GENETIC CONTROL OF CHLOROPHYLL BIOSYNTHESIS IN *CHLAMYDOMONAS*

Analysis of Mutants at Two Loci Mediating the Conversion of Protoporphyrin-IX to Magnesium Protoporphyrin

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ABSTRACT

In this report we describe two nonallelic Mendelian protoporphyrin accumulating mutants *br*$_s$-*1* and *br*$_c$-*1*. Results of experiments with these mutants lead us to postulate that porphyrin biosynthesis branches into light and dark steps between protoporphyrin-IX and magnesium protoporphyrin. We hypothesize that the *br*$_c$ locus controls a dark step while the *br*$_s$ locus either controls a step in the main pathway before the branch or mediates the preparation of the magnesium ion for its insertion into protoporphyrin-IX. The *br*$_s$-*1* mutant is thought to be light sensitive because a block prior to the branch point in the porphyrin pathway prevents chlorophyll formation in either the light or the dark. The *br*$_c$-*1* mutant, which also accumulates protoporphyrin in the dark, forms chlorophyll and chloroplast lamellae when transferred to the light, showing that function of the porphyrin pathway is normal in the light.

Granick (1948 *a*) first isolated a mutant of the green alga *Chlorella* that was blocked in chlorophyll biosynthesis and accumulated protoporphyrin-IX (PROTO) which is known to be a precursor of the iron porphyrin heme. Based on the study of this mutant and a mutant accumulating magnesium protoporphyrin (Granick, 1948 *b*), Granick (1948 *c*) then formulated a scheme in which chlorophyll and heme share a common pathway from the synthesis of δ-aminolevulinic acid (ALA) to PROTO. Magnesium is incorporated into PROTO (Granick, 1948 *b*) to form magnesium protoporphyrin (Mg-PROTO) which is then converted to protochlorophyllide (PCHLD) through a series of steps defined by characterizing additional mutants (Granick, 1950, 1961; Ellsworth and Aronoff, 1968 *a*, 1969; Aronoff et al., 1971). PCHLD is photoconverted to chlorophyllide (CHLD) in most plants (Boardman, 1966), but some other plants, including *Chlamydomonas reinhardtii* and *Chlorella*, can carry out the reduction enzymatically in the dark (Granick, 1950, 1967). Esterification of CHLD with phytol yields chlorophyll *a* (Wolff and Price, 1957; Ellsworth and Aronoff, 1968 *b*). Analysis of porphyrin mutants isolated in the photosynthetic bacterium *Rhodopseudomonas* support essentially the same biosynthetic pathway up to PCHLD (Lessie and Sistrom, 1964; Lascelles and Altshuler, 1969; Richards and Lascelles, 1969).

Although experiments with *Chlorella* and *Rhodopseudomonas* mutants have told us much about the pathway of chlorophyll biosynthesis, little has been learned about the genetic control of the pathway because one cannot do genetic analysis in either organism. Studies of barley mutants blocked in chlorophyll biosynthesis have demonstrated that nuclear genes control certain steps of the pathway in higher plants (von Wettstein, 1961; Boynton and Henningsen, 1967; von Wettstein et al., 1971; Gough, 1972; Henningsen et al., 1973). Four structural genes have been shown to control the conversion of PROTO to Mg-PROTO, and another structural gene controls a step between Mg-PROTO and PCHLD. In addition three loci which exert a regulatory role on the pathway have been described (Foster et al., 1971). Recently von Wettstein et al., (1974) have demonstrated that mutants at two of these regulatory loci can enhance the amounts of specific porphyrins accumulated by a structural gene mutant blocked between PROTO and Mg-PROTO and a second mutant blocked between Mg-PROTO and PCHLD. In spite of obvious difficulties in the genetic analysis of the seedling lethal structural gene mutations, von Wettstein and colleagues have laid the groundwork for our understanding of the genetic control of chlorophyll biosynthesis.

We feel that *Chlamydomonas reinhardtii* may even be a better system in which to study the genetics of chlorophyll biosynthesis, since: (*a*) It has well defined Mendelian and non-Mendelian (chloroplast) genetics. (*b*) Its life cycle is extremely short. (*c*) It can be handled like a bacterium in terms of growth, incubation, and identification of mutations. (*d*) Its chloroplast functions including photosynthesis are dispensable if acetate is provided as a carbon source. Although several pigment mutants of *Chlamydomonas* have been isolated previously (Sager, 1955; Sager and Zalokar, 1958), including the yellow mutant (*y-1*) and a brown mutant (*br*) which accumulates PROTO, this organism has heretofore not been exploited to investigate the genetic control of chlorophyll biosynthesis. Recently we embarked on such a study in *Chlamydomonas* and have already isolated many mutants which block chlorophyll synthesis and accumulate porphyrin intermediates. In this paper we report on mutants at two gene loci responsible for the conversion of PROTO to Mg-PROTO. Experiments with these mutants suggest that PROTO may act to regulate chlorophyll biosynthesis in *Chlamydomonas* and that the conversion of PROTO to Mg-PROTO may occur by separate light and dark reactions.

MATERIALS AND METHODS

Strains

A mating type plus (mt^+) stock derived from strain 137c of *Chlamydomonas reinhardtii* was used as the wild-type stock for mutagenesis to obtain pigment mutants. The *y-1* mutant was obtained from Dr. P. Siekevitz of Rockefeller University. This mutant was repeatedly cloned in darkness for yellow phenotype before use. Two arginine-requiring mutants *arg-2* and *arg-7* were also used.

Culture Methods

High salt medium (HS) (Sueoka, 1960) with 2 g/liter of sodium acetate·$3H_2O$ (HSA) was used routinely. Difco agar (1.5 g/liter) (Difco Laboratories, Detroit, Mich.) was added to make solid medium for plating experiments. Cells were grown mixotrophically at 25°C in 300 ml liquid medium in 500 ml flasks on a New Brunswick gyrotory shaker (New Brunswick Scientific Co., Inc., New Brunswick, N.J.) under ~15,000 lx of cool white fluorescent light, or on solid medium under ~6,000 lx of cool white fluorescent light. Cells were grown heterotrophically at 25°C either by wrapping the HSA flasks with black electrical tape or by placing the HSA plates in a dark box. In some experiments, flasks were wrapped in six layers of amber colored cellophane (no. 3159 Tango, Dennison Mfgr. Co., Farmington, Mass.) to screen out wavelengths below 550 nm absorbed by PROTO, yielding a light intensity of ~3,500 lx.

Viable Cell Counts

Cells from each sample were counted in a hemocytometer and then plated on HSA medium after appropriate dilution. The viable cell count is defined as the ratio of the number of colonies appearing on the plates per the number of cells plated as determined by hemocytometer counts.

Mutagenesis

Mutations were induced by irradiating a 5 ml cell suspension (5×10^6 cells/ml) in a small petri dish bottom (diam 5 cm) with a germicidal UV lamp (Sylvania G8T5, Sylvania Electric Products, Inc., New York) mounted in a ChromatoVue CC-20 cabinet (Ultra-Violet Products, Inc., San Gabriel, Calif.) at a distance of 20 cm for 30 s (11,000 ergs·cm^{-2}·s^{-1}) while the cell suspension was stirred by a magnetic stirring bar. The irradiated cells were kept in suspension in the dark for 12 h to prevent photoreactivation. They were then diluted, plated on HSA plates under room light at 10^5 cells per plate, and incubated in the dark. Survival under these conditions was 1–2% as determined by viable cell counts. Mutants

were selected first by visual examination of the plates for colonies with color differences. Brownish colonies are observed among viable cells at a frequency of about 1×10^{-5}.

Putative mutants were grown heterotrophically in liquid culture and their in vivo spectra measured with a Hamamatsu R636 photomultiplier (Hamamatsu Corp., Middlesex, N.J.) in the ZR-21 integrating sphere attachment of a Zeiss DMR-21 split-beam recording spectrophotometer (Carl Zeiss, Inc., New York). Isolates whose spectra differed markedly from those of wild-type cells are kept for further analysis. All operations were carried out under a dim green safe light to prevent photoconversion of any PCHLD present.

Quantitative Analysis of Chlorophylls

Chlorophyll content was determined from both in vivo and in vitro measurements. Total chlorophyll was estimated from 80% acetone (vol/vol) extracts of pelleted cells and measured in the Zeiss spectrophotometer without the sphere. The equation of Arnon (1949) was used to calculate total chlorophyll:

$$\text{Total chlorophyll} = 20.2\ OD_{645} + 8.02\ OD_{663}.$$

To correct the extinction values in Arnon's equation for in vivo chlorophyll determinations, the in vivo peak values of chlorophyll *a* (675 nm) and chlorophyll *b* (650 nm) were compared with the absorption values of 80% acetone extracts at 663 nm (chlorophyll *a*) and 645 nm (chlorophyll *b*) of the same wild-type cells over a range of cell concentrations. The modified equation for in vivo determination of total chlorophyll is:

$$\text{Total chlorophyll} = 24.24\ OD_{650} + 14.04\ OD_{675}.$$

To compare the carotenoid pigments in dark-grown cells of wild-type and the br_s-*1* and br_c-*1* mutants, cells were extracted in an acetone-petroleum ether mixture (1:2, vol/vol) and the chlorophyll and carotenoid pigments partitioned into petroleum ether by washing the extract with water to remove the acetone. The pigments were then separated on sucrose thin-layer plates (Chasson and Wickliff, 1964) by use of a solvent system consisting of *n*-propanol, benzene, and petroleum ether (0.15:35:65, vol/vol) into four discrete bands which were eluted in acetone and analyzed spectrophotometrically. Band 1, nearest the origin, was identified as chlorophyll *b* (λ max 650, 465 nm); band 2, as chlorophyll *a* (λ max 663, 430 nm); band 3 as xanthophylls (λ max 475, 442, and 415 nm); and band 4, near the solvent front, as carotenes (λ max 468, 438, and 415 nm).

Porphyrin Extraction and Determination

The procedures used were based on the solvent extraction methods of Falk (1964). Cells grown heterotrophically were harvested by centrifugation, and both the cells and the medium were extracted with a mixture of ethyl acetate and glacial acetic acid (3:1, vol/vol) at the ratio of 20 ml for 1 g of wet cells and 1 vol per 4 vol of medium. After 30 min of extraction with frequent shaking, the cell debris and the growth medium were removed from the ethyl acetate portion by centrifugation and liquid partition, respectively. The ethyl acetate portions were pooled, and washed twice with 0.5 vol of saturated aqueous sodium acetate and once with 0.1 vol of 3% aqueous sodium acetate. The sodium acetate layers were combined for uroporphyrin determination.

Protoporphyrin and coproporphyrin were extracted completely from the ethyl acetate layer by 15% (wt/vol) HCl. Porophyrins were extracted from the HCl fraction into 1 vol of ethyl ether after the HCl fraction was adjusted to pH 4 by adding saturated sodium acetate. The ethyl ether portion was washed twice with 0.5 vol of saturated sodium acetate and once with 0.1 vol of 3% sodium acetate before it was extracted exhaustively with 0.36% (wt/vol) HCl to remove coproporphyrin. Extraction with 10% (wt/vol) HCl removed the PROTO from the ethyl ether layer.

The sodium acetate layer collected from washing the ethyl acetate extract was adjusted to pH 3.2 with concentrated HCl and extracted with three portions of ethyl acetate. The uroporphyrin was then extracted from the ethyl acetate portion by 2% (wt/vol) HCl. The extraction process was carried out in dim light under a hood. Absorption spectra of all porphyrin fractions were measured immediately in the Zeiss recording spectrophotometer to minimize loss due to denaturation. Specific porphyrins were identified by comparing the observed absorption maxima with published values of purified porphyrins as given by Falk (1964). Extraction of porphyrins was considered complete only after no fluorescence could be observed under a long wave (Sylvania F8T5) UV lamp.

To determine the concentrations of specific porphyrins, the OD values of their Soret peaks were first corrected by the equation of Falk (1964):

$$OD_{corr} = \frac{2\ OD_{max} - (OD_{430} + OD_{380})}{k}$$

The *k* factors used for specific porphyrins were those given by Falk (1964). The corrected OD values were then divided by the molar extinction coefficients of the specific porphyrins (Falk, 1964) to give their molar concentrations. Ether extracts of porphyrins were also separated chromatographically on both silica gel thin-layer plates and aluminum oxide-impregnated paper by use of the solvent system B of Gough (1972).

Genetic Analysis

Standard procedures (Ebersold and Levine, 1959) were used for genetic analysis with some modifications to accommodate the light-sensitive nature of the mutants. Mutants to be crossed were grown on HSA plates in the dark. For gametogenesis the cells were suspended in

nitrogen-free medium (Gillham et al., 1974) and put under ~6,000 lx cool white fluorescent light. After the gametes had been allowed to differentiate (5–8 h) they were mated for 4 h under the same light conditions. The mating mixture, plated on HS plates containing 4% agar, was incubated overnight in the light, following which the plates were put in darkness for zygotes to mature. Zygotes were germinated under orange light in a cardboard box with a window covered by six layers of amber cellophane placed under ~6,000 lx cool white fluorescent light. This provided the light (~1,400 lx) for the germination of zygotes but did not affect the viability of the mutants. Zoospore colonies produced by the meiotic products of the zygote were grown up either in the dark or under very dim light (< 10 lx). The phenotype of each zoospore colony was then compared under mixotrophic and heterotrophic growth conditions on HSA plates. Different gene combinations can be identified and the three classes of tetrads separated on the basis of their phenotypes under the two growth conditions (see Table III).

Selection of Diploids for Complementation Tests

A new quick diploid method was used to test for complementation between different pigment mutants. After gametes of two mutants were put together, the mating mixture was plated on HSA plates and incubated in the dark. After 7–10 days in the dark, two sizes of colonies were visible on the plates. The small colonies were formed by gametes and the large colonies by spontaneous vegetative diploids (Ebersold, 1963, 1967). Zygotes did not germinate. Green diploid colonies indicated complementation while yellow-brown diploid colonies indicated no complementation. All presumptive diploid colonies were isolated and confirmed to be mt^- as expected since Gillham (1963) and Ebersold (1967) have shown that mt^- is dominant to mt^+.

Analysis of the Greening Process

Cells of appropriate genotypes grown heterotrophically to a concentration of 3 to 4 × 10^6 cells/ml were harvested under dim light by centrifugation. They were resuspended in fresh HSA medium to a concentration of 3.5 × 10^6 cells/ml and divided into seven samples of 250 ml each. One sample of each genotype (time 0) was analyzed immediately for chlorophyll and PROTO content, and viable cell count, and fixed for electron microscopy. The rest of the samples were put on a shaker under white light (~15,000 lx) and processed as before at selected time intervals.

Electron Microscopy

Cells (approx. 10^8 cells) were pelleted by centrifugation and resuspended in a mixture of equal volumes of 4% glutaraldehyde (70% conc., Ladd Research Industries, Inc., Burlington, Vt.) in 0.004 M phosphate buffer (Na_2HPO_4-KH_2PO_4, pH 7.0) and HSA medium. After fixation for 2 h at 20°C, the cells were washed three times in an equal volume mixture of 0.004 M phosphate buffer and HSA medium. The cells were then postfixed in 2% OsO_4 in 0.1 M phosphate buffer (pH 7.0) for 2 h at 20°C, centrifuged, and washed twice in the same buffer prior to dehydration in ethanol and embedding in a low viscosity epoxy resin (Spurr, 1969). During dehydration and infiltration the cells were centrifuged and resuspended at each step. Sections were cut on a Cambridge ultramicrotome (Cambridge Instument Co., Inc., Ossining, N.Y.) with DuPont diamond knives (E. I. DuPont de Nemours & Co., Wilmington, Del.), mounted on naked 75 × 300 mesh grids, and contrasted with saturated uranyl acetate in 50% ethanol and lead citrate.

Median sections were analyzed of 10 cells of each genotype and treatment, showing a central nucleus with nucleolus inside and a peripheral chloroplast. The area occupied by the chloroplast was measured by placing a Lucite grid of standard area (1 cm rulings) over 18,000 × micrographs of whole cells. Two micrographs of 60,000 × through known areas of the chloroplast of each cell were analyzed by running a K & E map measure along the length of all chloroplast lamellae sectioned perpendicular to their surface and showing distinct profiles to determine the total membrane profile, and along the segments of these lamellae which were tightly opposed to one another to determine the total paired distance. Measurements were averaged and converted to microns of lamellar profile or paired distance per square micron of chloroplast area.

RESULTS

Descriptions of the Mutants

Mutant br_8-*1* grows well on HSA medium either in the dark or under dim light (< 10 lx cool white fluorescent light, < 10 lx red light, or ~1,400 lx amber light). Colonies on plates are yellowish-brown to the eye with no visible green color. Under the microscope, cells filled with reddish-brown pigment can be seen scattered randomly over the colonies. Cultures of br_8-*1* grown heterotrophically in liquid medium were yellowish-brown and spectra of both the cell suspension in vivo (cell concentration 5 × 10^6 cells/ml) and the cell-free medium gave peaks at 643, 585, and 530 nm, suggesting the presence of PROTO (Granick, 1948 *a*). In a concentrated cell suspension (6 to 8 × 10^7 cells/ml), a peak is seen at 670 nm indicating the presence of some chlorophyll *a* (Fig. 1 a), and the 530 nm PROTO shoulder is no longer visible, presumably being masked by carotenoids or increasing light scattering of the preparation. Dark-

grown cultures of br_s-*1* analyzed by the solvent extraction procedure of Falk (1964) were shown to contain PROTO, identified by absorption spectra (Fig. 1 *b*) and fluorescence emission spectra (λ max at 655, 603 nm). No other porphyrins besides chlorophyll were detectable by absorption spectrophotometry, where our limit of detection for uroporphyrin and coproporphyrin would be 0.018 and 0.02 nmol/ml, respectively. We consider br_s-*1* as a stringent mutant in terms of chlorophyll synthesis, since it does not turn green when exposed to light.

Mutants br_c-*1* and br_c-*2* are independently isolated and have identical phenotypes. Colonies grown heterotrophically appear yellowish-brown with just a tinge of green, and reddish-brown pigment-filled cells can be observed under the microscope. In heterotrophic liquid culture these two mutants resemble br_s-*1* except that they are slightly greener. In vivo spectra of dark-grown cell suspensions show peaks at 670, 650, and 585 nm (Fig. 1 *a*), suggesting the presence of both PROTO and chlorophyll. The 643 nm PROTO peak is masked by a peak at 650 nm which does not disappear when cells are exposed to bright light, suggesting that chlorophyll *b* and not photoconvertible PCHLD (Henningsen and Boynton, 1969) is present. When transferred to bright light, heterotrophic cultures of these two mutants turn green, and the green cells have in vivo peaks at 675, 650, 620, and 590 nm comparable to the spectra of wild type cells. PROTO was extracted and identified from both the dark and light grown cultures of br_c-*1* as described for br_s-*1* (Fig. 1 *b*, Table I). No other porphyrins besides chlorophyll *a* and *b* were detected by either absorption spectrophotometry or chromatography. Carotenoid pigments in br_s-*1* and br_c-*1* appear to be similar to those of wild type when in vivo spectra of whole cells are compared and when analyzed by sucrose thin-layer chromatography. We consider br_c-*1* and br_c-*2* as conditional mutants in terms of chlorophyll synthesis because they can only turn green in the light.

Analysis of the Greening Process

The greening process in the *y-1* mutant of *Chlamydomonas reinhardtii* has been described in

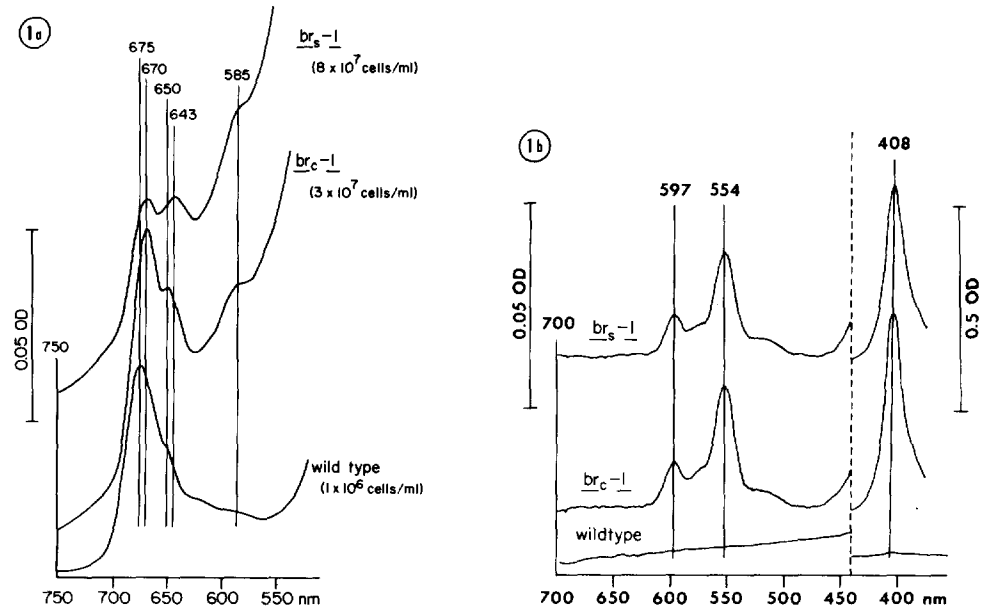


FIGURE 1 (*a*) Comparison of the in vivo absorption spectra of dark-grown cells of the br_s-*1* and br_c-*1* mutants and wild type. Absorption maxima at 643 and 585 nm are characteristic of protoporphyrin-IX. (*b*) Comparison of absorption spectra of the 10% HCl fractions of wild type, br_c-*1*, and br_s-*1* using the solvent extraction method of Falk (1964). Absorption maxima of the br_s-*1* and br_c-*1* extracts at 597, 554, and 408 nm agree well with published values for protoporphyrin-IX. Approximately the same amount of cells were used in each determination.

TABLE I

Comparison of Pigment Concentrations (Micromoles per 10^9 cells) in wild type, y-1, br_c-1, and br_s-1 under Different Growth Conditions

	Light grown		Dark grown			Amber light	
Genotype	chlorophyll	protoporphyrin	chlorophyll	Protochloro-phyllide	protoporphyrin	chlorophyll	protoporphyrin
Wild type	3.8	ND	2.5	—	ND	3.5	ND
*y-1**	3.6	ND	0.03	0.01	—	—	—
br_c-1	1.9	0.012	0.18	—	0.084	1.3	0.06
br_s-1	Lethal	Lethal	0.02	—	0.057	—	—

ND, undetectable.

* Data from Matsuda et al. (1971).

detail by Ohad et al. (1967 *a,b*). Plastids of dark-grown *y-1* cells accumulate large amounts of starch granules but have very little chlorophyll and few lamellae. The *y-1* mutant is thought to lack the enzyme necessary for the conversion of protochlorophyll to chlorophyll in the dark (Ohad et al., 1967 *a*). We calculate from data of Matsuda et al. (1971) that after seven generations of growth in the dark, *y-1* contains as PCHLD about 0.3%, and as chlorophyll about 0.8% of the chlorophyll in the light (Table I). When *y-1* is exposed to light, synthesis of chlorophyll proceeds rapidly together with the formation of chloroplast membranes and the disappearance of starch granules. Plastid differentiation continues for 9–10 h in the light until *y-1* cells are indistinguishable from wild type both in terms of pigment level, chloroplast structure, and function.

In dark-grown br_s-1 cells, the chloroplast closely resembles that of the dark-grown *y-1* mutant and is highly lobed, filled with big starch granules, and has few lamellae (Fig. 5). When a dark-grown culture of br_s-1 is transferred to light (~15,000 lx), PROTO decreases rapidly in the first 2 h but no chlorophyll is accumulated (Fig. 2). No increase of chloroplast lamellae occurs in cells grown in light for 24 h (Fig. 6). Viability of the mutant cells is not affected during the first 24 h exposure to light, but all cells were dead by 48 h (Fig. 3). Since br_s-1 is incapable of forming significant amounts of chlorophyll, the decrease of PROTO observed may be due to photodestruction.

Dark-grown cells of br_c-1 have about 8% of the chlorophyll present in dark-grown wild-type cells (Table I). Ultrastructurally, cells of this mutant are indistinguishable from dark-grown br_s-1 and *y-1* cells (Figs. 7, 12). When a dark-grown br_c-1 culture is transferred to light, PROTO decreases rapidly in the first 2 h with no chlorophyll increase (Fig. 2) suggesting that the accumulated PROTO is not converted to chlorophyll upon exposure to light. Subsequently, PROTO decreases at a faster rate in br_c-1 than in br_s-1 (Fig. 3) which may signify conversion of some accumulated PROTO to chlorophyll in br_c-1. To demonstrate this conclusively one would have to follow the greening process in dark-grown cells where PROTO had been labeled by [^{14}C]ALA feeding and measure the

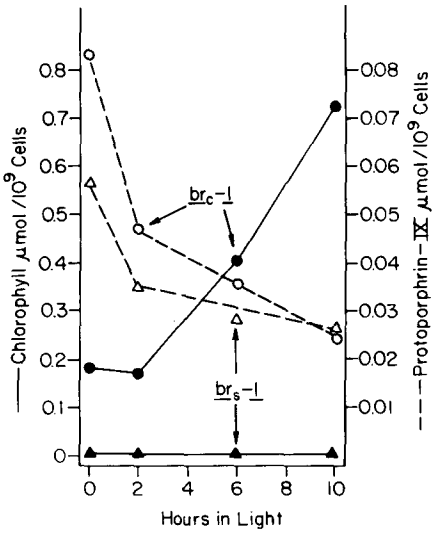


FIGURE 2 Molar comparison of chlorophyll increase and protoporphyrin-IX decrease after transfer of dark-grown cells of br_s-1 and br_c-1 to bright light (~15,000 lx). Protoporphyrin-IX was extracted and analyzed as described in the Methods section using the solvent extraction procedure of Falk (1964). Chlorophyll was determined from in vivo spectra.

incorporation of label into newly formed chlorophyll. However, conversion of accumulated PROTO cannot be responsible for all the chlorophyll increase since new chlorophyll accumulates at a faster rate than PROTO disappears.

6 h after a culture of br_c-*1* is transferred to light, starch granules in the chloroplast have disappeared, chlorophyll concentration has more than doubled and new lamellae have been formed adjacent to the chloroplast envelope (Figs. 9, 13). Both the chlorophyll concentration and the membrane profile reach half of their maximum values by 12 h, and the lamellae begin to pair over short distances (Figs. 10, 14). Extensive pairing of the lamellae is evident after 24 h exposure to light (Figs. 11, 15), by which time the cell number has almost doubled and the chlorophyll concentration is about 12 $\mu g/10^7$ cells. Synthesis of chlorophyll continues until the concentration stabilizes at 16.8 $\mu g/10^7$ cells in a mixotrophic culture near stationary phase. At this time, the membrane profile is 4.45 $\mu m/\mu m^2$ plastid area, the paired distance is 1 $\mu m/\mu m^2$ plastid area (Fig. 4) and the PROTO concentration is about ½ of the maximum (Table I) reached under heterotrophic growth. The chloroplast in these br_c-*1* cells resembles that from a light-grown wild-type or *y-1* cell except that there are somewhat fewer lamellae and less extensive pairing (Figs. 8, 16). It is interesting that about 75% of the chlorophyll accumulation, 75% of the membrane increase and 20% of the pairing of a mixotrophically grown br_c-*1* cell have taken place after 24 h of exposure to light (Fig. 4), while the greening process is virtually completed in the *y-1* mutant by 10 h under similar conditions (Ohad et al., 1967 *b*). In general, the increase of chloroplast membranes in br_c-*1* parallels the increase of chlorophyll in a manner similar to that reported for greening of the *y-1* mutant (Ohad et al., 1967 *b*) and etiolated barley (Henningsen and Boynton, 1974); however, grana formation proceeds at a significantly slower rate in br_c-*1* than in either of the other two systems. During the greening process, the in vivo chlorophyll *a* peak of br_c-*1* cells

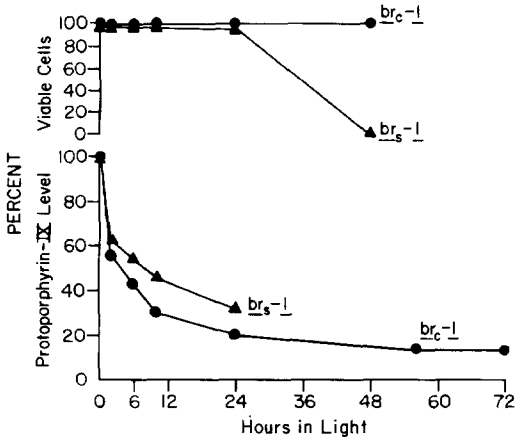


FIGURE 3 Relative decrease of protoporphyrin-IX and cell viability after transfer of dark-green cells of br_s-*1* and br_c-*1* to bright light (~15,000 lx).

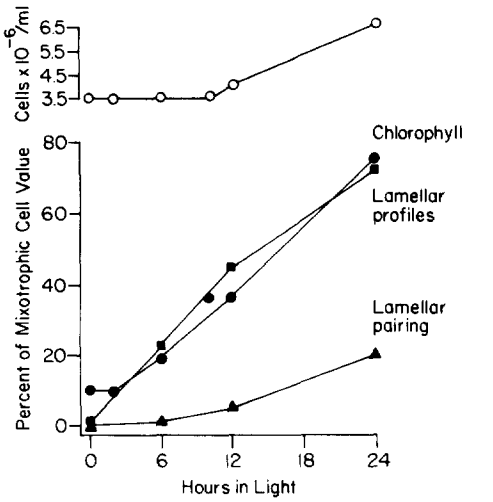
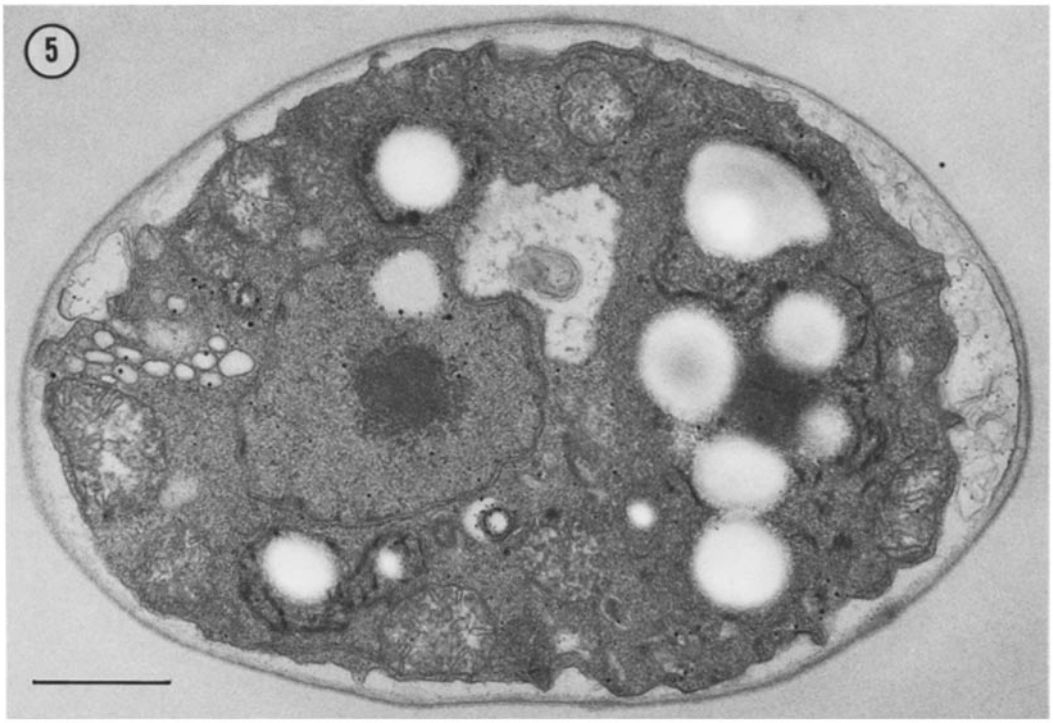


FIGURE 4 Increase of chlorophyll, chloroplast lamellae, and pairing during greening of br_c-*1* mutant. Data are expressed as a percentage of the values in mixotrophically grown br_c-*1* cells; chlorophyll 16.8 $\mu g/10^7$ cells, chloroplast lamellae 4.45 $\mu m/\mu m^2$ plastid area, and paired distance 1 $\mu m/\mu m^2$ plastid area. The increase of cell number during greening is also shown.

FIGURE 5 Median section of a dark grown cell of the mutant br_s-*1*. The chloroplast is highly lobed, has few lamellae, a poorly defined pyrenoid, and is filled with starch granules. (Bar in Figs. 5–10 equals 1 μm). × 18,000.

FIGURE 6 Median section of a br_s-*1* cell after 24 h illumination (~15,000 lx). No increase in chlorophyll or chloroplast lamellae has occurred. × 18,000.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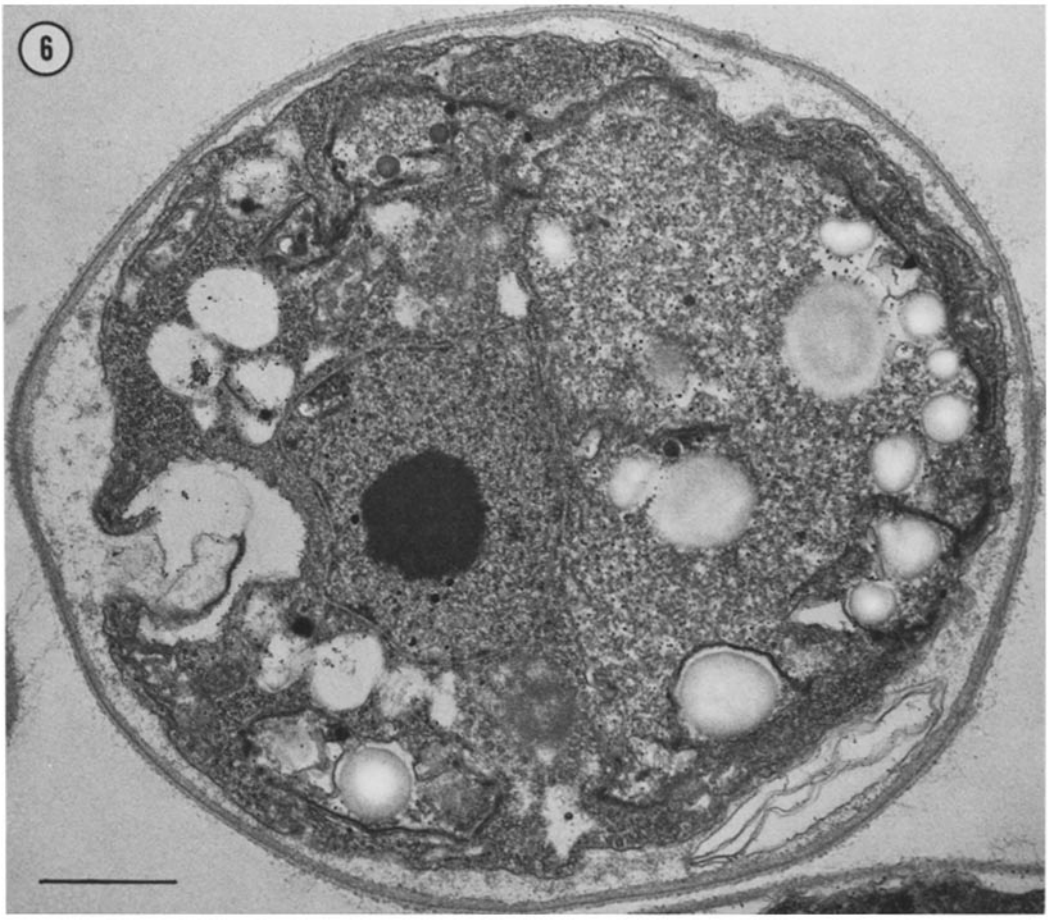
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gradually shifts from 670 to 675 nm in a fashion similar to that described in barley by Henningsen and Boynton (1974). No appreciable loss of viability is observed during the greening of the br_c-*1* mutant (Fig. 3).

Genetic Analysis

The phenotypically similar br_c-*1* and br_c-*2* mutants were crossed to the *arg-2* and *arg-7* mutants, mapping close together on linkage group I, to obtain double mutants for selecting diploids (Ebersold, 1963; Gillham, 1963), and to study the mode of inheritance of br_c-*1* and br_c-*2*. In crosses, both br_c-*1* and br_c-*2* segregate as single Mendelian genes (two mutant:two wild type in 17 and 18 tetrads, respectively) and are probably not on linkage group I (Table II). The cross between br_c-*1* and br_c-*2* produced only parental ditype (PD) tetrads (Table III) indicating that the two mutants are allelic. Since yellow mutations arise spontaneously at a high frequency (Sager and Tsubo, 1962) and because of the obvious phenotypic similarities between *y-1* and br_c-*1*, it was necessary to prove beyond doubt that br_c-*1* is neither allelic nor a double mutant with *y-1*. Since not a single yellow segregant was detected in the 35 zygotes analyzed, it is very unlikely that, barring close linkage, either br_c-*1* or br_c-*2* is a double mutant with *y-1*. Results of crosses between br_c-*1* and *y-1* showed that these two mutants are not linked because the ratio of PD tetrads to nonparental ditype (NPD) tetrads is close to 1:1 ($P > 0.5$), and the NPD to tetratype (T) tetrad ratio significantly exceeds 1:4 ($P < 0.01$) (Barratt et al., 1954).

Results of the cross between br_c-*1* and br_s-*1* (Table III) indicate that these two mutants are nonallelic since many recombinant wild-type progeny are observed. Results from the cross between br_s-*1* and *y-1* (Table III) also show that br_s-*1* is not linked to *y-1*, because the PD:NPD ratio approximates 1:1 ($P > 0.5$) and the NPD:T ratio significantly exceeds 1:4 ($P < 0.01$).

Complementation was tested between br_c-*1* and br_c-*2* using diploids selected from a cross of br_c-*1* *arg-7* and br_c-*2* *arg-2* double mutants. Complementation tests of other mutants were done by a faster and more convenient method (see Materials and Methods). Results of the complementation tests (Table III) agree with the results of the recombination analysis and show that: br_c-*1* and br_c-*2* behave as noncomplementing alleles; br_c, br_s, and *y* are functionally distinct genes; and each of these mutant alleles is recessive to its wild-type counterpart.

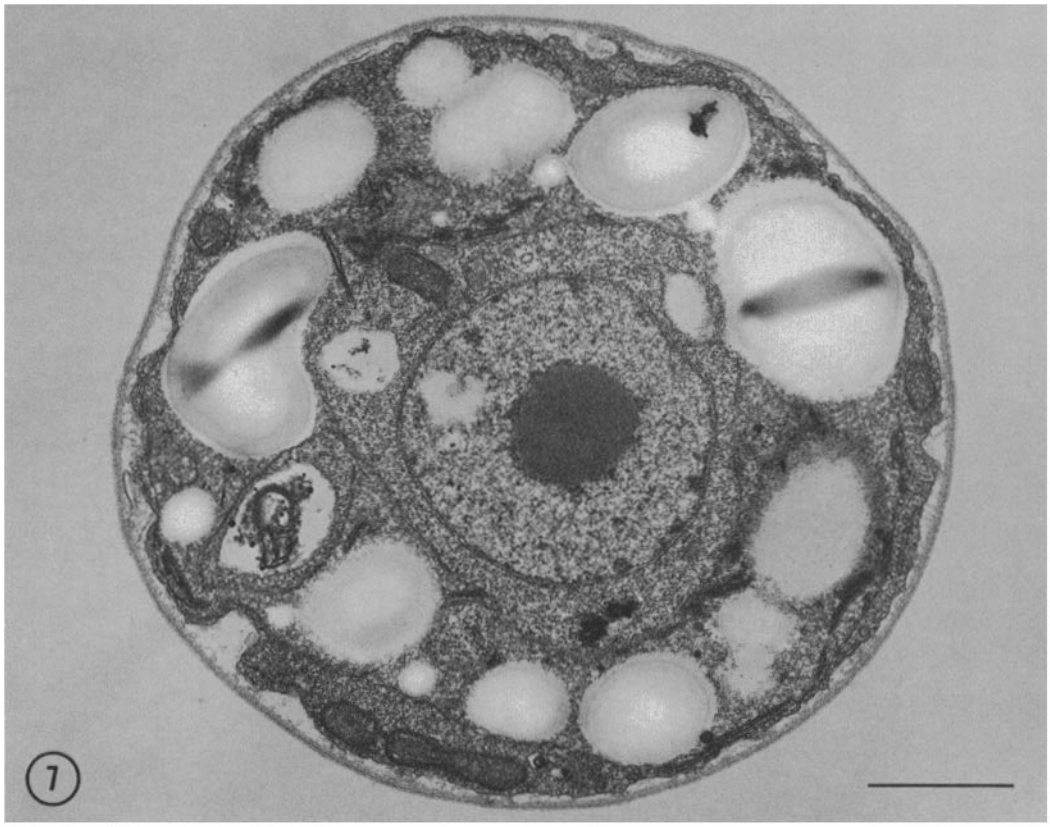
DISCUSSION

Mutants accumulating PROTO and certain other porphyrins have been reported to be photosensitive (Bendix and Allen, 1962). We have found that dark-grown cells of a stringent PROTO accumulator br_s-*1* can survive up to 24 h exposure to light (~15,000 lx), during which most of the accumulated PROTO is photodestroyed, but are killed by 48 h exposure. No chlorophyll or chloroplast membranes are formed during this time. The inability of br_s-*1* to form either chlorophyll or chloroplast lamellae in the light illustrates once again the close coupling between these two processes (cf. Ohad et al., 1967 *b*; Henningsen et al., 1973). In spite of the fact that *C. reinhardtii* can dispense with photosynthetic electron transport, certain Calvin cycle enzymes and chloroplast protein synthesis when acetate is present as a carbon source (Levine and Goodenough, 1970) it apparently cannot survive in the light in the absence of chlorophyll, and no bleached mutants which survive in the light have been reported.

The conditional PROTO-accumulating mutants br_c-*1* and br_c-*2* are exceptions to the rule that PROTO mutants in algae are light sensitive (Bendix and Allen, 1962). Dark grown cells of br_c-*1* and br_c-*2* have about 5% of the chlorophyll of wild type and accumulate as much PROTO as the stringent br_s-*1* mutant. Upon transfer to light, br_c-*1* and br_c-*2* turn green and the accumulated PROTO gradually disappears. In continuous light culture the mutant's phenotype stabilizes with half the normal chlorophyll content and ½ of the maximal

FIGURE 7 Median section of a dark grown br_c-*1* mutant cell. Large starch granules occupy most of the chloroplast and there are few lamellae. Chlorophyll concentration is 1.6 $\mu g/10^7$ cells. × 18,000.

FIGURE 8 Median section of a mixotrophically grown cell of the mutant br_c-*1* (~15,000 lx). Note the well developed chloroplast with pyrenoid. Chloroplast lamellae are largely organized into two-disc grana with occasional multi-disc grana. Chlorophyll concentration is 16.8 $\mu g/10^7$ cells. × 12,000.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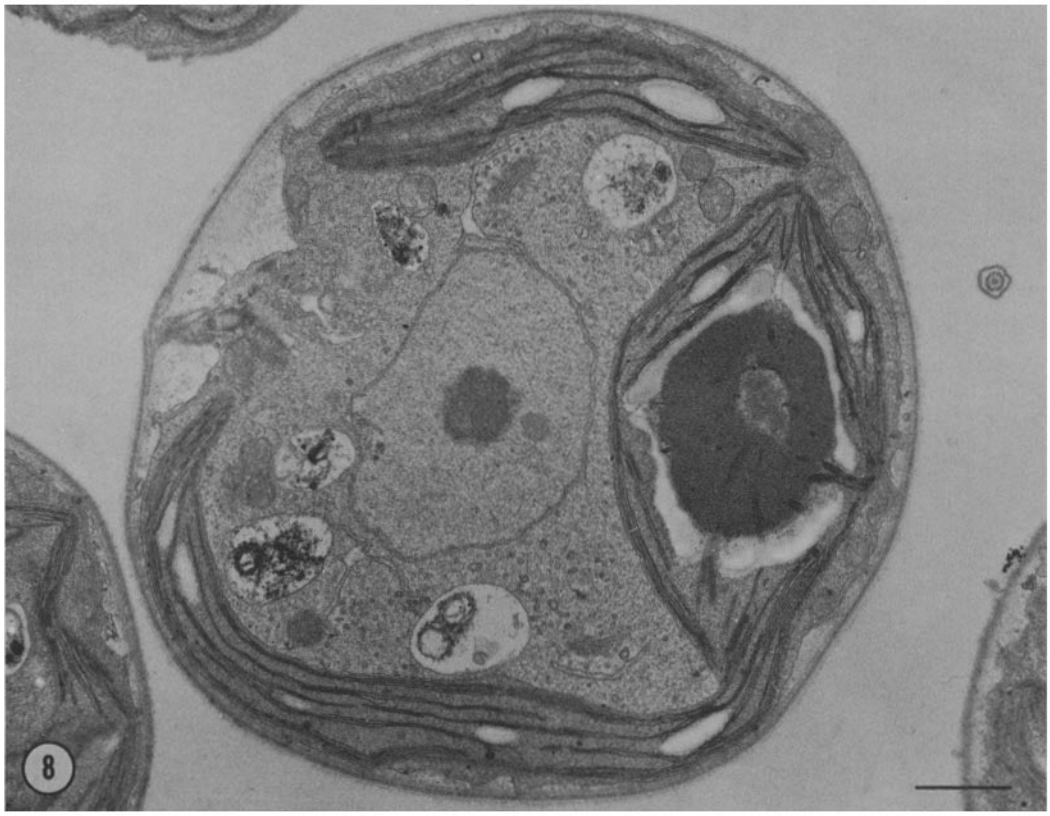
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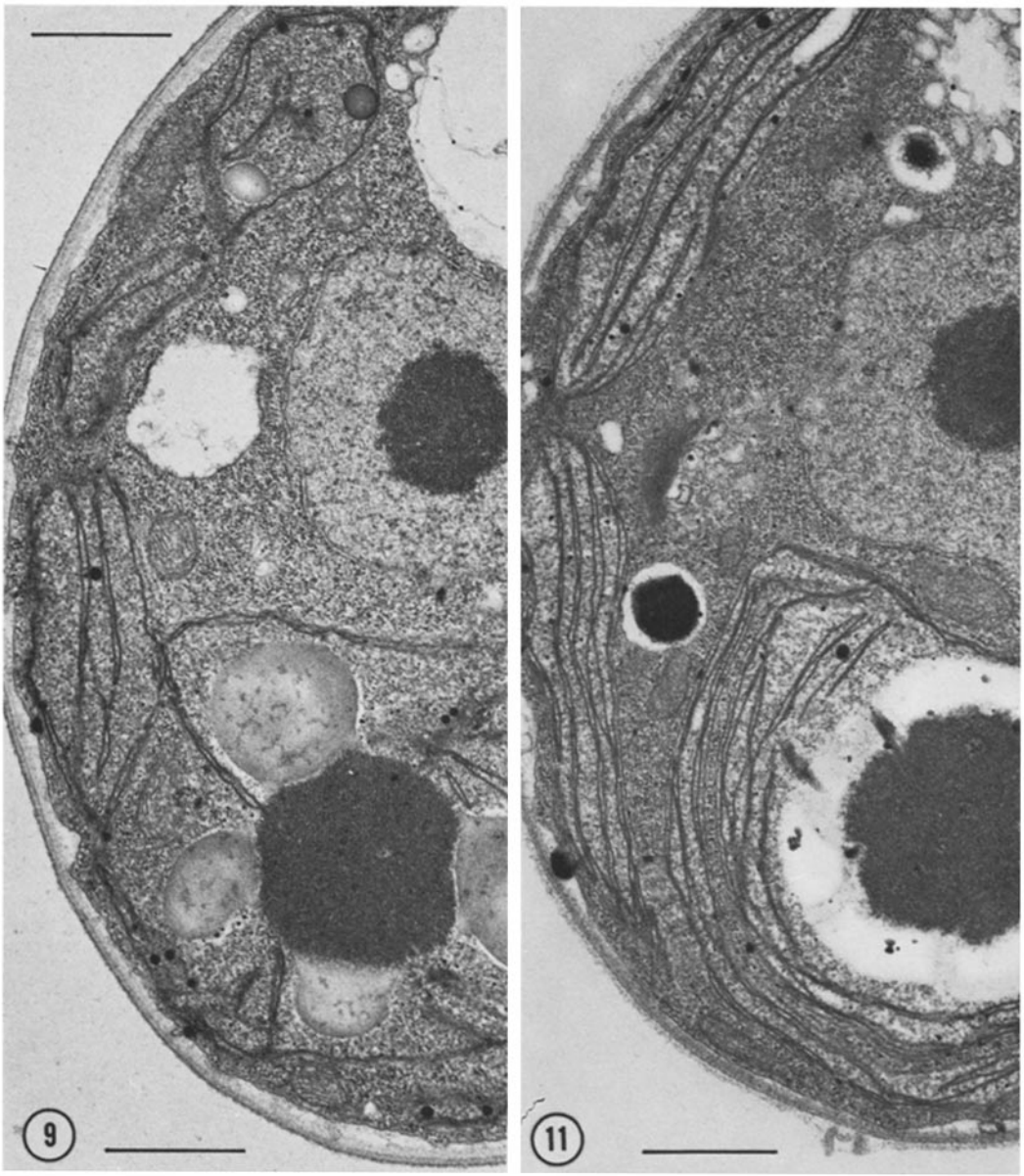
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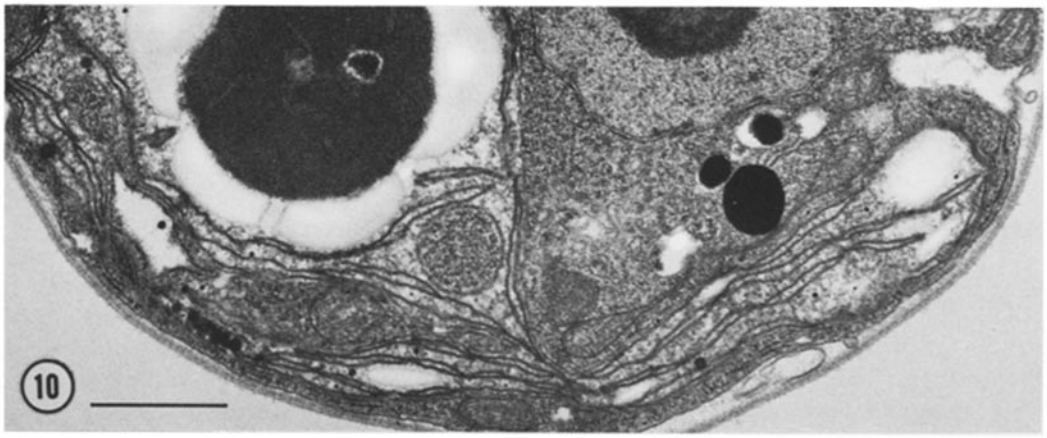
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dark PROTO accumulation. Events associated with greening may serve to protect br_c-*1* from irreversible light damage, since no appreciable loss of viability is observed following exposure to light. In contrast, cells of br_s-*1*, which fail to green, are killed by 48 hr light exposure.

Wild-type cells of *C. reinhardtii* and *Chlorella* are green in the dark because of their ability to reduce enzymatically PCHLD to CHLD (Granick, 1967). The yellow mutants of *Chlorella* (Granick, 1950) and *Chlamydomonas* (Sager, 1961) resemble higher plants in being unable to form chlorophyll in the dark, and instead are reported to accumulate small amounts of PCHLD. Upon exposure to light, both yellow mutants form normal amounts of chlorophyll. Ohad et al. (1967 *a*) concluded that the pathway for chlorophyll biosynthesis in the dark is normal in the *y-1* mutant except for the absence of the enzyme which reduces PCHLD to CHLD.

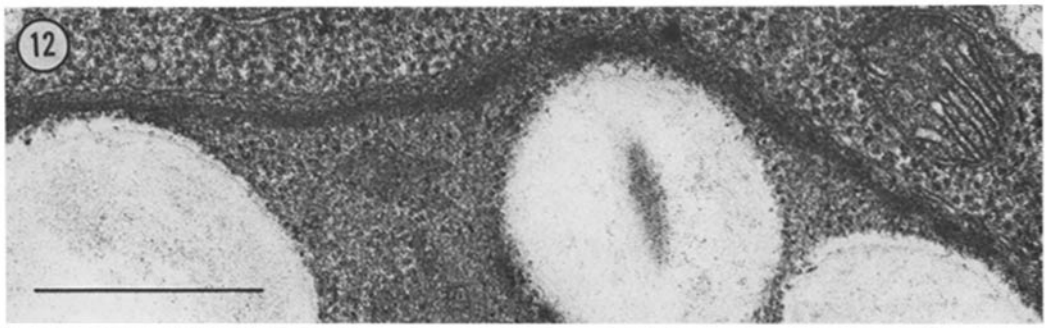
The conditional mutant, br_c-*1*, like *y-1*, lacks any substantial amount of chlorophyll in the dark and readily greens when exposed to light. Both br_c-*1* and *y-1* have starch-filled plastids with few lamellae when dark-grown and undergo similar morphological changes during the greening process (Ohad et al., 1967 *b*). At first we thought that br_c-*1* might be an allele of *y-1* which accumulates PROTO. However, genetic analysis (Tables II, III) clearly indicates that br_c-*1* is a single gene mutation which is functionally distinct from and unlinked to *y-1*. Furthermore, br_c-*1* and *y-1* differ in their initial and final chlorophyll concentrations during greening (Table I, Fig. 17), dark-grown br_c-*1* accumulates no detectable amounts of PCHLD when chromatographed against the PCHLD accumulated by *y-1*. The double mutant br_c-*1* *y-1* is phenotypically identical to br_c-*1* indicating that the br_c locus controls a step in the pathway prior to that controlled by the *y* locus. In contrast to the high reversion rate of *y-1* (Sager, 1959) br_c-*1* is stable genetically.

In higher plants (Granick, 1967; Bogorad, 1966; Foster et al., 1971; Gough, 1972) and in the yellow mutants of *Chlorella* (Granick, 1967; Beale, 1971) and *Chlamydomonas* (Granick, 1967) PCHLD accumulated in the dark is hypothesized to regulate the porphyrin pathway by preventing the formation of ALA. Our calculations reveal that the level of PCHLD accumulation in the dark is 0.6% of the chlorophyll formed in the light in barley (cf. Henningsen and Boynton, 1969, 1974), and 0.3% in the *y-1* mutant of *Chlamydomonas* (cf. Matsuda et al., 1971 and Table I). Gough (1972) summarizes evidence that regulation of the chlorophyll biosynthetic pathway may differ in algae and in higher plants. Barley mutants blocked at various steps in the pathway prior to PCHLD do not accumulate detectable amounts of porphyrin intermediates in darkness. Such porphyrins are accumulated only when these mutants are fed ALA (Gough, 1972) or are combined with regulatory mutants (von Wettstein et al., 1974). Thus in higher plants several of the intermediates in the chlorophyll biosynthetic pathway including PROTO are thought to exert a regulatory effect on the synthesis of ALA (Gough, 1972, von Wettstein et al., 1974). In contrast, mutants of *Chlorella* blocked between PROTO and Mg-PROTO accumulate large amounts of PROTO (Granick, 1948 *a*). Since the ALA-synthesizing enzyme is thought to be the enzyme under regulation in the porphyrin pathway (Granick, 1967) both PCHLD and PROTO might either inhibit the activity of this enzyme or repress its synthesis in the two systems. However, the regulatory effectiveness of PROTO

FIGURE 9 Median section showing part of a br_c-*1* cell after 6 h of illumination (~15,000 lx). Most of the starch granules have disappeared. New lamellae have been formed adjacent to the chloroplast envelope and very little pairing of the lamellae can be observed. Chlorophyll concentration is 2.4 $\mu g/10^7$ cells. × 18,000.

FIGURE 10 Median section showing part of a br_c-*1* cell after 12 h of illumination (~15,000 lx). Both chlorophyll content (6.5 $\mu g/10^7$ cells) and chloroplast lamellar profile (2.3 $\mu m/\mu m^2$ plastid area) have reached half of their maximum values. Pairing of the lamellae over short distances can be observed. × 18,000.

FIGURE 11 Median section showing part of a br_c-*1* cell after 24 h of illumination (~15,000 lx). Both the chloroplast lamellar profile and pairing have increased significantly over the level reached at 12 h. The chlorophyll concentration is 12 $\mu g/10^7$ cells. × 18,000.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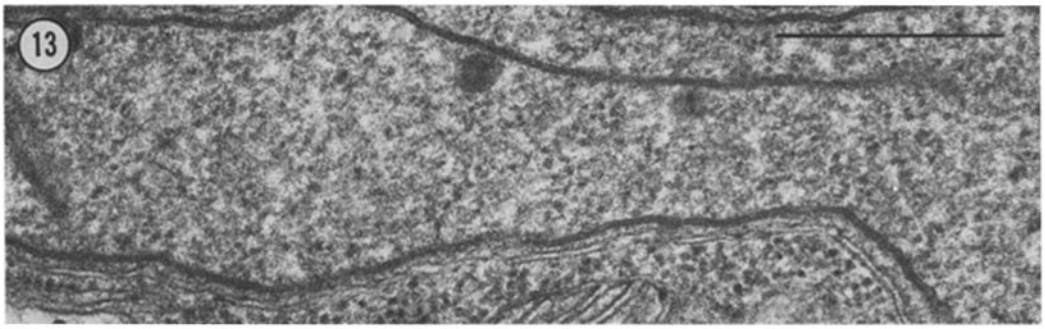
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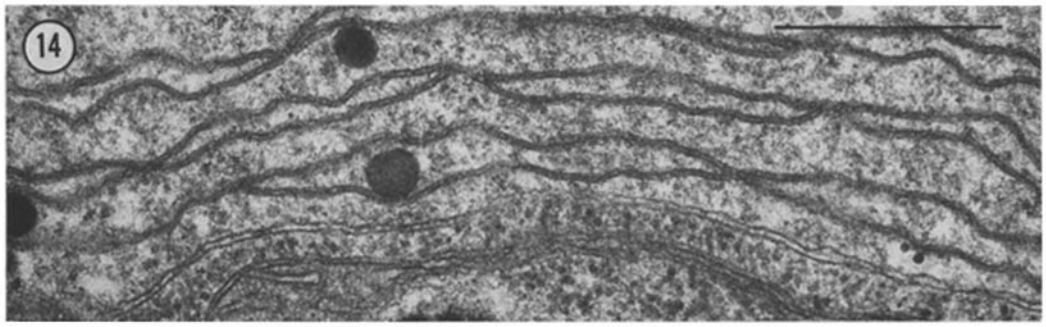
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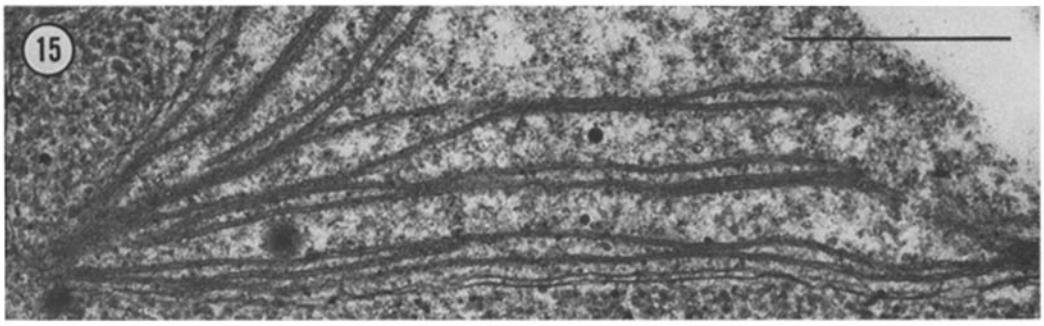
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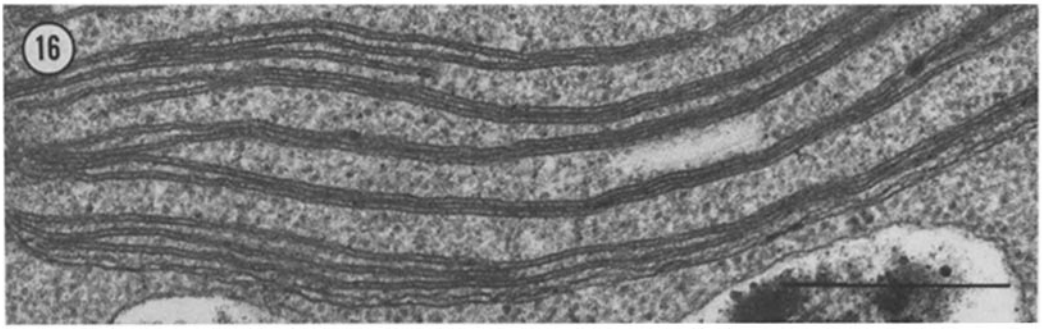
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would appear to differ markedly in higher plants and green algae.

When a dark grown culture of br_c-*1* is transferred to light, rapid photodestruction of PROTO occurs during the first 2 h before chlorophyll accumulation begins (Fig. 2). Molar comparison of chlorophyll increase and PROTO decrease in a greening culture of br_c-*1* (Table I, Fig. 2) points out the insignificant contribution of the PROTO preexisting in the dark to the synthesis of chlorophyll in the light. The porphyrin pathway of both br_s-*1* and br_c-*1* growing in the dark may be inhibited by the PROTO accumulated, since the level of PROTO fails to reach the level of chlorophyll synthesized by wild type in the dark. For reasons enumerated below, we postulate that PROTO inhibits the porphyrin pathway in *Chlamydomonas* as has been suggested in higher plants (Gough, 1972) although larger amounts of PROTO than of PCHLD appear to be required in *Chlamydomonas* to achieve the same level of inhibition. (*a*) The rate of chlorophyll increase of br_c-*1* during greening in the presence of PROTO is slower than that in the *y-1* mutant which does not accumulate PROTO (Fig. 17). (*b*) When br_c-*1* is grown in flasks under amber light, PROTO accumulation increases fivefold and chlorophyll concentration is reduced 30% below that of a culture grown in white light (Table I). Under the amber light conditions a wild-type culture has the same amount of chlorophyll as a culture grown in white light. (*c*) We have recently identified a regulatory mutant which enhances PROTO accumulation of both br_c-*1* and br_s-*1* about 30-fold, while having a wild-type phenotype by itself (Wang, Boynton, Gillham, and Gough, in preparation). This mutant

TABLE II

Tetrad Analysis of Crosses between the Conditional Mutants br_c-1, br_c-2 and the Arginine Requiring Mutants arg-2, arg-7

Cross	Tetrad type	Gene pair			
		br_c-*1* : *arg-2*	br_c-*1* : *arg-7*	br_c-*2* : *arg-2*	br_c-*2* : *arg-7*
br_c-*1 arg-2*$^+$ *mt*$^+$	PD	3			
×	NPD	2			
br_c-*1*$^+$ *arg-2 mt*$^-$	T	1			
br_c-*1 arg-7*$^+$ *mt*$^+$	PD		4		
×	NPD		3		
br_c-*1*$^+$ *arg-7 mt*$^-$	T		4		
br_c-*2 arg-2*$^+$ *mt*$^+$	PD			1	
×	NPD			1	
br_c-*2*$^+$ *arg-2 mt*$^-$	T			1	
br_c-*2 arg-7*$^+$ *mt*$^+$	PD				8
×	NPD				3
br_c-*2*$^+$ *arg-7 mt*$^-$	T				4

PD, parental ditype; NDP, nonparental ditype; and T, tetratype tetrads.

FIGURE 12 View through a small part of a dark grown br_c-*1* cell showing starch granules inside the chloroplast and a single disc adjacent to the chloroplast envelope. (Bar in Figs. 12–16 equals 0.5 μm). × 60,000.

FIGURES 13–16 Views through parts of br_c-*1* cells showing the increase in lamellar profile and pairing at different stages of greening during illumination (~15,000 lx). Fig. 13, 6 h; Fig. 14, 12 h; Fig. 15, 24 h and Fig. 16, mixotrophically grown cell. × 60,000.

TABLE III

Tetrad Analysis and Complementation Tests of the PROTO-accumulating Mutants br_s-1, br_c-1, br_c-2 and the Yellow Mutant y-1

Parental genotypes	Parental phenotype: Mixotrophic	Parental phenotype: Heterotrophic	Cross	Tetrad type	Zoospore phenotype and segregation ratios: Mixotrophic	Zoospore phenotype and segregation ratios: Heterotrophic	Number of tetrads	Complementation in diploids
Wild type	DG	DG	br_c-1 br_c-2$^+$ mt^+	PD	4G	4B	23	
br_c-1	G	B	×	NPD	2DG:2G	2DG:2B	0	No
br_c-2	G	B	br_c-1$^+$ br_c-2 mt^-	T	1DG:3G	1DG:3B	0	
br_s-1	—	B						
y-1	DG	Y	br_c-1$^+$ br_s-1 mt^+	PD	2G:2–	4B	14	
			×	NPD	2DG:2–	2DG:2B	7	Yes
			br_c-1 br_s-1$^+$ mt^-	T	1DG:1G:2–	1DG:3B	20	
			br_c-1 y-1$^+$ mt^+	PD	2DG:2G	2Y:2B	10	
			×	NPD	2DG:2G	2DG:2B	11	Yes
			br_c-1$^+$ y-1 mt^-	T	2DG:2G	1DG:1Y:2B	12	
			br_s-1 y-1$^+$ mt^+	PD	2DG:2–	2Y:2B	11	
			×	NPD	2DG:2–	2DG:2B	9	Yes
			br_s-1$^+$ y-1 mt^-	T	2DG:2–	1DG:1Y:2B	8	

Phenotype abbreviations: DG, dark green; G, green; B, brown; Y, yellow; –, lethal. Tetrad types: PD, parental ditype; NDP, nonparental ditype; T, tetratype.

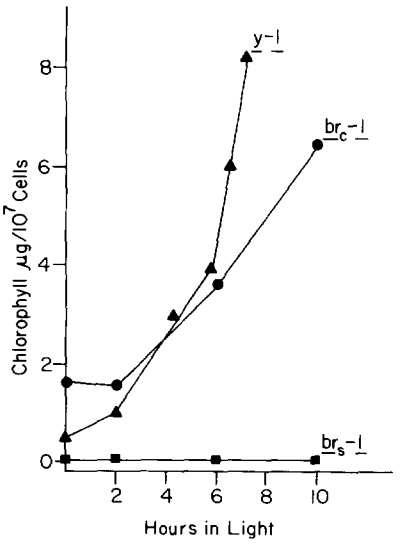

FIGURE 17 Comparison of chlorophyll increase after transfer of dark grown cells of br_s-1, br_c-1, and y-1 to light. Data for y-1 have been replotted from Fig. 18 *b* of Ohad et al., 1967 *a*.

may be similar to the *tig-d* regulatory mutant in barley described by von Wettstein et al. (1974).

In spite of the well recognized fact that magnesium is inserted into the PROTO molecule to form Mg-PROTO, the manner in which this reaction proceeds in vivo is still unknown (Burnham, 1969). In barley, mutants in four functionally distinct loci accumulate PROTO when they are fed ALA and these loci appear to control steps between PROTO and Mg-PROTO (Gough, 1972). As suggested by von Wettstein et al. (1971), these loci may either code for synthetic enzymes or for specific structural molecules to which these enzymes and/or their substrates must bind. In addition, if magnesium must be activated in preparation for its insertion, blocking this process may also cause PROTO accumulation.

The existence of yellow mutants in both *Chlorella* and *Chlamydomonas* illustrates that, in these two species, the reduction of PCHLD to CHLD can occur via separate light and dark reactions. We feel that the behavior of br_c-1 which accumulates PROTO in the dark and turns green in the light may be best explained by postulating that the porphyrin pathway splits into separate light and dark reactions at some step between PROTO and Mg-PROTO. Blockage of the dark reaction by br_c-1 would cause PROTO accumulation. Since the double mutant of br_c-1 and br_s-1 has the phenotype of br_s-1 (Table III), we think br_s-1 is controlling a step in the pathway prior to the step controlled by br_c-1.

Two alternative models seem most likely to us

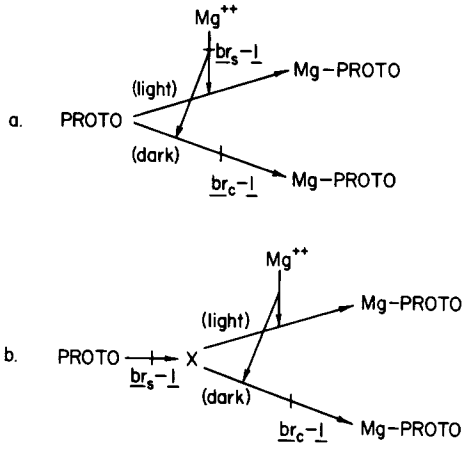

FIGURE 18 Two alternative models illustrating the roles of the br_s and br_c loci in converting protoporphyrin to magnesium protoporphyrin. In model *a*, the br_s locus mediates the activation of the magnesium ion for its insertion into the protoporphyrin molecule, and the br_c locus controls the dark reaction converting protoporphyrin to magnesium protoporphyrin. In model *b*, the br_s locus controls the conversion of protoporphyrin to porphyrin X which either has the same absorption spectrum as protoporphyrin or is accumulated in extremely small amounts. The br_c locus controls the dark reaction converting porphyrin X to magnesium protoporphyrin.

to explain the roles of the br_s and br_c genes in porphyrin biosynthesis (Fig. 18). In the first model the br_c locus would control a dark reaction converting PROTO to Mg-PROTO and the br_s locus would control a side reaction which involves the activation of magnesium (Fig. 18 *a*). In the second model, an intermediate porphyrin (X) between PROTO and Mg-PROTO is postulated which must either have the same absorption spectrum as PROTO, or be accumulated in trace amounts (Fig. 18 *b*). The br_s gene product would catalyze the conversion of PROTO to X and the br_c gene the conversion of X to Mg-PROTO via the dark pathway. A precedent for chemically dissimilar Mg-porphyrin intermediates having similar absorption spectra appears in the work of Ellsworth and Aronoff (1968 *a*). If more detailed chemical analysis reveals that the porphyrins accumulated by br_c-*1* and br_s-*1* are different, this will provide strong evidence for the second model.

Our evidence with br_s-*1* and br_c-*1* suggests that the step between PROTO and Mg-PROTO is divided into light and dark reactions. Whether the pathway from Mg-PROTO to PCHLD remains divided into separate light and dark branches can be tested by our ability to isolate specific classes of new mutants.

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