

CHLOROPLAST STRUCTURE AND FUNCTION  
IN *ac-20*, A MUTANT  
STRAIN OF *CHLAMYDOMONAS REINHARDI*

III. Chloroplast Ribosomes and Membrane Organization

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ABSTRACT

The fine structure of the *ac-20* strain of *Chlamydomonas reinhardi* is described. Cells grown mixotrophically in the presence of acetate have a highly disordered chloroplast membrane organization and usually lack pyrenoids. Chloroplast ribosome levels are only 5–10% of wild-type levels. Cells grown phototrophically without acetate possess more chloroplast ribosomes and have more normal membrane and pyrenoid organization. Chloroplast ribosome levels rise rapidly when cells are transferred from acetate to minimal medium, whereas membrane reorganization occurs only after a lag. These results, combined with earlier studies of the photosynthetic properties of the mutant strain, suggest that proper membrane organization, Photosystem II activity, and ribulose-1,5-diphosphate carboxylase formation are dependent on the presence of chloroplast ribosomes. Other chloroplast components tested are unaffected by a 10-fold reduction in levels of chloroplast ribosomes.

INTRODUCTION

It has been shown in the two preceding papers (11, 22) that the photosynthetic properties of *ac-20*, a mutant strain of *Chlamydomonas reinhardi*, vary depending on whether the cells are grown phototrophically on minimal medium or mixotrophically in the presence of acetate. Phototrophic cells possess ribulose-1,5-diphosphate (RuDP) carboxylase and Hill activities that are lower than those of the wild type; in mixotrophic cells, these same activities are much more markedly reduced. Other photosynthetic activities of the strain tested are apparently normal under both growth conditions.

In this paper, the fine structure of phototrophically and mixotrophically grown *ac-20* is presented, and the structural changes that occur during light-to-dark and light-to-light transfer experiments (22) are followed. Most of the properties of the mutant

strain can be interpreted as the consequence of a marked reduction in levels of chloroplast ribosomes.

MATERIALS AND METHODS

*Organisms and Culture Conditions*

The wild-type strain (137c) and the mutant strain *ac-20* of *C. reinhardi* were grown as described by Togasaki and Levine (22). The mutant strain *ac-31* was grown mixotrophically for 48 or 72 hr under identical culture conditions. Light-to-light and light-to-dark transfer experiments were performed as described by Togasaki and Levine (22).

*Electron Microscopy*

Fixation and embedding procedures were performed as previously described (10), except that a

$4 \times 10^{-3}$  M potassium phosphate buffer, pH 7, replaced the collidine buffer in most experiments, and infiltration times were shortened to 2 days. During transfer experiments, samples were commonly taken every 2 hr and stored in cold phosphate buffer overnight following a 2 hr fixation period; the remaining preparative procedures were performed on all the samples the following day. Specimens were examined with a Hitachi HU 11C electron microscope.

#### *Ribosome Determination: Cut-and-Weigh Technique*

Uniform thin sections exhibiting gray interference colors were prepared. Cells were randomly photographed at original magnifications of  $42,500 \times$  and enlarged photographically to  $107,250 \times$ . The resulting prints were cut with scissors and the cuttings were separated into four "fractions:" chloroplast stroma, disc membranes, DNA, and starch. All other cuttings were discarded unless cytoplasmic ribosomes were to be counted, in which case areas of cytoplasmic ground substance were reserved. At least 10 micrographs were cut for each sample, representing perhaps 20–30  $\mu^2$  of chloroplast area.

To estimate chloroplast ribosomes, the ribosomes in the stroma cuttings were counted, the cuttings were weighed, and a value of ribosomes/stroma weight was calculated. By a similar procedure, cytoplasmic ribosome density in the ground substance was measured.

To estimate chloroplast membrane density, the weights of the four fractions were added to give "total chloroplast weight," and a value of membrane weight/total chloroplast weight was then calculated.

#### *RNA Determinations: Acridine Orange Fluorescence*

Techniques developed by Dr. Christopher Woodcock were followed. Cells were dried on gelatin-coated slides, fixed for 5 min in formalin-acetic acid, soaked for 5 min in 70% ethanol, and soaked for 5 min in 100% ethanol to remove fixative and chlorophyll. The slides were then dried and placed in a 0.05% acridine orange solution in a 0.02 M sodium acetate buffer, pH 4.5. After 15 min, they were removed from the stain and rinsed for 10 min in three changes of the buffer. Wet mounts were examined with a Leitz ortholux fluorescence microscope equipped with a xenon lamp, a blue BG 12 filter, an immersion 95  $\times$  objective with an iris diaphragm, and an orange filter.

#### *Ribosome Determinations: Sucrose Density Centrifugation*

The procedure for ribosome isolation and characterization described by Hooper and Blobel (8) was followed exactly, except that 5 ml linear gradients from 15 to 30% sucrose were used instead of exponential gradients, and the gradients were spun for 105 min in an SW 50 rotor of a Model L ultracentrifuge (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.) at  $45.7 \times 10^3$  rpm.

#### *Ribosomal RNA Determinations*

The ribosomal RNA's from cytoplasmic and chloroplast ribosomes were isolated and identified by polyacrylamide gel electrophoresis (12). Cells were harvested and suspended in 0.25 M Tris-HCl, pH 7.5, containing 0.25 M sucrose, 0.25 M KCl, and 0.25 M  $MgCl_2$ . The cell concentration was adjusted to  $8-9 \times 10^8$ /ml, and the cells were broken at 280 kg/cm<sup>2</sup> in a French pressure cell (American Instrument Co., Silver Spring, Md.). Deoxycholate (in the buffer) was added to the broken cell preparation to give a final concentration of detergent of 1%. The preparation was then centrifuged for 5 min at 10,000 g. The supernate was preserved. Sodium dodecyl sulfate was added to a final concentration of 2.5%. A maximum of 50  $\mu$ l of the preparation was placed on top of a 10 cm column containing 2.6% polyacrylamide (5% cross-linked). The buffer for electrophoresis was that of Loening (12), but without ethylenediaminetetraacetate. The electrophoresis was run for 1.5 hr with a current of 5 ma/column. The gels were scanned with a scanning device attached to a Zeiss PMQ II spectrophotometer, and their absorbance at 260 nm was displayed on a recorder. Sedimentation (S) values were obtained by running RNA's of known S values on duplicate gels.

## RESULTS

#### *Wild-Type C. reinhardi*

The fine structure of the wild-type *C. reinhardi* chloroplast has been described in several other reports (6, 14, 18). A representative field is shown at high magnification in Fig. 1. Chloroplast discs (D) are organized into grana-like stacks (S) that exhibit a characteristic anastomosing pattern (6). Chloroplast ribosomes (cr), smaller than the cytoplasmic ribosomes (cyr) (14), are densely packed within the chloroplast stroma. The structure of the wild-type pyrenoid has been described in detail by Ohad et al. (14).

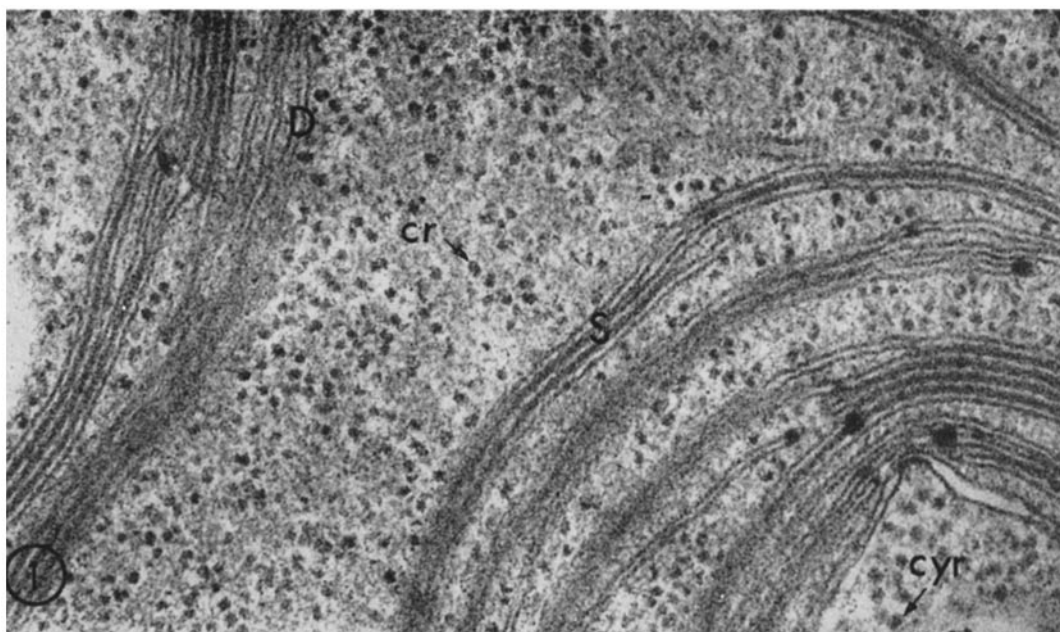


FIGURE 1 Portion of a chloroplast from a wild-type cell. Membrane discs (*D*) are commonly found in stacks (*S*) of from two to five discs. Chloroplast ribosomes (*cr*) are visibly smaller than cytoplasmic ribosomes (*cyr*).  $\times 98,000$ .

### *Mixotrophic ac-20*

Except for the chloroplast, the fine structure of a mixotrophically-grown *ac-20* cell is indistinguishable from a wild-type cell. The structure of the mixotrophic *ac-20* chloroplast is shown at high magnification in Fig. 2 and in survey view in Fig. 3. Several features of this chloroplast are distinctly different from the wild-type chloroplast, and these will be considered separately.

**CHLOROPLAST MEMBRANE ORGANIZATION:** The chloroplast membrane organization of mixotrophic *ac-20* is distinct not only from the wild type but also from all other green mutant strains of *C. reinhardtii* that have been studied (5, 6). It is characterized by a seeming lack of any kind of patterning; in a given chloroplast section, a very different morphology is encountered from one region to the next.

Three general kinds of chloroplast membrane conformation can be recognized. The first, and least frequent, is an apparently normal, stacked configuration of chloroplast discs (Fig. 2, arrow). The second, and dominant, conformation is the single unfused disc (Figs. 2 and 3). Such discs are not arranged in the orderly, concentric array found

in other mutant strains of *C. reinhardtii* where single discs predominate (5, 6); rather, they lie in a seemingly haphazard array within the chloroplast, approaching each other in certain regions and meandering through the stroma without any apparent orientation in other regions (Figs. 2 and 3). The third, and unique, membrane conformation takes the form of masses of vesicles, commonly located in the chloroplast interior (Fig. 3). They are reminiscent of vesicles found in the proplastid-like structure of a dark-grown yellow mutant strain of *C. reinhardtii* (14), but they are more tightly aggregated. Moreover, it is recalled that mixotrophic *ac-20* is grown in the light and has almost wild-type levels of chlorophyll (22).

The total amount of membrane in mixotrophic *ac-20* chloroplasts is about half that of wild-type chloroplasts, as estimated both by inspection of electron micrographs and by the cut-and-weigh assay.

**THE PYRENOID:** The pyrenoid of mixotrophic *ac-20* is conspicuous by its absence. When present, it is very small or rudimentary. A stellate array of pyrenoidal tubules can sometimes be recognized (Fig. 4), but these tubules are devoid of the granular pyrenoid ground substance that normally



FIGURE 2 Portion of a chloroplast from a mixotrophic *ac-20* cell. Membrane discs (*D*) are commonly single, but limited stacking does occur (arrow). Chloroplast ribosomes (*cr*) are sparse compared with wild type (Fig. 1); cytoplasmic ribosomes (*cyr*) are at the wild-type level.  $\times 98,000$ .

surrounds them (14). The inability to form a normal pyrenoid is a unique characteristic of the *ac-20* strain of *C. reinhardtii*, although other pyrenoidless green algae have been reported (2, 13, 15). Since starch formation proceeds normally in these algae and in *ac-20*, it appears unlikely that an organized pyrenoid structure is essential for starch synthesis in the Chlorophytes (4, 17).

**CHLOROPLAST RIBOSOMES:** The chloroplast stroma of mixotrophic *ac-20* (Fig. 2) is markedly deficient in chloroplast ribosomes (*cr*) when compared with that of the wild type (Fig. 1). This ribosome deficiency is also evident in Figs. 3 and 4. No other mutant strain of *C. reinhardtii* that has been studied exhibits this deficiency (5, 6), indicating that it is not some usual (secondary) property of a photosynthetically inactive chloroplast.

It is evident in Figs. 2-4 that the mixotrophic *ac-20* chloroplast is not ribosomeless. The level of chloroplast ribosomes was measured by the cut-

and-weigh assay in five separate experiments and was found to average 8% of the wild-type level; the actual values are given in Fig. 5. Thus, the *ac-20* mutation effects a 92% inhibition of chloroplast ribosome formation under mixotrophic growth conditions. No reduction is evident in levels of cytoplasmic or mitochondrial ribosomes.

To confirm the electron microscope observations, three independent demonstrations of the chloroplast ribosome deficiency in mixotrophic *ac-20* were made.

The first approach involved ribosome isolation and characterization on sucrose density gradients, following the techniques of Hooper and Blobel (8). Mixotrophic *ac-20* cell preparations were here compared with *ac-31* cells and not wild-type cells; the disposition of chloroplast membranes in the *ac-31* strain (5) most closely resembles that of mixotrophic *ac-20*, and it was felt that the degree of cell breakage with the French pressure cell and the

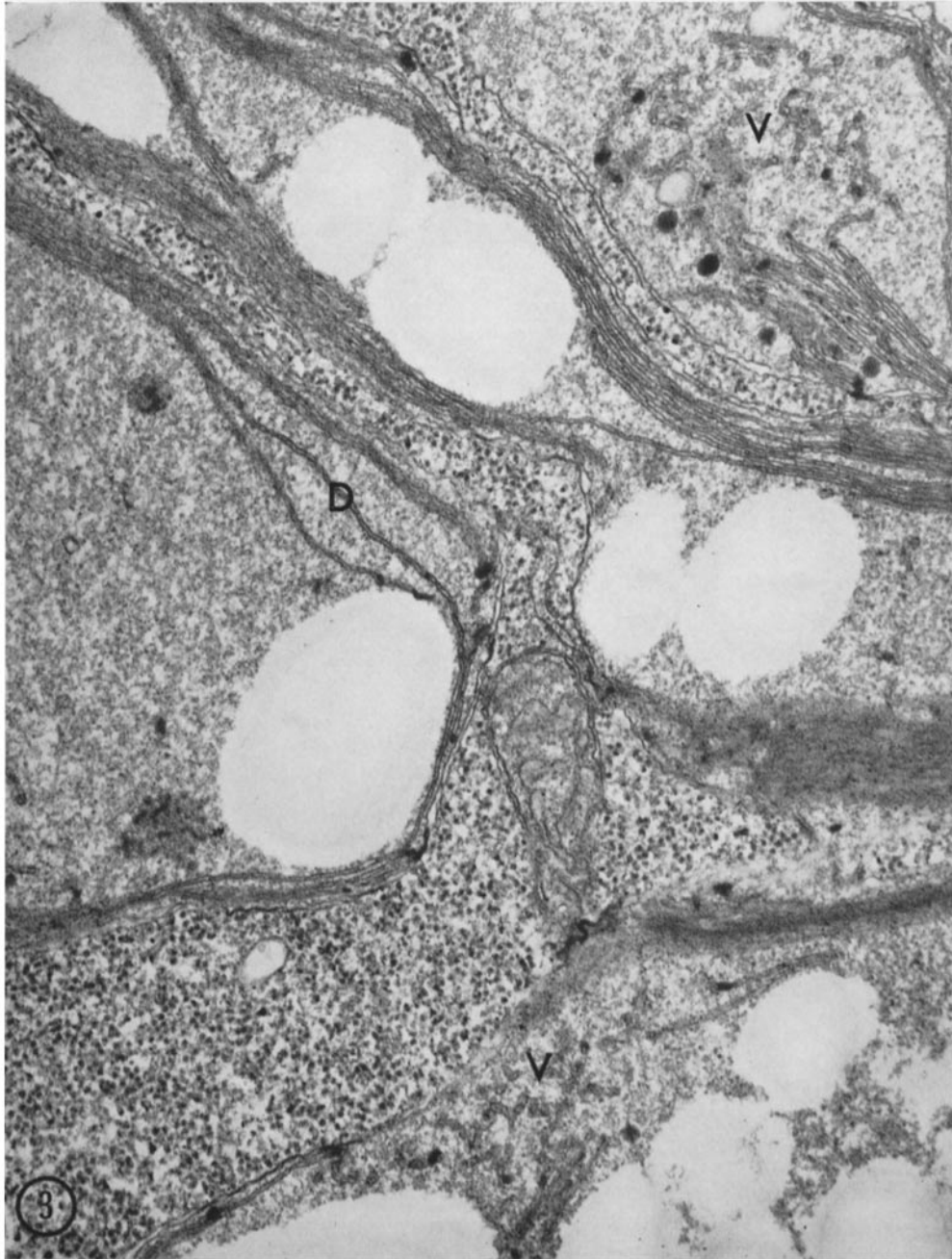


FIGURE 3 Survey view of a mixotrophic *ac-20* cell, showing several lobes of the chloroplast. Membrane discs (*D*) are largely unstacked. Two clusters of vesicles are seen at *V*. The paucity of chloroplast ribosomes is evident.  $\times 44,000$ .

number of ribosomes trapped in chloroplast membrane debris would be most similar if *ac-20* and *ac-31* were compared. The chloroplast ribosome density of *ac-31* is comparable to that of the wild type (Fig. 5 and ref. 6).

The resultant sucrose gradients were analyzed at 260 nm, and typical optical density patterns are shown in Fig. 6. The top profile was obtained from *ac-31*. Two distinct peaks are present. Using almost identical experimental conditions, Hooper and Blobel (8) demonstrated that the larger peak in such gradients has a sedimentation coefficient of 80S and the smaller peak (arrow) a coefficient of 68S; Hooper and Blobel further demonstrated that each peak corresponds to a distinct class of ribosomes, and they conclude that the slower-sedimenting ribosomes derive from the chloroplast. In the lower gradient profile of Fig. 6, obtained from mixotrophic *ac-20*, the slower-sedimenting ribosomes are seen to be absent.

A second independent demonstration of the

chloroplast ribosome deficiency in mixotrophic *ac-20* has utilized the fluorescence properties of acridine orange, a dye that fluoresces a brilliant orange when combined with RNA (3); the paler, yellow-green fluorescence of the dye-DNA complex cannot be detected in *C. reinhardtii* until the RNA fluorescence is removed with ribonuclease digestion. In the wild type (Fig. 7), the fluorescence of the dye-RNA complex covers the entire cell except the pyrenoid, and the chloroplast cannot be distinguished from the cytoplasm. In mixotrophic *ac-20* (Fig. 8), however, the chloroplast can be readily visualized as a dark, nonfluorescent cup-shaped area surrounding a brilliantly-fluorescent cytoplasm. The absence of a pyrenoid in mixotrophic *ac-20* is also apparent when Figs. 7 and 8 are compared.

The third independent approach demonstrates a chloroplast ribosomal RNA deficiency in mixotrophic *ac-20*. Dr. Stefan Surzycki from this laboratory has developed techniques for vis-

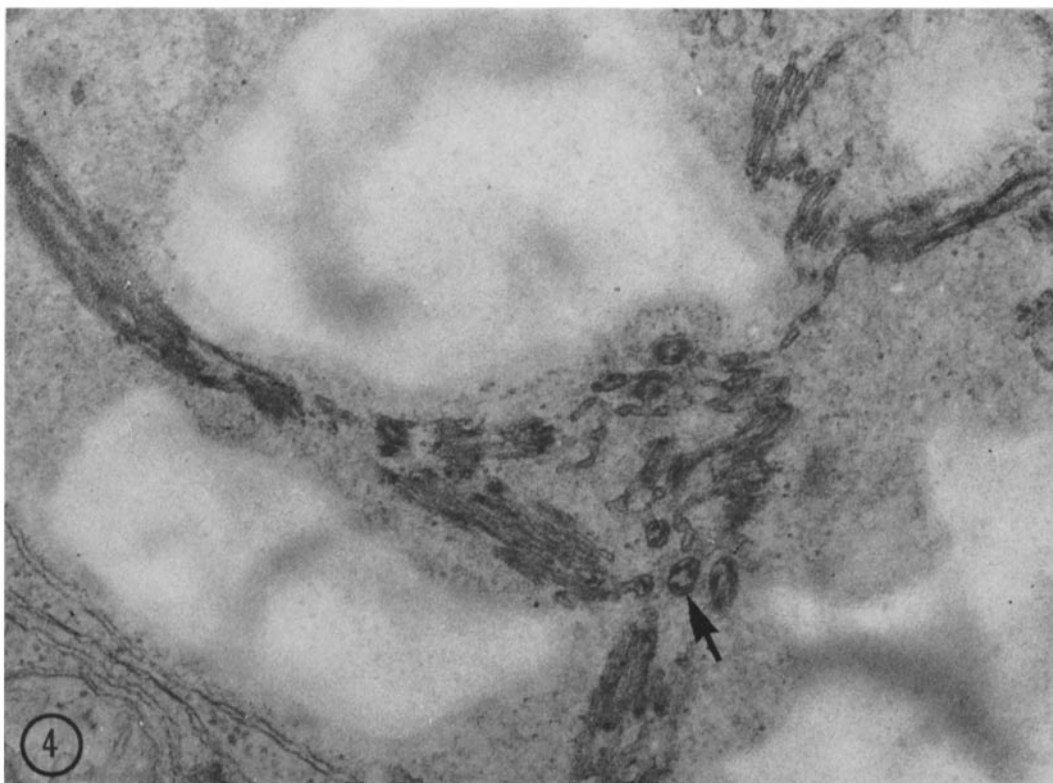


FIGURE 4 Rudimentary pyrenoid in a mixotrophic *ac-20* cell. Membranous elements radiate from a focal region that contains several pyrenoidal tubules (arrow), but the pyrenoidal ground substance is absent. Chloroplast ribosomes are sparse.  $\times 66,000$ .

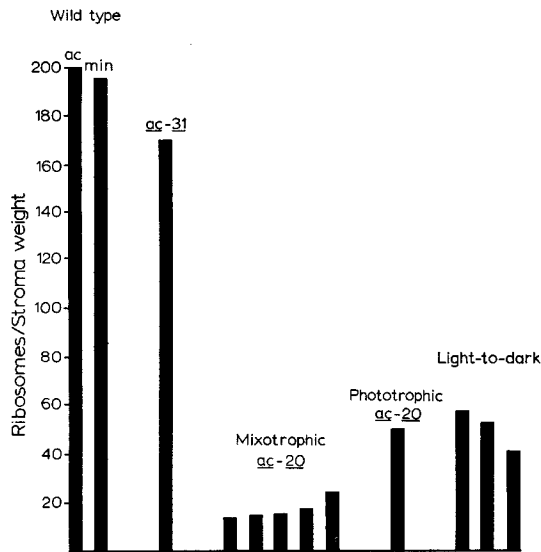


FIGURE 5 The levels of chloroplast ribosomes, as determined by the cut-and-weigh assay, for the following cell types: wild type grown on acetate (*ac*) and minimal (*min*) medium; *ac-31* on acetate medium; mixotrophic *ac-20* (five separate experiments); phototrophic *ac-20*; and mixotrophic *ac-20* transferred to minimal medium and incubated in the dark for 12 hr (light-to-dark, three separate experiments).

ualizing the RNA species of *C. reinhardi* by polyacrylamide gel electrophoresis and by methylalbumin-kieselguhr (MAK) column chromatography. He finds (Fig. 9) a marked deficiency in 16S and 23S ribosomal RNA species when mixotrophic *ac-20* is compared with mixotrophic wild-type preparations by gel electrophoresis. The 18S and 25S species of cytoplasmic ribosomal RNA are present in normal amounts in the mutant strain (Fig. 9). Similarly, he finds (21) that a 5S species of chloroplast ribosomal RNA is greatly reduced in the mutant strain, whereas the corresponding cytoplasmic 5S RNA species is present in normal amounts.

#### Phototrophic *ac-20*

When *ac-20* is grown on a minimal medium, a very different chloroplast ultrastructure is observed. A representative field is shown in Fig. 10.

**CHLOROPLAST MEMBRANE ORGANIZATION AND PYRENOID:** The chloroplast membranes are as carefully ordered in phototrophic *ac-20* cells (Fig. 10) as they are disordered in mixotrophic cells (Figs. 2 and 3). The discs are arranged in concentric, regular arrays of stacks, and most of the

stacks contain two discs. The phototrophic *ac-20* chloroplast also appears to contain more membrane; certainly the membrane density (i.e., membrane per unit chloroplast area) is greater in the phototrophic cells, although it is not so great as in wild type. Since cells grown on minimal medium are considerably smaller than cells grown on an acetate-supplemented medium in *C. reinhardi*, it is difficult to judge whether the total membrane content of the phototrophic cells is greater than that of the mixotrophic cells; this point remains undecided until a method to measure total chloroplast membrane per cell can be devised.

The pyrenoid of phototrophic *ac-20* cells is somewhat smaller than that of the wild type, but it is always present, in direct contrast to mixotrophically-grown cells.

**RIBOSOMES:** The chloroplast-ribosome levels of phototrophic *ac-20* are about two to four times greater than those of mixotrophic cells and about

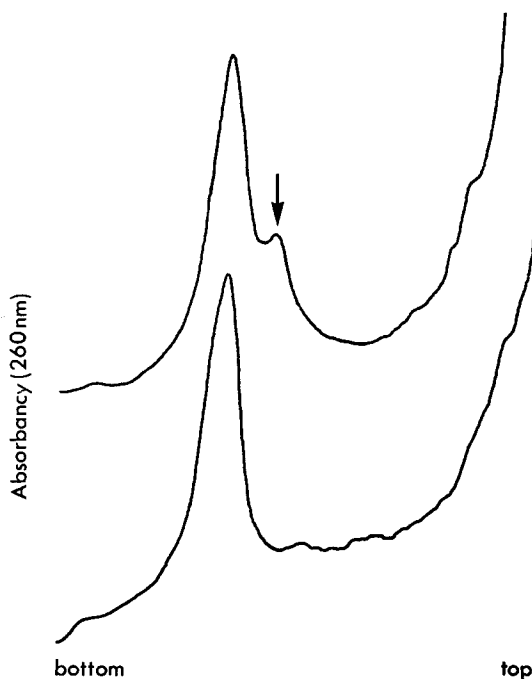


FIGURE 6 Optical density tracings of sucrose density gradients containing ribosome preparations, analyzed with an ISCO Fractionator and UV Analyzer (Instrument Specialties Co., Lincoln, Neb.). The upper profile, obtained from *ac-31*, exhibits two distinct peaks. The smaller, slower-sedimenting peak (arrow) is identified as deriving from chloroplast ribosomes. This peak is absent from the lower profile, obtained from mixotrophic *ac-20*.

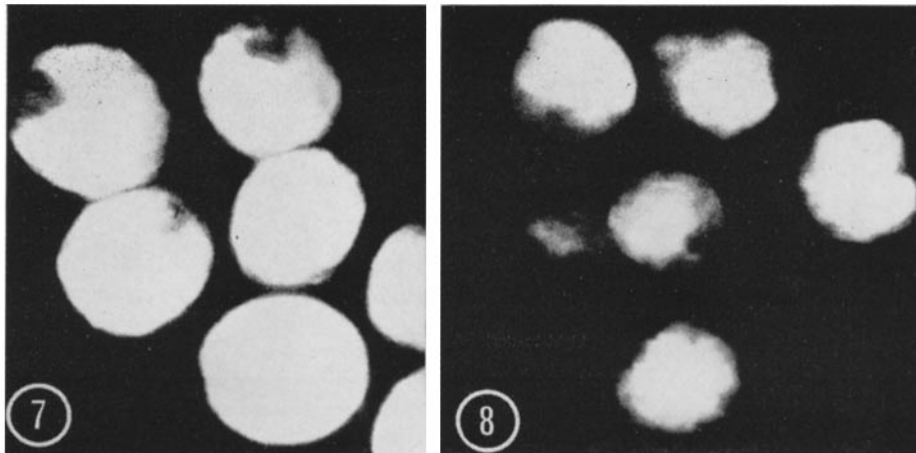


FIGURE 7 Wild-type cells stained with acridine orange. The orange fluorescence appears white in the micrograph. Only the pyrenoid is nonfluorescent.  $\times 1,600$ .

FIGURE 8 Mixotrophic *ac-20* cells stained with acridine orange. The cup-shaped chloroplast is nonfluorescent and appears as a dark, hoodlike structure shading the periphery of each cell.  $\times 1,600$ .

four times less than those of the wild type. This is indicated quantitatively in Fig. 5, and is evident when electron micrographs of phototrophic cells are inspected (Fig. 10).

#### *Fine Structure During Light-to-Dark Transfer Experiments*

**RIBOSOMES:** During the course of a 12 hr dark incubation following the transfer of mixotrophic *ac-20* cells to minimal medium, the low initial level of chloroplast ribosomes slowly increases, such that by the end of 12 hr the ribosome level found in phototrophic cells is attained. The time course for this ribosome recovery is shown in Fig. 11, as determined by the cut-and-weigh method, and the final levels attained in three separate experiments are indicated in Fig. 5. Cells in the process of recovery are shown in Figs. 12 and 13; when these figures are compared with Fig. 2, the increase in chloroplast ribosomes is apparent.

Cells maintained in the dark on minimal medium are deprived of any exogenous energy or carbon source; moreover, it is evident with the electron microscope that *C. reinhardi* does not appear to utilize its starch reserves during such a dark incubation. Thus the slow ribosome formation occurs even though the cells are apparently incapable of appreciable net protein synthesis. A possible explanation for this anomalous observation is that

protein turnover occurs during the dark incubation period, and that the ribosomal proteins are synthesized at the expense of existing proteins in the cell. A second explanation is that the assembly of chloroplast ribosomes during the light-to-dark incubation period does not require protein synthesis, implying that the proteins are already present but are somehow prevented from forming ribosomes as long as mixotrophic growth conditions are imposed.

**CHLOROPLAST MEMBRANE AND PYRENOID:** Within a few hours after mixotrophic *ac-20* is subjected to a light-to-dark transfer, the vesicular membrane profiles that are prominent in the mixotrophic chloroplasts virtually disappear. Accompanying this decrease in vesicles is a gradual transformation in the organization of the chloroplast membrane.

The haphazard membrane arrangement that characterizes mixotrophic *ac-20* chloroplasts is replaced by a more orderly, concentric arrangement, but the stacking pattern of phototrophic cells is not acquired. Instead, membrane is found either as single, unfused discs or as wide, banded stacks of many discs. As the dark incubation progresses, these wide stacks become prominent; the size of the stacks may become enormous, and their continuity with the rest of the chloroplast membrane system becomes limited (Figs. 12 and 13). Moreover, the stacks take on a disordered





FIGURE 9 Polyacrylamide gel electrophoresis (performed by Dr. S. J. Surzycki) of ribosomal RNA from *ac-20* (top) and wild-type (bottom) cells of *C. reinhardi* grown mixotrophically. The 16S and 23S species are markedly deficient in the *ac-20* preparation.

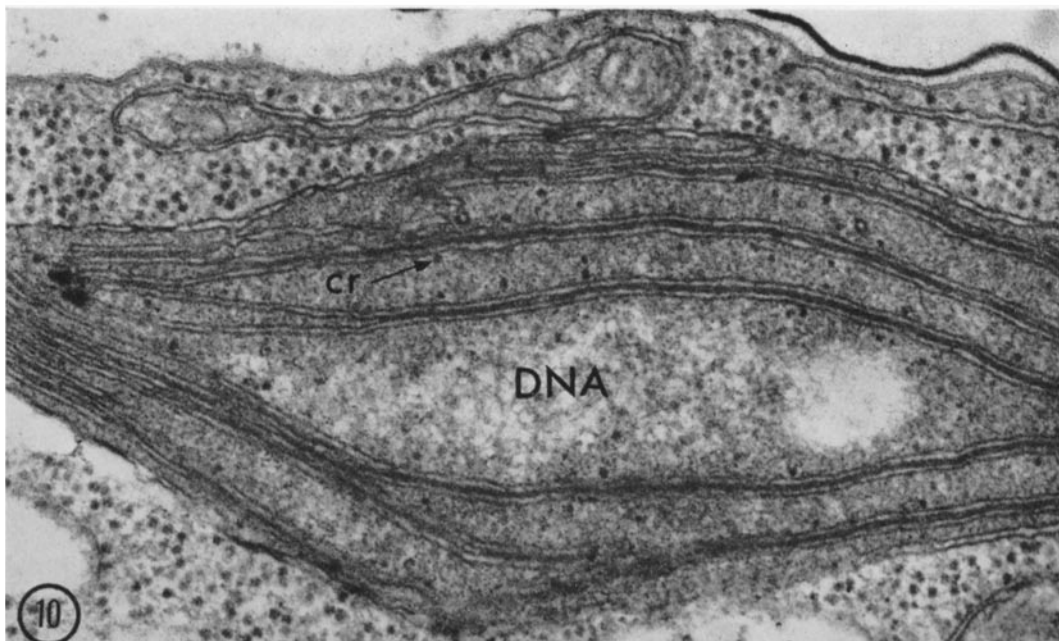


FIGURE 10 Portion of a chloroplast from a phototrophic *ac-20* cell. The membrane discs are in regularly ordered stacks, commonly of two discs. A region of chloroplast DNA is indicated. Chloroplast ribosomes (*cr*) are more numerous than in mixotrophic cells (Fig. 2).  $\times 77,000$ .

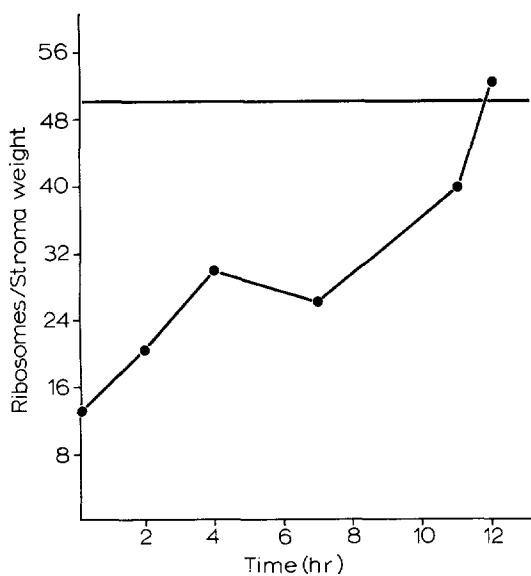


FIGURE 11 Levels of chloroplast ribosomes during the 12 hr dark incubation period following a light-to-dark transfer, as determined by the cut-and-weigh assay. The ribosome level of phototrophic *ac-20* is indicated by the horizontal line.

appearance: the intradisc space often becomes swollen or collapsed, and in fact the stacks appear more as irregular membrane "piles" than as an association of chloroplast discs (Figs. 12 and 13).

As a control, mixotrophically-grown wild-type cells were subjected to an identical light-to-dark transfer, and no change from the normal wild-type chloroplast membrane pattern was observed after 12 hr.

A partial recovery of pyrenoids occurs during the dark-incubation period: many more "attempted" pyrenoids (Fig. 4) can be recognized, and a number of pyrenoids typical of phototrophic *ac-20* can be found by the end of 12 hr.

**EFFECT OF LIGHT FOLLOWING A DARK INCUBATION:** When cultures are returned to the light following a 12 hr dark incubation, there is little further change in the level of chloroplast ribosomes, which is to be expected. A dramatic change occurs in the membrane conformation, however. Even after 1 hr in the light (during which time the starch reserves are practically depleted), the dormant, disorganized membrane "piles" take on an orderly, regular appearance (Fig. 14). The large stacks, in turn, give the

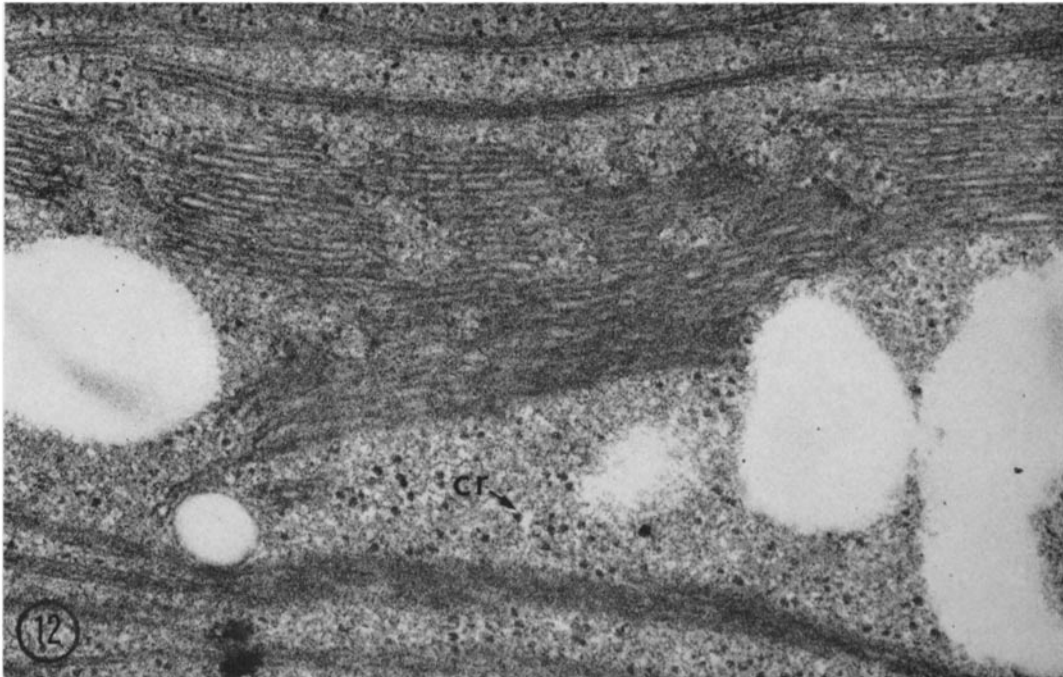


FIGURE 12 Portion of a chloroplast from a mixotrophic *ac-20* cell transferred to minimal medium and incubated in the dark for 7 hr. A large, disordered accumulation of membranous material is evident, as is the recovery of chloroplast ribosomes (*cr*).  $\times 77,000$ .

appearance of "shaking themselves out;" as seen in Fig. 14, smaller stacks appear to form from the larger ones.

By the end of 2 hr in the light, the giant grana are largely gone, and in their place one finds an orderly, concentric array of stacked discs: the chloroplast phenotype of phototrophic *ac-20* is already established. Pyrenoid development also goes rapidly to completion during these first few hours in the light. As noted previously (22), none of these changes is accompanied by changes in the chlorophyll content of the system.

One derives the impression from these observations that, during the dark-incubation period, certain potentialities for proper chloroplast membrane organization are being stored up but cannot find expression until light is provided; the light then triggers, or implements, a rapid series of events that result in the chloroplast phenotype of phototrophic *ac-20*. Thus chloroplast membrane organization, like carboxylase synthesis (22) and Photosystem II-associated activity (11), appears to be a light-dependent aspect of the *ac-20* recovery process.

#### *Fine Structure During Light-to-Light Transfers*

**RIBOSOMES:** The recovery of chloroplast ribosomes occurs much more rapidly in light-to-light than in light-to-dark transfers. The kinetics of ribosome recovery were estimated by the cut-and-weigh assay in three separate light-to-light experiments, and the resultant plots are shown in Fig. 15. The rates of recovery are different for each experiment, a result that can be explained in part by the inherent problem of sampling error in the assay. Nonetheless, it is apparent that ribosome recovery begins immediately after transfer, and that there is commonly an "overshoot" past the phototrophic *ac-20* level, as might be expected if the mixotrophic growth conditions were effecting a suppression of ribosome formation.

**CHLOROPLAST MEMBRANE AND PYRENOID:** Unlike chloroplast ribosome formation, membrane reorganization and pyrenoid formation are not apparent immediately after transfer in a light-to-light experiment. Instead, a lag of about

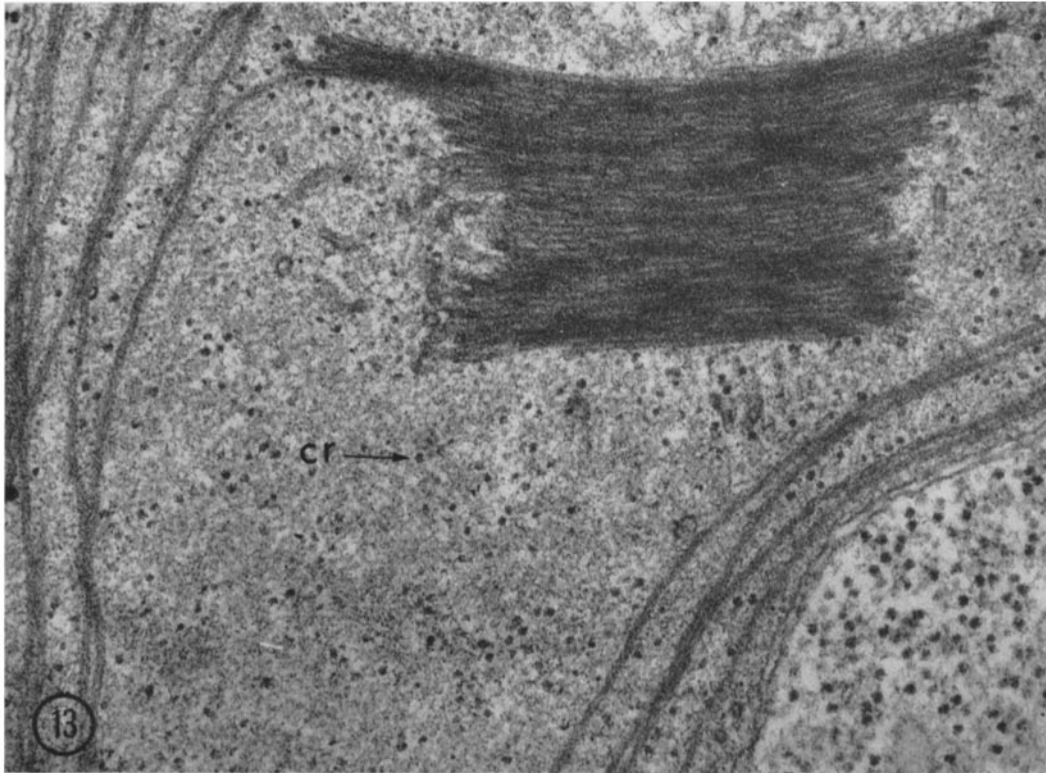


FIGURE 13 Portion of a chloroplast from a mixotrophic *ac-20* cell transferred to minimal medium and incubated in the dark for 11 hr. A compact mass of membrane is continuous with the disc system. Chloroplast ribosomes (*cr*) have recovered to the minimal level.  $\times 77,000$ .

4-6 hr is observed, during which time vesicles disappear and wide bands of discs begin to form. In other words, it appears that the phenotype of the light-to-dark *ac-20* chloroplast is beginning to develop. This trend is then reversed, and by 8-10 hr, small sized stacks and well developed pyrenoids predominate.

In two light-to-light experiments, Hill reaction rates were monitored every 1 or 2 hr, as described previously (11), and duplicate samples were fixed for electron microscopy. In both experiments, the acquisition of an orderly chloroplast membrane organization was found to coincide with the onset of a linear recovery of Hill activity. In light of observations reported previously (5), it cannot be said that the acquisition of normal disc organization causes the resumption of normal Hill activity, but rather that the two parameters are intimately related.

#### DISCUSSION

In the transfer experiments described in the two preceding papers, it is established that the *ac-20* "recovery" phenomenon requires two distinct processes. The first process can occur in the dark and in the presumed absence of net protein synthesis, whereas the second process requires light and involves *de novo* protein synthesis. In light-to-light transfer experiments, a 4-8 hr lag occurs before this second process can begin, whereas no lag occurs when dark-incubated cells are returned to the light. Together, these results indicate that the first, light-independent process must occur before the light-dependent recovery of Hill and carboxylase activity can begin.

The fine structural observations reported in this paper indicate that at least one of the essential events that occurs during the light-independent process is the formation of chloroplast ribosomes. Thus, recovery of chloroplast ribosomes from the

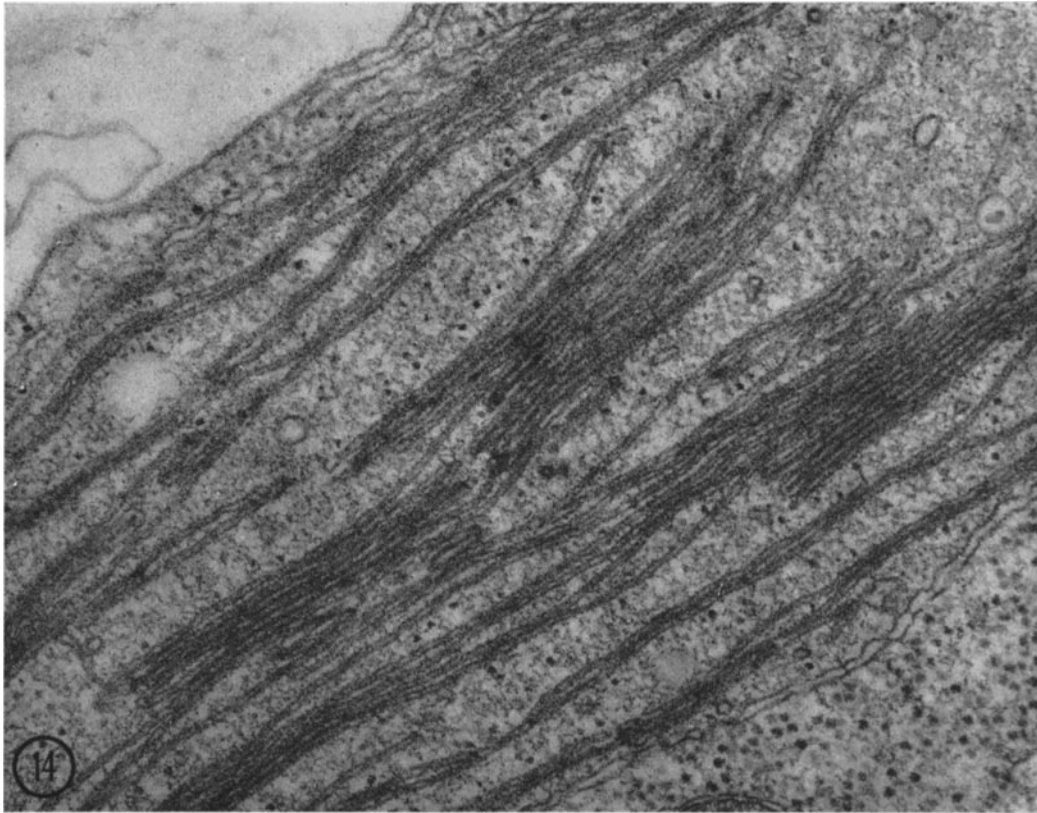


FIGURE 14 Portion of a chloroplast from a mixotrophic *ac-20* cell transferred to minimal medium, incubated in the dark for 12 hr, and exposed to light for 1 hr. Large, orderly stacks of discs, presumably derived from the membrane "piles" as seen in Figs. 12 and 13, appear to be giving rise to the small, regularly aligned stacks characteristic of phototrophic *ac-20* cells (Fig. 10).  $\times 75,000$ .

mixotrophic to the phototrophic *ac-20* level occurs during the dark-incubation period in a light-to-dark transfer experiment; it also occurs during the 4–6 hr "lag" period in a light-to-light experiment. The other, light-dependent aspects of *ac-20* recovery—RuDP carboxylase formation, cytochrome 559 activity, and chloroplast membrane reorganization—all occur only after chloroplast ribosome levels have substantially increased, suggesting that the formation of these ribosomes is required for the light-dependent processes to take place.

This interpretation of the *ac-20* syndrome is favored by the knowledge that ribosomes participate in protein synthesis. However, it clearly relies heavily on circumstantial evidence. It could be argued that the recovery of ribosomes is simply a less complex, and hence more rapid, aspect of the

*ac-20* recovery phenomenon and that it therefore always occurs first, without having any direct, causal relationship to the recovery of Hill activity, nor to the even more "delayed" recovery of carboxylase activity.

This interpretational dilemma has apparently been resolved by some recent experiments with rifampicin, an antibiotic that prevents chloroplast RNA formation in *C. reinhardi* by inhibiting the chloroplast DNA-dependent RNA polymerase (19). Wild-type cells grown in the presence of the antibiotic for 4 generations lose their chloroplast ribosomes, presumably by dilution, and the fine structural characteristics of these cells mimic those of mixotrophically-grown *ac-20* cells to a striking degree (20). Moreover, *C. reinhardi* cells grown in the presence of spectinomycin, an inhibitor of protein synthesis on 70S bacterial ribosomes (1),

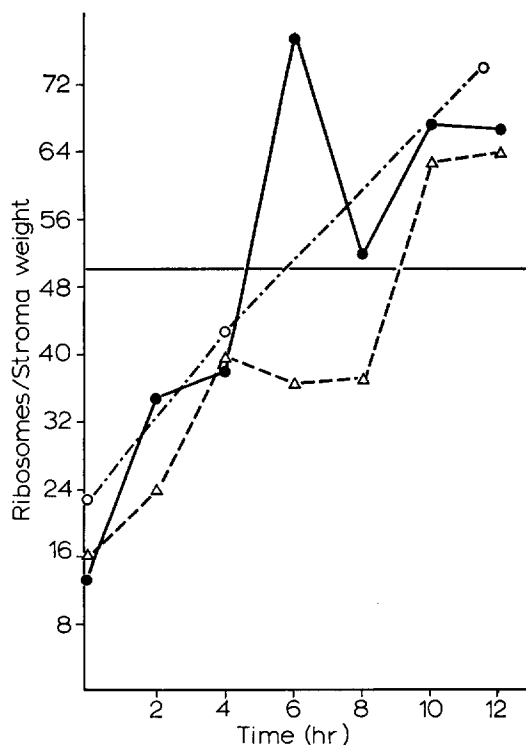


FIGURE 15 Levels of chloroplast ribosomes during three separate light-to-light transfer experiments, as determined by the cut-and-weigh assay. The ribosome level of phototrophic *ac-20* is indicated by the horizontal line.

lose both their Hill activity and their RuDP carboxylase activity, but retain normal chlorophyll levels (20). Finally, Hooper and coworkers have shown that the antibiotic chloramphenicol binds specifically to 70S chloroplast ribosomes of *C. reinhardtii* (8), and that in the presence of chloramphenicol, regreening cells of the *C. reinhardtii* *y-1* strain do not recover normal chloroplast membrane organization or Hill activity (9). Thus, the interference with chloroplast ribosome formation or activity in *C. reinhardtii*, whether by mutation or by treatment with a specific drug, produces a well-defined syndrome involving both photosynthetic and fine structural lesions, and we conclude that the ribosome reduction or inactivation and the syndrome are directly associated with one another.

The activities of various enzymes of the carbon reduction cycle in mixotrophic *ac-20* are given in Table II of Togasaki and Levine (22), and a survey of many other chloroplast components

found in the mutant strain is presented in Table III of Levine and Paszewski (11). These results are summarized in Table I of the present paper, where they can now be related to the fine structural observations that have been presented. It is apparent that many chloroplast components are not affected when levels of chloroplast ribosomes are reduced by more than 90% in mixotrophic *ac-20*, whereas a few chloroplast components are sharply affected. We conclude that the components in the "not affected" column do not depend on normal levels of chloroplast ribosomes for their synthesis, implying that these components are synthesized on cytoplasmic ribosomes in *C. reinhardtii*. Experiments with mitochondria have indicated a similar situation: in many organisms, mitochondria appear to import much, if not most, of their enzymatic apparatus from the cytoplasm (16).

It is seen in Table I that chlorophyll, carotenoid,

TABLE I  
Chloroplast Components Affected and not Affected in Mixotrophic *ac-20*

Affected	Not affected
	1. Phosphoriboisomerase
	2. Phosphoribulokinase
	3. 3-PGA kinase
1. RuDP carboxylase	4. G-3-P dehydrogenase (NAD)
	5. G-3-P dehydrogenase (NADP)
	6. Triosephosphate isomerase
	7. FDP aldolase
	8. Total quinone
	9. Plastocyanin
2. Cytochrome 559	10. Cytochrome 553
3. Q, the quencher of fluorescence of Photosystem II	11. Cytochrome 564
	12. P700
	13. Ferredoxin
	14. Ferredoxin-NADP reductase
	15. Chlorophyll (reduced by half)
	16. Carotenoid (reduced by half)
4. Membrane organization	17. Membrane formation (reduced by half)
5. Pyrenoid formation	18. Eyespot formation
	19. Starch synthesis

and chloroplast membrane are all reduced by about one half in mixotrophic *ac-20* cells compared to wild-type cells. In contrast, RuDP carboxylase and Hill activity are reduced at least 10-fold (11, 22) as are chloroplast ribosomes. These results could be explained by proposing that both the chloroplast and cytoplasmic protein-synthesizing systems contribute to the synthesis of chloroplast membrane and pigments, but this point remains undecided. Certainly the chloroplast ribosome system is not solely responsible for the synthesis of chlorophyll and membrane in *C. reinhardi*, a conclusion reached independently by Hooper et al. (9).

We stress that our results do not necessarily establish a direct nuclear control over chloroplast ribosome formation. Although the *ac-20* gene has been localized to linkage group XIII (7) and is thus presumably a nuclear gene, the *ac-20* muta-

tion could affect chloroplast ribosome formation quite indirectly, for example, by limiting the chloroplast's exogenous supply of a nucleotide essential for RNA synthesis, or by greatly stimulating ribonuclease activity within the chloroplast.

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