treated leaves are shown in Figures 1 and 2. When the leaves were treated with the inhibitors at the onset of illumination, they exhibited a lag period in ALA accumulation followed by a linear phase (Figs. 1C, 2C). The lag period in DA-treated leaves is approximately 65 to 75 min while that in LA-treated leaves is approximately 100 min. Addition of DA to the leaves 1 h prior to illumination reduced the lag period to 45 min (data not shown). Preilluminating the leaves for 2 h prior to addition of DA or preincubation of the leaves with DA for 2 h prior to illumination completely eliminated this lag period (Fig. 1). Similar treatment of LA-treated leaves resulted in a shortening in the lag period but not in elimination (Fig. 2). Preincubating leaves for up to 3 h with LA shortened the lag period to 30 min but did not abolish it. The lag period in Chl accumulation in untreated leaves was longer than the lag in ALA accumulation in inhibitor-treated leaves, even when the inhibitors were added at the start of the illumination (Fig. 2).

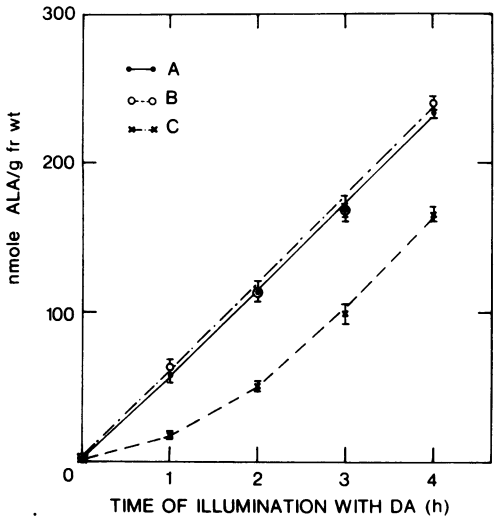

FIG. 1. The kinetics of ALA accumulation in etiolated barley leaves subjected to illumination in the presence of 10 mM DA. Etiolated barley leaves were preincubated for 2 h: A, in the dark with DA; B, in the light without DA; C, in the dark without DA; and then were illuminated for up to 4 h in the presence of DA.

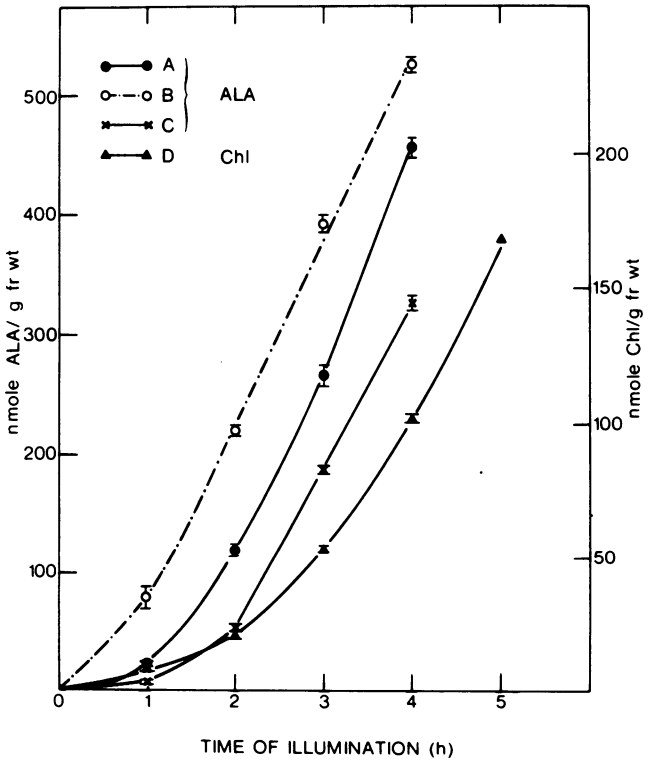

FIG. 2. The kinetics of ALA and Chl accumulation in etiolated barley leaves subjected to illumination in the presence or absence of 25 mM LA. Etiolated barley leaves were preincubated for 2 h: A, in the dark with LA; B, in the light without LA; C and D, in the dark without LA. The leaves were then exposed to illumination: A, B, C in the presence of LA, and D in the absence of LA, for up to 4 h.

Effect of Different Concentrations of DA on DA Uptake. Increasing the concentration of DA up to 10 mM caused a linear increase in the amount of DA found in the tissue after 4 h of incubation in the dark. In each case, about 12% of the DA applied was taken up (Fig. 3).

The DA extracted from the treated leaves was converted to a

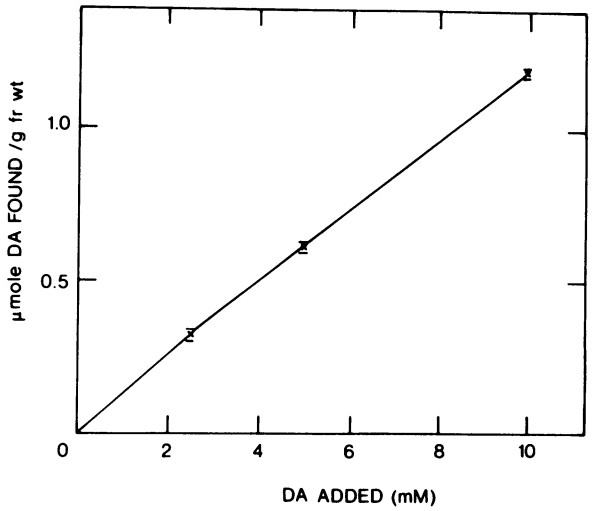

FIG. 3. The effect of various concentrations of DA on its uptake by etiolated barley leaves. Etiolated leaves were incubated with various concentrations of DA in the dark for up to 4 h. The determination of DA was as described in "Materials and Methods."

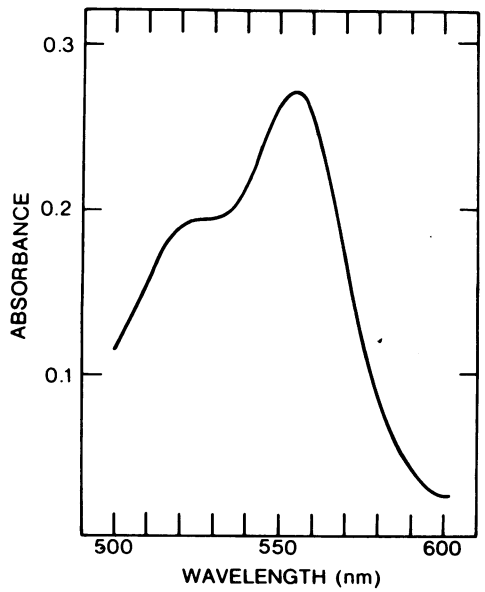

FIG. 4. Absorption spectrum of the reaction complex formed from DA-ALA-pyrrole with Ehrlich's reagent.

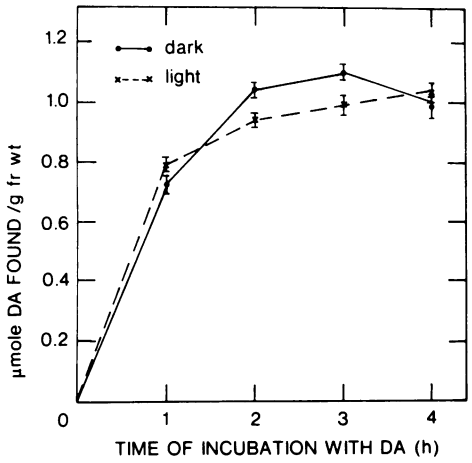

FIG. 5. The kinetics of DA uptake by etiolated barley leaves in the light or dark. Etiolated leaves were incubated with 10 mM DA for up to 4 h, and DA was determined at the indicated points.

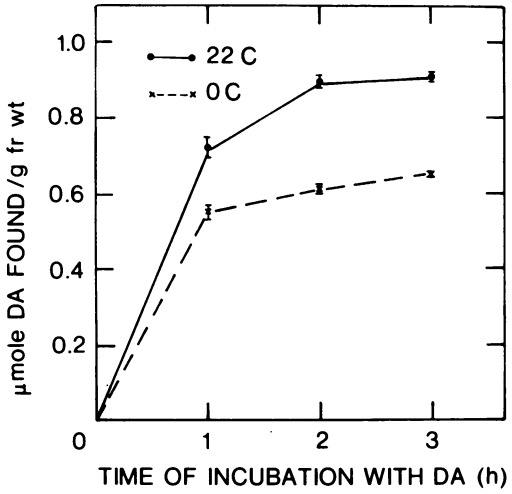

FIG. 6. The kinetics of DA uptake by etiolated barley leaves at 0 and 22 C. Etiolated leaves were incubated with 10 mM DA for up to 3 h in the dark, and DA was determined at the indicated points.

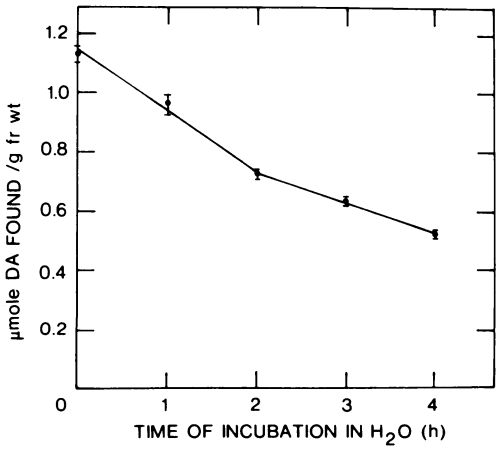

FIG. 7. The kinetics of DA breakdown in etiolated barley leaves in the dark. Etiolated barley leaves were incubated with 10 mM DA for 2 h in darkness, washed with water and then incubated in water for up to 4 h in the dark. DA was determined at the indicated points.

pyrrole (5); the absorption spectrum which the pyrrole yielded upon reaction with Ehrlich's reagent (Fig. 4) was identical to that of ALA-acetylacetone pyrrole (14). However, these pyrroles differed in their chromatographic mobility: the R_F of the DA-ALA pyrrole was 0.16 and 0.03 in 1-propanol: 1-butanol:5% NH_4OH (1:2:1) and in 1-butanol:10 N NH_4OH:H_2O (49:1:50), respectively, while the R_F of the ALA-acetylacetone pyrrole was 0.37 and 0.16, respectively.

Kinetics of DA Uptake in the Light and in the Dark. The uptake of DA into the leaves saturated by the second h of incubation in darkness or in the light (Fig. 5). The kinetics of uptake were similar under both sets of conditions (Fig. 5).

Kinetics of DA Uptake at 0 C and 22 C in the Dark. The amount of DA taken up by the tissue at 0 C was about 72% of that taken up at 22 C during a 3-h incubation in the dark (Fig. 6). After the first h, the tissue incubated at 22 C contained 78% of the total DA measured after 3 h, while the tissue incubated at 0 C contained 85%. The ratio of DA taken up at 0 C to that at 22 C was 0.77 at 1 h of incubation. These results indicate that most of the DA is taken up during the first h of incubation, and this uptake is relatively temperature insensitive.

Metabolism of DA. More than 50% of the DA taken up during a 2-h preincubation disappeared during a subsequent 4-h incubation in the dark (Fig. 7). These results suggest that barley leaves are apparently capable of metabolizing DA.

DISCUSSION

DA is a keto acid which accumulates in the serum of humans afflicted with hereditary tyrosinemia (12). Our studies indicate that DA, like LA, is metabolized by etiolated barley leaves. The elimination of the lag phase in light-stimulated ALA accumulation by DA may be linked to the product(s) of its metabolism.

It is interesting that DA treatment is just as effective as a preirradiation treatment in abolishing the lag phase in ALA biosynthesis (Fig. 1). It is well-known that the abolition of the lag phase in Chl synthesis is a phytochrome-mediated response (17). The action of Pfr and the treatment with DA may both lead to the formation of a factor which is necessary for rapid ALA accumulation in the light. Wolff and Price (18) reported that pretreating etiolated bean leaves with red light increased the respiratory rate during a subsequent incubation period with glucose in the dark. We found (14) that DA treatment stimulates the evolution of $^{14}CO_2$ from etiolated barley leaves fed [^{14}C]glucose in the dark. It is possible that both DA treatment and Pfr action lead to an acceleration of glucose metabolism which would then result in a diminution or elimination of the lag phase in ALA production.

Treatment of leaves with LA was also found to shorten, but not eliminate, the lag phase in ALA accumulation (Fig. 2). These results indicate both a similarity and difference between these two keto acids. The possibility that these two keto acids are metabolized to ALA should be considered. Although LA is known to be an ineffective precursor of ALA (1, 16), similar data for DA are lacking.

It has been proposed that the effect of Pfr in eliminating the lag phase in ALA accumulation in irradiated leaves is to increase the capacity to make ALA (11, 13). This increased capacity has been attributed to the synthesis of the enzyme(s) which make ALA (7, 15). However, both LA and DA have been reported to partially inhibit protein synthesis in etiolated barley leaves (14), and these leaves show no lag phase in ALA synthesis after preincubation with DA, especially when preirradiated. Unless the synthesis of such enzyme(s) is specifically resistant to the effect of these inhibitors, it seems unlikely that the lag phase represents the time required for synthesis of this enzyme(s).

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