THE EFFECTS OF INHIBITORS OF RNA AND PROTEIN SYNTHESIS ON CHLOROPLAST STRUCTURE AND FUNCTION IN WILD-TYPE *CHLAMYDOMONAS REINHARDI*

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ABSTRACT

Wild-type cells of the unicellular green alga *Chlamydomonas reinhardi* have been grown for several generations in the presence of rifampicin, an inhibitor of chloroplast DNA-dependent RNA polymerase, spectinomycin and chloramphenicol, two inhibitors of protein synthesis on chloroplast ribosomes, and cycloheximide, an inhibitor of protein synthesis on cytoplasmic ribosomes. The effects of cycloheximide are complex, and it is concluded that this inhibitor cannot give meaningful information about the cytoplasmic control over the synthesis of chloroplast components in long-term experiments with *C. reinhardi*. In the presence of acetate and at the appropriate concentrations, the three inhibitors of chloroplast protein synthesis retard growth rates only slightly and do not affect the synthesis of chlorophyll; however, photosynthetic rates are reduced fourfold after several generations of growth. Each inhibitor produces a similar pattern of lesions in the organization of chloroplast membranes. Only rifampicin prevents the production of chloroplast ribosomes.

INTRODUCTION

The unicellular green alga, *Chlamydomonas reinhardi*, possesses a single large chloroplast that contains DNA and 68S, bacterial-like ribosomes (for review, see reference 22). Investigations in this laboratory have focused on the role that this protein-synthesizing apparatus plays in constructing the structural and functional components of the chloroplast (1, 10, 11, 17, 23, 32–35). The present paper reports a study of the structural and functional lesions produced when wild-type *C. reinhardi* cells are grown for several generations in the presence of antibiotics that specifically inhibit transcription or translation, and hence protein synthesis, in the chloroplast. The antibiotics used are rifampicin, an inhibitor of the chloroplast's DNA-dependent RNA polymerase (32), and chloramphenicol and spectinomycin, two inhibitors of chloroplast protein synthesis (5, 6, 15). Cycloheximide, an inhibitor of protein synthesis in the cytoplasm (15, 30), is also used in an attempt to estimate the cytoplasm's contribution to the production of a normal chloroplast.

Long-term growth experiments that utilize antibiotics must be performed and interpreted with considerable caution. First, a concentration of antibiotic must be chosen that is high enough to inhibit RNA or protein synthesis but not high enough to be generally toxic to the cells during the course of the 48–72-hr growth periods required to dilute out existing chloroplast components by cell division. In particular, concentrations of antibiotic must be used that do not affect mitochondrial pro-

tein synthesis, for one assumes that *C. reinhardi* cannot survive without functional mitochondria (33). It is important to establish that the cells are not dying, since the loss of cellular activities and of structural integrity proceeds in a most nonspecific manner in moribund cells. Second, it has been found that when wild-type cells are exposed to an antibiotic for long periods of time, they sometimes make an adjustment to the exposure. For example, after 24 hr of growth in 3 μg/ml of spectinomycin, cells acquire a resistance to this concentration of the antibiotic (the origin and nature of this resistance has not yet been determined). One cannot, however, go to higher concentrations of spectinomycin because these are toxic to the cells during the first 24 hr of the experiment.

Despite these complications, long-term growth experiments can provide certain kinds of information—notably, information as to the effects of the inhibitors on chloroplast ribosome levels and chloroplast fine structure—that cannot be obtained in short-term experiments with wild-type cells (1).

MATERIALS AND METHODS

Culture of the Organisms

The plus and minus mating types of the wild-type strain 137c of *C. reinhardi* were used with comparable results. The *spa-2* mutant strain was used in certain experiments; its properties are described in the Results section.

For experiments investigating the effects of spectinomycin, chloramphenicol, or cycloheximide, cells were grown overnight in 300 ml volumes of Tris-acetate-phosphate (TAP) medium (13) contained in 500-ml Erlenmeyer flasks. These were agitated on rotary shakers in the light (2500 lux) at 26°C. The following morning, the culture was diluted twofold and divided between two 500-ml flasks, one serving as the control. All procedures were performed under sterile conditions. The following stock solutions (in water) of antibiotics were prepared the day of the experiment and sterilized by passage through a Millipore filter (Millipore Corp., Bedford, Mass.): spectinomycin (a gift from Upjohn Co., Kalamazoo, Mich.), 3 mg/ml; chloramphenicol (Sigma Chemical Co., St. Louis, Mo.), 2.5 mg/ml; and cycloheximide (Sigma Chemical Co.), 3 mg/ml. Appropriate aliquots of these were added to the experimental flask to give the final antibiotic concentrations cited in the text. Growth was continued for an additional 30–96 hr; where growth was continued for more than 48 hr, cells were transferred to fresh medium containing fresh antibiotic. Cell number during the growth period was determined with the aid of a hemacytometer.

The above procedures were modified in experiments investigating the effects of rifampicin, for the reasons given in the Results section. Two 300-ml volumes of TAP medium were each inoculated in the dark with 10 ml of a culture of wild-type cells whose growth had been synchronized (19) with a light-dark cycle for three generations. The cells in the inoculum were in the final hour of the dark cycle. To the experimental flask was also added a sample of a rifampicin stock solution prepared by adding 125 mg rifampicin (Mann Research Labs Inc., New York) to 5 ml of sterile 0.01 M KH_2PO_4, pH 4.5, and agitating in the dark with a magnetic stirrer for 8 hr to insure proper solution of the antibiotic. Both control and experimental flasks were maintained in darkness throughout the growth period. The cultures were then exposed to light for 2.5 hr before harvesting.

Measurement of Photosynthetic Parameters

Total chlorophyll and chlorophylls *a* and *b* were determined by a modification (2) of the method of Mackinney (26). Photosynthetic CO_2 fixation was measured as described previously (8).

Electron Microscopy

Cells were fixed and embedded as previously described (10, 18), and thin sections were examined with a Hitachi HU-11C electron microscope. Procedures for making ribosome counts from electron micrographs have been described previously (10).

RESULTS

Control Cells

The growth rate, chlorophyll content and chlorphyll *a*:*b* ratio, rate of photosynthetic CO_2 fixation, chloroplast ribosome content, and chloroplast membrane organization of wild-type *C. reinhardi* cells grown mixotrophically—in the light in the presence of a source of fixed carbon (acetate)—have been described in several other publications from this laboratory (3, 8–10, 21, 24, 35). Representative values for most of these parameters are given in Table I.

In experiments investigating the effects of rifampicin, control cells were grown heterotrophically—in the dark in the presence of acetate—for 3–4 days and were then exposed to light for 2.5 hr. The rationale for these growth conditions will be presented shortly. Control cells grown under such conditions have almost normal levels of chloro-

TABLE I

Chlorophyll Content, Photosynthetic Capacity, and Ribosome Levels in C. reinhardi Cells Grown in the Absence and in the Presence of Antibodies

Antibiotic	Strain, growth condition	Mean doubling time	Divisions before harvest	μg Chl/10^6 cells	Chl *a*:*b*	μmoles CO_2 fixed/hr				Chloroplast ribosomes		Cytoplasmic ribosomes
						·mg Chl	*% control*	*·10^6 Cells*	*% control*	*No. per stroma weight*	*% control*	*No. per ground substance weight*
		hr	*No.*									
Control	wild-type, mixotrophic	11	5.5	3.6	2.3	121		0.436		200		405
Control	wild-type, heterotrophic + 2.5 hr light	14	4	2.8	2.5	124		0.356		271		538
Control	*spa-2*, 10 μg/ml spectinomycin, mixotrophic	12	ca. 4	*	2.1	103		*		209		585
Rifampicin (250 μg/ml)	wild-type, heterotrophic + 2.5 hr light	20	3.5	1.3	2.1	25	(20%)	0.032	(9%)	43	(16%)	440
Spectinomycin (25 μg/ml)	*spa-2*, mixotrophic	12	ca. 10	*	2.0	29	(28%)	*		238	(114%)	610
Chloramphenicol (100 μg/ml)	wild-type, mixotrophic	17	3	3.0	2.2	36	(30%)	0.108	(25%)	208	(104%)	450
Cycloheximide (1 μg/ml)	wild-type, mixotrophic	48	1	7.6	2.8	126	(104%)	0.955	(220%)	368	(194%)	645

* Not possible to obtain data; see text.

phyll and rates of CO_2 fixation (Table I), and most of them exhibit the normal pattern of chloroplast membrane organization found in mixotrophically grown cells, namely, anastomosing stacks of from 2 to 10 thylakoids (9). Perhaps 20% of the cells in such cultures, however, exhibit the distinct chloroplast phenotype illustrated in Fig. 1. The chloroplast membranes are organized into what appear to be short, segmented thylakoids that frequently fold back on themselves. Cells that contain such membrane aggregates do not contain normal thylakoid stacks; they thus appear to represent a distinct population of cells within the culture. Because of their morphological similarity to "yellow" strains of *C. reinhardi* (16, 17, 29), it is likely that these cells represent a "yellow" clone within our wild-type stock. Despite their anomalous membrane organization, such cells possess abundant chloroplast ribosomes (Fig. 1); indeed, as seen in Table I, heterotrophically grown wild-type cells of *C. reinhardi* exhibit higher levels of chloroplast ribosomes than do their light-grown counterparts.

In experiments investigating the effects of spectinomycin it was found, as mentioned in the Introduction, that wild-type cells were killed by a concentration of 5 μg/ml of spectinomycin but acquired a resistance to 3 μg/ml of spectinomycin. To obtain meaningful data with this antibiotic it was therefore necessary to use a strain that was already resistant to low spectinomycin levels and that would tolerate a wider range of concentrations of the antibiotic. These requirements were met by the *spa-2* strain of *C. reinhardi*, recently isolated from the wild-type strain in this laboratory by J. J. Armstrong. Cells of this strain can be grown in liquid TAP cultures in the presence of 10 μg/ml of spectinomycin, and their growth rates, chloroplast fine structure, and photosynthetic capacities are indistinguishable from those of the wild-type cells (Table I). Therefore, the control cells in spectinomycin experiments are *spa-2* cells grown on 10 μg/ml of spectinomycin, and the experimental cells, to be described shortly, are *spa-2* cells grown on 25 μg/ml of spectinomycin. Concentrations of 50–100 μg/ml of spectinomycin are required to kill *spa-2* cells on liquid TAP medium.[1]

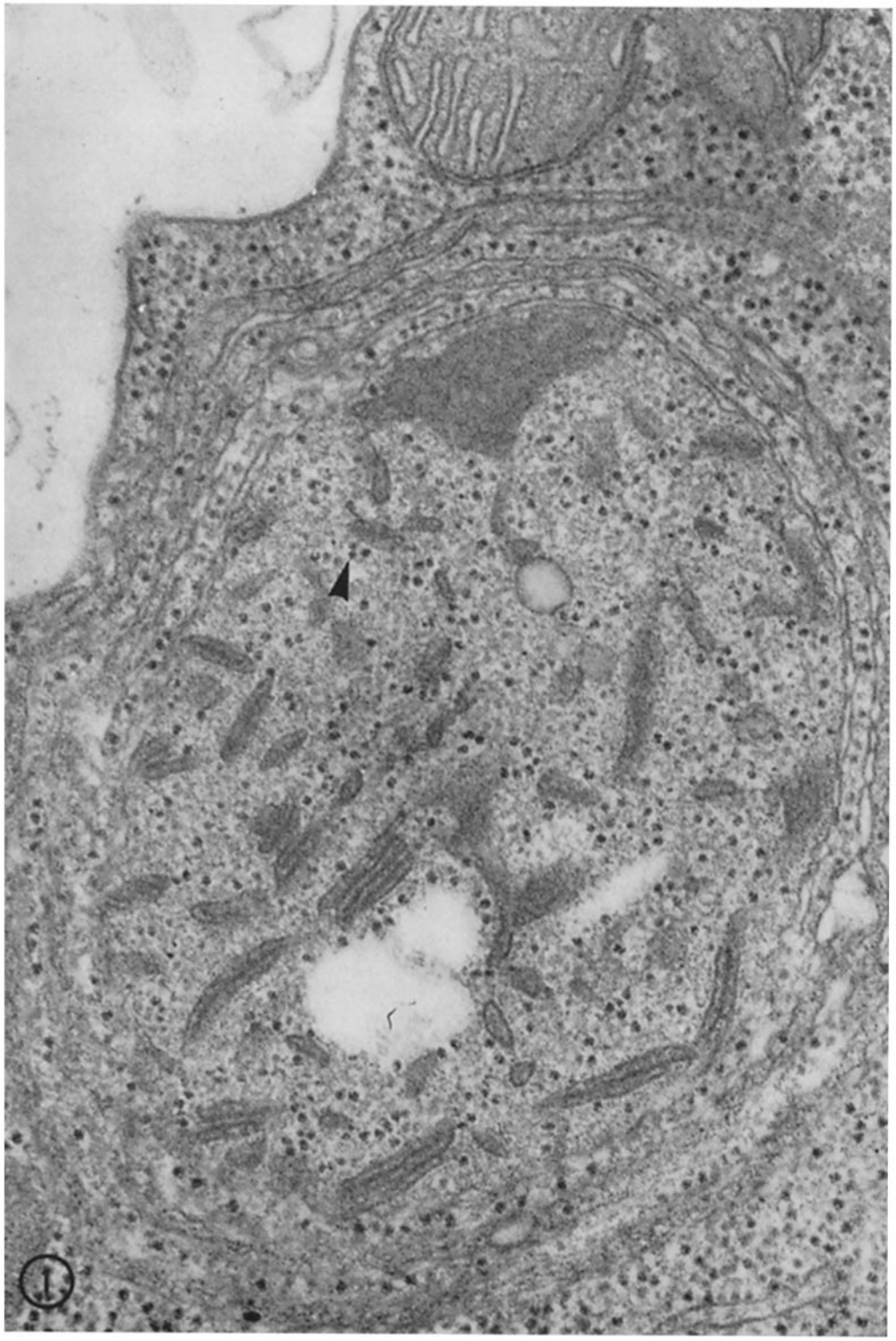
The major drawback to working with *spa-2* cells is that they become paralyzed during growth in liquid cultures, whether or not spectinomycin is present in the medium. It can be seen with the electron microscope that normally constructed but very short flagella are formed by such cells. Because they cannot swim, daughter cells tend to remain within the mother wall after cell division and, as a result, the culture comes to contain large clumps of cells that cannot be accurately counted with a hemacytometer. Growth rates can therefore only be estimated approximately (Table I), and it is impossible to give reliable data on chlorophyll content per cell. It is not known whether there is any relationship between the strain's paralysis and its resistance to low levels of spectinomycin.

Cells Grown in the Presence of Rifampicin

Rifampicin interacts with the bacterial (14), and apparently also with the *C. reinhardi* chloroplast (32) DNA-dependent RNA polymerase in such a way as to prevent the transcription of bacterial and chloroplast DNA. The antibiotic interacts with the polymerase only when the polymerase is not associated with its DNA template (36). In order to see a maximal effect of rifampicin on chloroplast DNA transcription it is, therefore,

[1] The genetics of the *spa-2* strain and the spectinomycin-binding properties of *spa-2* chloroplast ribosomes will be the subject of separate communications from this laboratory (S. J. Surzycki and W. Burton, manuscripts in preparation), which demonstrate that the *spa-2* mutation is inherited in a Mendelian fashion and that 68S ribosomes isolated from *spa-2* cells bind spectinomycin in the same way as wild-type 68S ribosomes.

FIGURE 1 Portion of a cell from the wild-type strain of *C. reinhardi* grown heterotrophically in the dark for 4 days and exposed to light for 2.5 hr before being fixed. Chloroplast ribosomes (arrowhead are at the normal wild-type level (Table I). Chloroplast membranes exist largely as segmented stacks of thylakoids, suggesting that the cell derives from a "yellow" clone (16, 17, 29) within the wild-type population. Perhaps 20% of the cells in the sample exhibit this phenotype; the remainder are indistinguishable from mixotrophically grown wild-type cells illustrated in other publications (7, 9, 10, 12, 18). × 95,000.



necessary to start with cells whose chloroplast DNA-dependent RNA polymerase is in the "unattached" state. This is accomplished by inoculating the culture with cells whose growth has been synchronized on a minimal medium by a light-dark cycle (1, 19). Cells are taken from the end of a 12 hr dark cycle; at this time they are engaged in no detectable chloroplast RNA synthesis (Surzycki and Hastings, manuscript in preparation) and thus the chloroplast DNA-dependent RNA polymerase is free to interact with rifampicin.[2]

Two other features of long-term growth experiments with rifampicin should be noted. First, the experiments are carried out in the dark under heterotrophic growth conditions because the antibiotic, a bright red-orange in color, has the effect of screening out a great deal of the light available to a control culture; moreover, light appears to inactivate rifampicin with time. Second, the heterotrophically grown cultures are exposed to 2.5 hr of light before being harvested. This step is taken because it has been shown (R. P. Levine and D. Graham, unpublished observations) that, after three to four generations of heterotrophic growth, wild-type *C. reinhardi* cells cannot carry out a maximal rate of photosynthesis until after they are exposed to light for about 2.5 hr.

CHLOROPLAST RIBOSOMES: Cells grown heterotrophically in the presence of 250 μg/ml of rifampicin under conditions described above contain only a fraction of the level of chloroplast ribosomes found in heterotrophically grown control cells. This is seen in Fig. 2 and in the chloroplast ribosome counts given in Table I. A slight reduction is also found in counts of cytoplasmic ribosomes in rifampicin-grown cells (Table I); however, since the value obtained is still within the normal range for cytoplasmic ribosomes (Table I) and since the tight packing of cytoplasmic ribosomes renders them very difficult to count, this observation remains of uncertain significance. Certainly the 18% reduction in levels of cytoplasmic ribosomes, if real, is an effect that is very different from the 84% reduction in levels of chloroplast ribosomes.

On the assumption that rifampicin brings about a 100% cessation of chloroplast ribosomal RNA synthesis (32), and that existing chloroplast ribosomes are lost from the cells by the dilution that accompanies cell division, one can calculate that, after 3.5 generations, cells should possess perhaps 9% of the control levels of chloroplast ribosomes. That they possess 16% of the control levels can be attributed to such considerations as an incomplete atibiotic-enzyme interaction and sampling errors; in other words, the discrepancy between observation and theory does not appear great.

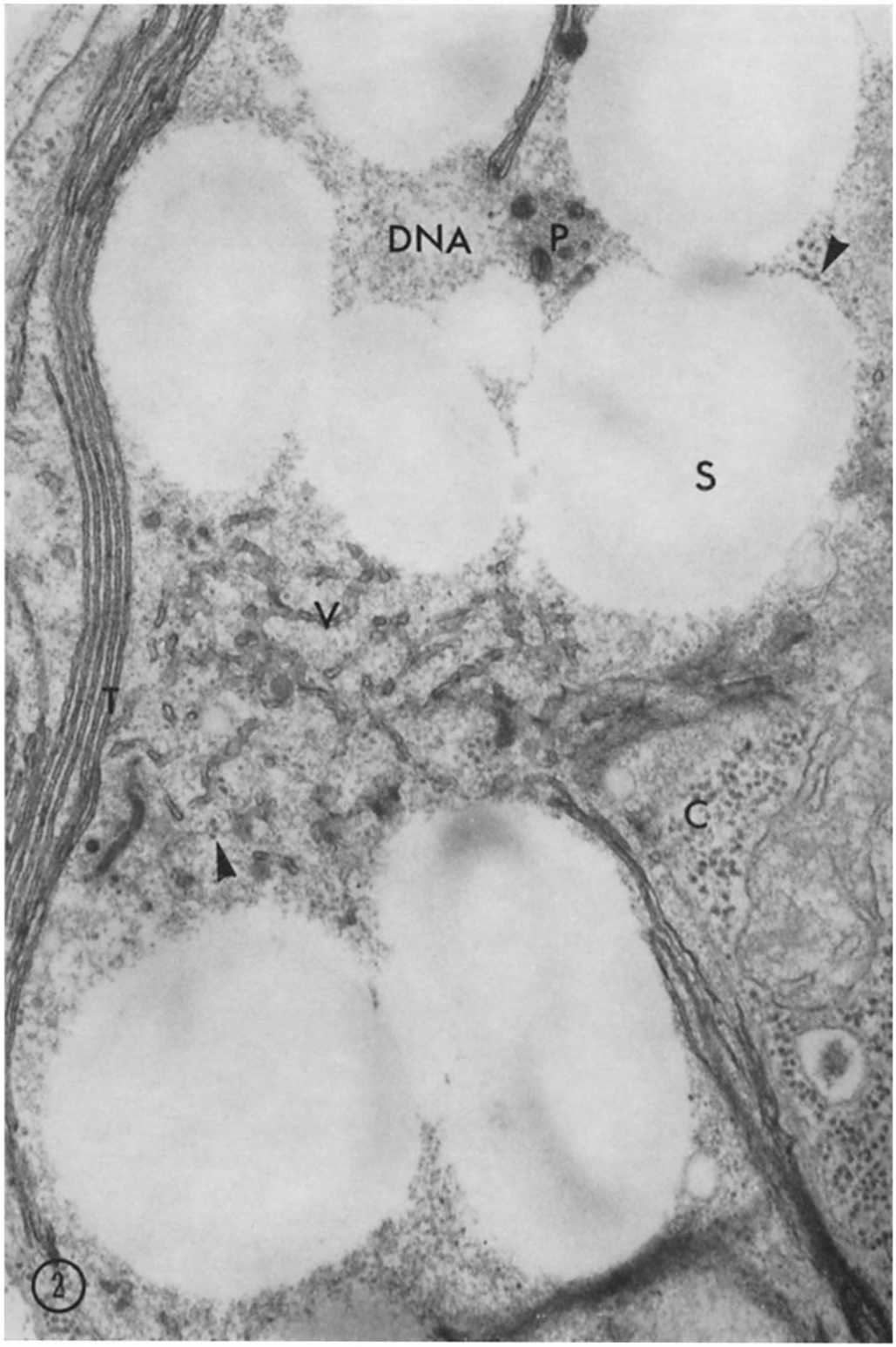
CHLOROPLAST FINE STRUCTURE: Growth in the presence of rifampicin brings about a severe disorganization of chloroplast membranes in wild-type cells. A branching system of membranes fills the chloroplast interior of virtually every cell (Fig. 2); these membrane profiles will be referred to as vesicles since they give a vesiculate appearance in section. A comparison of Figs. 1 and 2 indicates that the vesicles are quite different in morphology from the truncated thylakoids observed in a small portion of the heterotrophically grown control cells.

Although vesicles are the prominent membrane configuration, chloroplasts of rifampicin-grown cells typically contain some normal thylakoids as well. Most commonly, these exist as single, unstacked thylakoids (Fig. 2); they are also found in wide stacks beneath the chloroplast envelope (Fig. 2).

When control wild-type cells are grown in the

[2] Synchronized cells taken at the end of a dark cycle were also used by Surzycki in his in vitro experiments with rifampicin, a fact that was inadvertently omitted from his published results (32).

FIGURE 2 Portion of a cell from the wild-type strain of *C. reinhardi* grown heterotrophically in the dark for 4 days in the presence of 250 μg/ml rifampicin and exposed to light for 2.5 hr in the presence of rifampicin before being fixed. Chloroplast ribosomes (arrowheads) are sparse (Table I). Chloroplast membrane is seen as single thylakoids (*T*) and as masses of tubular profiles that appear as vesicles (*V*). A region of DNA (*DNA*) lies next to a pyrenoid (*P*) included in grazing section. Starch (*S*) levels are high. Ribosomes are abundant in the cytoplasm (*C*). The outer membrane of the mitochondrion in this section is absent; this is a fixation artifact which is seen only in occasional cells in the sample and which is unrelated to the effect of the antibiotic. × 68,000.


DNA
P
S
V
T
C
2

dark, pyrenoid formation is apparently abated: the chloroplasts contain much smaller pyrenoids than do the chloroplasts of light-grown wild-type cells. A similar reduction in pyrenoid size, and hence in the frequency with which pyrenoids are encountered in thin sections, is observed in cells grown in the presence of rifampicin; the antibiotic does not, however, appear to bring about any additional change in the cells's pyrenoid-forming capacity.

Starch production is stimulated by heterotrcphic growth conditions in both control and rifampicin-treated cells (see also reference 29).

Growth in rifampicin is without effect on the structure of mitochondria at the concentrations used. The fine structure of other cell organelles (except the chloroplast) is also normal. This statement applies as well to cells grown in the presence of spectinomycin and chloramphenicol.

PHOTOSYNTHETIC CAPACITY: Heterotropically grown wild-type cells typically contain about half the chlorophyll of light-grown cells, a difference that is rapidly erased when the cells are exposed to light. Under heterotrophic conditions, rifampicin-grown cells contain somewhat less chlorophyll than the control cells (Table I), but their ability to form chlorophyll has by no means been blocked by the antibiotic. The ratio of chlorophyll *a* to chlorophyll *b* in these cells also lies in the normal range (Table I).

Photosynthetic capacity, measured as the ability of cells to carry out a light-stimulated incorporation of $^{14}CO_2$ into carbohydrate, is greatly reduced by growth in the presence of rifampicin (Table I). When measured on a cell basis, the photosynthetic capacity has been reduced to 9% of the control; the reduction is less great (20%) when calculated on a chlorophyll basis since the cells are chlorophyll deficient.

Cells Grown in the Presence of Spectinomycin

Experiments with spectinomycin were carried out with the *spa-2* strain of *C. reinhardi* grown on 25 μg/ml of spectinomycin; the properties of this strain are described in an earlier section of this paper.

CHLOROPLAST RIBOSOMES: Long-term growth in the presence of spectinomycin has no effect on the ability of cells to form chloroplast ribosomes (Fig. 3 and Table I). Cytoplasmic ribosome formation is also unaffected by spectinomycin (Table I).

CHLOROPLAST FINE STRUCTURE: Growth in spectinomycin produces occasional cells whose chloroplasts contain vesiculate masses that resemble those found in rifampicin-grown cells. Commonly, however, thylakoid formation proceeds normally. The thylakoids that are formed may fuse into normal stacks or into high stacks beneath the chloroplast envelope (Fig. 3), but most characteristically they are found in the unstacked configuration (Fig. 3). The single thylakoids usually lie in the chloroplast interior, each following an independent, meandering course through the chloroplast stroma.

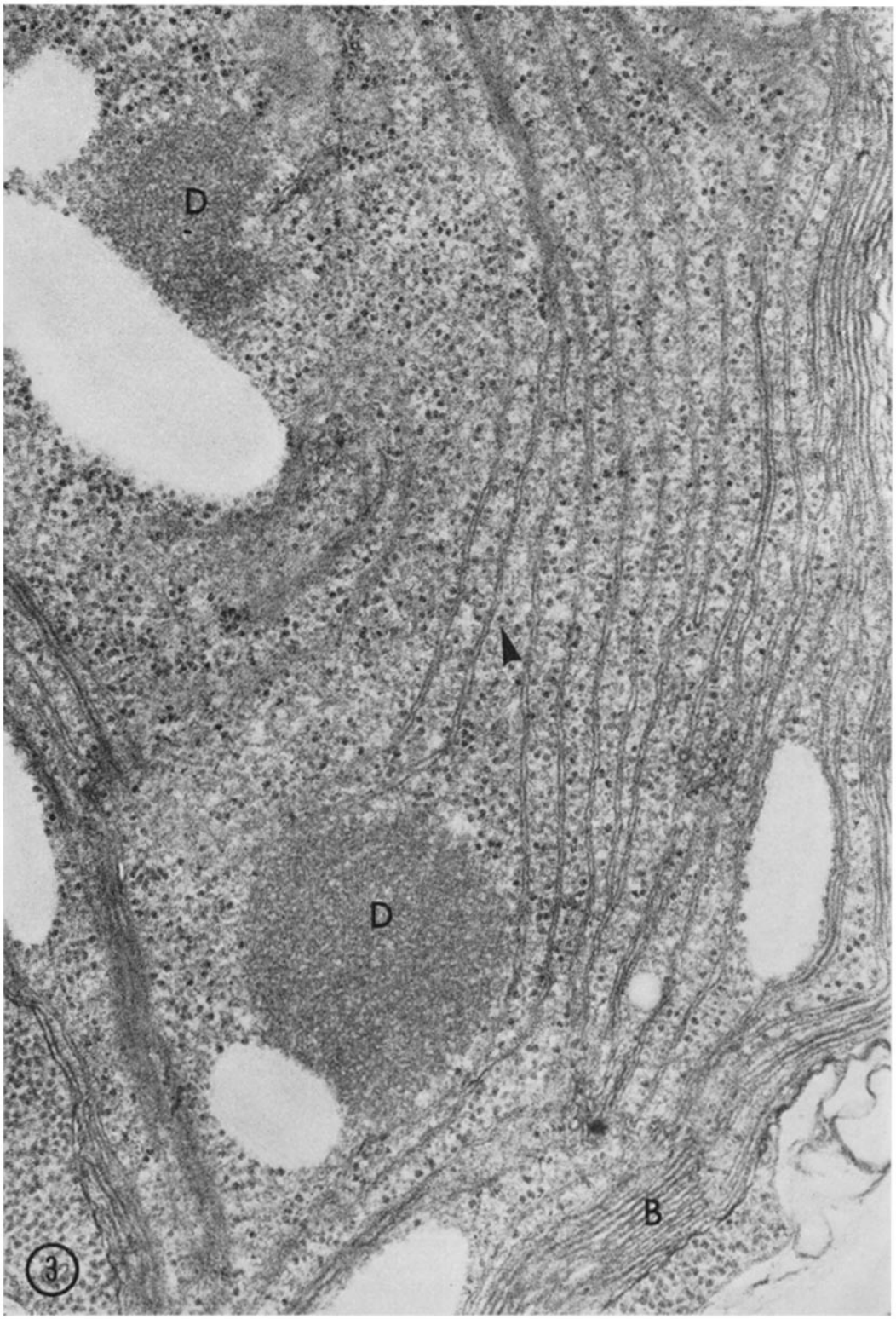
In cells exposed to toxic levels of spectinomycin (5 μg/ml for wild-type and 100 μg/ml for *spa-2*), a very different membrane phenotype is observed. Wide bands of perhaps 20–23 thylakoids aggregate beneath the chloroplast envelope, and the intrathylakoid spaces become irregularly swollen and collapsed so that the whole structure has a "ruffled" appearance.

Pyrenoid formation appears to be disrupted by growth in spectinomycin. The pyrenoids are small and hence are encountered less frequently in section compared to sections of control cells. Incursions of chloroplast stroma are frequently made into the pyrenoid matrix such that the matrix has a mottled appearance and lacks its usual polygonal symmetry (7, 29).

Starch formation suffers no apparent interruption during growth in spectinomycin.

PHOTOSYNTHETIC CAPACITY: Because of the "clumping" phenomenon described earlier, accurate values for the amount of

FIGURE 3 Portion of a cell from the *spa-2* strain of *C. reinhardi* grown mixotrophically for 5 days in the presence of 25 μg/ml spectinomycin. Chloroplast ribosomes (arrowhead) are at the normal wild-type level (Table I). Thylakoids stack in wide bands (*B*) beneath the chloroplast envelope and course singly through the stroma. This cell has recently completed mitotic division and exhibits two "dense bodies" (*D*) that characterize the mitotic chloroplast of *C. reinhardi* (7). × 79,000.


D
D
B
3

chlorophyll per cell cannot be given for *spa-2* cells grown in the presence of 25 μg/ml spectinomycin. The cultures appear fully green, however, and when wild-type cells are grown in spectinomycin, they contain normal levels of chlorophyll even when the concentrations of spectinomycin are toxic.

Long-term growth in 25 μg/ml of spectinomycin produces *spa-2* cells that fix CO_2 at 28% of the rate of *spa-2* cells grown on 10 μg/ml of spectinomycin (Table I). If the concentration of spectinomycin is raised to 100 μg/ml, cells fix CO_2 at only 4% of the control rate, but such cells are also moribund. Presumably, with sufficiently experimentation, a concentration of spectinomycin between 25 and 100 μg/ml could be found that would exert a greater inhibitory effect on the cells's photosynthetic capacity than does 25 μg/ml without simultaneously killing the cells, but for the purposes of the present experiments it was sufficient that the cells' photosynthetic capacity be inhibited fourfold.

Cells Grown in the Presence of Chloramphenicol

CHLOROPLAST RIBOSOMES: As with spectinomycin, growth on 100 μg/ml of chloramphenicol has no effect on the cells's ability to form chloroplast, or cytoplasmic, ribosomes (Fig. 4 and Table I).

CHLOROPLAST FINE STRUCTURE: In three different experiments, three different degrees of chloroplast membrane disorganization were observed in chloramphenicol-grown cells. In all cases, vesicle formation was prominent (Fig. 4). The vesicles are indistinguishable from those formed by rifampicin-grown cells (Fig. 2) and occasionally by cells in spectinomycin-grown cultures. Considerable variation was found, however, in the degree of thylakoid unstacking that accompanied this vesicle formation; the source of this variation is not understood. In one experiment, considerable unstacking was observed, none was was found in a second experiment, and an intermediate level was found in a third. Normal stacks and high stacks beneath the chloroplast envelope are also found in chloramphenicol-grown cells.

Pyrenoid formation is disrupted by growth in chloramphenicol in the same way that it is disrupted in spectinomycin-grown cells (Fig. 5). Starch formation is normal.

PHOTOSYNTHETIC CAPACITY: The ability to synthesize chlorophyll and to produce chlorophylls *a* and *b* in normal proportions is unaffected by chloramphenicol (Table I).

Growth on 100 μg/ml of chloramphenicol for three generations produces a 70% inhibition in CO_2 fixation rates (Table I); theoretically, one would predict an 87% inhibition by dilution concomitant with cell division. The discrepancy between theory and observation may be caused by a choice of too low a concentration of chloramphenicol, by the fact that chloramphenicol apparently begins to lose its inhibitory effects by 48 hr (see following section), or, probably, by a combination of the two effects. A comparable inhibition of photosynthesis was observed in each of the three chloramphenicol experiments performed even though variation in chloroplast structure was observed.

Cells maintained in the Presence of Cycloheximide

Exposure of wild-type *C. reinhardi* cells to low concentrations (1 μg/ml) of cycloheximide produces some bizarre effects. For the first 12 hr, cell division is completely blocked and no change is observed in cell size or in levels of chlorophyll per cell. By 18 hr, an increase in cell size is perceptible. By 24 hr, a few cells have divided (cell number may increase by 30%) and the remaining cells are even larger; chlorophyll per cell values are very high, being in some experiments five times higher than the control. By 48 hr, the cell number of the culture has roughly doubled. Typically, this doubling results from a portion of the cells in the culture undergoing three or four successive mitotic divisions so that clusters of 16 or 32 cells form within greatly distended mother walls; the remaining cells, those that have not yet undergone division, are enormous, perhaps five times the size of normal wild-type cells. The increase in size is accompanied by a dramatic increase in size of all the cells' organelles: with the electron microscope, one observes immense nuclei, a great proliferation of rough endoplasmic reticulum, huge chloroplasts containing giant pyrenoids, and so on. If cells are resuspended in fresh cycloheximide at the end of 24 hr, the same sequence of events is observed.

Since cytoplasmic protein synthesis is reportedly blocked by cycloheximide, such increases in total cell mass were difficult to understand. It was found, however, that the increases could be prevented if cells were grown in both cycloheximide (1 μg/ml)

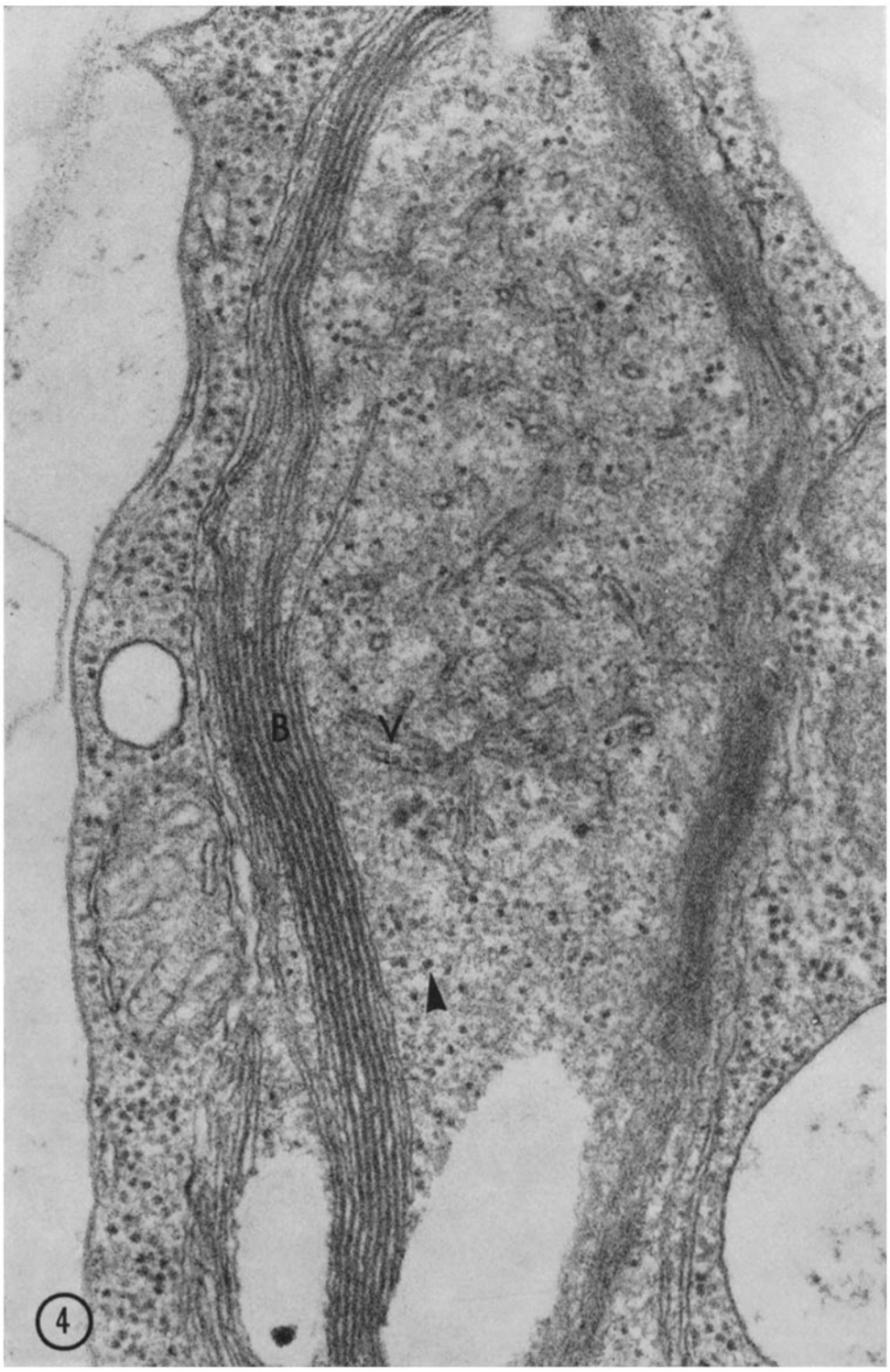


FIGURE 4 Portion of a cell from the wild-type strain of *C. reinhardi* grown mixotrophically for 48 hr in the presence of 100 μg/ml chloramphenicol. Chloroplast ribosomes (arrowhead) are at the normal wild-type level (Table I). Chloroplast membrane is found in wide bands (*B*) beneath the chloroplast envelope and in aggregates of tubular vesicles (*V*). × 97,000.

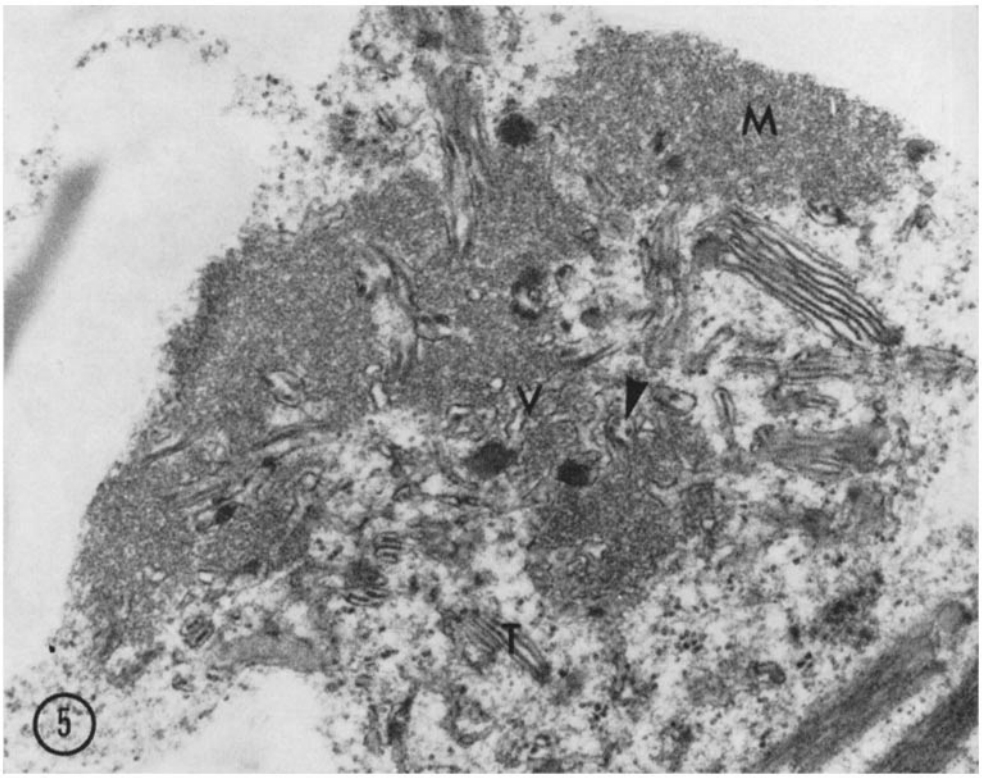


FIGURE 5 Pyrenoid from a wild-type cell of *C. reinhardi* grown mixotrophically for 48 hr in the presence of 100 μg/ml chloramphenicol. Normal pyrenoid structure has been greatly disrupted: the pyrenoidal matrix (*M*) is interrupted by incursions of chloroplast stroma, and most of the normal pyrenoidal tubules (arrowhead) are replaced by vesicles (*V*) and truncated thylakoids (*T*). × 63,000.

and chloramphenicol (100 μg/ml), Such cells undergo no cell division and synthesize no chlorophyll during the first 24 hr of growth. By 48 hr, some cell division begins to occur, and if the culture is allowed to grow for 5 days, it eventually reaches the stationary phase of growth.

Summarizing these observations, it appears that cycloheximide is able to effect a complete block over cytoplasmic protein synthesis for a 12 hr period; a slow, and apparently unregulated, protein synthesis then commences, one that is totally inhibited by chloramphenicol; and finally, a protein synthesis proceeds that is insensitive to both inhibitors. The simplest interpretation of the second "phase" of this sequence is to suggest that messenger RNA's that are normally translated on cytoplasmic ribosomes are able, under the stress of cycloheximide inhibition, to find their way into the chloroplast and be translated on chloroplast ribosomes. The simplest interpretation for the final "phase" of the process is that, after 48 hr, resistant cells arise in the culture, or the antibiotics lose their inhibitory properties, or both. Other, more complex, interpretations can also be envisaged. The important point is that cycloheximide can be used as a reliable inhibitor of cytoplasmic protein synthesis in *C. reinhardi* only during a 12 hr experiment, and since no cell division occurs during the first 12 hr in cycloheximide, a meaningful growth experiment with cycloheximide cannot be designed for *C. reinhardi*.

The data in Table I indicate that chloroplast ribosomes, cytoplasmic ribosomes, amount of chlorophyll per cell, and photosynthetic capacity are all either unaffected or show an increase in cells that have been exposed to cycloheximide for 48 hr. Chloroplast membrane organization is also normal except that most of the thylakoids are in

stacks of two. In view of the most unusual sequence of events that occurs during the 48 hr period, no information can be gleaned from these data as to the role of cytoplasmic protein synthesis in forming a chloroplast in *C. reinhardi*.

DISCUSSION

The experiments reported in this paper establish that wild-type cells of *C. reinhardi* can be grown mixotrophically or heterotrophically for periods of 48–96 hr in the presence of appropriate concentrations of antibiotics that inhibit protein synthesis in the chloroplast. Under these conditions the cells remain viable and their growth rates are comparable to those of control cells (Table I). Their ability to fix CO_2 by photosynthesis, on the other hand, is progressively lost as component(s) of their existing photosynthetic apparatus are diluted by successive cell divisions. Thus it is concluded that protein synthesis in the chloroplast of *C. reinhardi* is required to construct a functional photosynthetic apparatus. The loss of photosynthetic ability is never complete in these experiments, since it was deemed more important to work with healthy cells whose chloroplast protein synthesis is not completely inhibited than to work with fully inhibited cells that might also be dying.

With the exception of chlorophyll, no attempt was made in these experiments to determine which specific components of the photosynthetic apparatus are affected by the antibiotics. Such determinations can only be made in a meaningful way in short-term experiments such as those reported in the accompanying paper (11), in the paper of Armstrong et al. (1), and in the paper of Hoober et al. (16). In long-term experiments, the apparent loss of a given component may be the secondary result of the loss of a first component rather than the direct effect of inhibiting protein synthesis (see, in this regard, the papers of Smillie et al. [31] and Linnane and Stewart [25]). This point is well illustrated by the case of chlorophyll in rifampicin-grown wild-type cells. Such cells consistently contain only about half the chlorophyll of control cells (Table I). However, this deficiency is apparently a secondary result of the highly disordered chloroplast membrane conformations produced by growth in rifampicin, since in short-term experiments rifampicin has no inhibitory effect over chlorophyll synthesis (1).

An inhibition of protein synthesis on chloroplast ribosomes by spectinomycin or chloramphenicol that is sufficient to effect a 70–80% inhibition of photosynthesis has no effect on levels of chloroplast ribosomes (Table I). In the case of the spectinomycin experiments, this was true even after 10 generations of growth in the presence of the antibiotic. It is not known whether the ribosomes that are formed in the presence of these inhibitors possess all the ribosomal proteins in normal amounts, but if any proteins are missing, their absence does not prevent the construction of an intact ribosomal particle. Thus it is concluded that at least most of the proteins that make up chloroplast ribosomes in *C. reinhardi* are synthesized outside the chloroplast, presumably on cytoplasmic ribosomes. Similar conclusions have been reached for the proteins of mitochondrial ribosomes in fungi (4, 20, 27, 28).

The fact that chloroplast ribosomes continue to be made even though chloroplast protein synthesis is inhibited for as long as 10 generations would suggest that the chloroplast DNA-dependent RNA polymerase is also synthesized outside the chloroplast. Direct assays of the enzyme are necessary to confirm this inference.

The experiments with rifampicin reported in this paper demonstrate that a block in chloroplast DNA transcription leads to a dramatic cessation in the production of chloroplast ribosomes (Table I). Surzycki (32) has shown that long-term growth in rifampicin produces a dramatic loss in the cells' 16S and 23S RNA species, and Hoober and Blobel (15) have demonstrated that the 68S ribosomes of *C. reinhardi* contain 16S and 23S RNA's. Taken together, these results indicate that at least the bulk of the 68S ribosomes in *C. reinhardi* can be equated with ribosomes that are located within the chloroplast. They also confirm Surzycki's conclusion (32) that information for the synthesis of chloroplast ribosomal RNA resides exclusively in chloroplast DNA in *C. reinhardi*.

Growth in rifampicin, chloramphenicol, or spectinomycin brings about a similar pattern of membrane disorganization: small vesicles form, thylakoids do not stack properly, and wide membranous bands form beneath the chloroplast envelop. Such membrane conformations are not produced when wild-type *C. reinhardi* cells are grown in the presence of cycloheximide; they do not form when the photosynthetic capacity of cells is curtailed by gene mutation (8, 9, 12) or by growth in the presence of an herbicide such as 3-(3,4-dichlorophenyl)1,1-dimethylurea (19); nor

do they form if cells are exposed to toxic concentrations of the inhibitor spectinomycin. It is therefore concluded that the inhibition of chloroplast protein synthesis brings about a disruption in the ability of *C. reinhardi* to form a normally constructed chloroplast; moreover, this disruption produces a specific set of morphological lesions. Similar conclusions have also been reached by Hoober et al. (16) in their study of the effects of chloramphinicol on the re-greening process in the *y-1* mutant strain of *C. reinhardi*.

Summarizing, the results of long-term growth experiments indicate that the chloroplast protein-synthesizing system in *C. reinhardi* is required for the proper organization of chloroplast membranes, proper pyrenoid formation, and the formation of a functional photosynthetic apparatus. In an earlier study of *ac-20*, a mutant strain of *C. reinhardi* that possesses drastically reduced levels of chloroplast ribosomes, the cells were shown to possess rudimentary pyrenoids, vesiculate or unstacked chloroplast membranes, and a defective photosynthetic capacity (10, 23, 35). The present paper shows that an *ac-20* "syndrome" can be simulated in wild-type cells by inhibiting their chloroplast protein synthesis, thus supporting our earlier conclusion (10) that the *ac-20* syndrome is produced as a consequence of the strain's low levels of chloroplast ribosomes.

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