ISOLATION OF CYTOPLASMIC AND
CHLOROPLAST RIBOSOMES AND THEIR
DISSOCIATION INTO ACTIVE SUBUNITS
FROM CHLAMYDOMONAS REINHARDTII

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ABSTRACT

A mixture of cytoplasmic (80S) and chloroplast (70S) ribosomes from Chlamydomonas reinhardtii was freed of contaminating membranes by sedimentation of the postmitochondrial supernatant through a layer of 1.87 M sucrose. The purified ribosomes were separated into 80S and 70S fractions by centrifugation at a relatively low speed on a 10–40% sucrose gradient containing 25 mM KCl and 5 mM MgCl₂. Both the 80S and 70S ribosomes were dissociated into compact subunits by centrifugations in 5–20% high-salt sucrose gradients. The dissociations of both ribosomal species under these conditions were not affected by the addition of puromycin, indicating that the ribosomes as isolated were devoid of nascent chains. Subunits derived from the 80S ribosomes had apparent sedimentation coefficients of 57S and 37S whereas those from the 70S ribosomes had apparent sedimentation coefficients of 50S and 33S. In the presence of polyuridylic acid and cofactors, the 80S and 70S ribosomes incorporated [¹⁴C]phenylalanine into material insoluble in hot TCA. The requirements for incorporation were found to be similar to those described for eukaryotic and prokaryotic ribosomes. Experiments with antibiotics showed that the activity of the 80S ribosomes was sensitive to cycloheximide, whereas that of the 70S ribosomes was inhibited by streptomycin. The isolated subunits, when mixed together in an incorporation medium, were also active in the polymerization of phenylalanine in vitro.

INTRODUCTION

It has been known for some time that isolated chloroplasts are capable of incorporating amino acids into proteins (see reference 1). Many workers have shown that chloroplast ribosomes of both higher plants (2–5) and algae (6–10) have a sedimentation coefficient of approximately 70S and differ from the cytoplasmic 80S ribosomes in many aspects (see reference 1). These observations imply that the detailed but not the overall mechanism of protein synthesis in the chloroplast may be different from that in the cytoplasm. Before this question can be resolved, it is first necessary to obtain active 80S and 70S ribosomes as well as their subunits in a relatively pure state.

In Chlamydomonas reinhardtii, the 80S and 70S ribosomes sediment closely as two peaks when the postmitochondrial supernatant is centrifuged on a sucrose gradient (7, 10). These two species of ribosomes are present in the ratio of approximately 3:1, and it has been suggested that the 70S species is derived from the chloroplasts (7). This is supported by the observations that mutant strains of C. reinhardtii, which are deficient in chloroplast ribosomes as seen under the electron
microscope, also have reduced amounts of the 70S ribosomes\textsuperscript{1} (10, 12, 13). In this paper we present an improved method for the isolation of purified ribosomes from the extract of \textit{C. reinhardtii} and the fractionation of these ribosomes into the 80S and 70S species with little cross-contamination. Both of these species can be dissociated into their respective subunits by centrifugation in a high-salt sucrose gradient. The subunits thus isolated, as well as the monomers from which they are derived, are active in the incorporation of phenylalanine programmed by polyuridylic acid.

**MATERIALS AND METHODS**

**A. Conditions for the Culture of \textit{C. reinhardtii}**

The wild-type strain (137 c, mating type plus) of \textit{C. reinhardtii} was used in all the experiments described below. Cells of this strain were cultured in liquid Tris-acetate-phosphate medium at 25\textdegree C as described by Gorman and Levine (14). The culture was harvested at the exponential phase of growth (3-4 X 10\textsuperscript{8} cells/ml) by centrifugation at 0\textdegree C in a GSA rotor of the Sorvall RC-2B centrifuge (Ivan Sorvall, Inc., Newtown, Conn.) at 2,500 g for 5 min.

**B. Centrifugation Techniques and Fractionation on Sucrose Density Gradients**

Linear sucrose density gradients (12.5 ml) were prepared in cellulose nitrate tubes (9/\texttimes\ 3\texttimes\ 4 inches, Beckman Instruments, Inc., Fullerton, Calif.). All centrifugations of sucrose gradients were carried out with an SB 283 rotor of the International Equipment Company (IEC) centrifuge (B-60, Needham Heights, Mass.). The absorbance at 254 nm of each gradient was monitored with an Instrumentation Company (ISCO, Lincoln, Neb.) model D gradient fractionator and analyzer equipped with a flow cell of 0.5 cm light path. The absorbance profile was displayed with a 10-inch Bristol chart recorder (Bristol Div., American Chain & Cable Co., Waterbury, Conn.) such that the direction of centrifugation was from right to left. Monomers or subunits were recovered from the pooled fractions by centrifugation in a Spinco no. 40 rotor for 15 and 16 h, respectively, and were stored at -80\textdegree C in the form of pellets.

The apparent sedimentation constants of the monomers were designated as 80S and 70S based on earlier work (see reference 1). The apparent sedimentation constants of the subunits of these particles were determined by their sedimentation mobilities relative to the subunits of \textit{Escherichia coli} and rat liver ribosomes (see Results).

**C. Preparation of Purified Total Ribosomes**

Total ribosomes (cytoplasmic and chloroplast ribosomes) were prepared by a modification and extension of the procedure described by Hoober and Blobel (7). The cell pellet was washed once in a high-Mg\textsuperscript{2+} TKMD buffer, which contained 25 mM Tris-HCl (pH 7.5), 25 mM KCl, 25 mM MgCl\textsubscript{2}, and 5 mM dithiothreitol (DTT), and resuspended in the same buffer to a cell concentration of approximately 4-5 X 10\textsuperscript{9} cells/ml. The suspension was forced through a chilled French pressure cell maintained at a constant pressure of 4,800 lb/in\textsuperscript{2}. All subsequent operations were carried out at 4\textdegree C. The homogenate was centrifuged at 12,000 rpm for 10 min in a Sorvall SS-34 rotor, and 7.0 ml of the resulting postmitochondrial supernatant was layered over 2.5 ml of 1.87 M sucrose containing the high-Mg\textsuperscript{2+} TKMD buffer. After centrifugation at 40,000 rpm for 16 h in a Spinco No. 40 rotor, the ribosomes were sedimented to the bottom of the tube, whereas the photosynthetic membranes were retained at the 1.87 M sucrose interface. The surface of the pellet was rinsed twice with ice-cold deionized water, and either used directly or stored at -80\textdegree C for up to several weeks.

Ribosomal pellets prepared according to the above procedure contained a mixture of 80S and 70S ribosomes and were substantially free of contaminating proteins and photosynthetic membranes. The ratio of A\textsubscript{260} to A\textsubscript{230} of the postmitochondrial supernatant was between 1.40 and 1.50, and this value increased to 1.84-1.88 for the pellets.

**D. Separation of the 80S and 70S Ribosomes**

The ribosomal pellet was resuspended in ice-cold deionized water to a concentration of approximately 50 A\textsubscript{260} U/ml. About 0.5 ml of this was layered onto 10-40\% (wt/vol) linear sucrose gradients containing a low-Mg\textsuperscript{2+} TKMD buffer (same buffer as above, except that the Mg\textsuperscript{2+} concentration was

\textsuperscript{1} There is a possibility that a small fraction of the 70S peak might represent mitochondrial ribosomes; but, if present, they could not amount to more than a minor contamination since, compared with the chloroplast, mitochondria account for a much smaller fractional volume and have much lesser amounts of ribosomes as indicated by electron micrographs of whole cells of \textit{Chlamydomonas} (11).
lowered to 5 mM) and the gradients were centrifuged at 22,500 rpm for 15.5 h at 4°C. Fractions containing the 80S and 70S ribosomes (hatched areas in Fig. 2) were collected separately. In order to prevent dissociation of the ribosomes during subsequent centrifugation, the Mg2+ concentration in the pooled fractions was raised to 25 mM by the addition of appropriate amounts of a 1 M MgCl2 solution. The ribosomes were then sedimented by centrifugation as described in section E.

The RNA content of ribosomes was determined by a modification of the Schmidt-Tannhauser procedure (15). For both the 80S and 70S ribosomes it was found that 1 A450U of ribosomes contained 45 µg RNA. This value was used to compute the amount of ribosomes added to a reaction mixture.

E. Preparation of Subunits from 80S and 70S Monomers

Pellets of the 80S monomer obtained as above were resuspended in ice-cold deionized water and a high-salt compensating buffer was added such that the final ionic composition of the suspension was 50 mM Tris-HCl (pH 7.5), 500 mM KCl, 25 mM MgCl2, and 5 mM DTT. A sample (-20 A260 U) was layered onto a 5-20% (wt/vol) linear sucrose gradient which contained the 80S and 70S ribosomes (hatched fuged at 22,500 rpm for 15.5 h at 4°C. Fractions lowered to 5 mM) and the gradients were centrifuged in section C (see Fig. 4b). Subunits (L70 and S70) had the same ionic composition as the suspension, whereas L80 and S80 designate their counterparts in the chloroplast 70S ribosomes.

F. The High-Salt Puromycin Reaction

The high-salt puromycin reaction was carried out according to Blobel and Sabatini (16). The reaction mixture contained the following components: 40 µl of ribosomes in deionized water (3-5 A260 U), 10 µl of 10 mM puromycin (adjusted to pH 7.4 with KOH), and 50 µl of the appropriate compensating buffer. The ionic composition of the compensating buffer was twice the concentration of the sucrose gradient which had the same ionic composition as the suspension, and the gradient was centrifuged at 39,000 rpm for 3 h at 18°C. The separated large (L80) and small (S80) subunits were collected and pelleted as described in section C (see Fig. 4b). Subunits (L70 and S70) of the 70S monomers were prepared in the same way except that the KCl concentration was lowered to 400 mM (see Fig. 4d).

It was found that the addition of 5 mM DTT was necessary for the preparation of compact subunits from the 80S monomer since in its absence the subunits assumed smaller sedimentation coefficients and were unfolded. β-Mercaptoethanol can be substituted for the DTT.

G. Incorporation of [14C]Phenylalanine in the Presence of Polyuridylic Acid

The reaction mixture for the synthesis of polyphenylalanine contained the following components: Tris-HCl (pH 7.5), 25 µmol; KCl, 50 µmol; MgCl2, 6.25 µmol (for the 80S ribosomes or their subunits) or 11.25 µmol (for the 70S ribosomes or their subunits); DTT, 2.5 µmol; high-speed supernatant, 125 µl (6-8 mg protein/ml); pH 5 enzyme, 125 µl (10 mg protein/ml); creatine phosphokinase, 25 µg (1.87 U); phosphocreatine, 5 µmol; GTP, 0.25 µmol; ATP, 0.5 µmol; [14C]phenylalanine (383 µCi/µmol), 0.25 µCi; ribosomes, approximately 2.0 A260 U; polyuridylic acid (poly U), 200 µg; all in a final volume of 0.5 ml. All the components except poly U were mixed together at 0°C; the reaction was started by the addition of poly U and the immediate transfer of the mixture to a 37°C water bath. At 10, 20, 30, and 60 min, 50- or 100-µl samples of the mixture were pipetted onto Whatman 3 mM filter paper disks which were then extracted with hot 5% TCA, ethanol-ether (1:1), and ether as described by Mans and Novelli (17). Radioactivity was measured in 10 ml of Liquifluor-toluene mixture (40 ml Liquifluor, New England Nuclear, Boston, Mass., plus 960 ml toluene) with a Nuclear-Chicago Mark I scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.) which has an efficiency for 14C of 60%.

H. Preparations of the High-Speed Supernatant and the pH 5 Enzyme

The high-speed supernatant was prepared by centrifugation of the postmitochondrial supernatant (see section C) at 40,000 rpm for 4 h in a Spinco no. 40 rotor. The upper two-thirds of this supernatant was removed with a Pasteur pipette, passed through a Sephadex G-25 column, and stored at -196°C in 1.0-ml samples.

The pH 5 enzyme was prepared by the method of Falvey and Staehelin (18). 1 g wet weight of packed
cells was suspended in 2.0 ml of 10 mM Tris-HCl (pH 7.5) containing 5 mM MgCl₂ and 5 mM DTT, and was disrupted by a French pressure cell at 4,800 lb/in². This homogenate was centrifuged at 17,000 rpm for 15 min in a Sorvall SS-34 rotor, and the supernatant from this was centrifuged at 40,000 rpm for 3 h in a Spinco no. 40 rotor. The upper two-thirds of this supernatant was diluted with 2 vol of ice-cold 5 mM DTT and the pH was adjusted to about 5.1 by the dropwise addition of 1 M acetic acid. The precipitate was collected by centrifugation at 10,000 rpm in a Sorvall SS-34 rotor for 10 min and redissolved in the low-Mg²⁺ TKMD buffer to a protein concentration of about 10 mg/ml. The pH of the enzyme preparation was adjusted to 7.5 by the addition of 1 M KOH and the suspension was stored in 1.0-ml portions at -196°C.

I. Chemicals and Solutions

Chemicals were purchased from the following sources: ATP, GTP, DTT, creatine phosphate, creatine phosphokinase (E.C. 2.7.3.2., 75 U/mg), bovine serum albumin, chloramphenicol, cycloheximide, and streptomycin sulfate from Sigma Chemical Co., St. Louis, Mo.; [¹⁴C]phenylalanine (sp act 383 µCi/µmol), Liquifluor, and Protosol from New England Nuclear, Boston, Mass.; poly U from Miles Research Div., Miles Laboratories, Inc., Elkhart, Ind.; Sephadex G-25 from Pharmacia Fine Chemicals, Inc., Piscataway, N.J.; ultrapure grade sucrose (ribonuclease-free) from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N.Y.; and puromycin hydrochloride from Nutritional Biochemicals Corp., Cleveland, Ohio.

Stock solutions of sucrose were passed first through a 1.2 μm and then through a 0.45 μm Millipore filter (Millipore Corp., Bedford, Mass.). Stock solutions of KCl, Tris-HCl, and MgCl₂ were filtered through a 0.45 μm Millipore filter.

RESULTS

A. Separation of 80S and 70S Ribosomes

During disruption of C. reinhardtii by a French pressure cell the outer envelope of the chloroplast is damaged. The 70S ribosomes leak out of the chloroplast and are obtained together with the cytoplasmic 80S ribosomes in the postmitochondrial supernatant. To obtain a separation of these two species of ribosomes with minimum cross-contamination we took advantage of a previous observation (7, 10) that the 70S ribosomes, but not the 80S ribosomes, undergo a progressive reduction in sedimentation rate upon lowering of the Mg²⁺ concentration. The results in Fig. 1a show that an improved separation between the two species could indeed be obtained when the Mg²⁺ concentration was lowered from 25 to 5 mM. It should be noted that the 70S ribosomes did not sediment as a symmetrical peak; even at 25 mM Mg²⁺ there was a slower sedimenting shoulder which was resolved into an additional peak at 5 mM Mg²⁺ (Fig. 1a). Isolation of these two peaks which had apparent sedimentation coefficients of 56S and 59S, and subsequent recentrifugation under the conditions as in Fig. 4, showed that the 56S and 59S peaks were composed primarily
of the small and the large subunit, respectively, of the 70S ribosomes.

Further improvement of separation between the 70S and 80S species was obtained when the centrifugation was performed at a reduced speed (22,500 rpm for 15.5 h) and at 5 mM Mg$^{2+}$. Under these conditions (Fig. 1 b, right panel) the 70S ribosomes sedimented as a single broad peak of approximately 61S clearly separated from the 80S ribosomes.

The results illustrated in Fig. 1 a and b suggested that the 70S ribosomes sedimented as an equilibrium mixture of 70S monomers and their two subunits. This equilibrium was shifted to the subunits by low Mg$^{2+}$ concentration and/or by increasing pressure generated at a higher centrifugation speed (19-24). Thus, at 5 mM Mg$^{2+}$ and a high centrifugation speed (39,000 rpm for 5.2 h), the 70S ribosomes dissociated completely; because this occurred during the course of the centrifugation, the subunits assumed abnormally high apparent sedimentation coefficients of 59S and 56S (Fig. 1 a, right panel). At the same Mg$^{2+}$ concentration, however, the pressure generated at lower centrifugation speed (Fig. 1 b, right panel) was not sufficient to effect a complete shift of the 70S monomers toward subunits, and the equilibrium mixture sedimented as a single broad peak at about 61S. Furthermore, it should be noted that at this lower sedimentation pressure the 80S ribosomes sedimented as a sharp peak whereas at the higher pressure (Fig. 1 a, right panel) there was some broadening of the peak due to incipient dissociation resulting in an overlap with the 70S ribosomes.

From these data we adopted the low-Mg$^{2+}$ and low-pressure procedure for the separation of the 80S and 70S ribosomes (Fig. 2). Separate recentrifugations of the pooled 80S and 70S fractions under the hatched areas shown in Fig. 2 indicated less than 5% of cross-contamination (data not shown). The amount of ribosomes in the polysome region as determined in gradients centrifuged for a shorter time amounted to less than 10% (data not shown).

B. Dissociation of 80S and 70S Ribosomes into Active Subunits

Active subunits from ribosomes have been obtained by a variety of procedures. Methods for the dissociation of prokaryotic ribosomes, to which the chloroplast ribosomes are related, have been known for some time, and usually involve dialysis of ribosomes against buffers containing low concentrations of Mg$^{2+}$ (25). Eukaryotic ribosomes, on the other hand, have only recently been dissociated into active subunits and several methods have been used (16, 18, 26-32), the most frequent of which employs a high concentration of monovalent ions in the presence of Mg$^{2+}$ (16, 18, 26, 29-32). Detailed studies from several laboratories (16, 24, 29-31) have established that the ribosomal monomer occurs in an equilibrium with its two subunits. This equilibrium can be shifted toward full dissociation depending upon the parameters shown in Fig. 3. However, the parameters which effect complete dissociation into compact subunits will, beyond their threshold values, ultimately lead to the unfolding and inactivation of these subunits (16, 30, 31, 33). Furthermore, ribosomes which contain nascent chains, i.e., monosomes

![Figure 2: Preparative sucrose gradient for the separation of 80S and 70S ribosomes. 20 μg U of purified ribosomes were layered on the gradient as described in the text. The areas under the peaks cannot be quantitatively compared with each other, since at high concentrations absorbance readings traced by the ISCO analyzer are not linear.](image-url)
FIGURE 3 Schematic diagram of the relationships among monosomes, monomers, subunits, and derivatives of subunits. L and S are the large and small subunits of ribosomes; PM, puromycin.

and polysomes, do not participate in this equilibrium because the presence of nascent chains stabilizes the subunit interaction for unknown reasons. Only after artificial release of the nascent chains by puromycin (16, 27, 29, 31, 34) or natural release by in vitro readout of messenger RNA (18, 26, 30) do the resulting ribosomes behave like the monomers.

Fig. 4 b and d show that both the purified 80S and 70S ribosomes from *C. reinhardtii* dissociated into compact subunits at optimal ionic conditions (see below). Addition of puromycin did not significantly increase the amount of subunits (Fig. 4 a and c), indicating that both species of ribosomes were essentially free of nascent chains. Puromycin was therefore omitted in subsequent dissociation experiments. The results presented in Fig. 4 again show that the 80S and 70S ribosomal preparations were pure; the amount of cross-contamination was less than 5%.

We have studied in detail two of the parameters (Fig. 3) which affect the equilibrium between monomers and compact subunits: the monovalent and divalent ion concentrations. In the first series of experiments, the Mg\(^{2+}\) concentration in the sucrose gradient was held constant at 25 mM and the KCl concentration was varied from 25 to 700 mM. At 25 mM KCl (Fig. 5 a), the 80S monomer sedimented as a single peak with no apparent dissociation into subunits. With increasing KCl concentration there was a progressive reduction of the 80S peak and a concomitant dissociation into subunits which was complete at 500 mM. The absorbance profiles obtained between 200 and 300 mM show several not well resolved peaks sedimenting slower than 80S. These peaks probably contained an equilibrium mixture of monomers and subunits obtained at these intermediate KCl concentrations, concentrations which were below the threshold value necessary for complete dissociation. Similar anomalous patterns have been reported by other workers (22–24, 30, 31).

In contrast to the 80S monomer, the 70S mono-
FIGURE 5 Effects of different concentrations of KCl on the dissociation of 80S and 70S monomers at 25 mM Mg$_{2+}$. 80S or 70S monomers were suspended in buffers containing 50 mM Tris-HCl (pH 7.5), 25 mM MgCl$_2$, 5 mM DTT, and different concentrations of KCl as indicated in the figure. The ribosome suspensions were layered on 5-20% linear sucrose gradients containing the same ionic compositions as those of the suspensions. The gradients were centrifuged at 39,000 rpm for 1.8 h at 18°C. (a) 80S, 1.06 $A_{260}$ U; and (b) 70S, 1.02 $A_{260}$ U.

FIGURE 6 Effects of different Mg$_{2+}$ concentrations on the compactness of 80S and 70S subunits at 500 mM KCl. Ribosomes were suspended in high-salt buffers containing 50 mM Tris-HCl (pH 7.5), 500 mM KCl, 5 mM DTT, and different concentrations of MgCl$_2$ as shown in the figure. The ribosome suspensions were layered onto 5-20% linear sucrose gradients containing the same high-salt buffers. The gradients were centrifuged at 39,000 rpm for 8 h at 18°C. (a) 80S, 1.30 $A_{260}$ U; and (b) 70S, 1.23 $A_{260}$ U.

mer (Fig. 5 b) was already partially dissociated at the lowest KCl concentration tested (23 mM). Complete dissociation was obtained at 300-400 mM. Further increase in the KCl concentration to 700 mM did not seem to affect the sedimentation values of the subunits from both the 80S and 70S monomers.

The effects of decreasing Mg$_{2+}$ concentrations on the compactness of the 80S subunits were examined at 500 mM KCl. Fig. 6 a shows that the L$^{40}$ and S$^{40}$ exhibited differential sensitivity toward changes in Mg$_{2+}$ concentrations. The S$^{40}$ remained compact even when the Mg$_{2+}$ concentration was reduced to 5 mM whereas the L$^{40}$ began to unfold already at 15 mM. With decreasing Mg$_{2+}$ concentration in the gradient there was a concomitant reduction in the amount of compact L$^{40}$. At 5 mM Mg$_{2+}$, the unfolded derivative of the L$^{40}$ sedimented as a distinct peak slightly slower than the compact S$^{40}$. This unfolded derivative was not clearly resolved from the S$^{40}$ peak at the intermediate Mg$_{2+}$ concentrations (15-10 mM).

The subunits of the 70S monomer did not undergo any apparent unfolding as the Mg$_{2+}$
concentration was reduced from 25 to 5 mM (Fig. 6b). At lower concentrations (2.5 mM-0), however, both subunits also sedimented slower in the sucrose gradient and were presumably unfolded (data not shown).

The sedimentation coefficients of the compact subunits of both the 80S and 70S monomers were determined in a 5-20% linear sucrose gradient containing 50 mM Tris-HCl (pH 7.5), 500 mM KCl, 25 mM MgCl₂, and 5 mM DTT. Subunits derived from rat liver and *E. coli* were used as standards. Assuming sedimentation coefficients of 60S and 40S for the large and small subunits of rat liver ribosomes (35) and 50S and 30S for those of *E. coli* ribosomes (25), the following values were obtained: L₈₀, 57S; S₈₀, 37S; L₇₀, 50S; and S₇₀, 33S. The ratio of L₈₀ to S₈₀ varied between 1.75 and 1.95 whereas that of L₇₀ to S₇₀ was between 2.5 and 3.0, as measured by A₂₅₄.

In the scheme presented in Fig. 3 the isolated compact subunits should reassociate into monomers under the appropriate conditions. To achieve this, we lowered the KCl concentration to 25 mM. Under these conditions the two subunits derived from the 80S ribosomes reassociated into monomers (Fig. 7c). The two subunits of the 70S ribosomes, however, remained separated (Fig. 7f), and may require higher Mg²⁺ concentrations to achieve reassociation. It also can be seen that each subunit preparation showed little cross-contamination by its respective partners (Fig. 7a, b, d, and e).

**C. [¹⁴C]Phenylalanine Incorporation by Monomers and Isolated Subunits In Vitro**

Both the 80S and 70S monomers, collected as shown in Fig. 2, were active in polyphenylalanine synthesis in an in vitro system. The data in Table I indicate that the requirements were similar to those of other in vitro plant systems which have been examined (36-41). The requirements for GTP, ATP, and an ATP-generating system, as well as for the pH 5 enzyme fraction, were only partial. The high-speed supernatant was not required, indicating that perhaps all the protein factors and tRNA necessary for the phenylalanine incorporation were present in the pH 5 fraction. The polyphenylalanine-synthesizing activities of the monomer varied somewhat, depending on the preparations. The amounts of phenylalanine incorporated per milligram RNA after 60 min of incubation were 630-1,600 pmol for the 80S ribosomes and 424-1,100 pmol for the 70S ribosomes.
TABLE I
Requirements for \(^{14}C\)Phenylalanine Incorporation by 70S and 80S Ribosomes

| Conditions | 70S | 80S |
|------------|-----|-----|
| Complete   | 6,436 | 11,181 |
| - Ribosomes | 276 | 380 |
| - Poly U    | 366 | 374 |
| - High-speed supernatant | 8,727 | 13,115 |
| - High-speed supernatant and pH 5 enzyme | 1,514 | 2,683 |
| - pH 5 enzyme | 152 | 144 |
| - GTP       | 1,065 | 7,879 |
| - ATP       | 2,074 | 3,793 |
| - Phosphocreatine and creatine phosphokinase | 2,068 | 8,255 |

Experiments were performed as described in the text. Amounts of ribosomes used: 80S, 2.04 \(A_{260}\) U; 70S, 1.86 \(A_{260}\) U.

Boardman et al. (36) reported that the chloroplast ribosomes of tobacco leaves required higher Mg\(^{2+}\) concentration for optimum amino acid incorporation as compared with the cytoplasmic ribosomes of the same plant. Similar results were obtained with chloroplast and cytoplasmic ribosomes of \(C.\) reinhardtii as assayed with the phenylalanine-incorporating system (Fig. 8). The higher Mg\(^{2+}\) optimum observed with the 70S ribosomes was in the range of that reported for isolated yeast mitochondrial ribosomes (42) and for \(E.\) coli ribosomes (43).

The isolated subunits (cf. Fig. 4c and d) when mixed together were also active in polyphenylalanine synthesis in vitro. For the purpose of comparison, we assayed monomers and subunits derived from the same monomer preparation simultaneously (Fig. 9). The amounts of phenylalanne incorporation per milligram RNA after completion of the reaction (60 min of incubation) were: 80S monomer, 1,070 pmol; 80S subunit mixture, 950 pmol; 70S monomer, 495 pmol; 70S subunit mixture, 583 pmol. These results show that the recombined subunits had approximately the same activity as the monomer preparation from which they were derived, indicating that the high-salt treatment did not impair their activity.

From the above calculations it appeared that not all ribosomes had participated in the polymerization reaction. To obtain quantitative data on this point we exposed the ribosomes after incorporation to the dissociation conditions given in Fig. 4. It can be seen (Fig. 10a) that approximately 35\% of the subunits derived from the 80S ribosomes had reassociated into salt-resistant monosomes after synthesis of polyphenylalanine in the presence of poly U. The monosome peak contained most of the radioactivity in the sucrose gradient, and only after the addition of puromycin were these monosomes dissociated into subunits with the concomitant release of the labeled polyphenylalanine. Similar results were obtained with the subunits derived from the 70S ribosomes (Fig. 10b) except that the amount of subunits participating in polyphenylalanine synthesis was only about 8\%. Since no extensive investigation was made to establish the optimal conditions for incorporation, this low activity may not be the result of a large number of nonfunctional ribosomes or subunits but may be caused by some other deficiencies in the system. Assuming a molecular weight of \(4.5 \times 10^6\) for the 80S ribosomes (44) and \(2.7 \times 10^6\) for the 70S ribosomes (25), it can be calculated that an average of about 3.8 and 8.7 phenylalanine residues, respectively, are polymerized on each monosome.

Sucrose gradient analyses of the amino acid incorporation mixture with either monomers or subunits failed to show the formation of active ribosomes larger than monosomes. Since it is known that the sedimentation pattern of a ribosome-poly U mixture depends on the molar ratio of these two components (45), and since the incubation mixture used in our experiments contained a large excess of poly U over ribosomes, it was not surprising that only monosomes were detected.

D. Effects of Antibiotics on the \(^{14}C\)Phenylalanine Incorporation by the 80S and 70S Monomers

In vivo and in vitro experiments with a number of antibiotics have indicated that the 80S and 70S ribosomes exhibit different sensitivities toward inhibition of antibiotics (see reference 1). Cycloheximide has been shown to affect protein synthesis with the eukaryotic 80S ribosomes, whereas chloramphenicol and streptomycin exert their effects on the prokaryotic 70S ribosomes (see reference 46). Similar results were obtained with the 80S and 70S ribosomes from \(C.\) reinhardtii in
the poly U-directed polyphenylalanine synthesis (Table II). Cycloheximide inhibited the 80S system by approximately 50%. However, there was only a low level of inhibition by chloramphenicol in the 70S system. This observation is in agreement with results obtained with E. coli ribosomes, where it was shown that the degree of inhibition by chloramphenicol depended to a great extent on the base composition of the synthetic messenger RNA (47). In our experiments the most complete inhibition of the 70S system was achieved with streptomycin. This finding is consistent with the observation that this antibiotic blocks protein synthesis on bacterial ribosomes (46) and also interferes with the synthesis of several chloroplast components during the light-induced chloroplast development in Euglena gracilis (48).

DISCUSSION
Under our preparation conditions almost all the ribosomes in the 80S and 70S peaks (Fig. 2) were devoid of nascent chains as demonstrated by the lack of a puromycin effect (see Results). The amount of ribosomal materials which sedimented faster than 80S was between 5 and 10%, depending on the preparations, and this material consisted of small polysomes of the 80S type. Since it is difficult to imagine that approximately 90% of the cytoplasmic ribosomes and almost all of the chloroplast ribosomes of the cells were inactive in vivo, the apparent lack of active ribosomes must be an artifact of our isolation procedure. Since we have recently found (manuscript in preparation) that it was possible to increase the proportion of the polysomal materials to 30–40% by previous cooling of the culture in acetone-dry ice before harvest or by the addition of 80S- and 70S-specific antibiotics which block polypeptide chain elongation, a large proportion of the 80S and 70S monomers must have been derived from the runoff of subunits from polysomes in vivo, perhaps as a result of slow cooling (49), from the 25°C temperature of the culture to the 0°C of the harvesting. The most
striking example of the accumulation of runoff ribosomes during slow cooling has been obtained with chicken embryo in which the resulting monomers subsequently crystallized into sheets of tetramers (30).

Fig. 3 summarizes the results presented in this paper concerning the relationships among monomers, monomers, and subunits. The dynamic equilibrium between a monomer and its constituent subunits is governed by a least five variables (16, 24, 29): (a) KCl concentration, (b) Mg$^{2+}$ concentration, (c) temperature, (d) centrifugation speed (pressure), and (e) ribosome concentration. At a constant centrifugation speed and a fixed amount of ribosomes, there is a narrow range of ionic concentrations and temperature in which dissociation of monomers into active subunits occurs. Beyond this range, the active subunits may be irreversibly converted into unfolded derivatives which have smaller sedimentation coefficients and which are inactive. In order to ensure that the latter reaction does not occur, it is necessary to establish the ionic conditions and temperature required for complete dissociation in each system.

The effects of KCl and Mg$^{2+}$ concentrations in the monomer ↔ subunit equilibrium are antagonistic: an increase in the KCl concentration favors dissociation whereas the reassociation of subunits is facilitated by high Mg$^{2+}$ concentration. Several workers (16, 24, 29, 30) have shown that there is a critical K$^+$:Mg$^{2+}$ ratio necessary for complete dissociation. In addition, Faust and Matthaci (31) have also stressed the importance of an absolute KCl concentration. Table III lists the ionic conditions used for the isolation of subunits from the 80S and 70S monomers of *C. reinhardtii* compared with those used for the isolation of active subunits from other sources. The 80S ribosomes of *C. reinhardtii* differ strikingly from all other eukaryotic ribosomes investigated, in that the subunits are stable only in a very narrow range of K$^+$:Mg$^{2+}$ ratios. Approximately 95% of the monomers dissociate into subunits at 500 mM KCl and at a K$^+$:Mg$^{2+}$ ratio of 20. The latter value is about five times lower than the usual value found for other cytoplasmic ribosomes from either plant or animal sources (Table III). Inactivation of the L$^{48}$ occurred when the Mg$^{2+}$ concentration was de-
FIGURE 10 Sucrose gradient analyses of subunits after [14C]phenylalanine incorporation. (a) The [14C]phenylalanine incorporation mixture (1 ml) containing 5.92 A260 U of LS0 and 2.92 A260 U of S80 was incubated at 37°C for 90 min and then chilled on ice to stop the reaction. 350 µl of the solution was mixed with 50 µl of 10 mM puromycin (pH 7.0) followed by 400 µl of compensating buffer so that the final ionic composition was 50 mM Tris-HCl (pH 7.5), 500 mM KCl, 25 mM MgCl2, and 5 mM DTT. After an incubation period of 10 min at 0°C, 400 µl (0.89 A260 U of L80 and 0.44 A260 U of S80) of the reaction mixture was layered onto a high-salt 5-20% linear sucrose gradient containing 500 mM KCl and was centrifuged at 39,000 rpm for 1.8 h at 18°C. A second sample was processed in the same way except that 50 µl of deionized water was used instead of puromycin. After centrifugation, the gradient was fractionated into 0.5 ml portions. 0.1 ml of 1% bovine serum albumin was added to each fraction as a carrier, followed by 0.2 ml of 2 N KOH. After incubation at 37°C for 30 min, 0.2 ml of 2 N HCl was added to neutralize the base. The proteins were precipitated with 4 ml of ice-cold 10% TCA. After being kept at 4°C overnight, the precipitate was washed twice with 5 ml of ice-cold 5% TCA, dissolved in 0.5 ml of Protosol, and the radioactivity was measured in 8 ml of the Liquifluor-toluene mixture with a Nuclear-Chicago Mark I scintillation counter. The counting efficiency for 14C in this system was about 81%. 695 cpm corresponds to the incorporation of 1 pmol of phenylalanine. A control sample was also incubated in the same reaction mixture except without poly U. After 90 min a sample containing 0.89 A260 U of L80 and 0.44 A260 U of S80 was layered onto a high-salt buffered gradient as described above. This gradient was not processed for radioactivity. (b) Experiments were carried out and processed for counting as in a. The following amounts of ribosomes were layered onto the gradients: minus poly U, 0.89 A260 U of L70 and 0.44 A260 U of S70; plus poly U, 1.08 A260 U of L70 and 0.53 A260 U of S70; plus poly U and puromycin, 1.08 A260 U of L70 and 0.53 A260 U of S70.
creased to a level below 25 mM (Fig. 6a). In contrast to the 80S ribosomes, subunits of the 70S ribosomes can tolerate a wider variation in the K⁺:Mg²⁺ ratio without being converted to slower sedimenting derivatives (Fig. 6b).

It was shown previously that the L₈₀ and S₃₀ of C. reinhardtii contain 2₅S and 1₈S RNA, respectively (7, 10). In a high-salt sucrose gradient, both the L₈₀ and the S₃₀ sedimented slightly slower than their counterparts in rat liver ribosomes.

| Table II |
| Effects of Antibiotics on the In Vitro [¹⁴C]Phenylalanine Incorporation by 80S and 70S Monomers |

| Conditions   | 80S  | 70S  |
|--------------|------|------|
| Control      | 100  | 100  |
| + Cycloheximide | 54.5 | 95.5 |
| + Chloramphenicol | 100  | 84.5 |
| + Streptomycin | 96.5 | 19.5 |

Experiments were carried out as described in Materials and Methods. The following concentrations of antibiotics were used: cycloheximide, 100 μg/ml; chloramphenicol, 85 μg/ml; streptomycin sulfate, 100 μg/ml. 100% activity corresponds to 915 pmol of phenylalanine incorporated/mg RNA for the 80S and 753 pmol phenylalanine incorporated/mg RNA for the 70S.

The large subunit of the latter contains 2₈S RNA (54) and this may account for the differences in sedimentation coefficients. With chloroplast 70S ribosomes, the L₇₀ and the large subunit of E. coli ribosomes sedimented at the same position in a high-salt sucrose gradient whereas the S₇₀ sedimented faster than its counterpart in E. coli ribosomes. Boardman et al. (36) obtained the following sedimentation coefficients for subunits from the chloroplast and cytoplasmic ribosomes of tobacco leaves: cytoplasmic large subunit, 5₈S; cytoplasmic small subunit, 3₅S; chloroplast large subunit, 5₁₆S; and chloroplast small subunit, 3₅S. These values are in excellent agreement with those reported here for the subunits of 80S and 70S ribosomes of C. reinhardtii.

The sedimentation coefficients of the high-salt-derived subunits reported in this paper are higher than those found previously (7). These discrepancies may be attributed to different methods of preparing the subunits. Hoober an Blobel (7) obtained their subunits at 50 mM KCl and 0.1 mM Mg²⁺ or at 50 mM KCl without Mg²⁺, whereas it is now known that with mammalian ribosomes, chelation of Mg²⁺ by EDTA leads to the formation of inactive subunit derivatives which move slower than active subunits obtained by high-salt treatment (26, 30, 33).

The subunits of both the cytoplasmic and chloroplast ribosomes spontaneously reassociated.

| Table III |
| Ion's Conditions for the Isolation of Active Subunits from Ribosomes of Various Sources |

| Source of ribosomes              | K⁺   | Mg²⁺ | K⁺/Mg²⁺ |
|----------------------------------|------|------|---------|
| Rat liver and muscle             | 880  | 12.5 | 70.5    |
| Rat liver                        | 500  | 5    | 100     |
| Rat and mouse liver              | 500  | 2-3  | 166-250 |
| Dog pancreas                     | 500  | 3    | 166     |
| Sea urchin                      | 240  | 5    | 48      |
| Mouse plasmacytoma tumors       | 300  | 5    | 60      |
| Chicken embryo                   | 500  | 5    | 100     |
| Mouse lymphoma cells             | 500  | 5    | 100     |
| Murine plasma cells              | 500  | 5    | 100     |
| Pea seedlings                    | 500  | 5    | 100     |
| Rice embryo                     | 500  | 4    | 125     |
| Neurospora crassa                | 800  | 7    | 114     |
| Yeast mitochondria               | 500  | 10   | 50      |
| Chlamydomonas 90S                | 500  | 25   | 20      |
| Chlamydomonas 70S                | 500  | 5-25 | 20-100  |

* NH₄Cl was used instead of KCl.
to form active ribosomes when they were incubated under conditions for amino acid incorporation. The monosomes, which carried nascent polyphenylalanine chains, were susceptible to dissociation in high salt only after the chains had been removed by puromycin treatment (Fig. 10). These observations confirm the earlier work on mammalian (16, 18, 26, 34), yeast (55) and Neurospora crassa (53) ribosomes, and give further emphasis on the role played by the nascent chain in the stabilization, under high ionic strength conditions, of subunit couples in both the 80S and 70S systems.

The polyphenylalanine-synthesizing activities of the 80S monomer and its subunits were less than those of other eukaryotic ribosomes (16, 18, 26, 34). Falvey and Stachelin (18) estimated that 50-60% of their mouse liver subunit preparation was active in in vitro polypeptide synthesis, and each active ribosome couple had about 15-20 polyphenylalanine residues. With our subunit preparation from the 80S ribosomes, approximately one-third of the subunits reassociated into active ribosomes and there were about 4 polyphenylalanine residues per monosome.

Previous investigations on chloroplast protein synthesis have been carried out mainly with isolated chloroplasts (see reference 1) or chloroplast extracts (3, 56-58). Only a few attempts have been made to isolate purified ribosomes and to test for their protein synthesis capacity in a reconstituted system in vitro. Eisenstadt and Brawerman (59) first reported that isolated Euglena chloroplast ribosomes are able to incorporate amino acids into protein. They characterized the ribosomes as having a sedimentation coefficient of 60S containing 14S and 19S RNA (60). Subsequent investigations by Rawson and Stutz (8), however, provided clear evidence that intact chloroplast ribosomes in fact sediment at 70S and contain 22S and 17S RNA. With higher plants, both Boardman et al. (36) and Van Kammen (61) obtained active chloroplast ribosome fractions from tobacco leaves but the preparation was 50% contaminated by cytoplasmic ribosomes. In all these cases, the activities of the chloroplast ribosomes were uniformly low. Recently, Hadziyev and Zalik (38) and Avadhani and Buetow (41) reported the isolation of active chloroplast polyribosomes from wheat and Euglena, respectively.

In this paper, we describe a procedure for the purification of chloroplast 70S ribosomes from extracts of C. reinhardtii. The purified preparation was contaminated by less than 3% of the 80S ribosomes and it could synthesize polyphenylalanine in an in vitro system with activities, at completion of incorporation, of 424-1,100 pmol phenylalanine/mg RNA. These activities are within the range of those reported for isolated active mitochondrial ribosomes (42) and are 4-20 times higher than those of related plant systems (36, 59).

Subunits prepared from the 70S monomers by high-salt dissociation still retain their capacities to incorporate phenylalanine in vitro. It was estimated that about 8% of the subunits reassociated into monosomes, and each monosome had approximately 8-9 polyphenylalanine residues. Similar values were also obtained with the monomers from which the subunits were derived (unpublished results). Therefore, the fact that only a small amount of the subunits were active in vitro could not be an artifact resulting from exposure to high salt. In Euglena, it has been reported (62) that the 50S and 30S subunits of chloroplast ribosomes when mixed together failed to incorporate phenylalanine programmed by poly U. However, a heterologous mixture of chloroplast 30S subunits and E. coli 50S subunits showed some incorporation activity, suggesting that the 50S subunits of the chloroplast ribosomes were preferentially inactivated by the isolation procedure.

It is also known that under certain conditions, both subunits of the E. coli ribosomes may be inactivated without a change in their sedimentation values, and the inactivated subunits may be reactivated by heat treatment (63, 64). We are at present investigating the possibility of increasing the yield of active 70S ribosomes by similar treatments. After the completion of this work, Grivell and Groot (65) reported the successful isolation of active chloroplast ribosomes and subunits from spinach leaves. Their experiments, however, were carried out with supernatant factors from E. coli.

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