THE EFFECTS OF INHIBITORS OF RNA AND PROTEIN SYNTHESIS ON THE RECOVERY OF CHLOROPLAST RIBOSOMES, MEMBRANE ORGANIZATION, AND PHOTOSYNTHETIC ELECTRON TRANSPORT IN THE ac-20 STRAIN OF CHLAMYDOMONAS REINHARDI

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ABSTRACT

The ac-20 strain of Chlamydomonas reinhardi is characterized by low levels of chloroplast ribosomes when grown mixotrophically. Cells can be transferred to minimal medium and their ribosome levels increase. If, at the time of transfer, cells are exposed to chloramphenicol, an inhibitor of protein synthesis in the chloroplast, or cycloheximide, an inhibitor of protein synthesis in the cytoplasm, ribosome recovery is not affected; however, recovery is blocked by exposure to rifampicin, an inhibitor of chloroplast DNA-dependent RNA polymerase. It is therefore concluded that ac-20 cells suffer from an impaired chloroplast ribosomal RNA synthesis. Mixotrophic ac-20 cells are also characterized by low rates of photosynthetic electron transport, disorganized chloroplast membranes, and a small pyrenoid. If chloramphenicol is applied to transferred cells whose chloroplast ribosome levels have already recovered, recovery of photosynthetic electron transport and of structural integrity does not occur. Under the same conditions, cycloheximide has no effect on recovery. It is concluded that the structural and photosynthetic lesions in ac-20 are a secondary consequence of the low levels of chloroplast ribosomes. Finally, we present evidence that recovery of photosynthetic electron transport requires the transcription of chloroplast DNA. This transcription is apparently triggered by light.

INTRODUCTION

In a previous study of the ac-20 strain of the unicellular green alga, Chlamydomonas reinhardi (6, 12, 18), it was proposed (6) that the primary effect of the ac-20 mutation was to bring about a dramatic reduction in the cells' ability to produce chloroplast ribosomes. It was further proposed that, as a consequence of their greatly reduced levels of chloroplast ribosomes, the ac-20 cells were unable to synthesize certain chloroplast-specific components in normal amounts. These components include several electron-carrier molecules in the photosynthetic electron transport chain (12), the enzyme ribulose-1,5-diphosphate carboxylase (18), and factor(s) necessary for normal chloroplast membrane organization and pyrenoid formation (6).

Our proposal that the ac-20 syndrome results from defective chloroplast protein synthesis was based largely on the sequence of events we observed in so-called transfer experiments. The ac-20 mutation is more strongly expressed if cells are grown mixotrophically (in the light on an
acetate-supplemented minimal medium) than if grown phototrophically (in the light on minimal medium). Therefore, when ac-20 cells are transferred from mixotrophic to phototrophic conditions, a fourfold increase in chloroplast ribosome levels occurs within a few hours. This increase is always followed by, rather than accompanied or preceded by, an increase in levels of the affected chloroplast components. It thus appeared likely that the affected components were dependent on the chloroplast ribosomes for their synthesis.

To further evaluate this interpretation of the ac-20 syndrome and, in so doing, to further establish the protein-synthesizing capacity of the chloroplast of C. reinhardtii, experiments have been performed in which antibiotic inhibitors of chloroplast and cytoplasmic protein synthesis are presented to ac-20 cells at the time of transfer to minimal medium. The effects of the antibiotics on the recovery of ribosomes and of affected chloroplast components are then studied. The antibiotics used are rifampicin, an inhibitor of DNA-dependent RNA polymerization in the chloroplast (16), chloramphenicol and spectinomycin, two inhibitors of protein synthesis on chloroplast ribosomes (2, 3, 8), and cycloheximide, an inhibitor of cytoplasmic protein synthesis (8, 13).

The results of the experiments reported in this paper indicate that (a) the primary lesion in ac-20 cells is a defective synthesis of chloroplast ribosomal RNA and not a defective synthesis of chloroplast ribosomal protein; (b) the recovery of chloroplast membrane organization and photosynthetic electron transport is dependent on protein synthesis on chloroplast ribosomes and is independent of cytoplasmic protein synthesis; (c) the recovery of photosynthetic electron transport involves a light-triggered transcription of chloroplast DNA that is distinct from the transcription of information required for chloroplast ribosomal RNA formation. In a separate communication, Togasaki will report results of similar experiments with ac-20 that monitor the recovery of ribulose-1,5-diphosphate carboxylase in the presence of antibiotics.

MATERIALS AND METHODS

Cells of the ac-20 strain were grown mixotrophically as described previously (18), except that the concentration of sodium acetate in the culture medium was increased to 0.3% and fresh acetate-supplemented minimal medium (15) was added periodically to the culture as growth proceeded, to assure that the amount of acetate available to the cells did not become limiting.

For transfer experiments, the final volume of the mixotrophic culture was 1–3 liters, depending on how many cells were needed, and the culture had been growing 48–72 hr. Cells were harvested, washed once in minimal medium, and resuspended in minimal medium. All procedures were performed under sterile conditions. Aliquots of 300 ml of the resuspended cells were placed in 500-ml Erlenmeyer flasks. For light/–dark transfer experiments, the flasks were wrapped with aluminum foil and placed on rotary shakers for 12–16 hr. The foil was then removed, exposing the cells to light for the final stages of the experiment. For light-to-light transfer experiments, the flasks were placed directly in the light. The light intensity was 2000 lux from daylight fluorescent lamps, except in certain experiments with rifampicin to be described below.

For experiments using antibiotics, stock solutions of antibiotics were freshly prepared as described in the previous paper (4); appropriate samples were added at the times indicated, to give the following final concentrations: rifampicin (Mann Research Labs., Inc., New York), 250 µg/ml; chloramphenicol (Sigma Chemical Co., St. Louis, Mo.), 100 µg/ml; spectinomycin (a gift from the Upjohn Co., Kalamazoo, Mich.), 3 µg/ml; cycloheximide (Sigma Chemical Co.), 1 µg/ml.

As pointed out in the previous paper (4), rifampicin is red-orange in color and acts to screen out some of the light available to a culture of cells. In certain experiments with rifampicin, therefore, a culture containing the antibiotic and a control culture surrounded by a bath of a rifampicin solution at the same concentration were both illuminated from below at a light intensity of 8000 lux. Results of such experiments were the same as those carried out at lower light intensities.

At the times indicated in the text, cells were harvested. A portion of the culture was fixed for electron microscopy (6) and the remainder was washed and disrupted by sonication as previously described (11). The resulting chloroplast fragments were used to assay rates of Hill reaction, using 2,6-dichlorophenol-indophenol (DPIP) as an electron acceptor, with a Model 14 Cary recording spectrophotometer (Cary Instruments, Monrovia, Calif.) in the IR mode (7, 14). In some cases, Hill reaction rates were also measured with whole cells, using p-benzoquinone as electron acceptor and monitoring light-induced O₂ evolution with a Clarke-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio).

Procedures for counting chloroplast ribosomes from electron micrographs were as described elsewhere (6).
RESULTS

Chloroplast Ribosomes

Mixotrophically grown ac-20 cells (Fig. 1) possess low levels of chloroplast ribosomes (6). When such cells are transferred to minimal medium and placed either in the light (a light-to-light transfer) or in the dark (a light-to-dark transfer), their levels of chloroplast ribosomes increase approximately fourfold within a 12 hr period, the rate of increase being more rapid in the light than in the dark (6). A sensitive measure of this increase is provided by the cut-and-weigh technique whereby areas of chloroplast stroma are cut from electron micrographs, the number of chloroplast ribosomes present in the cuttings is counted, the cuttings are weighed, and a value of ribosomes per stroma weight is calculated (6). Typical values for control cells in a transfer experiment are given in Table I.

It is seen in Table I that if rifampicin is given to ac-20 cells at the time they are transferred to minimal medium, virtually no recovery of chloroplast ribosomes occurs during the ensuing 12 hr. In contrast, if either chloramphenicol or cycloheximide is given at this time, recovery of chloroplast ribosomes is not affected. The values given in Table I are taken from light-to-dark transfer experiments, but comparable results are obtained in light-to-light transfer experiments, as illustrated in Figs. 3–5.

Chloroplast Membrane Organization

Mixotrophically grown ac-20 cells exhibit three abnormal patterns of chloroplast membrane organization (6): membrane is found in vesicles, in single, unstacked thylakoids (Fig. 1), and in stacked thylakoids where the stack size tends to be larger and the stacks less confluent than in wild-type cells (Fig. 1) (5). The relative proportions of these three types of membrane organization vary considerably from one cell to the next and also from one culture to the next. For example, vesicles may be a prominent feature of cells in one experiment and be only infrequently encountered in cells from another experiment. The basis for this variation is not known, but it necessitates a careful examination of control cultures along with each antibiotic-treated culture before the degree of membrane recovery in a transfer experiment can be assessed.

Following a light-to-light transfer, chloroplast membrane reorganization proceeds after a lag of about 6 hr. By 10–12 hr, membranes are arranged in orderly stacks of two thylakoids; no vesicles and few single thylakoids or high stacks are observed (Fig. 2). If either rifampicin or chloramphenicol is given to the ac-20 cells at the time of a light-to-light transfer, no recovery of membrane organization occurs during the ensuing 10–12 hr, although the membranes undergo certain rearrangements.

In the case of rifampicin-treated cells, the thylakoids tend to lie together (Fig. 3) but they do not stack; in the case of chloramphenicol-treated cells, the membranes associate into poorly defined aggregates (Fig. 4). On the other hand, cycloheximide does not prevent the assumption of an orderly stacking pattern (Fig. 5).

When ac-20 cells are subjected to a light-to-dark transfer, a dramatic transformation in chloroplast membrane organization takes place during the ensuing 12–16 hr in the dark. Most striking is the accumulation of huge aggregates of membrane that we have termed membrane “piles” (6). In the present series of experiments we have found that such piles accumulate even when rifampicin, cycloheximide, or chloramphenicol is present; in other words, pile formation is not prevented by any of the inhibitors of protein synthesis that we have used.

After the 12–16 hr dark incubation that follows a light-to-dark transfer, cells are routinely returned to the light. When control cells are exposed to light, the membrane piles rapidly disperse and appear to give rise to smaller stacks that arrange themselves in the orderly, two-thylakoid arrays found in “recovered” cells (6). The membrane piles also disappear in the antibiotic-treated cells after exposure to light, but the course of events that follows their dispersal depends on the antibiotic and on the time the antibiotic is given to the cells.

If either rifampicin or chloramphenicol is given to cells at the start of a light-to-dark transfer experiment and if the cells are placed in the light at the end of 12–16 hr, the membrane does not come to assume an orderly organization as in the control. Instead, the membrane piles disperse and the membrane returns to its unstacked, disordered, mixotrophic state. If, on the other hand, the cells are transferred to minimal medium and allowed to incubate in the dark for 12–16 hr before either rifampicin or chloramphenicol is added, the anti-
Biotics have no effect on the ensuing light-stimulated membrane reorganization; after several hours in the light the treated cells are indistinguishable from the controls.

Cycloheximide has quite a different effect. Regardless of when it is administered, it is without effect on chloroplast membrane organization: in its presence, membrane piles accumulate in the dark and give rise, in the light, to orderly arrays of small stacks.

**Pyrenoid Formation**

We previously reported that a pyrenoid is either lacking or rudimentary in mixotrophic \textit{ac-20} cells (6). Further examination of this point indicates that in fact most mixotrophic \textit{ac-20} cells probably possess rudimentary pyrenoids, but because of their small size, they are only rarely encountered in section. If 100 cells of comparable size are scored for the presence or absence of pyrenoid material in thin section, 40 are found to exhibit pyrenoids when the cells are from a wild-type culture, whereas seven exhibit pyrenoids when the cells are from a mixotrophic \textit{ac-20} culture.

This type of counting procedure is meaningful only if cells are the same size: large pyrenoids in small cells will, for example, be encountered more frequently than large pyrenoids in large cells, and so on. Since \textit{ac-20} cells undergo a cell division during the course of a transfer experiment (6), it is not possible to give a quantitative estimate of pyrenoid frequencies as they are observed during a transfer experiment. Qualitatively, however, it is quite apparent that pyrenoids undergo a dramatic increase in size during a transfer experiment, that both chloramphenicol and rifampicin prevent this increase, and that cycloheximide does not affect the increase. As reported previously (6), pyrenoid recovery proceeds during the dark period of a light-to-dark transfer experiment.

**Hill Activity**

The Hill reaction measures the light-induced reduction of an electron acceptor where water is the electron donor. In this paper, Hill reaction rates are given for the photoreduction of DPIP by chloroplast fragments. These experiments were also performed with whole cells using \textit{p}-benzoquinone as the electron acceptor, and comparable results were obtained. Thus the effects of the inhibitors that are reported below cannot be attributed to antibiotic-produced differences in the way chloroplast membranes fragment when subjected to sonic disintegration.

As reported by Levine and Paszewski (12), mixotrophic \textit{ac-20} cells have low rates of Hill activity, commonly one-fourth the rates of wild-type cells. In Figs. 6–9 of the present paper it is seen that the rates of mixotrophic \textit{ac-20} cells vary between 45 and 85 \textit{m\mu}oles of DPIP reduced/hr per mg chlorophyll (chl). A comparable range of variation in rates is observed in wild-type cells from one experiment to the next. Following transfer to minimal medium, Hill reaction rates in \textit{ac-20} cells increase, after a lag, in the light; in the dark, the rates do not increase, but after light is provided, recovery proceeds without a lag (12). In the present experiments, only initial and final rates were measured for a given set of experimental conditions. Therefore, the straight lines in Figs. 6–9 do not represent the actual kinetics of recovery.

Fig. 6 shows that if rifampicin is given to mixotrophic \textit{ac-20} cells at the start of a light-to-light transfer experiment, recovery of Hill activity is inhibited although not, in this experiment, inhibited completely. This experiment was carried out at high light intensities in order to make certain that the red color of the rifampicin was not screening out light and thereby preventing recovery. The high light intensities may, however, produce a partial breakdown of the inhibitor. Other difficulties in working with rifampicin are discussed in the preceding paper (4). It is also seen in Fig. 6 that if rifampicin is added at the end of the dark incubation period that follows a light-to-dark transfer, a complete inhibition of recovery of Hill activity is effected.

When chloramphenicol is given to cells at either the onset of a light-to-light transfer experiment or before providing light in a light-to-dark transfer, no recovery of Hill activity occurs (Fig. 7). There is some indication that chloramphenicol may be inhibitory to the photosynthetic process itself in short-term experiments (1), and we therefore repeated these experiments with a second inhibitor of chloroplast protein synthesis, spectinomycin. As seen in Fig. 6, identical results were obtained.

Fig. 9 shows that cycloheximide is without effect on the recovery of Hill activity, whether given at the beginning of a light-to-light transfer or at the end of a light-to-dark incubation.
Figure 1. Portion of an ac-30 cell grown mixotrophically. Chloroplast ribosomes (arrowheads) are sparse. Thylakoids (T) are unstacked except for a wide band (B) beneath the chloroplast envelope. × 67,000.
Figure 2  Portion of an ac-20 cell transferred from mixotrophic to phototrophic conditions and incubated in the light for 8 hr. Chloroplast ribosomes (arrowheads) have recovered (compare with Fig. 1). Thylakoids are in the process of becoming ordered into the concentric arrays of two-thylakoid stacks that typify phototrophic ac-20 cells (6). A grazing section of the pyrenoid (P) is included. × 72,000.
**Table I**

Effect of Antibiotics on the Recovery of Chloroplast Ribosomes in ac-20 Cells during Light-to-Dark Transfer Experiments, as Determined by the Cut-and-Weigh Technique

<table>
<thead>
<tr>
<th>Antibiotic added</th>
<th>Initial chloroplast ribosome level (ribosomes/stroma weight)</th>
<th>Final chloroplast ribosome level 16 hr after transfer</th>
<th>% inhibition by antibiotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>110</td>
<td>--</td>
</tr>
<tr>
<td>Rifampicin (250 μg/ml)</td>
<td>20</td>
<td>30</td>
<td>89</td>
</tr>
<tr>
<td>Chloramphenicol (100 μg/ml)</td>
<td>20</td>
<td>106</td>
<td>4</td>
</tr>
<tr>
<td>Cycloheximide (1 μg/ml)</td>
<td>20</td>
<td>114</td>
<td>0</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The experiments reported in this paper indicate that the low levels of chloroplast ribosomes found in mixotrophic ac-20 cells are the consequence of defective chloroplast DNA-dependent RNA synthesis. Surzycki has shown (16) that the 5S, 16S, and 23S species of chloroplast ribosomal RNA (rRNA) are among the products of chloroplast DNA-dependent RNA synthesis and that these species of rRNA are greatly reduced in amount in mixotrophic ac-20 cells; it therefore seems most reasonable to conclude that a primary, if not the primary, lesion suffered by mixotrophic ac-20 cells is a reduced ability to synthesize chloroplast rRNA. This lesion is apparently not accompanied by a reduction in the synthesis of chloroplast ribosomal proteins or in the synthesis of the chloroplast DNA-dependent RNA polymerase enzyme. A sizable store of these proteins must exist in mixotrophic ac-20 cells, for when chloroplast rRNA synthesis is stimulated by transfer to minimal medium, chloroplast ribosomes assemble in the presence of either chloramphenicol or cycloheximide. Moreover, if cells are presented with either rifampicin or chloramphenicol (but not cycloheximide) at the start of a light-to-dark transfer experiment and are exposed to light after 12 hr, the large piles disperse but they do not give rise to normal membrane structures. Thus the membrane piles do not represent, as was previously suggested (6), stores of membrane material that are "potentiated" for the formation of normally organized thylakoids once light is provided.

In spite of the fact that the visible alterations in membrane conformation that occur during the dark period are not morphological manifestations of the recovery process, it is nonetheless clear that alterations in ac-20 chloroplast membranes that depend upon chloroplast protein synthesis do occur during the dark period. This is demonstrated by the observation that if either rifampicin or chloramphenicol (but not cycloheximide) is given at the start of the dark period, normal membrane configurations never form in the ensuing light period, whereas if either rifampicin, chloramphenicol, or cycloheximide is given to cells at the end of the dark period, membrane reorganization proceeds normally when the cells are exposed to light. Thus the process of membrane reorgani-
FIGURE 3 Portion of an ac-20 cell transferred from mixotrophic to phototrophic conditions and incubated in the light in the presence of 250 µg/ml rifampicin for 11 hr. No recovery of chloroplast ribosomes (arrowheads) has occurred (compare with Figs. 1 and 2). Thylakoids associate in large bundles but stacking does not usually occur between these thylakoids. × 71,000.
FIGURE 4 Portion of an ac-20 cell transferred from mixotrophic to phototrophic conditions and incubated in the light in the presence of 100 μg/ml chloramphenicol for 10 hr. Chloroplast ribosomes (arrowheads) have recovered (compare with Figs. 1 and 2). A short region of normal stacking (S) is included in the field, but most of the membrane lies in ill-defined aggregates (the poor definition of these aggregates is not the result of an oblique sectioning angle; most cells in the sample exhibit a preponderance of similar profiles). A region of chloroplast DNA is indicated (DNA). × 71,000.
FIGURE 5  Portion of an ac-30 cell transferred from mixotrophic to phototrophic conditions and incubated in the light in the presence of 1 μg/ml cycloheximide for 12 hr. Chloroplast ribosomes (arrowheads) have recovered (compare with Figs. 1 and 3). Thylakoids are in the process of becoming ordered into two-thylakoid stacks. × 71,000.
zation in ac-20 appears to involve two phases: a light-independent, synthetic phase that is dependent on protein synthesis in the chloroplast but not in the cytoplasm, and a light-dependent, organizational phase that is independent of protein synthesis, during which the “potentiated” membranes formed in the dark are rapidly arranged into their more orderly morphological configurations. The existence of these two phases cannot be readily detected in light-to-light experiments because, in such experiments, synthesis and organization occur concurrently.

The stimulation of membrane reorganization by light apparently involves an interaction between light and chloroplast membranes that is not mediated by RNA or protein synthesis. Our experiments also indicate a second role for light in the recovery process. If cells are exposed to rifampicin, chloramphenicol, or spectinomycin (but not cycloheximide) at the end of the dark period (a time, it will be recalled, when chloro-

**Figure 6** The effect of 250 μg/ml rifampicin on the recovery of DPIP-Hill activity in mixotrophic ac-20 cells transferred to minimal medium at 0 hr. Open symbols represent values from a light-to-light transfer experiment, and closed symbols represent values from a light-to-dark transfer experiment. Control values are indicated by circles and solid lines. Values from rifampicin-treated cells are symbolized by hexagons and dashed lines. For the light-to-dark transfer experiment, the reaction rates at the beginning and at the end of the dark incubation period are given; the two values are connected by a dotted line. The time that light is provided in the light-to-dark transfer experiment is indicated. Rifampicin was added to the experimental cultures at the times indicated by the thinner arrows.

**Figure 7** The effect of 100 μg/ml chloramphenicol on the recovery of DPIP-Hill activity in mixotrophic ac-20 cells transferred to minimal medium at 0 hr. Symbols are as described for Fig. 6 except that values from chloramphenicol-treated cells are symbolized by squares, and the thinner arrows indicate the times of addition of chloramphenicol.

**Figure 8** The effect of 3 μg/ml spectinomycin on the recovery of DPIP-Hill activity in mixotrophic ac-20 cells transferred to minimal medium at 0 hr. Symbols are as described for Fig. 6 except that values from spectinomycin-treated cells are symbolized by triangles, and the thinner arrows indicate the times of addition of spectinomycin.

plast ribosomes have already formed), no recovery of Hill activity occurs, even though membrane reorganization is not blocked. Thus light is apparently required to trigger, and perhaps also to sustain, a chloroplast-located protein synthesis.
that produces a photosynthetic apparatus capable of greatly stimulated Hill activity. The fact that rifampicin produces a complete block in recovery of Hill activity under these experimental conditions strongly suggests that light is first required to stimulate a DNA-dependent RNA synthesis within the chloroplast, one that cannot or does not occur in the dark. The RNA so produced is apparently then translated on chloroplast ribosomes (unless these are blocked by chloramphenicol or spectinomycin). Whether light is also required for the translational phase of the process cannot be determined from these experiments. We believe, then, that our experiments indicate the existence of a light-stimulated messenger RNA synthesis from chloroplast DNA in *Chlamydomonas reinhardi*.

In summary, it appears that the *ac-20* mutation produces a primary defect in chloroplast rRNA synthesis; it may produce a more generalized inhibition of all chloroplast DNA-directed RNA synthesis, but our experiments were not designed to examine this possibility. As a consequence of this defective rRNA synthesis, chloroplast ribosomes are not produced at normal levels and normal synthesis of proteins required for the Hill reaction, for membrane organization, and for pyrenoid formation cannot occur. Once cells have been transferred to minimal medium and chloroplast ribosome levels have increased, chloroplast protein synthesis also increases. The synthesis of component(s) required for normal membrane organization and pyrenoid formation can apparently occur in either the light or the dark, but recovery of Hill activity appears to require a light-stimulated transcription of chloroplast DNA and thus occurs only in the light. Cytoplasmic protein synthesis appears not to be involved in any of the aspects of the *ac-20* recovery process that we have studied; indeed, the absence of any effect of cycloheximide is most dramatic, for in short-term synchronous-culture experiments with *C. reinhardi*, cycloheximide produces a rapid and complete inhibition of most synthetic processes (1).

The existence of a Mendelian gene that exerts an apparent control over chloroplast DNA transcription is potentially of considerable interest, as we have discussed elsewhere (6, 10). However, the strong influence of mixotrophic versus phototrophic growth conditions over the expression of the *ac-20* mutation leads us to question how direct this "control" really is.

Initial experiments on the effects of chloramphenicol and cycloheximide on recovery of Hill activity were performed in this laboratory by Dr. A. Paszewski. We acknowledge the excellent technical assistance of Miss Diane Dwyer and the continued interest in this research given by Dr. S. J. Surzycki and Miss J. J. Armstrong.

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