STUDIES ON THE STRUCTURE AND CELLULAR LOCATION OF VARIOUS RIBOSOME AND RIBOSOMAL RNA SPECIES IN THE GREEN ALGA CHLAMYDOMONAS REINHARDI

D. P. BOURQUE,* J. E. BOYNTON AND N. W. GILLHAM
Departments of Botany and Zoology, Duke University, Durham, North Carolina 27706, U.S.A.

SUMMARY
Under ionic conditions effecting little or no subunit dissociation, Chlamydomonas reinhardi contains 2 major classes of ribosomes with generic sedimentation velocities of 83 and 70s and 3 minor classes with sedimentation velocities of 66, 54, and 41s. Ribosomal RNAs with sedimentation velocities of 25, 23, 18, 16 and 5s have been identified. The 70-s ribosomes are in the chloroplast and contain 23-, 16- and 5-s ribosomal RNA whereas the 83-s ribosomes are in the cytoplasm and contain 25-, 18- and 5-s ribosomal RNA. Numbers of chloroplast ribosome particles counted in electron micrographs of wild type C. reinhardi and the ac-20 and y mutants have been compared with relative amounts of 70-s ribosomes determined by sucrose gradient sedimentation and amounts of 23-, 16- and 5-s ribosomal RNA determined by gel electrophoresis. In response to reduced concentrations of magnesium the 70-s ribosomes of wild type are susceptible to a progressive reduction in sedimentation velocity whereas the 66-s ribosomes of the mutant ac-20 are not. Chlorophyll synthesis and the formation of the chloroplast lamellar system do not appear to be correlated with the relative amounts of chloroplast ribosomes.

INTRODUCTION
Chloroplasts of green plants are thought to possess ribosomes and ribosomal RNA (rRNA) distinct from their counterparts in the cytoplasm. Although chloroplast ribosomes are reported to have a range of different sedimentation velocities, the most frequently encountered value is 70s, in contrast to cytoplasmic ribosomes which exhibit less heterogeneity and usually have values of 80s (Boardman, Francki & Wildman, 1966; Clark, Matthews & Ralph, 1964; Odintsova, Brusco & Golubeva, 1967; Stutz & Noll, 1967; Svetalo, Philippovich & Sissakian, 1967). Chloroplast ribosomes appear to contain 23- and 16-s RNA whereas cytoplasmic ribosomes contain 25- and 18-s RNA (Stutz & Noll, 1967; Loening & Ingle, 1967), despite conflicting reports (Eisenstadt & Brawerman, 1964; Spencer & Whitfield, 1966) which probably arise from the susceptibility of the chloroplast rRNA to degradation (Bourque, 1970; Ingle, 1968; Svetalo et al. 1967). In general, the chloroplast ribosomes have certain physical

* Present address: Department of Botany, University of California, Los Angeles, California 90024, U.S.A.
similarities to bacterial ribosomes whereas the cytoplasmic ribosomes of green plants resemble the cytoplasmic ribosomes of other eukaryotic species (Loening, 1968).

Hoober & Blobel (1969) have demonstrated the existence of both 68- and 80-s ribosomes in a yellow mutant \( y \) of the green alga *Chlamydomonas reinhardi* which, unlike wild type, cannot form chlorophyll and chloroplast lamellae when grown in the dark (Ohad, Siekevitz & Palade, 1967). Hoober & Blobel (1969) have also shown that the 68-s ribosomes of the \( y \) mutant contain 23- and 16-s rRNA and that the 80-s ribosomes contain 25- and 18-s rRNA. Based on the electron microscopic observations of Ohad et al. (1967) that the chloroplast ribosomes of *Chlamydomonas* seen in thin sections appear smaller than the cytoplasmic ribosomes, Hoober & Blobel (1969) have suggested that the 68-s ribosomes are in the chloroplast. From studies of the *ac-20* mutant of *Chlamydomonas*, which has few chloroplast ribosomes (Johnson, 1968), Goodenough & Levine (1970) have recently presented evidence that the 70-s ribosomes and the 23- and 16-s rRNA are, indeed, of chloroplast origin. The present paper quantitatively documents this localization by studies with wild type cells of *C. reinhardi* and the \( y \) mutants and demonstrates that several minor classes of ribosomes can be observed in addition to the 70- and 83-s ribosomes. In the \( ac-20 \) mutant, the 70-s ribosomes are entirely replaced by a minor class sedimenting at 66-s which contains 23- and 16-s rRNA. Finally, we confirm the observations of Hoober & Blobel (1969) that chloroplast ribosomes of *Chlamydomonas* can undergo progressive decreases in sedimentation velocity in response to sequential reductions in magnesium ion concentration, prior to their actual dissociation. Similar changes are not observed in the case of 83-s ribosomes.

**MATERIALS AND METHODS**

**Organisms and culture conditions**

The wild type strain of *Chlamydomonas reinhardi*, \( 137C \), and 2 mutants derived from it were used in these experiments. The mutant, \( ac-20 \), which grows rapidly when supplied with sodium acetate as a carbon source, but slowly when given \( CO_2 \) as a carbon source, was the kind gift of Dr. R. P. Levine in 1968. A mating type minus (\( mt^- \)) clone of this mutant was used for all experiments. The spontaneous yellow mutant, \( y \), isolated by us appears to be similar to the \( y \) mutant studied by Hoober & Blobel (1969) and Ohad et al. (1967).

The alga was grown at 25 °C in 300-ml shake cultures to late logarithmic phase (3-5 × 10^6 cells per ml) on the high salt medium of Sueoka (1960) with (HSA) or without (HS) the addition of 2 g/l of sodium acetate as follows: mixotrophic growth, in HSA medium under cool white fluorescent light (about 3500 lx); heterotrophic growth, in HSA medium in the dark in flasks covered with black electrical tape; phototrophic growth, in HS medium under cool white fluorescent light (about 3500 lx) and gassed with 5% carbon dioxide in air.

**Ribosomes**

Ribosomes were prepared by a modification of the method of Hoober & Blobel (1969). Cells were harvested from shake cultures by centrifugation at 4080 g for 5 min, washed and resuspended in solution A of Hoober & Blobel (1969) to a concentration of 5 × 10^6 cells per ml. All subsequent steps were carried out at 4 °C. The cells were passed through a French pressure cell at 2-413 × 10^6 N m^-2 (3800 lb in^-2) gauge pressure) to disrupt both whole cells and chloroplasts and the broken cell suspension was sedimented at 12 000 g for 10 min. The supernatant was removed and Triton X-100 was added to a final concentration of 2.5%. One to 2 ml aliquots of this
Ribosomes of Chlamydomonas reinhardi

supernatant were then layered on to 38-ml linear 10–30 % sucrose gradients containing solution A. The gradients were centrifuged for 14 h at 23 500 rev/min in the Spinco SW 27 rotor at 6 °C. Gradients were analysed by monitoring absorbance at 254 nm with an ISCO Model D fractionator equipped with an ultraviolet analyser having a flow cell of 1-cm light path. Under our conditions the cytoplasmic ribosomes, which have a sedimentation velocity of 83 s (Keller, 1970; Sager & Hamilton, 1967), move about 80 % of the length of the gradients (see p. 171). We have assumed the fastest sedimenting ribosome peak to be 83 s in all genotypes since this peak moves the same distance in gradients of wild type and the y and ac-20 mutants in any given run. The 83-s peak of each gradient serves as an internal standard for determining the s-values of the other classes of ribosomes by linear extrapolation. Areas under peaks on the recorder charts were estimated for both ribosomes and rRNA by making a Xerox copy of the tracing, extrapolating the tails of each peak to the baseline, cutting the peaks out, and weighing them.

Isotopic labelling of ribosomes from ac-20

Cells of ac-20 growing mixotrophically in 300-ml shake cultures (4.5 × 10^6 cells per ml) were pelleted and resuspended in 300 ml of HS medium containing 30 μCi of [3H]adenine (Amersham-Searle, specific activity 4.86 mCi per micromol). At various times after addition of the isotope, cells were harvested and ribosomes prepared for sucrose gradient analysis as described previously. Absorbance of the gradients was monitored as before and 1-ml fractions collected for radioactivity determinations. An 0.1-ml aliquot from each fraction was transferred to a glass-fibre filter (Whatman, GF/C, 2.4 cm) and dried thoroughly. The filters were then soaked successively in 2 changes of cold 10 % trichloroacetic acid (TCA) for 30 min; once in cold 5 % TCA for 15 min; and finally in cold acetone for 15 min. After drying, the filters were transferred to vials containing scintillation fluid (1 l. toluene; 5 g 2,5-diphenyloxazole; 0.3 g 1,4-bis 2-(5-phenyloxazolyl)-benzene) and counted in a Packard 3320 Tri-Carb spectrometer having a counting efficiency of 47 % for tritium.

Ribosomal RNA

About 3×10^6 cells were pelleted by centrifugation and frozen in a dry ice-acetone bath. Highly intact rRNA was obtained by 3 successive extractions with phenol in solution A of Hoober and Blobel containing 1 % sodium dodecyl sulphate and 50 mg/ml bentonite (Bourque, 1970). Separation of the different species of high molecular weight rRNA on disk gels was achieved by a modification of Peacock & Dingman’s (1967) method (Bourque, 1970). The gels (2-5 % acrylamide plus 0-5 % agarose) were run at 5 mA/gel for 2 h, and then scanned for absorbance at 260 nm in a Gilford Spectrophotometer equipped with a model 2410 Linear Transport. The 4- and 5-8 RNAs were resolved on 10 % acrylamide gels. RNA from E. coli and Jack Bean (Canavalia ensiformis) was used to establish s-values for Chlamydomonas rRNA (Bourque, 1970). Molecular weight estimates of 25, 23, 18 and 16 s (13, 105, 0.67 and 0.56 × 10^6 Daltons, respectively) rRNA were in close agreement with those determined by Loening (1968) for Chlamydomonas.

Chlorophyll determinations

Chlorophyll was assayed by in vivo spectrophotometry of whole cells using a Zeiss DMR 21 recording spectrophotometer equipped with an integrating sphere attachment to correct for light scattering. Suspensions were scanned from 750 to 375 nm and total chlorophyll was estimated from the in vivo peak values for chlorophyll a (678 nm) and chlorophyll b (650 nm). These extinction values were corrected with standard curves to the comparable absorbances for 80 % acetone extracts at 663 and 645 nm respectively and total chlorophyll estimated by the formula of Arnon (1949). Both chlorophyll determinations and fixation for electron microscopy of heterotrophically grown cells were carried out under a dim green safelight.

Electron microscopy

Cells were pelleted by centrifuging 10 ml of culture for 5 min at 4080 g and resuspended in a mixture of equal volumes of 4 % glutaraldehyde (Fisher biological grade 50 %, redistilled and...
filtered over activated charcoal to bring pH to about 7 in 0.004 M Sorensen’s Na₂HPO₄-KH₂PO₄ phosphate buffer, pH 7.0, and HSA medium. After fixation for 2 h at 20 °C, the cells were washed by centrifuging and resuspending them 4 times in an equal volume mixture of 0.004 M phosphate buffer, pH 7.0, and the HSA medium. The cells were postfixed in 2 % OsO₄ buffered in 0.1 M phosphate, pH 7.0, for 1.5 h at 20 °C, centrifuged and resuspended in 0.1 M phosphate buffer, pH 7.0, for a single washing prior to dehydration in ethanol and embedding in a new low viscosity epoxy resin (Spurr, 1969). During dehydration and infiltration the cells were centrifuged and resuspended at each step. Silver-grey sections, cut with duPont diamond knives on a Cambridge ultramicrotome, were mounted on naked 75 x 300 mesh grids and contrasted with saturated uranyl acetate in 50 % ethanol and Reynold’s lead citrate.

Micrographs were taken on a Siemens Elmiskop 101 of approximately median sections of 10 randomly selected cells of each sample showing a centrally located nucleus and peripheral chloroplast. Cell and organelle areas were measured from low-magnification micrographs of whole cells (18000 x ) whereas chloroplast lamellae and chloroplast and cytoplasmic ribosomes were estimated on higher-magnification micrographs of the same cells (60000 x ). Lucite grids of standard area (0.5- or 1.0-cm² rulings) were placed over the micrographs and the corners of the grid squares falling within the particular area of interest counted (Weibel & Gomez, 1962; Weibel, Kistler & Scherle, 1966).

The number of cytoplasmic ribosomes per cell section was estimated by counting the number of electron-dense particles of ribosome size falling within 75 0.5-cm² squares (0.525 μm²) of cytoplasm not obstructed by other cellular components in each of 10 cells of the sample. This value was then expressed as the number of ribosomes per μm² of cytoplasm. To estimate the total number of cytoplasmic ribosomes per cell section, the area occupied by the chloroplast, nucleus, mitochondria and vacuoles was subtracted from the total cell area and the resulting net cytoplasmic area in each cell multiplied by the number of ribosomes per μm² of cytoplasm. Minor cytoplasmic areas occupied by the Golgi, lysosomes, endoplasmic reticulum, etc. were ignored in our calculations.

Similarly, the number of chloroplast ribosomes was determined by counting the number of electron-dense particles of ribosome size within 75 squares (0.525 μm²) of chloroplast stroma unobstructed by starch grains, pyrenoid or lamellae in each of 10 cells. This value was then expressed directly as the number of ribosomes per μm² of chloroplast stroma. To estimate the total number of ribosomes per chloroplast section, the area occupied by the lamellae, the pyrenoid and starch grains was subtracted from the total chloroplast area and the resulting net stroma area in each cell multiplied by the number of ribosomes per μm² of stroma. Chloroplast area occupied by DNA was ignored in our calculations.

Finally, we extrapolated our data per μm² section to μm³ volume, to approximate the total number of cytoplasmic and chloroplast ribosomes in a hypothetical cell. We assumed that the Chlamydomonas cell was approximately spherical in shape and that the cytoplasm in the cup formed by the chloroplast was also nearly spherical. Consequently, the 2-dimensional electron micrograph of the cell was treated as a circle containing a smaller circle of cytoplasm. The mean area in μm² occupied by whole cells of a given genotype (A₁) was used to calculate the radius (r₁) of this circle since r₁ = (A₁/π). The value r₁, was then used to calculate the volume in μm³ of a whole hypothetical cell. The mean area in μm² occupied by cytoplasm (A₂) was used to calculate a radius (r₂) of the circle of cytoplasm. From this radius the gross cytoplasmic volume was estimated. This value was subtracted from the volume of the whole cell to give the volume of the chloroplast.

The gross cytoplasmic volume could not be used directly to estimate the total number of cytoplasmic ribosomes because this volume included mitochondria, vacuoles, and the nucleus. Consequently, net cytoplasmic area (A₃) was calculated by subtracting the mean area occupied by these structures from the total cytoplasmic area. A third radius (r₃) was then calculated using A₃, which was used to establish the net cytoplasmic volume of the hypothetical cell. The volume of chloroplast stroma was obtained by determining the mean percentage area of the chloroplast-section occupied by stroma and using this percentage to estimate the volume of the chloroplast accounted for by stroma. The number of ribosomes per μm³ of cytoplasm and stroma was then calculated. The total number of cytoplasmic ribosomes in a given hypothetical cell was obtained by multiplying the μm³ net cytoplasm in that cell by the number of cytoplasmic ribosomes per μm³. Multiplication of the μm³ of stroma by ribosomes per μm³ stroma gave the total number of ribosomes per chloroplast for the hypothetical cell.
Table 1. Relative amounts of generic classes of ribosomes and rRNA from cells of wild type C. reinhardtii, and the y and ac-20 mutants

Sedimentation velocities of the different species of ribosomes were determined by sucrose density gradient centrifugation. In a separate experiment rRNA was fractionated on agarose-acrylamide gels and 4- and 5-s RNA on acrylamide gels.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Growth conditions</th>
<th>Generic classes of ribosomes, %</th>
<th>rRNA, %</th>
<th>Ratio of 4-s to 5-s RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>83 s</td>
<td>70 s</td>
<td>66 s</td>
</tr>
<tr>
<td>Wild type</td>
<td>Mixotrophic</td>
<td>62.4</td>
<td>37.6</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Heterotrophic</td>
<td>59.8</td>
<td>40.2</td>
<td>0.0</td>
</tr>
<tr>
<td>y</td>
<td>Mixotrophic</td>
<td>69.3</td>
<td>21.2</td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td>Heterotrophic</td>
<td>68.1</td>
<td>20.8</td>
<td>11.1</td>
</tr>
<tr>
<td>ac-20</td>
<td>Mixotrophic</td>
<td>87.5</td>
<td>0.0</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>Phototrophic</td>
<td>84.0</td>
<td>0.0</td>
<td>7.0</td>
</tr>
</tbody>
</table>

Table 2. Numbers of chloroplast and cytoplasmic ribosomes estimated from electron micrographs of cells from the same experiment in which ribosomes were analysed by density gradient centrifugation (see Table 1)

(Means with the standard errors are given for data from 10 cells in each case.)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Growth conditions</th>
<th>Ribosomes counted per μm² unobstructed section area</th>
<th>Total ribosomes estimated per cell section x 10³</th>
<th>Total ribosomes calculated per hypothetical cell x 10⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cytoplasm</td>
<td>Chloroplast</td>
<td>Chloroplast, %</td>
</tr>
<tr>
<td>Wild type</td>
<td>Mixotrophic</td>
<td>161 ± 7</td>
<td>115 ± 4</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>Heterotrophic</td>
<td>228 ± 15</td>
<td>134 ± 13</td>
<td>37</td>
</tr>
<tr>
<td>y</td>
<td>Mixotrophic</td>
<td>281 ± 9</td>
<td>222 ± 21</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>Heterotrophic</td>
<td>315 ± 11</td>
<td>241 ± 28</td>
<td>44</td>
</tr>
<tr>
<td>ac-20</td>
<td>Mixotrophic</td>
<td>154 ± 8</td>
<td>46 ± 4</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Phototrophic</td>
<td>309 ± 21</td>
<td>67 ± 4</td>
<td>18</td>
</tr>
</tbody>
</table>
Ribosomes of Chlamydomonas reinhardi

All parameters were measured on populations of cells of the various genotypes grown in a single experiment with the exception of the rRNA determination and the isotopic labelling of ac-20 which were done in independent experiments under the same environmental conditions.

RESULTS

To establish a common identity between the ribosomes seen in micrographs of the chloroplast, the 70-s ribosomes isolated in sucrose gradients and the 23- and 16-s rRNA determined by agarose-acrylamide gel electrophoresis, we compared these parameters in cells of wild type, ac-20 and the y mutant. We expected the comparisons to be definitive since Johnson (1968) reported the virtual absence of chloroplast ribosomes in mixotrophically grown ac-20 and Hoober & Blobel (1969) noted that their y mutant had a higher level of 68-s ribosomes when grown mixotrophically than when grown heterotrophically.

Wild type cells

In mixotrophically grown wild type cells 38% of the total ribosomes are 70-s as estimated from sucrose gradients (Fig. 1B, Table 1). By actual counts per unit area in the electron micrographs, 42% of the total ribosomes appear to be localized in the chloroplast (Table 2). However, the proportion of chloroplast ribosomes is reduced to 24% when estimated as the total number of ribosomes per cell section or to 35% when extrapolated to a volume basis (Table 2). The amount of 23- and 16-s rRNA is 28% of the total rRNA present in the cells (Fig. 1A, Table 1).

The relative amount of ribosomes in the 70-s region of our sucrose gradients of wild type is very reproducible (36.5 ± 1.6% in 10 experiments). However, we observe a 2.5-fold variation in the total amounts of ribosomes from these same gradients. This variation does not occur during centrifugation since the amounts of ribosomes are highly reproducible between gradients from a single extraction of wild type cells. We do not know whether this variation between experiments is due to incomplete extraction of ribosomes or to real differences in amounts of ribosomes in different populations of wild type cells. Accordingly, our analysis is restricted to the relative proportions of the various ribosome classes.

Since the precise sedimentation velocity of ribosomes in the 70-s region varies between 68 and 72s in wild type '70-s' must be considered as a generic term. Changes in sedimentation velocity of the 68-s ribosomes from the y mutant of Chlamydomonas were first noted by Hoober & Blobel (1969) in response to variations in the Mg²⁺

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Fig. 1. Ribosome (bottom) and rRNA (top) profiles from wild type cells of Chlamydomonas reinhardi and the ac-20 and y mutants. rRNA species were separated on agarose-acrylamide gels and their positions identified by scanning the gels for absorbance at 260 nm. Ribosomes were separated by sucrose gradient centrifugation and the different species were identified by monitoring the absorbance of the gradients at 254 nm. The absorbance scale (A) is designated for each tracing. A, B, wild type cells grown mixotrophically; C, D, y mutant grown heterotrophically; E, F, ac-20 grown mixotrophically; and G, H, ac-20 grown phototrophically.
### Table 3. Sedimentation velocities and percentage amounts of ribosomes of various classes from C. reinhardi isolated in different molarities of Mg²⁺

(Ribosomes extracted from mixotrophically grown wild type cells are compared to those obtained from the ac-20 mutant grown under the same conditions.)

<table>
<thead>
<tr>
<th>Generic class of ribosomes with range of s values</th>
<th>Mg²⁺ molarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type ac-20</td>
<td>Wild type ac-20</td>
</tr>
<tr>
<td>83 (68-73)</td>
<td>59.0</td>
</tr>
<tr>
<td>66 (56-61)</td>
<td>70.5</td>
</tr>
<tr>
<td>54 (50-55)</td>
<td>0.0</td>
</tr>
<tr>
<td>41 (38-43)</td>
<td>0.5</td>
</tr>
</tbody>
</table>

### Table 4. Cytological organization and chlorophyll content of wild type C. reinhardi and the y and ac-20 mutants used for isolation of ribosomes and ribosome counts

(Means with the standard errors are given for 10 cells in each case.)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Growth conditions</th>
<th>Area, μm²/median section</th>
<th>% of cell area occupied by Chloroplast</th>
<th>% of chloroplast area occupied by lamellar system</th>
<th>Total chlorophyll, µg/10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Mixotrophic</td>
<td>43.4 ± 1.8</td>
<td>33.9</td>
<td>33.9</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>Heterotrophic</td>
<td>37.0 ± 6.6</td>
<td>34.3</td>
<td>34.3</td>
<td>1.2</td>
</tr>
<tr>
<td>y</td>
<td>Mixotrophic</td>
<td>48.4 ± 3.1</td>
<td>34.2</td>
<td>34.2</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>Heterotrophic</td>
<td>25.8 ± 1.8</td>
<td>20.9</td>
<td>20.9</td>
<td>0.1</td>
</tr>
<tr>
<td>ac-20</td>
<td>Mixotrophic</td>
<td>52.8 ± 2.6</td>
<td>30.0</td>
<td>30.0</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>Phototrophic</td>
<td>50.5 ± 3.5</td>
<td>33.7</td>
<td>33.7</td>
<td>2.0</td>
</tr>
</tbody>
</table>
concentration of the isolation medium and sucrose gradients. Similarly, we have found that, as the Mg\(^{2+}\) concentration is sequentially reduced, the sedimentation velocity of the '70-s' ribosomes of wild type shifts progressively from 72 to between 61 and 56s at 0.005 M Mg\(^{2+}\) (Table 3). Below 0.005 M Mg\(^{2+}\) the '70-s' ribosomes appear to dissociate into the 2 subunits, however, the sedimentation velocities of both the large and small subunits in *C. reinhardi* need to be firmly established to substantiate this.

Similar changes in the sedimentation velocity of 83-s ribosomes do not occur under these conditions although the 83-s ribosomes, like the 70-s ribosomes, begin to dissociate below about 0.005 M Mg\(^{2+}\). Parallel experiments with the ac-20 mutant grown mixotrophically confirm our findings for the 83-s ribosomes of wild type (Table 3). Since ac-20 has very low levels of 66-s ribosomes and no 70-s ribosomes, any changes in the sedimentation velocity of the 83-s ribosomes should be evident. Dissociation of 83-s ribosomes takes place in ac-20 before any changes in their sedimentation velocity are noted. The 66-s class of ribosomes from ac-20 does not appear to undergo the changes in sedimentation velocity observed with the 70-s ribosomes from wild type (Table 3).

Wild type cells grown heterotrophically in the dark show no marked change in the relative levels of 70-s ribosomes or of 23- and 16-s rRNA compared to cells grown mixotrophically in the light (Table 1). No shift is observed in the ratio of 4-s to 5-s RNA and no new low-molecular weight RNA species are observed. A modest increase in the density of ribosomes per \(\mu m^2\) of cytoplasm (Table 2) combined with a small reduction in the net area of chloroplast stroma (Table 4) results in a decline in the percentage of chloroplast ribosomes per cell section and per hypothetical cell volume (Table 2). Chlorophyll is reduced about 2-fold and the area of chloroplast occupied by lamellae is correspondingly reduced, although the total area of the cell occupied by the chloroplast is about the same as in mixotrophically grown wild type cells (Table 4, Figs. 5–8).

**\(y\) mutant**

Our \(y\) mutant grown mixotrophically differs from wild type by having both 66- and 70-s ribosomes present in a 1:2 ratio (Fig. 1b, Table 1). Both the 66- and the 70-s ribosomes probably contain 23- and 16-s rRNA since no new rRNA species are observed in gels of the \(y\) mutant (Fig. 1c, Table 1) and since the 66-s ribosomes isolated from a streptomycin-resistant mutant contain only 23- and 16-s rRNA (Gillham, Boynton & Burkholder, 1970). Although the chloroplast of our \(y\) mutant contains the same relative amounts of ribosomes as wild type, the density of ribosomes is almost double that of wild type in both the cytoplasm and the chloroplast (Table 2). Cells of \(y\) mutants grown in the light are indistinguishable from those of wild type in ultrastructural organization (compare Figs. 9 and 5) as has been reported previously by Ohad *et al.* (1967).

Heterotrophic growth of our \(y\) mutant brings about dramatic ultrastructural changes (Fig. 11), similar to those described by Ohad *et al.* (1967) which are not perceived in heterotrophically grown wild type cells (Fig. 7). Chlorophyll and the chloroplast lamellar system are reduced about 10-fold compared to mixotrophically grown *
cells while the area of the chloroplast and the total cell area are reduced 2- to 3-fold (Table 4).

The relative amounts of 66- and 70-s ribosomes do not change although the relative amounts of 23- and 16-s rRNA appeared to drop slightly in a separate experiment (Figs. 1c, d, 12; Table 1). While the density of chloroplast ribosomes per $\mu m^2$ of stroma area remains the same, the total number of ribosomes per chloroplast section and per hypothetical chloroplast volume is reduced almost 2-fold (Table 2), paralleling the 50% reduction in net chloroplast stroma (Table 4). The density of cytoplasmic ribosomes increases slightly and the net cytoplasmic area declines to a greater extent resulting in a moderate reduction in total number of cytoplasmic ribosomes per cell section and hypothetical cell volume (Table 2).

**ac-20**

Johnson (1968) reported that only very few ribosomes are seen in the chloroplast of mixotrophically grown cells of *ac-20*. We have demonstrated very low levels of 23- and 16-s rRNA in *ac-20* grown both mixotrophically and phototrophically (Bourque, Boynton & Gillham, 1969; Figs. 1e, f; Table 1). Recently, Goodenough & Levine

![Fig. 3. Profiles of 4- and 5-s RNA of mixotrophically grown cells of wild type (A) and ac-20 (B) separated on 10% acrylamide gels. Gels were scanned for absorbance (A) at 260 nm.](image-url)
Ribosomes of Chlamydomonas reinhardi (1970) have also demonstrated a great reduction in 23- and 16-s rRNA in mixotrophically grown ac-20 cells in addition to mentioning a reduction of a species of 5-s RNA. The latter may correspond to the increase in the ratio of 4-s to 5-s RNA which we observe (Fig. 2, Table 1). Goodenough & Levine (1970) have also found that ac-20 grown mixotrophically forms greatly reduced levels of 70-s ribosomes and that the chloroplast ribosomes are reduced to about 10% of the wild type level on a unit area basis.

We find that cells of both mixotrophically and phototrophically grown ac-20 have no ribosomes which sediment at 70s, but rather have small numbers sedimenting in the 66-, 54- and 41-s regions of the gradients (Fig. 1F, H; Table 1). These 3 classes together comprise about 15% of the total ribosomes of the ac-20 cell.

In electron micrographs of ac-20, chloroplast ribosomes comprise 23% of the total ribosomes per unit area in mixotrophically grown cells and 18% of the total in phototrophically grown cells (Figs. 14, 16; Table 2). These values drop to 11 and 7% respectively when calculated per cell section and to 14 and 9% respectively when extrapolated to hypothetical cell volume (Table 2).

If differences in cytoplasmic ribosomes are ignored and the chloroplast ribosomes of ac-20 grown mixotrophically expressed as a percentage of chloroplast ribosomes of wild type on a unit area basis, we obtain a value of 29% compared with the 10% value of Goodenough & Levine (1970). Although they show about a 3-fold increase in chloroplast ribosomes per unit area when cells of ac-20 are grown phototrophically over the level observed in mixotrophically grown cells, we observe only a 1.4-fold increase. This increase is accompanied by a greater than 2-fold increase in cytoplasmic ribosomes (Table 2). When the increase in chloroplast ribosomes is expressed relative to the increase in cytoplasmic ribosomes, a net decrease in chloroplast ribosomes is seen under our phototrophic growth conditions (Table 2). Extrapolation of these data to total numbers of chloroplast and cytoplasmic ribosomes per cell section and hypothetical cell volume, results in an even greater increase in the proportions of cytoplasmic ribosomes under phototropic conditions. Our failure to observe a large increase in chloroplast ribosomes per μm² in ac-20 under phototrophic conditions may result because the level of chloroplast ribosomes in our mixotrophically grown cells is already 3-fold higher than observed by Goodenough & Levine (1970).

When cells of ac-20 were transferred from mixotrophic to phototrophic conditions and the newly synthesized rRNA labelled by adding [3H]adenine to the media, all ribosome classes were labelled (Fig. 3). Up to 22 h following transfer to phototrophic conditions, cytoplasmic ribosomes were labelled at more than twice the rate of chloroplast ribosomes (Fig. 4). The transfer experiment substantiates our independent electron-microscopic observations that cytoplasmic ribosomes in phototrophically grown cells of ac-20 increase more than chloroplast ribosomes.

Since electron microscopy reveals that the mitochondria of both wild type cells and the 2 mutants contain few ribosome-like particles, and since mitochondria occupy only a small fraction of the area in our median cell sections (Table 4), we believe the loss of 70-s ribosomes in ac-20 to be strictly correlated with the reduction in chloroplast ribosomes.
Fig. 3. Optical density (---) and radioactivity (-----) profiles of ribosomes of ac-20 cells transferred from mixotrophic conditions in the absence of [3H]adenine to phototrophic conditions in the presence of 30 μCi [3H]adenine and harvested at 7 h (A) and 12 h (B) following transfer.
We find that cells of ac-20 grown either mixotrophically or phototrophically contain
the same amount of chlorophyll (Table 4) although the organization of the chloroplast
lamellar systems differs markedly (compare Figs. 13, 15). Whereas Goodenough &
Levine (1970) report that unpaired randomly oriented disks are the most prevalent
lamellar configuration in mixotrophically grown cells of ac-20, we find that the mutant
forms an extensive and highly ordered lamellar system consisting mostly of abnor-
mally large diameter stacks. These resemble the giant grana described in a number of
chloroplast mutants of higher plants (see Appelqvist, Boynton, Henningsen, Stumpf
& von Wettstein, 1968). The disks in these stacks may be either loosely aggregated or
fused together in the fashion of typical grana (Fig. 14). We have not observed the mas-
eses of vesicles described by Goodenough & Levine (1970), but do concur in their
observation that the pyrenoid is absent. In fact, the chloroplast phenotype of our
mixotrophically grown ac-20 cells most closely resembles that of Goodenough &
Levine’s mixotrophically grown ac-20 cells which had been transferred to minimal
medium and incubated in the dark for several hours.

When ac-20 is grown phototrophically a pyrenoid is formed and the chloroplast
lamellar system consists almost entirely of pairs of fused disks (Figs. 15, 16). Photo-
trophically and mixotrophically grown cells of ac-20 have chloroplasts of similar size
but the lamellar system of the former is somewhat more extensive resulting in a higher
lamellar density per chloroplast (Table 4) as has been suggested by Goodenough &

Fig. 4. Relative rates of incorporation of [3H]adenine into ribosomes of ac-20 cells fol-
lowing transfer of the mutant from mixotrophic to phototrophic growth conditions.
Each point represents the ratio of the radioactivity in a 0.1-ml aliquot of the gradient to
the absorbance of that aliquot at 254 nm in the peak tube for both the 66- and 83-s
ribosome classes. Values for the 7- and 12-h ratios are the average of duplicate deter-
minations, one set of which are shown in Fig. 3, and the 22-h values are from a single
determination.
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Differences between our experimental results with mixotrophic cells of ac-20 and those of Goodenough & Levine (1970) may arise for one or both of the following reasons. First, the mixotrophic growth conditions used by U. W. Goodenough (personal communication) for ac-20 differ from ours in that her cells are provided with 2.7 g/l of sodium acetate and this medium is replenished twice during the 48-h growth period. The phenotype of ac-20 may depend upon the amount of acetate present in the medium at the time the cells are harvested. Secondly, we have recently found that our ac-20 mt− stock is a double mutant in which both mutations affect chloroplast ribosomes (Boynton, Gillham & Burkholder, 1970). Since this double mutant stock has the same ribosome phenotype as the original ac-20 mutation which lacks 70-s ribosomes, but forms small amounts of 66- and 54-s ribosomes, ac-20 is epistatic to the new mutant. This new mutant appears to have arisen spontaneously in our ac-20 mt− stock and by itself forms the same low level of 66-s ribosomes as ac-20, but in addition accumulates large amounts of 54-s ribosomes. Because these 54-s ribosomes contain only 23-s RNA, we presume them to be large subunits of the chloroplast ribosome.

DISCUSSION

Our results together with those of Bourque et al. (1969), Hoober & Blobel (1969), Goodenough & Levine (1970) and Surzycki, Goodenough, Levine & Armstrong (1970) establish that chloroplast ribosomes in wild type cells of the green alga Chlamydomonas reinhardtii have a generic sedimentation velocity of 70s and contain 23- and 16-s rRNA. Cytoplasmic ribosomes have a sedimentation velocity of 83s (Sager & Hamilton, 1967) and contain 25- and 18-s rRNA (Hoober & Blobel, 1969; Bourque et al. 1969; Goodenough & Levine, 1970).

If each ribosomal subunit contains one high molecular weight RNA species, the theoretical absorbance ratio of 25- to 18-s rRNA is 1.94 when calculated from the molecular weight ratios of these species and 1.88 for 23- to 16-s rRNA. Observed absorbance ratios of 25- to 18-s rRNA range from 1.29 to 2.00 for different gels and genotypes whereas the ratios of 23- to 16-s rRNA range from 0.93 to 1.51. These departures from expectation may be attributed to specific degradation of 25- and 23-s rRNA into high molecular weight pieces which presumably contribute to the absorbance of 18- and 16-s rRNA since no other significant absorbances are detected in the gels (Bourque, 1970). The 23-s species appears to be the more susceptible of the 2 to degradation as noted by Loening (1968) who obtained very low yields of this component.

Several species of low molecular weight RNA have been observed in Chlamydomonas by Surzycki & Hastings (1968) with methylated albumin kieselguhr (MAK) columns and in leaves of Vicia faba by Dyer & Leech (1968) with MAK columns and polyacrylamide gels. We only find 4- and 5-s species of RNA in Chlamydomonas on 10% polyacrylamide gels and these are separated completely. The higher ratio of 4- to 5-s RNA observed in ac-20 compared to wild type probably results from the reduction in chloroplast ribosomes and the 5-s RNA associated with them. However, the alternative explanation that the 4-s RNA has been selectively increased while the level of 5-s RNA
remains constant cannot be ruled out. Surzycki & Hastings (1968) observed the disappearance of a low molecular weight RNA in ac-20 which is present in wild type. Their results suggest that MAK columns may chemically separate two 5-s RNA species which do not separate electrophoretically on acrylamide gels.

Our studies and those of Hooper & Blobel (1969) demonstrate that chloroplast ribosomes of wild type cells are more sensitive to changes in magnesium concentration than cytoplasmic ribosomes. As the magnesium concentration is lowered, the chloroplast ribosomes undergo a series of changes which result in a progressive lowering of their sedimentation velocity. These changes may be the result either of an alteration in ribosome conformation or of a loss in proteins bound to the 70-s ribosomes. Dissociation of the chloroplast ribosomes into subunits appears to occur at magnesium concentrations which are similar to those required to effect dissociation of the 83-s ribosomes. This sensitivity to Mg$^{2+}$ ion concentration together with the susceptibility of chloroplast rRNA to degradation probably explains the heterogeneity in sedimentation velocity of chloroplast ribosomes reported previously for *Chlamydomonas* (cf. Gillham, 1969; Sager & Hamilton, 1967) and for other organisms such as *Euglena* (Eisenstadt & Brawerman, 1964; Mendiola, Kovacs & Price, 1968; Smillie, Scott, Graham, Greive & Tobin, 1968).

In *C. reinhardi* 5 generic classes of ribosomes (83, 70, 66, 54 and 41 s) are observed (Table 1) under conditions where there should be no appreciable subunit dissociation (0.025 M Mg$^{2+}$). These classes suffice to describe the ribosomes seen in wild type cells, the ac-20 and y mutants, and also in certain uniparentally inherited antibiotic-resistant mutants of *C. reinhardi* (Gillham, 1969) which contain 66-s ribosomes (Gillham et al. 1970).

Precise quantitative agreement between estimates of cytoplasmic and chloroplast ribosomes in cells from the same population made by electron microscopy and by sucrose gradient centrifugation would not necessarily be expected due to assumptions inherent in the analytical methods: (1) no selective loss of any particular class of ribosomes occurs during extraction or sucrose gradient centrifugation; (2) ribosomes in the chloroplast and the cytoplasm of the cell are equally well preserved and contrasted in the electron micrographs; (3) ribosomes of the nucleolus, which are ignored in our electron micrograph counts, do not contribute significantly to one or more of the ribosome classes in our sucrose gradients; (4) polysomes of the chloroplast and cytoplasm contain the same relative numbers of ribosomes since neither are resolved on the sucrose gradients, but both are accounted for in our rRNA and electron-microscope estimates; and (5) electron-microscopic data, which are essentially 2-dimensional, can be extrapolated to a valid 3-dimensional facsimile of the cell, its chloroplast, and its cytoplasm. The ribosome extractions, after all, were done on 3-dimensional cells.

Keeping these limitations in mind, the number of chloroplast ribosomes per unit area can be compared directly between genotypes. However, we feel it is more biologically relevant to express the chloroplast ribosomes as a percentage of the total ribosomes in the cell and then make intergenotypic comparisons. This also permits direct comparison with sucrose gradient data which, of necessity, express the different ribosome classes as a percentage of the total ribosomes. Neither electron-microscopic
comparison, however, takes into account changes observed in net chloroplast stroma or cytoplasmic area (Table 4) which may effect ribosome packing in the cells (Table 2). To circumvent this, we calculated the total number of ribosomes per cytoplasm and chloroplast section and extrapolated these figures to hypothetical cell volume. Total chloroplast ribosomes per plastid could then be compared directly or the total ribosomes of the chloroplast expressed as a percentage of the total ribosomes of the cell.

While none of these comparisons exactly fit with our sucrose gradient estimates made on the same cell samples, all reflect to a greater or lesser degree changes observed in the gradients. Where organelle areas and volumes vary profoundly within a genotype such as the y mutant grown under different conditions (Table 4), the aforementioned comparisons can give widely different estimates. The number of chloroplast ribosomes per unit area of the y mutant remains the same under both heterotrophic and mixotrophic conditions, when expressed directly or as a percentage of total ribosomes of the cell. However, the number of ribosomes in the chloroplast drops by 50% when expressed on either a total area or volume basis and to a lesser degree when expressed as a percentage of total cell ribosomes. We suspect that the asymmetrical nature of the chloroplast of *Chlamydomonas* may introduce an error in estimating the relative number of chloroplast and cytoplasmic ribosomes per cell on an area basis. Calculations based on the volume of a hypothetical cell are probably a better approximation, but our model cell may be only a crude representation of the actual living cell. If the shape of the cup formed by the chloroplast varies in different genotypes under different growth conditions this can cause the cell cytoplasm to depart from spherical shape. Such deviations from a geometrical model of a sphere inside a sphere can distort estimates of ribosomes per cell cytoplasm and per chloroplast.

We must conclude that attempts to establish causal relationships between changes in the amounts of chloroplast ribosomes or ribosomal RNA and specific chloroplast functions by means of correlation studies can be treacherous. This is particularly true in the case of the y mutant. When the y mutant is grown heterotrophically, we observe a 10-fold reduction in chlorophyll and in the area of the chloroplast lamellar system. If one measures the ribosomes per $\mu m^2$ of stroma, there is no reduction in chloroplast ribosomes when mixotrophically and heterotrophically grown cells are compared. However, there are 2-fold less chloroplast ribosomes in a hypothetical cell of the y mutant grown heterotrophically, when compared to a mixotrophically grown cell. This reduction in chloroplast ribosomes per hypothetical cell is correlated with a decrease in the size of the chloroplast but the packing of ribosomes within the chloroplast stroma does not change. Obviously, heterotrophic growth affects chlorophyll biosynthesis and synthesis of lamellae far more dramatically than chloroplast ribosome biosynthesis.

Comparison of cells of the y mutant and wild type grown heterotrophically reveals that the y mutant contains more chloroplast ribosomes than wild type by all our electronmicroscopic parameters. Yet heterotrophically grown wild type cells contain 8 times more chlorophyll and have a 4-fold more extensive lamellar system than the y mutant. Hence the 2-fold drop in chloroplast ribosomes in the y mutant grown heterotrophically is probably not directly related to the marked dedifferentiation of its chloroplast.
Our comparisons between heterotrophically grown wild type cells of C. reinhardi and cells of the y mutant may be particularly relevant to studies of light-induced greening in both Chlamydomonas and higher plants (Boardman, 1967; Bogorad, 1967; Gyldenholm, 1968; Ohad et al. 1967). In these investigations an increase in chloroplast ribosomes or rRNA of about 2-fold is noted concomitant with the light-induced synthesis of chlorophyll and chloroplast lamellae. Our results together with those of both Boardman (1967) and Bourque (1970) suggest that in both Chlamydomonas and higher plants no cause and effect relationship exists between the greening process and the increase in amount of chloroplast ribosomes. This, however, does not rule out the possibility that both events are triggered by the same stimulus when the plants are exposed to light.

Goodenough & Levine (1970) suggested that the large reduction in chloroplast ribosomes in their stock of ac-20 grown mixotrophically may effect a reduction of other chloroplast components such as ribulose diphosphate carboxylase (Togasaki & Levine, 1970). We feel that the caution with which these authors have alluded to chloroplast ribosome function is commendable. At present, one has no idea how many chloroplast ribosomes are enough to ensure proper functioning of the protein synthesizing system of the chloroplast. The total number of ribosomes calculated per chloroplast in hypothetical cells of the different genotypes grown mixotrophically varies from 22,000 in ac-20, to 53,000 in wild type, to 161,000 in the y mutant. On an area basis variations from 46 ribosomes per µm² in ac-20, to 115 in wild type cells to 222 in y cells are seen.

In terms of protein synthesis it is as yet difficult to say what these differences in numbers of ribosomes mean to the cell. Perhaps messenger RNA in the chloroplast is translated faster by chloroplast ribosomes when their number is reduced. Conversely, since the chloroplast ribosomes of ac-20 have a different sedimentation velocity than those of wild type (66 compared with 70s) and respond differently to Mg²⁺, one could speculate that their function in protein synthesis might be greatly impaired. What does seem clear is that per chloroplast of mixotrophically grown ac-20 there is probably a minimum of several thousand ribosomes.

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REFERENCES


Ribosomes of Chlamydomonas reinhardi


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**Note added in Proof**

Since submission of this paper, we have learned that the radiometer of our Beckman-Spinco L 265 B ultracentrifuge was defective at the time of these experiments, resulting in a lower temperature than indicated on the temperature gauge. We estimate that the actual rotor temperature was −4°C. Recently we have obtained excellent separation of ribosomes at a speed of 22,500 rev/min for 14 h at 2°C. Under these conditions, the 83-s ribosomes move about 90% of the length of the gradient.
ABBREVIATIONS ON PLATES

*cr* and *pr*, ribosomes in cytoplasm and chloroplast, respectively; *pe*, double-membrane plastid envelope.

Fig. 5. Section through a typical wild type cell of *C. reinhardtii* grown mixotrophically. The cup-shaped chloroplast with a well-developed lamellar system surrounds the periphery of the cell. × 16500.

Fig. 6. A portion of the wild type cell of Fig. 5 at high magnification showing ribosomes in both the cytoplasm and the chloroplast. × 165000.
Ribosomes of Chlamydomonas reinhardtii
Fig. 7. Section through a typical wild type cell grown heterotrophically. The well-developed lamellar system of the chloroplast persists despite the several generations of growth in the dark. Many starch grains are evident. $\times 16,500$.

Fig. 8. A portion of the wild type cell of Fig. 7 at high magnification showing ribosomes in both the cytoplasm and the chloroplast. $\times 165,000$. 
Fig. 9. Section through a typical cell of the y mutant grown under mixotrophic conditions showing a chloroplast with a well-developed lamellar system. × 16500.

Fig. 10. A portion of the y cell of Fig. 9 at high magnification showing ribosomes in both the cytoplasm and the chloroplast. × 165000.
Ribosomes of Chlamydomonas reinhardtii
Fig. 11. Section through a cell of the \( y \) mutant grown heterotrophically. The chloroplast has been reduced greatly in area and the lamellar system consists of only a few scattered paired disks. \( \times 16,500 \).

Fig. 12. A portion of the \( y \) cell of Fig. 11 at high magnification showing ribosomes in both the cytoplasm and the chloroplast. \( \times 165,000 \).
Fig. 13. Section through a typical cell of the ac-20 mutant grown under mixotrophic conditions showing a chloroplast with an extensive lamellar system. × 16500.

Fig. 14. A portion of the ac-20 cell of Fig. 13 at high magnification showing ribosomes in the cytoplasm. The number of ribosomes in the chloroplast appears to be greatly reduced. × 165000.
Fig. 15. Section through a typical cell of the ac-20 mutant grown under phototrophic conditions. The lamellar system of the cup-shaped chloroplast is organized largely into 2-disk grana in contrast to the grana stacks seen in the other genotypes. $\times 16500$.

Fig. 16. A portion of the ac-20 cell of Fig. 15 at high magnification showing ribosomes in the cytoplasm and their relative absence in the chloroplast. $\times 165000$. 

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