The Chloroplast and Cytoplasmic Ribosomes of Euglena

1. STABILITY OF CHLOROPLAST RIBOSOMES PREPARED BY AN IMPROVED PROCEDURE

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ABSTRACT

A new isolation procedure has resulted in an improved yield of stable 68S chloroplast ribosomes from Euglena gracilis var. bacillaris. Chloroplasts are isolated by suspending the cells in buffer I (sorbitol, 250 mM; sucrose, 250 mM; Ficoll, 2.5% [w/v]; magnesium acetate, 1 mM; bovine serum albumin, 0.01% [w/v]; mercaptoethanol, 14 mM; N-2-hydroxyethyl-piperazine-N′-2-ethanesulfonic acid, pH 7.6, 5 mM) and passing through a French press at less than 1500 pounds per square inch. The crude chloroplasts are purified by three washings with buffer II (sorbitol, 150 mM; sucrose, 150 mM; Ficoll, 2.5% [w/v]; magnesium acetate, 1 mM; bovine serum albumin, 0.01% [w/v]; mercaptoethanol, 14 mM; N-2-hydroxyethyl-piperazine-N′-2-ethanesulfonic acid, pH 7.6, 5 mM). Stable 68S chloroplast ribosomes are obtained when the isolated chloroplasts are resuspended in ribosome buffer (tris-HCl, pH 7.6, 10 mM; magnesium acetate, 12 mM; KCl, 60 mM) containing spermidine, 0.5 mM; mercaptoethanol, 14 mM; sucrose, 8% (w/w), passed through a French press at 4000 pounds per square inch and extracted with either 0.1% (w/v) sodium deoxycholate or 1.0% (v/v) Triton X-100. At 0 to 4°C in ribosome buffer, the purified 68S chloroplast monosome forms a 53S particle while the 55S particle, an expected product of monosome dissociation, cannot be detected. Spermidine and mercaptoethanol prevent the formation of 53S particles from 68S monosomes. The purified 53S particles derived from 68S monosomes contain 23S RNA as well as a significant amount of 16S RNA, suggesting that this particle may not be a true ribosomal subunit.

It is generally agreed that Euglena plastids contain bacteri-alike ribosomes which sediment as 68S particles (see ref. 2 for summary). Munns (25) and Scott et al. (32) were able to isolate and characterize Euglena chloroplast ribosomes, although their preparations were extremely unstable and disassociated into subunits when pelleted. Others find that the Euglena chloroplast ribosome preparations also contain a particle of 46 to 55S which often exceeds the amount of monosomes. A 30S particle, the expected product of monosome

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MATERIALS AND METHODS

Growth Conditions. Cultures of Euglena gracilis Klebs var. bacillaris Pringsheim were maintained on the pH 3.5 medium of Hutner (13). For chloroplast isolation, cultures were grown in 20-liter carboys containing 14 liters of Hutner medium with the B2 concentration reduced to 0.07 µg/l (low B2 medium). Sterile vitamin B2 was added aseptically after autoclaving the carboys for 3 hr at 15 psi. After cooling they were inoculated with 1 liter of late log phase cells grown on Hutner's medium (13). The cultures were harvested after 3 days at 27°C under 1000 ft-c. of illumination with daylight fluorescent tubes and continuous aeration; at this time the cell density was approximately 2 × 10^6 cells/ml and the concentration of Chl was about 15 pg/cell.

Buffers. Buffer I contains: sorbitol, 250 mM; sucrose, 250 mM; Ficoll, 2.5% (w/v); magnesium acetate, 1 mM; bovine serum albumin, 0.01% (w/v); mercaptoethanol, 14 mM; HEPES, 5 mM. The pH is adjusted to 7.6 with 1 N NaOH. Buffer II contains: sorbitol, 150 mM; sucrose, 150 mM; Ficoll, 2.5% (w/v); magnesium acetate 1 mM; bovine serum albumin, 0.01% (w/v); mercaptoethanol, 14 mM; HEPES, 5 mM. The pH is adjusted to 7.6 with 1 N NaOH. Buffer III contains: tris-HCl, pH 7.6, 10 mM; KCl, 60 mM; magnesium acetate, 12 mM; spermidine trihydrochloride, 0.5 mM; mercaptoethanol, 14 mM. Buffer IV contains: 8% sucrose (w/w) prepared in buffer III. Buffer V contains: tris-HCl, pH 7.6, 10 mM; KCl, 60 mM; magnesium acetate, 12 mM; spermidine trihydrochloride, 0.5 mM; mercaptoethanol, 7 mM. Buffer VI (15) contains: tris-HCl, pH 7.4, 100 mM; sodium acetate, 150 mM; magnesium acetate, 5 mM. Buffer VII (15) contains: tris-HCl pH 7.2, 10 mM; sodium acetate, 100 mM; disodium EDTA, 10 mM.

Isolation of Chloroplasts. Cultures were harvested at 0 to 2 C in the Szent-Gyorgyi and Blum KSB-R Sorvall continuous flow rotor at 8000 rpm with a flow rate of 1 to 1.5 l/min. All subsequent operations were performed at 0 to 4°C. The har-
vested cells were washed once with buffer I and were resuspended in 1.0 ml of buffer I per g of cells. The suspension was passed through a French pressure cell at no more than 1500 psi. The homogenate was immediately diluted with 2 volumes of buffer II per g of cells and was centrifuged for 2 min at 121g. The supernatant was centrifuged for 10 min at 700g. The crude chloroplast pellet was resuspended in 2 ml of buffer II per g of cells and centrifuged for 10 min at 700g to give the once-washed chloroplasts. These chloroplasts were further purified by two washings with buffer II at 500g. A flow chart of the chloroplast purification is shown in Figure 1.

**Isolation of Chloroplast Ribosomes.** The purified chloroplasts are resuspended in 0.5 ml of buffer IV per g of cells and centrifuged at 500g for 10 min. The chloroplast pellet is resuspended in 3 ml of buffer IV per g of pellet and passed through a French press at 4,000 psi. This suspension is made to a final concentration of 1% (v/v) Triton X-100 using a 20% (v/v) solution of Triton prepared in buffer III. The lysed chloroplasts are pelleted by centrifuging for 5 min at 3,000g. The supernatant is clarified by centrifuging at 20,000g for 15 min. The 20,000g supernatant is layered over 2 ml of 40% (w/w) sucrose in buffer III containing 1% (v/v) Triton X-100.

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**Flow Chart for Isolation of Chloroplast Ribosomes**

1. **Harvested cells**
   - Resuspend in 200 ml of buffer I; centrifuge 4,000g, 5 min.

2. **Washed cells**
   - Weigh and resuspend in buffer I (1 ml/g cells; French press at less than 1500 lbs/in² and immediately dilute with buffer II (2 ml/g cells).

3. **Cell lysate**
   - Centrifuge lysate, 121g, 2 min; discard pellet. Centrifuge supernatant, 700g, 10 min.

   - **Supernatant (cytoplasmic ribosomes)**
     - Crude chloroplast pellet
       - Resuspend chloroplasts in buffer II (2 ml/g cells) without disturbing paramylum pellet, decant; centrifuge suspension 121g, 2 min; discard pellet. Centrifuge supernatant 700g, 10 min.

   - **Supernatant (discard)**
     - Once-washed chloroplast pellet
       - Resuspend chloroplasts in buffer II (2 ml/g cells) without disturbing paramylum pellet, decant; centrifuge suspension 500g, 10 min; resuspend pellet in buffer II (2 ml/g cells) and repeat.

   - **Supernatant (discard)**
     - Three-times-washed chloroplast pellet
       - Resuspend chloroplasts in buffer IV (0.5 ml/g cells) without disturbing paramylum pellet, decant; centrifuge suspension 500g, 10 min. Resuspend pellet in buffer IV (3 ml/g chloroplasts) without disturbing paramylum pellet, decant. French press suspension, 4,000 lbs/in². Add DOC, 0.1% (w/v) or Triton X-100, 1.0% (v/v) to final concentration. Shake 15 min, 2°C; centrifuge 3,000g, 5 min, then recentrifuge supernatant 20,000g 15 min; combine pellets.

   - **20,000 g chloroplast pellet**
     - Repeat detergent extractions and centrifugations

   - **Twice-extracted chloroplast pellet**
     - Pellet ribosomes
     - Twiced-extracted ribosome supernatant

   - **Layer over a 2 ml cushion of 40% (w/w) sucrose in buffer III (add 1.0% final concentration Triton X-100 if used for initial detergent extraction), centrifuge 17 hrs, 25,000 rpm in Beckman SW27.1 rotor, discard supernatant.

   - **Ribosome supernatant**
     - Ribosome pellet
The ribosomes are sedimented by centrifugation for 17 hr at 25,000 rpm in a Beckman SW-27.1 rotor at 0 to 2 C. DOC4 at a final concentration of 0.1% (w/v) can be used in place of Triton X-100.

**Density Gradient Centrifugation of Ribosomes.** Linear gradients (12.5 ml) of 5 to 20% (w/w) sucrose in buffer V are prepared over a 3 ml cushion of 40% (w/w) sucrose in buffer V. The ribosome pellet is resuspended in buffer III and 5 to 20 A\textsubscript{260} are layered onto the gradient. The gradients are centrifuged for 15 hr at 25,000 rpm in the Beckman SW-27.1 rotor at 0 to 2 C. The gradients were analyzed as described by Noll (26). Both the puncturing device and the flow cell were maintained at 0 to 4 C. The flow rate through the spectrophotometer was 1.2 ml/min as suggested by Noll (26). Ribosomal fractions were collected from the gradients, and the ribosomes were recovered by centrifuging at 25,000 rpm overnight.

**Preparation of Radioactive Ribosomes from E. coli.** E. coli strain MRE 600 were grown at 37 C in M\textsubscript{f} medium (11) supplemented with 0.2% glucose (w/v) and 1.0 mg/ml of uridine. A culture grown overnight was inoculated into 500 ml of M\textsubscript{f} media containing glucose, uridine, and 1.0 mc of uracil-5' H. When the cell density had reached 1.0 A\textsubscript{600}, the cells were harvested at 0 to 4 C. The harvested cells were washed with an 8% (w/w) sucrose solution prepared in E. coli buffer (tris-HCl, pH 7.6, 10 mM; KCl 30 mM; magnesium acetate, 10 mM; spermidine trihydrochloride, 0.5 mM and mercaptoethanol 14 mM). The washed cells were resuspended in the same sucrose solution at a final concentration of 1.0 ml per g of cells. The cell suspension was passed through a French press at 6000 psi and was then diluted with 2 ml of the sucrose solution per g of cells, and 2 mg/ml DNase were added. The homogenate was centrifuged for 10 min at 15,000g, and the supernatant was layered over a 40% (w/w) sucrose solution made in the E. coli buffer. The ribosomes were pelleted by centrifugation for 16 hr at 25,000 rpm in the Beckman SW-27.1 rotor at 0 to 2 C. The ribosome pellet was washed once and resuspended in the E. coli buffer. The radioactive ribosomes were stored at -80 C. The cold trichloroacetic acid precipitated fractions from sucrose gradients were filtered onto glass fiber filters. Ten milliliters of toluene solution (PPO, 4 g; POPOP, 50 mg; and toluene, 1 liter) were added to the dried filters, and the radioactivity was determined in a Beckman LS-150 liquid scintillation counter.

**Electron Microscopy of Isolated Fractions.** The purified chloroplast pellet, the French-pressed chloroplast pellet or the detergent-extracted chloroplast pellet, was resuspended and fixed for 1 hr in 3% glutaraldehyde prepared in buffer II or IV. The procedure of Klein et al. (21) was used from this point on. The samples were postfixed in 2% osmium tetroxide for 90 min. After dehydration with increasing concentrations of ethanol and propylene oxide, the samples were embedded in Epon. Sections were stained with uranyl acetate or lead citrate or both. An RCA EMU 3 electron microscope was used to examine the sections.

**Isolation and Characterization of RNA.** The whole cell pellet, detergent-treated chloroplast pellet, and the ribosome pellet were used directly for the extraction of RNA. Ribosomal fractions collected from sucrose gradients were precipitated with 2 volumes of ethanol for 3 hr at -20 C. After centrifugation for 15 min at 20,000g, the pellets were used for the extractions of RNA.

RNA was extracted using the technique of Heizmann (15).

The pellets were resuspended in buffer VI made 2% with respect to sodium thioglycollate. After shaking for 10 min at 0 to 2 C, SDS was added to a final concentration of 2%. After 5 min 1 volume of phenol prepared according to Rawson et al. (29) (using buffer VI to saturate the phenol) was added, and the sample was deproteinized by shaking at 0 to 2 C for 10 min. The aqueous phase was recovered after centrifugation at 10,000 rpm, re-extracted with phenol if necessary, and the RNA was precipitated at -20 C by the addition of 2 volumes of ethanol.

A 0.5% agarose-2.5% polyacrylamide composite gel was prepared in the E-C slab gel electrophoresis system as described by Peacock and Dingman (27, 28). The RNA pellet was dissolved in buffer VII and 20 ml RNA solution were added to 10 ml of 60% (w/w) sucrose in buffer VII containing bromphenol blue as a marker. Twenty microliters of this mixture containing approximately 1.5 A\textsubscript{260} of RNA were applied to each slot. Electrophoresis was performed for 5 hr at a constant voltage of 200 v at 2 to 4 C. The gels were then stained with methylene blue and destained with running tap water as described by Peacock and Dingman (27). The gels were photographed through a red filter using polaroid type 55P/N 4x5 film and scanned with a Joyce-Lobel densitometer. The pictures were used to measure the electrophoretic mobility and to calculate the mol wt of the RNA using the relationship of Peacock and Dingman (27) with E. coli ribosomal RNA as a standard. The densitometer tracings were used to determine the relative amounts of each RNA species.

**RESULTS AND DISCUSSION**

**Electron Microscopy of Isolated Chloroplast Fractions.** The method we have used for the isolation of Euglena chloroplasts and chloroplast ribosomes is shown in Figure 1. The harvested cells are broken by passage through a French press at a maximum pressure of 1500 psi. This pressure does not break 100% of the cells but gives the best yield of isolated plastids; higher pressures cause the breakage of a large portion of the plastids. The chloroplasts are separated from whole cells, mitochondria, and cell debris by repeated washing in low salt buffer. Three washes were found to give chloroplast preparations with relatively low levels of contaminating cytoplasmic material.

Figure 2A shows a representative electron microscope field of the chloroplast fraction washed three times. It can be seen that the majority of the plastids are sacks of membranes from which the majority of the stromal material has been removed. The thylakoids resemble the membrane structure of the intact chloroplast. A small number of the plastids contain a densely staining stromal region between the thylakoid stacks. These chloroplasts resemble those found within normal cells and are judged to be relatively intact. It should be noted that, except for paramylum granules, the preparation is free of other contaminating cellular material.

At high magnification (Fig. 2, B and C) the stromal region of intact chloroplasts is seen to be composed of densely staining granules which are characteristic of the stromal region of normal chloroplasts. The thylakoids are not as tightly packed as they are in chloroplasts in intact cells indicating that some swelling has occurred. The chloroplasts of Figure 2, B and C, have a pyrenoid region. This region appears to be disorganized, and a break can be seen in the outer chloroplast envelope in the region of the pyrenoid (Fig. 2C). Holdsworth in Eremosphaera (16, 17) and Weatherbee and Schiff in Euglena (35) have presented evidence suggesting that the pyrenoid contains ribulose diphosphate carboxylase. Paramylum granules are photosynthetically synthesized as a sheath surrounding the pyrenoid external to the plastid membrane. The shear forces

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4 Abbreviations: DOC: sodium deoxycholate; SDS: sodium dodecyl sulfate.
FIG. 2. Electron micrographs of isolated three times purified chloroplast fractions. A: Representative field, arrows indicate intact chloroplasts, scale marker = 1 μm here and subsequently; B: chloroplast with intact pyrenoid (P) region; C: chloroplast with disrupted membrane (arrow) at pyrenoid (P); D: fraction after passage through the French press at 4000 psi; E: 20,000g chloroplast pellet from D prepared using 0.1% DOC.
developed during centrifugation probably remove the parame-
ylum from the plastid during the washing procedure ruptur-
ing the outer chloroplast envelope (Fig. 2C). This may enable
the stromal material to leak out yielding the empty plastids of
Figure 2A. Mechanical breakage of the isolated chloroplasts,
French pressing, sonication, or grinding with alumina, fails to
release a significant amount of ribosomal material, although
the chloroplast structure is completely disrupted and the chloro-
plant fragments form numerous vesicles (Fig. 2D). Densely
staining particles resembling ribosomes are seen adhering to
irregular masses which look similar to the stromal material.
Treatments with low concentrations of detergent releases the
ribosomes from the stromal and vesicular material; there is a
lack of stroma-like material in the detergent treated chloroplast
fraction (Fig. 2E).

Analysis of Isolated Chloroplast Ribosomes. To obtain high
yields of stable 68S monosomes, it was necessary to modify the
chloroplast isolation buffers of Vasconcelos et al. (34). The
addition of Mg++, mercaptoethanol, and bovine serum albumin
to the chloroplast isolation buffers increased the amount of
intact chloroplast monosomes which could be extracted from the
isolated plastids. Bovine serum albumin is thought to stabilize
the isolated plastids by preventing nonsomatic swelling (18) while (as will be described later) mercaptoethanol stabilizes the chloroplast monosome. Contrary to previous re-
ports (2, 25, 32), a chloroplast isolation buffer high in Mg++
is not necessary for the isolation of intact chloroplast mono-
somes.

A Euglena chloroplast ribosome preparation subjected to
sedimentation with radioactive E. coli monosomes is shown in
Figure 3A. The Euglena ribosomes were isolated as shown in
Figure 1 using Triton X-100. The main peak of plastic ribo-
somes sediments slightly slower than the 70S E. coli mono-
somes, and we have assigned it a sedimentation coefficient of
68S, the value currently accepted for the Euglena chloroplast
monosome (2, 30). By comparison with the E. coli monosome,
the other two peaks have apparent sedimentation coefficients
of 89S corresponding to the Euglena cytoplasmic monosome
(2, 30) and 53S, thought by other workers to be the large chlo-
roplast subunit (2, 22, 24, 25, 30, 32, 34). A shoulder corre-
sponding to the chloroplast ribosome dimer (30) is seen at 100S.
Near the top of the gradient, only a negligible amount of the
small chloroplast subunit (35S) is seen. Our inability to obtain
the expected 2:1 ratio of large to small chloroplast ribosomal
subunits could be explained by the failure of the small subunit
to sediment through the sucrose cushion or by the degradation
of the small subunit.

B.2-limited cells having a very low amount of paramyllum and
a high Chl content (5) gave the best yields of chloroplast ribo-
somes. Ribosomes isolated from B.2-sufficient cells having a
high paramyllum content yielded preparations which gave the
sedimentation pattern seen in Figure 3B. The major ribosome
species is the 53S particle and few chloroplast monosomes are
obtained. The majority of the ribosomes isolated from cells with
a low paramyllum content are 68S chloroplast monosomes
(Fig. 3, A and E), indicating that the high paramyllum content is
correlated with the isolation of the 53S particle as the major
ribosome species. During isolation of the plastids, the paramy-
lum grain may be ripped from the pyrenoid region (Fig. 2, B
and C), tearing the outer chloroplast membrane and, perhaps,
showering other plastids in the exposure of the ribo-
somes to an unfavorable ionic environment which brings about
the formation of the 53S particle.

To maintain the pH of the cell lysate above 7, as recom-
pended by Avadhani and Butelow (2), 100 mM tris-HCl must
be added to buffer I and II. Figure 3C shows the sedimenta-
tion pattern obtained when ribosomes are extracted from chlo-
roplasts which have been isolated using 100 mM tris-HCl rather
than 5 mM HEPES in buffers I and II. The main peak contains
the 53S ribosomal particles. There are negligible amounts of
89S and 68S material. Using 100 mM HEPES in place of tris,
similar patterns are obtained. The high ionic strength of these
buffers is thought to make the chloroplasts leaky. Since the 53S
particle is formed at low magnesium concentrations (25, 32),
the 68S monosomes are probably derived from intact chloro-
plasts which have maintained a favorable internal ionic environ-
ment. The absence of 89S cytoplasmic monosomes in these
preparations suggests that the cytoplasmic ribosomes adhere
or are bound to the outer chloroplast envelope and can only be
removed by treatments which would disrupt this membrane,
such as high salt washes. Rawson and Stutz (30) also report an
inability to obtain high yields of chloroplast monosomes in the
absence of cytoplasmic contamination.

Detergents are normally used to release the chloroplast ribo-
somes from the isolated plastids even though it has been re-
ported that DOC can induce the dissociation of the chloroplast
monosomes (25, 32). When Triton (1.0% [v/v]) is used, all of
the extractable material is released after shaking the broken
chloroplasts for 15 min in the presence of detergent. The total
ribosome yield is about 50 A280 per 100 g wet weight of cells.
When DOC (0.1% [v/v]) is used, it is not necessary to add the
detergent to the sucrose cushion. The first DOC extraction
yields 70 A280 per 100 g wet weight of cells and an additional
25 A280 of ribosomal material can be obtained by re-extracting
the 20,000g DOC-treated chloroplast pellet. A significant
fraction of the chloroplast ribosomes fail to sediment through
the 40% sucrose, since ribosomal RNA is extractable from the
membranous material found on the sucrose cushion.

Ribosomes extracted with DOC or Triton give similar pat-
terns (Fig. 3, A and D) at the time of analysis on a sucrose
gradient indicating that the effects of the two are equivalent.
Ribosomes isolated by a second DOC extraction show an in-
creased amount of 53S material relative to the 68S peak (Fig.
3E). The second detergent extraction may induce the forma-
tion of 53S particles. For preparation of ribosomes to be used
for subsequent biochemical analysis, a single detergent extrac-
tion is recommended.

Stabilization of Isolated Chloroplast Monosomes. Figure 4
demonstrates the stabilizing effect of spermidine and merca-
toethanol on the purified chloroplast monosomes. Chloroplast
monosomes were isolated as shown in Figure 1. The ribosome
pellet was resuspended in standard buffer (tris-HCl, pH 7.6,
10 mM; magnesium acetate, 12 mM; KCl, 60 mM) with additions
as indicated and analyzed by centrifugation through linear 5
to 20% sucrose gradients prepared in the resuspension buffer.
The sedimentation pattern of ribosomes prepared in standard
buffer is shown in Figure 4A. The main peak is the 68S chloro-
plast monosome with smaller peaks in the 53S and 89 to 100S
regions of the gradient. Comparison with Figure 3B indicates
that the absence of spermidine and mercaptoethanol during the
5-hr centrifugation does not affect the sedimentation pattern.

Three fractions (89–100S, 68S, and 53S) were collected from
the gradients, pelleted by overnight centrifugation and rean-
alyzed on another set of linear sucrose gradients. The 68S frac-
tion purified in standard buffer sediments as a 53S particle on
the second sucrose gradient (Fig. 4B). The same result is ob-
tained when the 68S particle is dialyzed against standard buffer,
indicating that the monosome conversion is not an artifact pro-
duced exclusively by high hydrostatic pressure as shown with
sea urchin ribosomes (19). This conversion is not a simple dis-
sociation of the monosome to its subunits since a dissociation
of the 68S monosome would be expected to give a 53:35S A280
Fig. 3. Sucrose gradient sedimentation analysis of isolated chloroplast ribosomes. Ribosomal pellets were resuspended in buffer III and layered over 5 to 20% (w/w) linear sucrose gradients prepared in buffer V. The gradients were centrifuged for 5 hr at 25,000 rpm in the Beckman SW-27.1 rotor at 0 to 2°C and analyzed as described by Noll (26). A: Chloroplast ribosomes, (15- μg) extracted from three times washed chloroplasts with Triton X-100 were mixed with 3H-uracil E. coli ribosomes (0.2 A260) and layered onto the gradient. B: Chloroplast ribosomes prepared from cells harvested prior to becoming B12-limited. Ribosomes were extracted from three times washed chloroplasts with Triton X-100. C: Chloroplast ribosomes extracted with DOC from three times washed chloroplasts prepared using 100 mM tris-HCl, pH 7.6, in buffer I and buffer II. D: Chloroplast ribosomes extracted with DOC from the 20,000g chloroplast pellet.
Fig. 4. Stabilization of isolated chloroplast monosomes by spermidine and mercaptoethanol. Ribosomal pellets were resuspended in standard buffer (tris-HCl, pH 7.6, 10 mM; KCl, 60 mM; and magnesium acetate, 12 mM) with additions as indicated and centrifuged through a 5 to 20% (w/w) linear sucrose gradient prepared in the resuspension buffer. The 89 to 100S, 68S, and 35S fractions were collected, and the ribosomes were recovered by overnight centrifugation. The purified particles were resuspended using standard buffer with additions as indicated and centrifuged through a second linear 5 to 20% (w/w) sucrose gradient prepared in the resuspension buffer. Gradients were centrifuged and analyzed as in Figure 3. A: Chloroplast ribosomes extracted with DOC were resuspended in standard buffer, centrifuged, and the fractions indicated by arrows were collected for further analysis. B: Sedimentation pattern of the 68S fraction purified in standard buffer. C: Sedimentation pattern of the 68S fraction purified in standard buffer containing 7 mM mercaptoethanol. D: Sedimentation pattern of the 68S fraction purified in standard buffer containing 7 mM mercaptoethanol and 0.5 mM spermidine. E: Sedimentation pattern of the 53S fraction purified in standard buffer containing 0.5 mM spermidine. F: Sedimentation pattern of the 53S fraction purified in standard buffer containing 0.5 mM spermidine. G: Sedimentation pattern of the 89 to 100S fraction purified in standard buffer containing 7 mM mercaptoethanol and 0.5 mM spermidine.
ratio of 2, the mol wt of the large subunit being twice that of the small subunit. The ribosomes of Figure 4B have a 53:35S ratio of 4.35, a value inconsistent with a simple dissociation of the monosome. The purification procedure used, centrifugation or dialysis, contains no step during which a preferential loss of the small subunit would be expected, suggesting that the 68S monosome is not dissociating into two stable subunits. If 68S monosomes are isolated and repurified using standard buffer containing 7 mM mercaptoethanol, most of the ribosomal material is found in the 53S region of the gradient (Fig. 4C).

Figure 4D shows that spermidine alone can partially stabilize the chloroplast monosome. When monosomes are purified in standard buffer containing 0.5 mM spermidine, a large amount of the isolated monosomes resediment as 68S particles. Addition of spermidine and mercaptoethanol to the standard buffer results in almost complete stabilization of the 68S monosome during purification (Fig. 4E).

When E. coli ribosomes are heated above 60°C, there is a conversion of the 70S monosome into a 50S particle without the appearance of the 30S ribosomal subunit as well as an increased absorbance of the ribosomal preparation (23). Spermidine increases the Tm of ribosomes indicating an inhibition of temperature-induced conformational changes (9), and also promotes the association of ribosomal subunits (8). Spermidine and mercaptoethanol probably stabilize the Euglena chloroplast ribosome by maintaining a tight association between the subunits preventing the spontaneous conformational changes and the loss of ribosomal proteins which are responsible for the production of a 53S particle without the appearance of a 35S subunit.

The transformation observed in the absence of spermidine and mercaptoethanol is a property of the chloroplast monosome. Purification of the 53S particle in standard buffer with or without spermidine and or mercaptoethanol always gives the sedimentation pattern of Figure 4F. The 53S particle is the major species indicating that it is stable under all of the conditions tested.

Purification of the 89 to 100S fraction in standard buffer containing spermidine and mercaptoethanol gives the sedimentation pattern of Figure 4G. The pattern is similar to that of Figure 4A. The chloroplast dimer, cytoplasmic monosome, chloroplast monosome, and the 53S particle are found. This cannot be explained as contamination due to an incomplete separation of the 89 to 100S region from the 68S region in the initial centrifugation since the other regions of the gradient can be isolated relatively free of contamination (Fig. 4, B to F). We conclude that the 68S material arises from the dissociation of the chloroplast dimer during purification. The resulting monosomes are then converted to 53S particles. Buffers containing spermidine and mercaptoethanol reduce the extent of the time-dependent formation of the 53S particles.

Chloroplast monosomes resuspended in buffer containing spermidine and mercaptoethanol can be stored at 0°C for at least 3 days and at −80°C for at least 1 month without major modifications in the sedimentation pattern or distribution of the three ribosomal species.

Characterization of Ribosomal RNA on Polyacrylamide Gels. The cytoplasmic and chloroplast ribosomal RNA of Euglena can be separated by electrophoresis on composite agarose polyacrylamide gels (4, 6, 7, 24). To fully characterize our subcellular fractions, we have analyzed the distribution of cytoplasmic and chloroplast RNA.

The RNA extracted from whole cells is resolved into four high mol wt species (Fig. 5A). The two minor bands co-migrate with E. coli ribosomal RNA indicating mol wt of 1.1 × 10⁶ (23S) and 0.55 × 10⁶ (16S). These are the chloroplast ribosomal RNAs (2, 5, 6, 15, 24, 25, 32, 34). Using the relationship of

![Fig. 5. RNA composition of Euglena subcellular fractions. RNA was extracted from whole cells or purified subcellular fractions. Electrophoresis on 0.5% agarose-2.5% polyacrylamide composite gels was for 5 h at a constant voltage of 200. The gels were stained with methylene blue and scanned with a densitometer. Mol wt × 10⁴ and s values are given above each peak. A: RNA extracted from intact green cells of Euglena; B: RNA extracted from the three times washed chloroplast fraction; C: RNA extracted from the 20,000g chloroplast fraction after two treatments with DOC (twice extracted chloroplast pellet, Fig. 1); D: RNA extracted from the ribosome pellet. Ribosomes were extracted from the three times washed chloroplasts using DOC.

Peacock and Dingman (27), the electrophoretic mobilities of the two major species indicate mol wt of 1.35 × 10⁶ (25S) and 0.85 × 10⁶ (20S); these are the cytoplasmic RNAs (2–6, 15, 24, 25, 30, 32, 34). Since the ribosome is composed of equimolar amounts of the large and small RNA, the absorbance ratio of large to small RNA should be 1.6 for the cytoplasmic species and 2 for the chloroplast species if degradation has not occurred (15, 29). The RNA of Figure 5A has a cytoplasmic ratio of 1.59 and a chloroplast ratio of 2.22 indicating that a negligible amount of degradation occurs during RNA extraction. In agreement with other workers, the chloroplast RNA represents 24% of the total cellular RNA (6, 7, 15, 25).

The RNA extracted from the three times washed chloroplast fraction is shown in Figure 5B, and the RNA extracted from the 20,000g pellet obtained after two extractions with 0.1% DOC is seen in Figure 5C. In both cases the amount of cytoplasmic RNA is small, an indication of the purity of the chloroplast preparations. The main peaks are the chloroplast ribosomal RNAs; the ratio of large to small RNA is close to 2, indicating that the isolated plastids contain equimolar amounts of the two ribosomal subunits. The RNA extracted from the twice-extracted chloroplast pellet (Fig. 5C) indicates that detergent treatment does not result in the preferential extraction of one of the ribosomal subunits or in the degradation of the ribosomal RNA.

The RNA extracted from the purified ribosomal pellet is shown in Figure 5D. The two main peaks represent the chloro-
The ribosomal particle in the 89 to 100S, 68S, and 53S regions of sucrose gradients have been isolated, purified by sedimentation in a second linear sucrose gradient, recovered by precipitation with ethanol and the RNA isolated and characterized on composite agarose acrylamide gels. The ribosomes in the 89 to 100S region of the sucrose gradients contain the four major ribosomal RNA species and a small amount of material with a mol wt of $0.77 \times 10^6$ (19S) (Fig. 6A). The presence of chloroplast and cytoplasmic ribosomal RNA in this region of the gradient in nearly equal amounts confirms the earlier observation that this region is composed of a mixture of chloroplast dimers and cytoplasmic monosomes.

Figure 6B shows the electrophoretic profile of the RNA isolated from the 68S region of the sucrose gradient. A small amount (12%) of cytoplasmic RNA is found. The major portion of the RNA represents the large, $1.1 \times 10^6$ (23S), and the small, $0.55 \times 10^6$ (16S) chloroplast ribosomal RNAs. The degradation products, $0.77 \times 10^6$ (19S) and $0.43 \times 10^6$ (14S) of the large ribosomal RNA are also seen and they are probably identical to the 19S and 14S RNA reported by Zeldin and Schiff (36).

The electrophoretic pattern of the RNA extracted from the 53S region of the gradient is shown in Figure 6C. The main peak has a mol wt of $1.1 \times 10^6$ (23S) corresponding to the large chloroplast ribosomal RNA. Degradation products of this RNA, $0.77 \times 10^6$ (19S) and $0.43 \times 10^6$ (14S) (14), are also present in significant amounts. Another peak with a mobility indicating a mol wt of $0.55 \times 10^6$ (16S) represents 17% of the total RNA isolated. Since an RNA of this mol wt is not normally observed as an RNase-produced degradation product of the ribosomal RNAs of *E. coli* (14), but is in fact identical with the expected value for RNA from the small subunit, we conclude that this 16S RNA arises from the RNA of the 35S small chloroplast ribosomal subunit. It might be argued that this RNA arises as a contaminant from the 35S portion of the gradient, but this is unlikely since there is a negligible amount of this ribosomal particle present in our preparations (Fig. 4). Another possibility is that the 16S RNA is extracted from 68S monosomes which have not been fully separated by the sucrose gradients from the 53S particles. If this were the case, it can be calculated that 43% of the RNA isolated from the 53S particle and therefore, 43% of the total absorbance in the 53S region of the sucrose gradient would be contributed by the tailing edge of the 68S monosome peak. This is unreasonable since the absorbance of the 68S monosome peak (Fig. 4F) associated with purified 53S particles is never of a sufficient magnitude to contaminate the 53S region to this extent. Indeed a distinct 68S peak is not usually found in these preparations (Fig. 4). It is unlikely that this 16S RNA is extracted from aggregated 35S ribosomes which sediment at 53S, since under the ionic conditions used, *E. coli* small ribosomal subunits do not aggregate to a significant extent (33). We suggest then, that the 53S region of the gradient has large chloroplast ribosomal subunits containing 23S RNA together with ribosomal particles having both 16S and 23S ribosomal RNA in the normal proportions. If the 53S particle contains all of the 16S RNA, this would explain the absence of the small ribosomal subunit after the transformation of the 68S monosome to the 53S particle in the absence of spermidine (Fig. 4).

**CONCLUSIONS**

A procedure has been developed for obtaining reproducibly high yields of 68S chloroplast monosomes only slightly contaminated with cytoplasmic material. These monosomes can

Fig. 6. RNA composition of purified ribosomal particles. Ribosomes were extracted from three times washed chloroplasts using DOC. The ribosomal particles were separated and purified by centrifugation through two linear 5 to 20% (w/w) sucrose gradients as described in the legend of Figure 4. Ribosomes were collected from the second gradient, precipitated with ethanol, and their RNA was extracted. Conditions of electrophoresis are described in the legend of Figure 5. Mol wt $\times 10^6$ and s values are indicated above each peak. A: RNA extracted from the 89 to 100S fraction; B: RNA extracted from the 68S fraction; C: RNA extracted from the 53S fraction.
be purified by centrifugation and stored for several weeks allowing studies to be carried out.

The 68S chloroplast monosomes can be extracted from plastids isolated in buffers low in Mg$^2+$ if the plastids remain intact. Chloroplast monosomes prepared from intact plastidals always contain a significant amount of contaminating 98S cytoplasmic monosomes. These 98S monosomes are removed by procedures which disrupt the plastids and result in the isolation of 53S particles rather than 68S chloroplast monosomes. The cytoplasmic monosomes are probably attached to the chloroplast outer envelope and they may be the site of synthesis of chloroplast-localized cytoplasmically synthesized enzymes as has been suggested for the yeast cytoplasmic ribosomes which are found bound to mitochondria (20).

In the absence of spermidine and mercaptoethanol, the purified 68S monosome is transformed into a 53S particle without the concomitant production of one of the expected products of monosome dissociation, a 35S particle. Consistent with this, the 53S particle derived from 68S monosomes contains a significant amount of 0.55 × 10$^3$ mol wt RNA (16S) in addition to the expected 1.1 × 10$^3$ mol wt species (23S). Similar 53S particles are also unable to reassociate with 35S subunits isolated from E. coli or Euglena ribosomes under conditions which allow the reassociation of the Euglena 30S and E. coli 50S subunit (22). These properties of the 53S particle derived from 68S monosomes suggest that it is not a ribosomal subunit but that it may be a monosome which has undergone a conformational change or has been stripped of a large portion of its proteins while retaining both rRNAs, resulting in a decreased sedimentation coefficient. Other explanations can be offered but further work is necessary to characterize the 53S particle derived from the 68S monosome (31).

In any case, the methods which have grown out of these studies provide, for the first time, a high enough yield of stable intact 68S Euglena chloroplast ribosomes to permit biochemical characterization of ribosomal proteins (12) and the binding of antibiotics (31).

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LITERATURE CITED


