The Absence of Protochlorophyll(ide) Accumulation in Algae with Inhibited Chlorophyll Synthesis

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ABSTRACT
Golenkinia, Chlorella protothecoides, and mutant C-2A' of Scenedesmus were grown in darkness and on media in which chlorophyll synthesis is reduced significantly. The pigments were analyzed by spectrophotometry or by paper chromatography and compared with similar extracts from light-grown algae and dark-grown beans. No protochlorophyll(ide) was present in the dark-grown algae indicating that chlorophyll synthesis is blocked by a mechanism other than feedback regulation of aminolevulinic acid synthesis by protochlorophyll(ide) which has been proposed for flowering plants.

FLOWERING plants and certain algae grown in darkness do not synthesize Chl; instead, they accumulate small amounts of the precursor, Pchl(ide). [No distinction will be made in this paper between Pchl and its unesterified form, Pchl(id).] Associated with this lack of Chl synthesis are significantly lower levels of intermediates in the synthetic chain, including the first compound committed to porphyrin synthesis, ALA1. Addition of exogenous ALA results in a restored capacity to make various porphyrins, in particular Pchl(id). Accordingly, it has been proposed that the accumulation of Pchl(id) (when photoreduction to Chl does not occur) retards the synthesis of ALA and other key intermediates or chloroplast membrane constituents (1, 5-7, 16).

Although Chl formation in most algae is unaffected by light, dark, or growth medium (provided that basic nutritional, temperature, etc., requirements are met), a number of strains have been reported which synthesize much less pigments when grown in darkness, or in media with high ratios of fixed carbon to available nitrogen, or both. Chlorella protothecoides (9, 11), Golenkinia minitissima (4), and Scenedesmus obliquus C-2A' (14) have been reported not to accumulate Pchl(id), but rather to synthesize Chl at a rate lower than usual when grown in conditions unfavorable to high levels of Chl synthesis. This contrasts with the absence of Chl and presence of Pchl(id) in flowering plants grown in darkness. If true, these findings indicate a different mechanism of Chl synthesis regulation in these algae, one not involving feedback control by Pchl(id). Acceptance of the pigment complement proposed for these three algae must be tentative because of the following reservations. (a) Data supporting the presence of Chl and absence of Pchl(id) during low pigment synthesis were presented only for the mutant of Scenedesmus (14), and even these were unaccompanied by controls using organisms known to accumulate Pchl(id) in the dark. The latter are necessary to show the adequacy of the methodology used to prevent photoconversion of Pchl(id) to Chl and to detect Pchl(id). (b) None of the algae were maintained in conditions inhibiting Chl synthesis long enough to dilute the pigments formed during growth in an environment favorable to Chl formation and/or to establish a steady-state pigment complement. (c) The most critically studied algae which require light for Chl synthesis have been shown to form Pchl(id), but not Chl in the dark. These include Chlamydomonas reinhardtii y-l (16), Chlorella regularis YG-1 (12) which forms both Chl and Pchl(id) when grown in darkness, Chlorella vulgaris C-10 (2), and Euglena (3). Except for Euglena, all are mutants of algae which normally synthesize Chl in darkness.

The experiments reported here were performed to determine the identity of the pigments formed in Golenkinia, C. protothecoides, and S. obliquus C-2A' during periods of inhibited Chl synthesis, to allow a more accurate assessment of the mechanism of regulation.

MATERIALS AND METHODS
Details of the maintenance, culture conditions, and media used for Golenkinia are described elsewhere (4). Cells with high Chl contents were obtained by growth in an autotrophic medium in the light for 6 days. Cells with low Chl levels were obtained by growth in the dark in the presence of 10 mM Na-acetate (4) for at least 3 weeks (representing at least 16 cell divisions or cycles), during which time they were transferred into fresh media at least three times. Media were sterilized by autoclaving.

Techniques for culturing C. protothecoides are given by Oh-Hama et al. (11). Solutions of mineral salts were autoclaved and combined aseptically with filter-sterilized glucose and/or urea. Light-grown cells with high Chl contents were obtained by growth for 6-7 days in a medium containing mineral salts and 0.5% urea. Inhibition of Chl synthesis was accomplished by growth in a medium containing mineral salts, 1% glucose, and 0.1% urea in darkness. Although darkness is not necessary for bleaching of cells in this medium, it is critical to the determination of the pigments formed. The technique for bleaching cells described previously (11) was modified by extending the time of bleaching from 5 days to 3 weeks, which included three transfers into fresh media.

S. obliquus C-2A' cultures with either high ("light") or low ("dark") levels of pigment were grown in a medium containing mineral salts, ferric citrate, 5.0 g/l glucose, and 0.5 g/l yeast extract (14). It is important to maintain this mutant at 28-30°C, since at 25 C and lower the mutation "leaks" and significant Chl is formed. These cultures were maintained in either light or darkness for 3 weeks and were subcultured three times.

Seeds of Phaseolus vulgaris (var. Red Kidney, Ky. Wonder Bush) were germinated in a mixture of Vermiculite and potting soil in darkness at 26-28°C. Harvesting was done at 7-8 days.

Pigments were extracted into 80-90% acetone containing MgCO3; occasionally this was preceded by treatment of the cells or tissue with boiling water. All manipulations up to this point were performed in darkness or under a green safelight (13). The efficacy of this technique in preventing photoconversion of Pchl(id) to Chl was monitored by subjectsing bean plants to similar lighting conditions, followed by extraction and examina-

1 Abbreviation: ALA; aminolevulinic acid.
tion for Pchl(ide). Subsequent operations were performed in dim laboratory light. Pigments were then transferred to fresh, peroxide-free diethyl ether and filtered through a sugarcane column (8). The bulk of contaminating lipids and carotenoids were removed by transfer into petroleum ether (boiling range 30-50°C), adsorption onto a column of sugarcane, and repeated washing with petroleum ether or benzene (8). The pigment was eluted with diethyl ether for spectrophotometry or chromatography. Chromatographic analysis was run on Whatman 3MM paper using a solvent system of either petroleum ether-ether-acetone (2:2:1) or n-hexane-benzene-acetone-methanol-water (130:70:50:20:2) (12) in darkness at 4°C. The spots were visualized with long wavelength UV. Visible spectra were obtained with a Cary 14 recording spectrophotometer.

RESULTS AND DISCUSSION

The inclusion of results from only one or two of the algae in each figure for comparison with bean Pchl(ide) is done for the sake of clarity; identical data were obtained for all three strains in each of the tests.

Spectra of the pigments isolated from “light”- and “dark”-grown algae, and from etiolated beans are presented in Figure 1. Bean Pchl(ide) shows a peak in the red region at 625 nm, in close agreement with the absorption peak at 623 nm previously found in ether (8). The spectra of light- and dark-grown algae are identical and the peak at 663-664 nm is indicative of Chl which has a published maximum at 660-662 nm in ether (15). There is no shoulder or peak at or near 623 nm.

Diagrams of chromatograms are presented in Figures 2 and 3. Both solvent systems permitted a distinction to be made between Chl and Pchl(ide) and show that the pigments isolated from “light”- and “dark”-grown algae are similar to each other and different from extracts from dark-grown beans. The solvent system used in the analysis represented by Figure 3 gave results and separations very similar to those published previously (12). There is no indication of Pchl(ide) in the extracts of algae. Additional support for this interpretation was provided by elution and subsequent spectrophotometric analysis of the spots indicated as Chl.

The spectra (data not presented) from 600 to 700 nm are similar to those obtained using crude extracts.

These data support the earlier view based on preliminary observations that Pchl(ide) does not accumulate in measurable quantities when these algae are grown in conditions which greatly reduce Chl synthesis. The data further suggest that even when pigment production is very low, the algae are capable of completing the synthetic pathway and producing some Chl. Each of these algae, like flowering plants, regulates Chl synthesis at ALA production (4, 9, 10). Unlike flowering plants, however, the regulation cannot be due to feedback control by Pchl(ide). It is of interest to contrast this difference with the many similarities of plastid structure and composition between flowering plants and green algae which have led to suggestions of evolutionary commonality.

LITERATURE CITED

3. Ellis H. S. and Golenkinia, C. prototheocides, and beans, and from light-grown Golenkinia and Chlorella. Solvent system was petroleum ether-ether-acetone (2:2:1). Abbreviations: Chl.; Golenkinia; Chlor.: Chlorella; Car.: carotenoids; Chlor.: chlorophyll; Protochlor.: Pchl(ide).

FIG. 1. Absorption spectra of pigments extracted from light- and dark-grown Golenkinia, or from dark-grown beans. The solvent for all spectra was diethyl ether.

FIG. 2. Representation of chromatography of pigments from dark-grown Golenkinia, C. prototheocides, and beans, and from light-grown Golenkinia and Chlorella. Solvent system was petroleum ether-ether-acetone (2:2:1). Abbreviations: Chl.; Golenkinia; Chlor.: Chlorella; Car.: carotenoids; Chlor.: chlorophyll; Protochlor.: Pchl(ide).

FIG. 3. As Figure 2. The solvent system employed was hexane-benzene-acetone-methanol-water (130:70:50:20:2).

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