

RIBOSOMES BOUND TO CHLOROPLAST MEMBRANES IN *CHLAMYDOMONAS REINHARDTII*

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ABSTRACT

The amount of chloroplast ribosomal RNAs of *Chlamydomonas reinhardtii* which sediment at 15,000 *g* is increased when cells are treated with chloramphenicol. Preparations of chloroplast membranes from chloramphenicol-treated cells contain more chloroplast ribosomal RNAs than preparations from untreated cells. The membranes from treated cells also contain more ribosome-like particles, some of which appear in polysome-like arrangements. About 50% of chloroplast ribosomes are released from membranes *in vitro* as subunits by 1 mM puromycin in 500 mM KCl. A portion of chloroplast ribosomal subunits is released by 500 mM KCl alone, a portion by 1 mM puromycin alone, and a portion by 1 mM puromycin in 500 mM KCl. Ribosomes are not released from isolated membranes by treatment with ribonuclease. Membranes in chloroplasts of chloramphenicol-treated cells show many ribosomes associated with membranes, some of which are present in polysome-like arrangements. This type of organization is less frequent in chloroplasts of untreated cells. Streptogramin, an inhibitor of initiation, prevents chloramphenicol from acting to permit isolation of membrane-bound ribosomes.

Membrane-bound chloroplast ribosomes are probably a normal component of actively growing cells. The ability to isolate membrane-bound ribosomes from chloramphenicol-treated cells is probably due to chloramphenicol-prevented completion of nascent chains during harvesting of cells. Since chloroplasts synthesize some of their membrane proteins, and a portion of chloroplast ribosomes is bound to chloroplast membranes through nascent protein chains, it is suggested that the membrane-bound ribosomes are synthesizing membrane protein.

INTRODUCTION

Chlamydomonas reinhardtii is one of the organisms that has been used extensively to study the biosynthesis of chloroplast membranes (1). Although much has been learned, the precise mechanism by which proteins are inserted into chloroplast membranes has not been determined.

Chloroplast ribosomes are found mainly in monomeric form in homogenates of *C. reinhardtii* (2-4). The number of free monomers in homoge-

nates is greatly decreased if cells are first treated with chloramphenicol for 10 min or more (3). Chloramphenicol inhibits protein synthesis by chloroplast ribosomes but does not inhibit protein synthesis by cytoplasm ribosomes (5). Chloramphenicol can stabilize polysomes (6-8) and induce formation of polysomes (9). Therefore, it was postulated that chloramphenicol caused an accumulation of polysomes at the expense of free

monosomes in chloroplasts of *C. reinhardtii* (3). In confirmation of this hypothesis, it was found that the amount of chloroplast ribosomal RNAs found in polysome regions of density gradient analyses of *C. reinhardtii* homogenates increased when cells had been treated with chloramphenicol (3). In our hands, however, although chloramphenicol produced a decrease in free chloroplast ribosome monomers, it did not increase the number of free chloroplast ribosomes recovered as polysomes. Thus, chloramphenicol produced an unaccounted loss of free chloroplast ribosomes. In contrast, it has been reported that there was no change in the number of chloroplast ribosomes (determined by electron microscopy) when cells of *C. reinhardtii* were grown mixotrophically for three generations in media containing chloramphenicol (10). Our investigation was carried out to resolve this discrepancy.

We found that the ribosomes which were unaccounted for in homogenates of cells treated with chloramphenicol could be recovered attached to chloroplast membranes. The nature of the membrane-ribosome association suggested a mechanism by which proteins could become incorporated into the membrane. A brief report of a portion of this investigation has been published elsewhere (11). A brief report of similar findings was published by Chua et al. (12), and a full report of their findings appeared before revision of this manuscript (13).

MATERIALS

Culture and Treatment of Cells

C. reinhardtii wild type 137 c (+) and the arginine-requiring mutant arg-1 (+), were cultured in liquid medium as already described (14). The arg-1 mutant was grown on arginine medium (14). Unless specifically stated, cells were treated under growth conditions in medium in which they were grown (14). Cells were treated with chloramphenicol or streptogramin at final concentrations of 100 $\mu\text{g}/\text{ml}$. A sample of streptogramin A/B was kindly supplied by Merck Chemical Div., Merck & Co., Inc., Rahway, N. J. It was used without further purification, and was added to the cultures as a 10 mg/ml solution in ethanol. Chloramphenicol was obtained from Calbiochem, La Jolla, Calif., and puromycin dihydrochloride was obtained from ICN Nutritional Biochemicals Div., Cleveland, Ohio. Cell RNA was labeled with [^3H]adenine (New England Nuclear, Boston, Mass.), essentially as described elsewhere (15).

Preparation of Subcellular Fractions

Cells were collected, washed, and broken in a French pressure cell at 4,000 lb/in² (14). Homogenates were centrifuged for 15 min at 16,000 rpm in a Sorvall SS 34 rotor (g_{avg} of about 15,000), and supernates (S-15) decanted. Pellets (P-15) were usually suspended in the same volume of homogenate from which they were collected.

Chloroplast membranes were prepared according to Hooper (16), with the following modifications. 20 ml of suspended P-15 fraction (equivalent to material from 32×10^9 cells) were layered on 20 ml of solution containing 0.6 M sucrose, rather than on a step gradient consisting of 10 ml of 0.5 M sucrose and 20 ml of 0.6 M sucrose. Each centrifuge tube in the membrane flotation step (see reference 16) was likewise loaded with material which had come from 32×10^9 cells. Solutions also contained 25 mM MgCl_2 , 25 mM KCl, 25 mM Tris, and 5 mM dithiothreitol, pH 7.5, in addition to various concentrations of sucrose. EDTA was not used at any point. Sucrose, wherever used, was reagent grade and ribonuclease-free (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.). P-15 and S-15 fractions, as well as membranes, were stored at -80°C after quick freezing in a bath of Dry Ice and acetone.

Electron Microscopy

Cells and membranes were collected by centrifugation, and were resuspended in 0.1 M phosphate buffer, pH 7.5. Glutaraldehyde was added to a final concentration of 4% (vol/vol), and samples were incubated at 0°C for 20 min. Cells and membranes were collected by centrifugation, and were resuspended in phosphate buffer. Osmium tetroxide was added to a concentration of 1%, and the samples were incubated 30 min at 0°C , at which time cells and membranes were collected by centrifugation. Pellets were dehydrated in a graded series of aqueous acetone, transferred to propylene oxide, and embedded in Epon. Sections were cut with a diamond knife on an LKB ultramicrotome. The sections were stained on grids with uranyl acetate and lead citrate (17), and were viewed with a Philips 300 electron microscope.

Analyses

Chlorophyll was determined spectrophotometrically (18). Cell number was determined with a hemacytometer. Relative quantities of ribosomes were determined by measuring the area under peaks of A_{254} scans of density gradients (14). To determine RNA content of cells, they were first extracted to remove interfering substances (14, 19). The residues were then extracted with alkali (14, 19), and the orcinol reaction (20) was carried out with these ex-

tracts. RNA content of membranes was determined similarly. Ribosomal RNAs in cell fractions were determined by gel electrophoresis as already described (4). Amounts of chloroplast or cytoplasm ribosomal RNAs were determined from the sum of areas under 16S and 23S peaks or 18S and 25S peaks, respectively, of A_{260} scans of gel electrophoretograms.

RESULTS

Association of Chloroplast Membranes and Chloroplast Ribosomal RNAs in Chloramphenicol-Treated Cells

Typical absorbance scans of sucrose density gradient analyses of S-15 fractions show a major absorbance maximum which is designated 83S, and corresponds to the location of monomeric cytoplasm ribosomes (4) (see Fig. 1 and also reference 4, Fig. 1). A second maximum at about 67S corresponds to the location of chloroplast monomeric ribosomes (see Fig. 1 and also reference 4, Fig. 1). Absorbance maxima attributable to polysomes are small compared to absorbance maxima due to ribosome monomers (Fig. 1). The amount of chloroplast 67S monomeric ribosomes found in S-15 fractions of chloramphenicol-treated cells is $41 \pm 9\%$ (SD four experiments) of that found in controls. The amount of cytoplasm 83S monomeric ribosomes is unchanged ($103 \pm 10\%$ of controls, SD four experiments). Total monomeric ribosomes (67S and 83S) decrease to $73 \pm 6\%$ (SD four experiments) of controls, and this decrease is accounted for by the decrease in

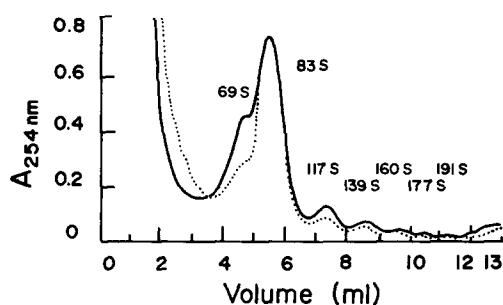


FIGURE 1 Effect of chloramphenicol treatment of cells on small polysomes in S-15 fraction. A 100 ml sample of culture containing 1.0×10^6 cells/ml of wild type was treated with $100 \mu\text{g/ml}$ of chloramphenicol for 1 h (\cdots) and a duplicate sample was not treated (—). Cells were collected, washed, resuspended in 5.0 ml, broken, and S-15 fractions prepared. Duplicate 1.0 ml portions of S-15 were layered on sucrose gradients and centrifuged for 2 h at 40,000 rpm.

67S chloroplast ribosomes. Chloramphenicol treatment of cells does not produce any change in the total amount of RNA found in cells comparable to the change in monomer ribosome content of S-15 fractions, since treated cells have $98 \pm 8\%$ (average deviation, two experiments) of the RNA found in untreated cells. Thus, the chloroplast ribosomes lost from the monomer fraction, or the RNAs they contain, must exist in the homogenate in some modified form. They are not accounted for as free chloroplast RNA, nor as chloramphenicol

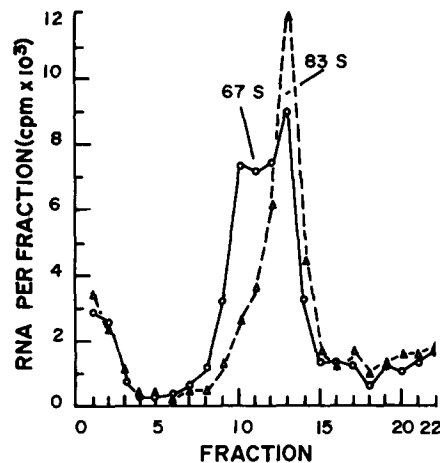


FIGURE 2 Absence of chloramphenicol particles and additional free RNA in homogenates of chloramphenicol-treated cells. 100 ml of medium containing 0.05 mCi of [^3H]adenine (6.3 Ci/mmol, generally labeled, obtained from New England Nuclear) were inoculated with wild type. After 2 days cells ($4 \times 10^6/\text{ml}$) were collected by centrifugation at room temperature, washed with fresh medium lacking labeled adenine, and were resuspended in 500 ml of fresh medium lacking labeled adenine. Incubation was continued until the culture reached a density of 4×10^6 cells/ml. A 150 ml portion of the culture was treated with $100 \mu\text{g/ml}$ of chloramphenicol under growth conditions for 1 h ($\Delta\text{---}\Delta$). A second 150 ml portion was treated identically except that addition of chloramphenicol was omitted ($\circ\text{---}\circ$). Cells were collected, washed, resuspended in 7.5 ml, S-15 fractions prepared, and 1.0 ml samples analyzed by sucrose density gradient centrifugation. Gradients were divided into 22 fractions. Six 0.1 ml samples were taken from each fraction. Three were extracted by the method used to determine radioactive protein. This included both hot and cold trichloroacetic acid extractions. The remaining three samples were extracted in the same way except that extraction with hot trichloroacetic acid was omitted. Radioactivity in RNA was determined as the difference between the two.

particles, since chloramphenicol produces no change in the amount of RNA found in particles smaller than 67S (Fig. 2). Nor are the unaccounted chloroplast ribosomes recovered as small polyribosomes (Fig. 1), as has been reported (3). The chloroplast ribosomes unaccounted for in chloramphenicol-treated cells are not recovered as small polysomes even when unfractionated cell homogenates, or S-15 fractions prepared from homogenates to which Triton X-100 is added (final concentration, 2.5%, vol/vol), are analyzed by density gradient centrifugation.

The RNA in chloroplast monomeric ribosomes that is unaccounted for in S-15 fractions of chloramphenicol-treated cells is largely recovered in P-15 fractions. Chloramphenicol treatment of cells produces a decrease in the chloroplast ribosomal RNA in S-15 fractions, and an increase in the P15 fractions (Table I). When chloroplast membranes are prepared from P-15 fractions, chloroplast ribosomal RNAs co-purify with the membranes, i.e., the amounts of chloroplast ribosomal RNAs relative to chlorophyll are approximately the same in P-15 and membrane fractions (Table II). This result indicates that all of the chloroplast ribosomal RNAs found in the P-15 fractions are associated with chloroplast mem-

TABLE I
Effect of Chloramphenicol Treatment of Cells on Distribution of Chloroplast Ribosomal RNAs in Centrifugal Fractions of Cell Homogenates

Centrifugal fraction analyzed	RNA content of fractions		Difference in RNA content of untreated and chloramphenicol treated
	Minus chloramphenicol	Plus chloramphenicol	
	<i>relative values</i>		
S-15	29 ± 2	15 ± 2	-14 ± 3
P-15	8 ± 1	17 ± 2	+9 ± 2

A portion of a culture containing 3×10^6 cells/ml of arg-1 was treated with chloramphenicol for 1 h, and a portion was left untreated. P-15 and S-15 fractions were prepared from duplicate portions of each. Duplicate portions of P-15 and S-15 fractions, each corresponding to equal numbers of cells (about 2×10^6) were treated with sodium dodecyl sulfate (SDS) and electrophoresed (see legend to Fig. 3). Gels were scanned at 260 nm, and relative amounts of 16S and 23S RNAs were determined. Dispersion is given as standard deviation of four electrophoretograms (duplicate electrophoretograms for each of two homogenates).

TABLE II
Association of Chloroplast Ribosomal RNAs with Chloroplast Membranes

Fraction analyzed	Untreated cells		Chloramphenicol-treated cells	
	Chloroplast RNA/chlorophyll	Cytoplasm RNA/chlorophyll	Chloroplast RNA/chlorophyll	Cytoplasm RNA/chlorophyll
P-15	0.07	0.37	0.27	0.45
Membranes	0.07	0.04	0.24	0.05

branes were prepared and chlorophyll determined as described in Methods. RNAs were determined as described in Methods and the legends to Table I and Fig. 3.

In contrast, cytoplasm ribosomal RNAs are removed during preparation of membranes, since the amount of cytoplasm ribosomal RNAs relative to chlorophyll in membrane preparations is only about 10% of that in P-15 fractions (Table II). The membranes from chloramphenicol-treated cells contain predominantly chloroplast ribosomal RNAs (Fig. 3). Usually membranes from treated cells contain about five times as much chloroplast ribosomal RNAs as membranes from untreated cells. Sometimes they contain 10 times as much. Membranes from chloramphenicol-treated cells contain about 20 μg RNA/mg chlorophyll.

Association of Ribosomes with Membranes

Membrane preparations from untreated cells have few ribosomes attached to membranes, while membranes from chloramphenicol-treated cells have many (Figs. 4 and 5). Ribosomes occur singly and in groups of two and more in membrane preparations from chloramphenicol-treated cells (Figs. 4 b and 5). Some groups of ribosomes appear in linear or circular arrays, which suggest that some ribosomes are present as polyribosomes. Both linear and circular arrays could be different aspects of the same polysome-like arrangements. The particles we identify as ribosomes stain more densely than the membranes. They are $175 \pm 30 \text{ \AA}$ (SD 10 particles) on their long axis. This size agrees reasonably well with the 200 \AA recently reported for ribosomes bound to chloroplast membranes (13). Our membrane preparations consist largely of chloroplast membranes, as identified by the presence of chloroplast remnants, and by the presence of thylakoid partitions, i.e., fused mem-

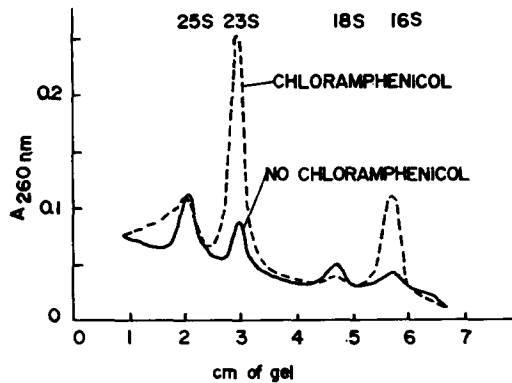


FIGURE 3 Ribosomal RNAs of chloroplast membranes from untreated and chloramphenicol-treated cells. 30 μ l of 20% SDS were added to 0.2 ml of membranes (116 μ g of chlorophyll) from untreated cells and was incubated at 40°C for 4 min (—). 0.2 ml of membranes (108 μ g/chlorophyll) from chloramphenicol-treated cells was treated in the same way (---). 75- μ l samples were electrophoresed at 5 mA per 0.6 \times 9 cm gel for 3 h.

branes. The thickness of thylakoid partitions is 115 ± 15 Å, in good agreement with the 120 ± 15 Å we measured from published micrographs of cells of *Chlamydomonas* (21). However, identification of specific pieces of membrane associated with ribosomes as chloroplast membrane is difficult. The possibility that some of the ribosomes that we see are bound to some other type of membrane, e.g., endoplasmic reticulum, cannot be ruled out. However, it is unlikely that a large proportion of the membrane-associated ribosomes is bound to endoplasmic reticulum, since the membrane preparations contain largely chloroplast ribosomal RNAs (Fig. 3).

The association of chloroplast ribosomes with chloroplast membranes suggests a resemblance to the association of cytoplasmic ribosomes with endoplasmic reticulum. Most ribosomes of endoplasmic reticulum can be removed by the combined action of puromycin and high concentrations of KCl (22). Therefore, the conditions which would release ribosomes from chloroplast membranes were studied to determine if these conditions are the same as required for release of ribosomes from endoplasmic reticulum.

Particles of principally two sizes are recovered when membranes from chloramphenicol-treated cells are treated with 1 mM puromycin in 500 mM KCl, and are analyzed on sucrose density gradients containing 25 mM KCl (Fig. 6 A). No appreciable amount of particles is released from membranes of

untreated cells (Fig. 6 B). The large particle released from membranes of chloramphenicol-treated cells sediments at about 58S, and the small one at about 45S. Sedimentation coefficients are only approximate values. They were calculated as already described, using 83S cytoplasmic ribosomes as reference (4). The distance sedimented by 83S ribosomes was determined in gradients which contained 25 mM KCl instead of 500 mM KCl. The concentration of KCl does not appreciably affect the rate of sedimentation, since subunits of chloroplast ribosomes sediment at approximately the same rate whether gradients contain 25 or 500 mM KCl. The 58S particle contains only 23S chloroplast ribosomal RNA. Neither the 45S nor the 58S particles are resolved into additional components when they are treated with 500 mM KCl and analyzed on gradients containing 500 mM KCl. We conclude that the 58S particle is the large subunit of chloroplast ribosomes, and suggest that the 45S particle is the small subunit. Only small amounts of particles which sediment at 67S and 83S are recovered when membranes from chloramphenicol-treated cells are treated with 1 mM puromycin in 500 mM KCl. Thus, we find that chloroplast ribosomal subunits do not reassociate when returned to 25 mM KCl, in agreement with the findings of Chua et al. (13).

$54 \pm 6\%$ (average deviation, two experiments) of the subunits (45S plus 58S) released by 1 mM puromycin in 500 mM KCl are released by 500 mM KCl alone, and $29 \pm 3\%$ are released by 1 mM puromycin alone. Less than 5% are released by 25 mM KCl in the absence of puromycin. Since more subunits are released by 1 mM puromycin in 500 mM KCl than are released by 1 mM puromycin and 500 mM KCl, each used separately, release of some particles may require both 1 mM puromycin and 500 mM KCl. The results we obtain with chloroplast membranes are similar to those obtained with endoplasmic reticulum (22), and *C. reinhardtii* chloroplast membranes (13), except that in our experiments some ribosomal particles are released by puromycin alone (Fig. 6 A).

After density gradient centrifugation of membranes treated with 1 mM puromycin in 500 mM KCl most membranes are found in a pellet at the bottom of the tube, but some are found suspended in the bottom quarter of the gradient. When we reisolate membranes by differential centrifugation after treatment with 1 mM puromycin in 500 mM

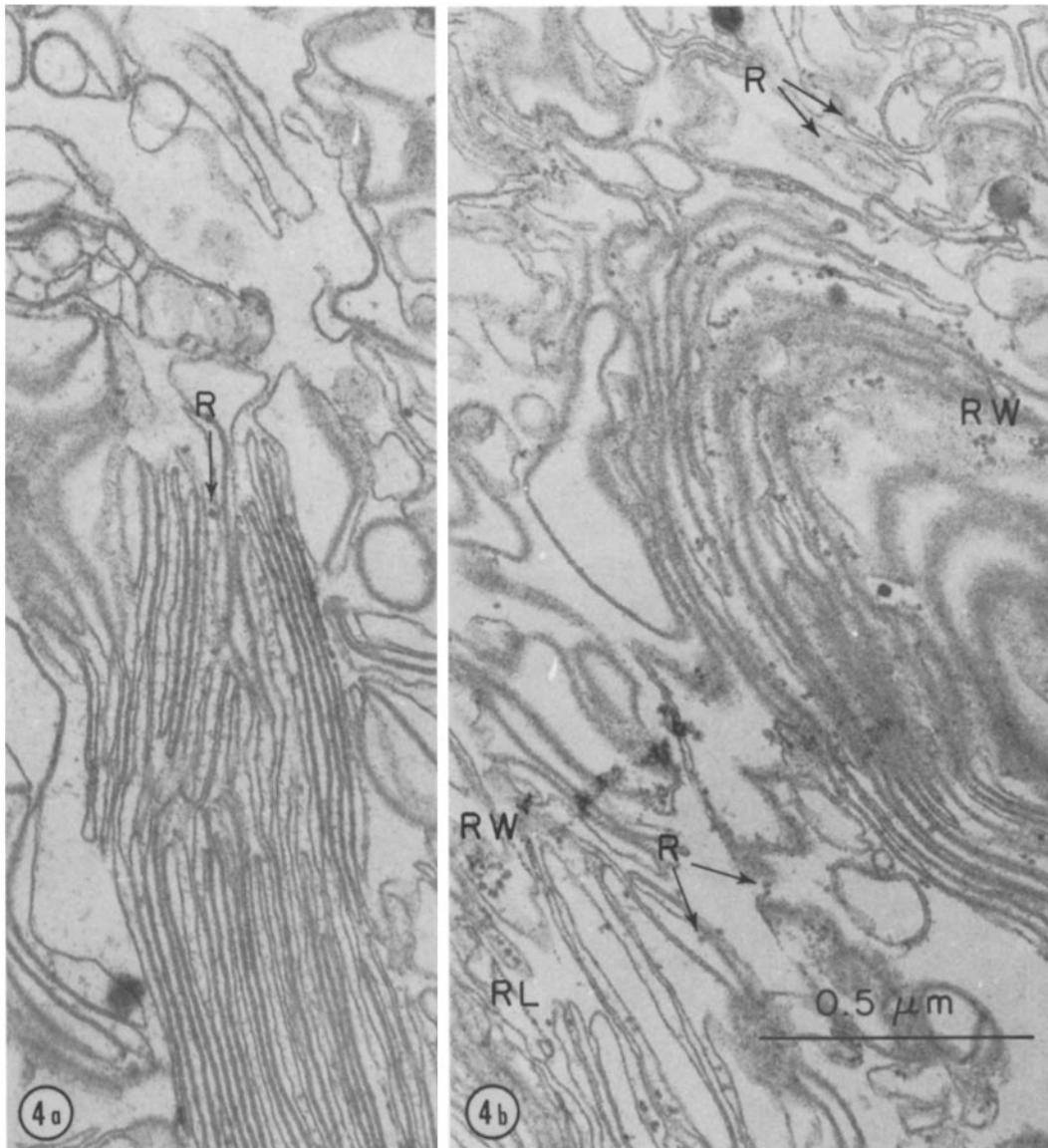


FIGURE 4 Isolated chloroplast membranes from untreated (a) and chloramphenicol-treated (b) cells. Ribosomes occur singly (*R*), in linear arrays (*RL*), or in whorls (*RW*). $\times 70,000$.

KCl, they have lost about 70% of their chloroplast ribosomal RNAs (Table III). However, the loss due to treatment with 1 mM puromycin in 500 mM KCl is estimated at only 50% of the chloroplast ribosomal RNAs originally present in the membranes (Table III). This value is calculated as the difference between RNA recovered from membranes treated with 25 mM KCl, and membranes treated with 1 mM puromycin in 500 mM KCl. The cytoplasmic ribosomal RNAs lost from the membranes by treatment with 1 mM

puromycin in 500 mM KCl account for only 10% of the total ribosomal RNAs lost.

The increased amounts of chloroplast ribosomal RNAs and chloroplast ribosomes in membrane preparations from chloramphenicol-treated cells do not appear to be an artifact of the membrane isolation procedure. First, ribonuclease does not release ribosomes from the membranes of chloramphenicol-treated cells (Fig. 6 C), as might be expected if ribosomes (present as polyribosomes) were merely entangled with membranes. Failure

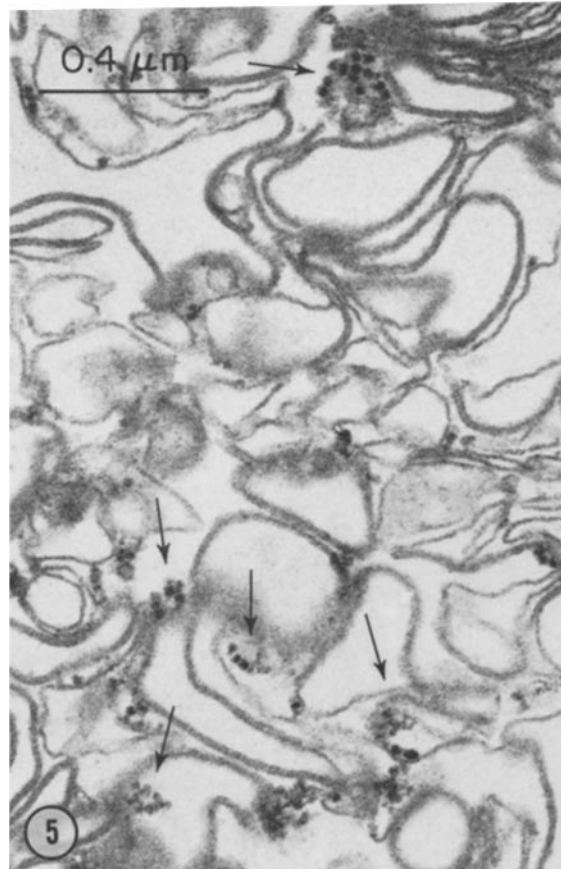


FIGURE 5 Isolated membranes from chloramphenicol-treated cells. Arrows indicate the location of poly-some-like arrangements of ribosomes associated with membranes. $\times 90,000$.

to detect release of ribosomes is not due to degradation of ribosomes by ribonuclease, since the enzyme has no effect on the amount or sedimentation rate of particles released by 1 mM puromycin in 500 mM KCl (Fig. 6 C). In parallel with results obtained with isolated membranes, the proportion of ribosomes associated with chloroplast membranes is greater in chloramphenicol-treated than untreated cells (Fig. 7). Cells were harvested and washed as for preparation of cell homogenates, and then were fixed. Ribosome-membrane associations in chloramphenicol-treated cells typically take the form of linear arrays in sections perpendicular to the membranes (Fig. 7 b). Some ribosomes are associated with membranes even in untreated cells, as might be expected, since membranes isolated from untreated cells contain some chloroplast ribosomal RNAs (see Fig. 3). However, micrographs of untreated cells are always identified correctly when scored blind, because most chloroplast

ribosomes are not associated with chloroplast membranes. Micrographs of chloramphenicol-treated cells contain many ribosomes associated with membranes, but also many that are not associated with membranes. For this reason, micrographs of chloramphenicol-treated cells are identified correctly only 70% of the time when scored blind. However, the result is compatible with our finding that 40% of chloroplast ribosomes in cell homogenates of chloramphenicol-treated cells are present in unbound form.

Effect of Streptogramin on Recovery of Free Chloroplast Ribosomes

Chloramphenicol presumably allows isolation of chloroplast ribosomes bound to chloroplast membranes by virtue of its ability to inhibit protein synthesis by chloroplast ribosomes. The simplest explanation is that chloramphenicol prevents run-

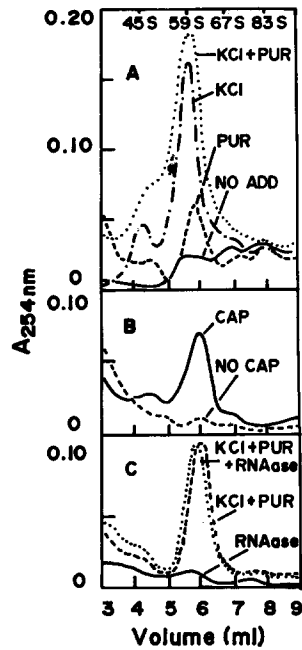


FIGURE 6 Release of chloroplast ribosomes from chloroplast membranes. (A) Release of ribosomes from membranes of chloramphenicol-treated cells by puromycin and KCl. Membranes containing 350 μg of chlorophyll were incubated for 15 min at 0°C followed by 15 min at 37°C in 1 ml of medium containing, 125 mM sucrose, 25 mM MgCl_2 , 5 mM dithiothreitol, 25 mM Tris, pH 7.5. In addition, reaction mixtures contained: 25 mM KCl (—); 1 mM puromycin and 25 mM KCl (---); 500 mM KCl (----); 1 mM puromycin and 500 mM KCl (···). Reaction mixtures were layered on sucrose density gradients containing 25 mM KCl, 25 mM MgCl_2 , 25 mM Tris, pH 7.5, and 5 mM dithiothreitol. The gradients were centrifuged for 4 h at 40,000 rpm. Sedimentation coefficients were calculated by comparison with the location of the 83S cytoplasmic ribosome peaks obtained by analysis of S-15 fractions. The top of the gradients were 0 ml and the bottoms at 13 ml. (B) Release of ribosomes from membranes of chloramphenicol-treated cells but not from membranes of untreated cells. Membranes were treated with 1 mM puromycin in 500 mM KCl and were analyzed as described in (A). The membrane sample from untreated cells (---) contained 170 μg chlorophyll and the sample from treated cells (—) contained 160 μg chlorophyll. (C) Lack of release of ribosomes by ribonuclease. 1 ml samples of membranes containing 180 μg of chlorophyll in medium containing 125 mM sucrose, 25 mM MgCl_2 , 25 mM Tris, pH 7.5, and 5 mM dithiothreitol were incubated for 15 min at 0°C with 25 mM KCl and 10 μg ribonuclease (—), for 15 min at 37°C with 1 mM puromycin in 50 mM KCl (---), or for 15 min at 37°C with 1 mM puromycin in 500 mM KCl followed by addition

TABLE III
Release of Chloroplast Ribosomes from Membranes by 500 mM KCl and 1 mM Puromycin

Additions to reaction mixture		Chloroplast RNAs retained by membranes	Chloroplast RNAs released from membranes due to KCl and/or puromycin
KCl	puromycin	%	%
mM	mM		
13	0	76 \pm 1	—
500	0	65 \pm 2	11
13	1	61 \pm 6	15
500	1	28 \pm 0	48

Replicate membrane samples containing 510 μg chlorophyll were incubated at 0°C for 15 min followed by 37°C for 15 min in 1 ml of reaction mixture containing 125 mM sucrose, 25 mM MgCl_2 , 25 mM Tris, pH 7.5, 5 mM dithiothreitol, and KCl and puromycin at the concentrations listed above. Reaction mixtures were cooled to 0°C and were centrifuged for 0.5 h at 15,000 g_{avg} . Supernates were carefully removed to avoid disturbing pellets. The latter were resuspended in 0.5 ml 250 mM sucrose, 25 mM MgCl_2 , 25 mM KCl, 25 mM Tris, pH 7.5, and 5 mM dithiothreitol, and chloroplast RNA contents were determined by gel electrophoresis after treatment of samples with SDS (see Methods and legends to Fig. 1 and Table I). A sample of membranes containing 510 μg chlorophyll in 0.5 ml of this medium was incubated at 0°C and 37°C as above. Its RNA content was determined and set equal to 100%. Figures are averages plus average deviations for two experiments.

off of nascent chains during harvesting and washing of cells (see Discussion). If chloroplast protein synthesis was inhibited by a substance which inhibited initiation, e.g., streptogramin (23), chloroplast ribosomes in cells and homogenates should be free, i.e., unattached to membranes. Addition of chloramphenicol to such cells should then have no effect on the amount of free ribosomes found in homogenates since membrane-bound ribosomes would have already been converted to free ribosomes during the incubation with streptogramin. In accordance with these expectations, the amount of free chloroplast monomeric ribosomes in homogenates is the same in untreated cells (Fig. 8 A), in cells treated with streptogramin (Fig. 8 C), and in cells that are treated with chloramphenicol after they are treated with streptogramin (Fig. 8 D). As usual, homogenates from

of 10 μg ribonuclease and incubation at 0°C for 15 min (···). The reaction mixtures were analyzed as in (A).

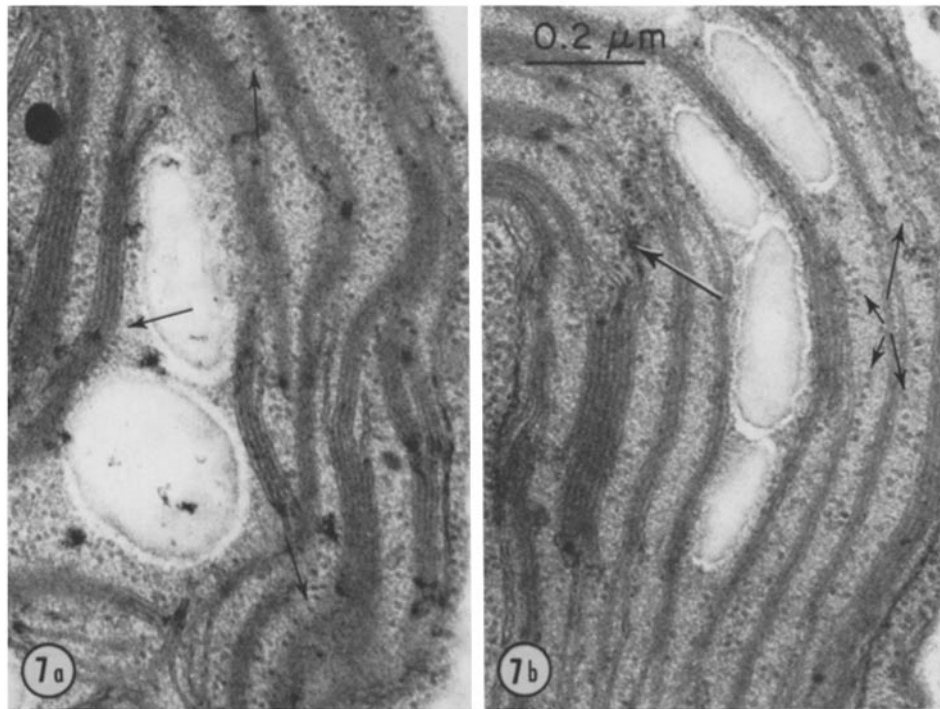


FIGURE 7 Effect of chloramphenicol treatment on distribution and arrangement of chloroplast ribosomes. Cells were chilled, collected, and washed in the same way as cells that were used for preparation of homogenates. Then they were fixed as described in Methods. Arrows indicate the location of groups of ribosomes which appear attached to membranes. (a) A portion of an untreated cell. (b) A portion of a chloramphenicol-treated cell. $\times 70,000$.

cells that are treated with only chloramphenicol have a decreased amount of free chloroplast monomeric ribosomes (Fig. 8 B).

DISCUSSION

Our studies show that under suitable conditions, i.e., in cells treated with chloramphenicol, it is possible to isolate from *C. reinhardtii* a large portion of chloroplast ribosomes attached to chloroplast membranes. This is demonstrated by the copurification of chloroplast ribosomal RNAs and membranes, the visualization by electron microscopy of the association between membranes and ribosomes, and by the partial requirement for puromycin to obtain release of ribosomes from membranes *in vitro*. Since electron micrographs of (harvested and washed) cells indicate that cells that have been treated with chloramphenicol contain more membrane-bound ribosomes, attachment of chloroplast ribosomes to membranes does not appear to be an artifact of isolation.

Ribosomes have been reported to be associated with chloroplast membranes both *in situ* (24) and

in isolated chloroplasts (25, 26). Recently, it has been reported that chloroplast ribosomes can be isolated attached to chloroplast membranes when *C. reinhardtii* is treated with chloramphenicol (12, 13). These last two reports are similar in many respects to the present one. However, in our work late log phase asynchronous cultures of *C. reinhardtii* are used instead of synchronous cultures. Thus, the ability to isolate membrane-bound chloroplast ribosomes is not only a property of the synchronous cultures. In the present work, ribosomes attached to membranes are also identified on the basis of both the RNA species contained by membranes and the sedimentation properties of particles released from the membranes. The RNA species determinations allowed us to estimate the percentage of bound ribosomes released by 1 mM puromycin in 500 mM KCl. Data on ribosomes associated with membranes in cells are also provided here, as well as inhibitor effects on the chloramphenicol-inhibited state.

In most respects, the requirements for release of ribosomes from chloroplast membranes we report

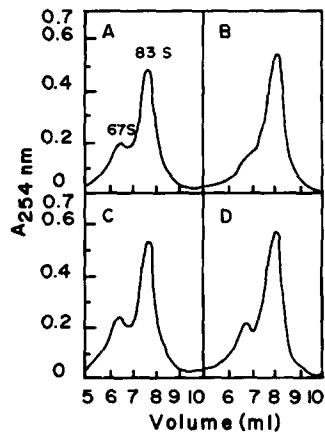


FIGURE 8 Effect of streptogramin on chloramphenicol-induced decrease in free chloroplast ribosomes. Replicate 100-ml samples of arg-1 (1.2×10^6 cells/ml) were taken. One was left untreated (A), one was treated with chloramphenicol for 1 h (B), one was treated with streptogramin for 1 h (C), and one was treated with streptogramin for 1 h at which time chloramphenicol was added and incubation was continued for a second hour (D). S-15 fractions were prepared, and analyzed by 4 h centrifugation at 40,000 rpm on sucrose density gradients.

are similar to the requirements for release of ribosomes from endoplasmic reticulum (22), and release from chloroplast membranes of synchronous cells of *C. reinhardtii* (13). Release is not effected by 25 mM KCl in the absence of puromycin. Large amounts of particles are released with 500 mM KCl alone, and still more particles are released by the combination of 1 mM puromycin in 500 mM KCl. In our experiments, ribosomes attached to chloroplast membranes are released as subunits as is reported for synchronous cultures of *C. reinhardtii* (13), and ribosomes attached to endoplasmic reticulum (22). Unlike the results with endoplasmic reticulum (22) and synchronous *C. reinhardtii* (13), our results show that a portion of membrane-bound chloroplast ribosomes are released with puromycin alone. The partial dependence on puromycin for release indicates that a portion of the membrane-bound chloroplast ribosomes in our preparations are attached to the membrane through nascent protein chains. Although micrographs indicate the presence of polysomes on membranes, we have not been able to demonstrate release of polysomes by treatment of membranes with 500 mM KCl plus 1 mM puromycin at 0°C. Release of polysomes might have

been expected since this procedure releases nascent protein from free polysomes without separation of messenger RNA and ribosomes (27).

It appears that there are two sites of attachment of ribosomes to the membranes, as is postulated for ribosomes bound to endoplasmic reticulum (22). Attachment at one site is broken by puromycin, presumably because of attachment of membrane and ribosome by the nascent polypeptide chain. A second type of attachment is broken by high concentrations of KCl. It has been suggested that the KCl-sensitive link is a direct electrostatic bond between ribosome and membrane (22). Thus, in our preparations some ribosomes are linked to the membrane by the nascent chain alone (released by puromycin alone), while some ribosomes are linked by electrostatic linkage alone (released by KCl alone). However, a small percentage of ribosomes may be linked by both the nascent chain and the electrostatic bond, because the sum of particles released by 1 mM puromycin in 500 mM KCl is somewhat greater than the sum of particles released by 500 mM KCl and 1 mM puromycin alone. Since it has been shown that ribosomes with nascent chains are resistant to dissociation with high concentrations of KCl (27, 28), the following possibility should be considered to explain KCl-dependent release. At least some of the ribosomes removed from the membrane by 500 mM KCl alone might not be attached to the membrane except in an indirect fashion, i.e., a ribosome is attached to a messenger RNA strand which is anchored to the membrane at another point by a ribosome with a nascent chain.

The simplest way to explain the effect of chloramphenicol on our ability to isolate ribosomes bound to chloroplast membranes is that in actively growing cells a large portion of ribosomes are bound to chloroplast membranes as polysomes. These bound ribosomes complete protein chains and are released from membranes during harvesting and washing of cells, even though cells are cooled to 0°C within 0.5 min. Chloramphenicol, which inhibits protein synthesis by inhibiting transpeptidation (23), prevents this "runoff." This explanation seems likely since most chloroplast ribosomes and cytoplasm ribosomes are recovered as monomers without nascent chains in the absence of chloramphenicol, i.e., most ribosomes in homogenates of *C. reinhardtii* are dissociated into subunits by high concentrations of KCl (2) (A. Michaels, unpublished experiments), and less than 5% react

with radioactive puromycin to give radioactive peptidyl puromycin (M. Margulies, unpublished experiments). Since it is improbable that ribosomes exist as monomers in actively growing cells, conversion of polysomes to monomers must occur. Probably this conversion happens during harvesting and washing cells, since electron micrographs of harvested cells that have not been treated with chloramphenicol contain few membrane-bound ribosomes. The alternate possibility that chloramphenicol actually causes an accumulation of chloroplast ribosomes on membranes *in vivo* was also considered. However, this hypothesis requires incomplete inhibition by chloramphenicol of transpeptidation on chloroplast ribosomes. Although such incomplete inhibition cannot be ruled out, it is felt that the presence of ribosomes as monomers in untreated cells argues strongly for the runoff hypothesis discussed above.

Our studies suggest that membrane-bound chloroplast ribosomes are functioning in protein synthesis. This view is supported by the observation that some of the membrane-bound ribosomes are attached to the membrane by nascent polypeptide chains. In addition, membrane-bound ribosomes are isolated only when cells have been treated with chloramphenicol, or when they have been treated with some other substance which blocks chain elongation by chloroplast ribosomes. Spectinomycin, another antibiotic which selectively binds to chloroplast ribosomes (29), appears to behave like chloramphenicol (30). Lastly, accumulation of chloroplast ribosomes in a state where they cannot initiate protein synthesis (by treating cells with streptogramin) vitiates the effect of chloramphenicol that allows us to isolate membrane-bound chloroplast ribosomes.

The similarity between ribosomes bound to chloroplast membranes and ribosomes bound to endoplasmic reticulum suggests a possible similarity in function. Ribosomes of endoplasmic reticulum are synthesizing secreted proteins (31-34), and also may be synthesizing membrane proteins (35, 36). In endoplasmic reticulum, secreted proteins are temporarily a part of the membrane and are directionally released into the cisternae of the reticulum (37, 38). It seems unlikely that chloroplasts are secreting proteins into the thylakoid lumen. Thus, it is tempting to speculate that ribosomes bound to chloroplast membranes are synthesizing proteins of the chloroplast membranes. Evidence has been presented that chloro-

plasts synthesize some of their membrane proteins (16, 39, 40). The hypothesis that membrane-bound chloroplast ribosomes synthesize membrane proteins has been made to explain the activity of chloroplast membranes in amino acid incorporation into protein *in vitro* (25). In this amino acid incorporation system, radioactive protein remained with the membranes after ribosomes were released with puromycin (25). Thus, the presence of ribosomes bound to chloroplast membranes in the way that ribosomes are bound to endoplasmic reticulum suggests a mechanism for the incorporation of newly synthesized protein into the structure of the chloroplast membrane.

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