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CHLOROPLAST CULTURE IX
CHLOROPHYLL(IDE) A BIOSYNTHESIS IN VITRO AT RATES HIGHER THAN IN VIVO*

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SUMMARY: Chlorophyll a is the plant pigment which in nature catalyzes the conversion of solar energy into chemical energy. By pretreating etiolated cucumber cotyledons with kinetin and gibberellic acid in the dark, it was observed that the plastids which were isolated from such tissues, and incubated in a cofactor-fortified medium, under a repetitive light-dark regime, were capable of synthesizing chlorophyll(ide) a from exogenous δ-aminolevulinic acid at a rate about twice as high as the highest rates observable in greening tissues in vivo.

INTRODUCTION:

The bioengineering of man-made membranes, more efficient than natural photosynthetic membranes in converting solar energy into chemical energy (1-3) requires a knowledge of what to bioengineer and how to bioengineer a particular structure. We have recently proposed that such man-made membranes may have to be patterned, at first, after the natural green membranes of plants before further useful structural modifications are envisioned (3). In order to pattern man-made membranes after the green plant membranes, an intimate knowledge of the biochemistry of the greening process is mandatory. In this respect, the induction of total chloroplast differentiation in vitro, would constitute an important first step in helping derive the extensive knowledge needed for duplicating the greening process in vitro. Such an undertaking would require among other things (a) a potent cell-free system, capable of the massive net synthesis of protochlorophyllide (Pchlide) of chlorophyll

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Abbreviations: ALA: δ-aminolevulinic acid; ATP: adenosine 5'-triphosphate; BSA: bovine serum albumin; Chl: Chlorophyll; Chl(ide): Chlorophyll(ide) which in turn refers to a mixture of Chl and Chlide; EDTA: ethylenediaminetetraacetatic acid.

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(Chl) and of thylakoid lipoproteins and (b) the coupling of plastidic and extra-plastidic thylakoid protein biosynthesis to pigment and lipid biosynthesis *in vitro*. As a part of a research effort aimed at inducing total chloroplast differentiation *in vitro*, we have just described a cell-free system capable of very high rates of net synthesis and accumulation of Pchlide (4). In this work, we report the biosynthesis and accumulation of chlorophyll(ide) *a* by isolated etiochloroplasts at rates about twice as high as the highest rates observable in greening tissues, i.e. *in vivo*.

MATERIALS AND METHODS:

The high rates of Chl *a* net synthesis and accumulation reported in this work, were achieved by first preincubating 3-day-old etiolated cucumber cotyledons with an aqueous solution of 0.5 mM kinetin and 2 mM gibberellic acid for 20 h. The etiochloroplasts were then isolated as described in (4) and were resuspended in a medium modified from that reported in (4) and which consisted of 0.5 M sucrose, 0.2 M Tris-HCl, pH 7.7, 20 mM $MgCl_2$, 2.5 mM EDTA, 1.25 mM methanol, 20 mM ATP, 40 mM NAD, 8 mM Methionine and 1% BSA. Each incubation consisted of 1 ml of plastid suspension (4-12 mg plastid protein), one additional ml of the suspension medium, 0.1 ml of 10 mM ALA and 0.9 ml of H_2O. The plastids were irradiated with white light (320 $\mu w\ cm^{-2}$) for 30 sec before incubation (5). Incubation was carried out at 28°C for 2 h on a reciprocating water bath operated at 50 oscillations per min. Chlorophyll(ide) *a* net synthesis and accumulation was induced by exposing the plastids to an alternating light-dark regime, which consisted of a 2.5 ms pulse of red actinic light, followed by 30 min of dark incubation. The red light pulse was generated by a Sun-pack model Auto 611 photographic flash unit (Berkey Marketing Co., Woodside, NY) (6) shielded by a long wavelength cut-off red filter, Turner No. 25, that excluded light below 585 nm. In this manner, the Pchlide which was synthesized from the added ALA in the dark, was converted into Chlide *a* by the brief red light treatment. During the subsequent dark incubation, Chlide *a* was converted into Chl *a* by esterification and more Pchlide was regenerated.

RESULTS AND DISCUSSION:

As depicted in Fig. 1, c,d, the isolated etiochloroplasts approximately quadrupled their Chl(ide) content during 2 h of incubation under the forementioned light-dark regime. For the sake of comparison, the Chl(ide) net synthesis and accumulation in excised cucumber cotyledons, which were greening at the highest rate observable in nature is reported in Fig. 1, a,b. The *in vitro* and *in vivo* rates of greening are compared quantitatively in Table I. For such a comparison to be meaningful, we have reported the rates on a unit Chl(ide) present before incubation, which in effect normalized the biosynthetic rates to the same number of plastids in both systems (7). It is apparent from Table I (exp. A,E) that the isolated plastids accumulated Chl(ide) *a* at a rate about twice as high as the highest rate of greening achievable *in vivo*.

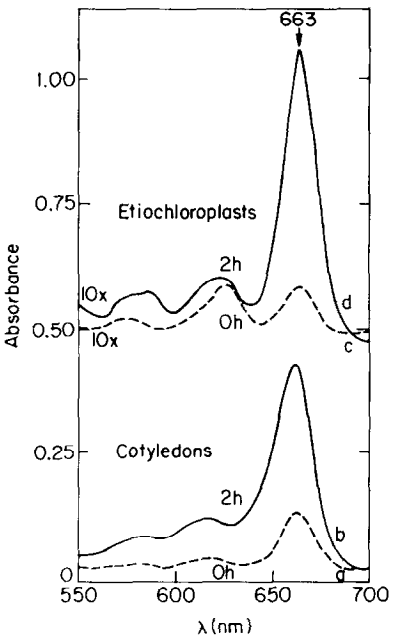

Fig. 1. Room temperature absorption spectra of the 80% acetone extracts of greening, excised cotyledons (a,b) and etiochloroplasts (c,d) before (a,c) and after (b,d) 2 h of incubation. Four-day-old etiolated cucumber cotyledons were excised with hypocotyl hooks and their lag-phase of Chl(ide) biosynthesis was eliminated by exposing them to 50 μw cm^{-2} of white fluorescent light, for 30 min followed by 3 h of dark incubation (15). Very rapid Chl accumulation was then initiated by exposing the pre-treated cotyledons to 320 μw cm^{-2} of white fluorescent light (15). Etiochloroplasts were isolated and incubated as described in Methods, under a repetitive light-dark regime that consisted of 2.5 ms of red actinic light followed by 30 min of darkness. In order to extract the pigments, 3 g of cotyledons were homogenized in 40 ml of acetone: 0.1 N NH_4OH (9:1 v/v) while 3 ml of plastids were extracted with 15 ml of this ammoniacal acetone solution (4,13). The absorption spectra of the acetone extracts were recorded on an Aminco spectrophotometer model DW-2, at an absorbance scale of 0 to 1.0, times the factors indicated on the spectra. In order to avoid overlap of the retraced spectra, the baselines were arbitrarily adjusted on the ordinate axis as evident from the absorbance values at 700 nm. The concentration of the incubated plastids was about 10 fold lower than the concentration of the plastids in the cotyledons as evidenced by the initial Chl(ide) content depicted in spectra a and c. The arrow points to the absorption maximum of Chl(ide) a.

It is noteworthy that the isolated plastids and the cotyledonary tissue exhibited different requirements for achieving their highest greening rates. First, the isolated plastids required much lower light intensities than the cotyledons (Table I, exp. A, B, E). Second, while the plastids did very well in the presence of exogenous ALA [no substantial tetrapyrrole biosynthesis occurs in the absence of added ALA in cell-free systems, (8,9)], the addition of ALA to the greening cotyledons was detrimental to Chl(ide) a accumulation, even under the low light intensities of exp. B. This is not surprising however as plant tissues are noteworthy for generating

Table I. Comparison of the Rates of Chl(ide) a net Synthesis by Etiochloroplasts In Vitro, to the Highest Rates Observable during Greening In Vivo. The Chls present in the acetone extracts described in Fig. 1, were extracted into hexane, while the Chlides remained in the hexane-extracted acetone fraction as described in ref. 14. The amounts of Chl a and Chlide a were determined by spectrofluorometry as described in ref. 14. The Δ change refers to the pigment content at the end of the incubation minus the pigment content before incubation. Chl(ide) refers to the total amount of Chl + Chlide.

Experiment	Treatments	Δ change after 2 h incubation nmoles/μmole Chl(ide) present before incubation		
		Chlide a	Chl a	Chl(ide) a
A	Etiochloroplasts were isolated from kinetin and Gibberellic acid-pretreated cucumber cotyledons as described in Methods and incubated in the presence of 0.33 mM ALA under a repetitive light dark regime that consisted of red actinic light followed by 30 min of darkness, as described in Fig. 1	2557.80	2172.96	4730.76
B	Cotyledons pretreated with kinetin and Gibberellic acid were incubated in the presence of 0.33 mM ALA under a repetitive light dark regime that consisted of 2.5 ms of red actinic light followed by 30 min of darkness.	148.90	-229.50	-80.60
C	Cotyledons pretreated with kinetin and Gibberellic acid were illuminated under 320 μw cm^{-2} of cool white fluorescent light for 2 h as described in Fig. 1	36.01	2109.88	2145.89
D	Cotyledons pretreated with water were illuminated under 320 μw cm^{-2} of cool white fluorescent light for 2 h as described in Fig. 1	-64.40	2327.37	2262.97
E	Etiolated cucumber seedlings were exposed to 50 μw cm^{-2} of cool white fluorescent light for 30 min followed by 3 h of dark incubation (15) to eliminate the lag phase in Chl(ide) a biosynthesis. The Cotyledons were excised with hypocotyl hooks and were illuminated under 320 μw cm^{2} of white light for 2 h, as described in Fig. 1.	35.17	2466.87	2502.06

their own ALA during greening and for failing to accumulate substantial amounts of Chl(ide) a from exogenous ALA, under even moderate light intensities (10, 11). Third, while pretreatment with hormones was required for achieving high Chl(ide) a biosynthetic rates in vitro, this was not observed to be the case in vivo, as if the tissue generated its own hormonal requirements during greening in the light (Table I, exp. A, C, D). These differences are exciting in their own right, and the understanding of the molecular basis which is responsible for these differences is likely to deepen our understanding of the regulation of the greening process in nature.

The massive amounts of Chlide a detectable in vitro, was not generated by the hydrolysis of endogenous Chl a but was synthesized de novo from exogenous ALA as

evidenced by the lack of Chlide a accumulation in dark controls, i.e., in etiochloroplasts incubated in complete darkness, with ALA, for 2 h (data not shown). It could have arisen also from the newly formed Chl a. This point is presently being investigated.

Finally the cell-free system described in exp. A (Table I) was not optimized for Chl(ide) b biosynthesis and accumulation. For example under the conditions of exp. A only 52.27 nmoles of Chl(ide) b per μmole of Chl(ide) a originally present before incubation were formed by the isolated plastids while 15 55.4 μmoles of Chl(ide) b were formed by the excised cotyledons in exp. E (Table I). We are now in the process of optimizing this cell-free system for massive Chl(ide) b biosynthesis and accumulation in vitro.

The cell-free system described in this work, promises to be very useful for studying the biochemistry of the greening process in vitro (12). This is particularly true since sophisticated spectrofluorometric instrumentation is no longer mandatory for such studies, as it used to be in the past (5, 13). Indeed as shown in Fig. 1, the greening process can now be reliably monitored in vitro, with a conventional spectrophotometer. Further improvements in the rate of greening of etiochloroplasts in vitro is in progress as well as an assessment of the degree of plastid differentiation which accompanies the high rates of greening just reported.

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