Assembly of Escherichia coli 50 S Ribosomes from Ribonucleic Acid and Protein Components

I. CHEMICAL AND PHYSICAL FACTORS AFFECTING THE CONFORMATION OF ASSEMBLED PARTICLES*

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SUMMARY

The *in vitro* assembly of Escherichia coli 50 S ribosomes from the ribonucleic acid and protein components of 50 S ribosomes was attempted. The two components were prepared by subjecting 50 S ribosomes to LiCl-urea treatment. The ribonucleic acid fraction contains 23 S RNA and 5 S RNA in equimolar amounts and is free from significant amounts of protein. The protein fraction is composed of all of the protein subunits of 50 S ribosomes. The reconstitution experiments were carried out in a standard system: 0.01 M Tris HCl, pH 7.5-0.3 M KCl-0.02 M magnesium acetate-0.006 M β-mercaptoethanol. The ribosomal RNA and protein components were incubated in the standard system at 42°C for 15 min. These conditions permitted the formation of 30 S ribosomes from their components with 72% recovery of the original activity, as measured by polyuridylic acid-directed phenylalanine incorporation. Under identical conditions, the RNA and protein components of 50 S ribosomes were assembled stoichiometrically into 37 S particles. In a search for a chemical agent that would effectively reassemble particles of more compact structure, we found that spermine or spermidine or both, in addition to Mg++, led to the formation of 45 to 48 S ribonucleoprotein particles with all of the macromolecular components of 50 S ribosomes. The 45 to 48 S particles and 37 S particles were inactive in terms of polyuridylic acid-directed phenylalanine incorporation. Under identical conditions, the RNA and protein components of 50 S ribosomes were assembled stoichiometrically into 37 S particles. In a search for a chemical agent that would effectively reassemble particles of more compact structure, we found that spermine or spermidine or both, in addition to Mg++, led to the formation of 45 to 48 S ribonucleoprotein particles with all of the macromolecular components of 50 S ribosomes. The 45 to 48 S particles and 37 S particles were inactive in terms of polyuridylic acid-directed phenylalanine incorporation, the peptidyltransferase reaction and peptide chain termination reaction. We also showed that 23 S ribonucleoprotein particles are assembled in the absence of Mg++ and polyamines. The particles continuously increase in sedimentation constant from 23 S to 26 S when Mg++ is gradually increased to 10^{-2} M. Above 10^{-2} M Mg++, 36 S particles appear and the sedimentation constant of the particles plateaus at 37 to 39 S at the concentration higher than 2 × 10^{-2} M Mg++. Spermine or spermidine, in addition to Mg++, further increased the sedimentation constant of the particles continuously to 45 to 48 S. The process is the reversal of the previously reported unfolding of 50 S ribosomes by citrate Mg++ treatment. NHCl in place of KCl, or the pH in a range between 7 and 8 had no significant effects on the ribosomal assembly. Mg++ or Mg++ plus polyamine(s) have to be added to the assembly system at zero time. The later those cations are added, the more unfolded are the resultant particles. The incorporation of 5 S RNA into the assembled ribonucleoprotein particles is stoichiometric and highly specific so that no significant incorporation of tRNA was observed.

The ribosome is a cytoplasmic organelle which provides a site for protein synthesis. When functioning, the ribosome exists as a complex of a large ribosomal subunit and a small ribosomal subunit and engages in the translation of a genetic message, retained in the nucleotide sequence of messenger RNA, into the corresponding specific sequence of a polypeptide. In Escherichia coli a large ribosomal subunit is a 50 S ribosome which consists of 1 molecule each of 23 S RNA (1), 5 S RNA (2), and approximately 35 different proteins (3). A 30 S ribosome, a small subunit, is made of 1 molecule each of 16 S RNA (1) and 21 distinct proteins (4). Certain specific proteins (split proteins) are dissociated from the ribosomal subunits when they are subjected to CsCl density gradient centrifugation (5). The resulting protein-deficient particles, which consist of ribonucleic acid and the remainder of proteins, are called CsCl core particles. Functionally active ribosomes can be reconstituted from CsCl core particles and split proteins (6, 7). The reconstitution process is so specific that only the homologous CsCl core and split proteins form functionally active particles.

The initial attempt at the total reconstitution of ribosomal subunits was made by mixing, in the cold, the ribonucleic acid and proteins prepared by LiCl-urea treatment (8, 9) of the sub-
units. The resultant ribonucleoprotein particles were, however, inactive in amino acid incorporation (10). The successful total assembly of 30 S ribosomes was achieved by Traub and Nomura (11), and required incubation at 37°C. Recently, active 50 S ribosomes were assembled from the mixture of 23 S RNA, 5 S RNA, and proteins in thermophilic bacteria (12), as well as in an E. coli (13) system. In the E. coli system, however, the protein fraction has to be kept in a mixture of albumin, oligonucleotides, and other ingredients. Attempts to purify ribosomal proteins resulted in a loss of activity in the reassembled particle. In the case of the thermophilic bacteria, the formation of active 50 S ribosomes takes 80 min of incubation at 60°C, a marked contrast to the rapid physiological process. Moreover, in vitro reassembled ribosomes sediment slightly slower than 50 S ribosomes in a sucrose gradient, indicating a partially unfolded structure. Therefore, it is still necessary to establish a reconstitution system in which functionally active, as well as physically intact, 50 S ribosomes can be assembled from the protein subunits and two RNA species of E. coli, a mesophilic organism, in order to explore the role of the individual ribosomal components in protein synthesis and the mode of assembly of ribosomes.

Our early efforts to assemble 50 S ribosomes from their ribonucleic acid and protein components led to the formation of particles that sedimented slower than 50 S ribosomes but had all the macromolecular components of 50 S ribosomes. A systematic search for the physical and chemical factors responsible for proper assembly of the particle enabled us to produce ribonucleoprotein particles of the same macromolecular composition as 50 S ribosomes and with a sedimentation constant very close to that of the original 50 S ribosomes.

MATERIALS AND METHODS

Chemical Reagents—β-Mercaptoethanol and dithiothreitol were products of Calbiochem. Spermide, spermidine, putrescine, tetrahydrofurfurate, and yeast concentrate were obtained from Sigma Chemicals. Yeast concentrate contains 10 to 20% NAD+, 5 to 10% NADP+, 1 to 3% coenzyme A, mixtures of nucleosides, nucleotides, coenzymes etc. Trinucleotide codons, AUG, UAG, and UAA were purchased from Miles Laboratories, Inc. [3P] i-[U-14C]phenylalanine (380 mCi per mmole) and sodium [3C]formate (56.8 mCi per mmole) were products of New England Nuclear. Purified peptide chain release factor Rr of E. coli was kindly supplied by A. L. Beaudet, Dr. C. T. Caskey's laboratory, National Heart Institute, National Institutes of Health. Formyltetrahydrofurfurate synthetase was a kind gift from Dr. Jesse C. Rabinovitz, Department of Biochemistry, University of California, Berkeley.

Phenol was redistilled before use. Pancreatic RNase and RNase-free DNase were purchased from Worthington Biochemicals. Other chemicals were the purest grades obtainable. All experiments were performed at 4°C unless otherwise specified.

The following buffer solutions were used in this work: Buffer A, 0.01 M Tris-HCl, 0.03 M NH4Cl, 0.01 M magnesium acetate, 0.006 M β-mercaptoethanol, pH 7.8; Buffer B, same as Buffer A except pH 7.5; Buffer C, same as Buffer A except that Mg2+ is 0.0003 M; Buffer D, 0.01 M Tris-HCl, 0.03 M NH4Cl, 0.001 M magnesium acetate, pH 7.5; Buffer E, 0.01 M Tris-HCl, 0.3 M KC1, 0.006 M β-mercaptoethanol, pH 7.5; Buffer F, same as Buffer E except that β-mercaptoethanol is replaced by 0.001 M dithiothreitol. Sucrose solutions for preparative gradient centrifugation were made in Buffer A or Buffer B and those for analytical gradient centrifugation were made in the same buffers without β-mercaptoethanol. Solutions of urea, sucrose and buffer systems were treated with bentonite. After this treatment, Mg2+ was added if needed and the pH was finally adjusted.

Preparation of 50 S and 30 S Ribosomes—E. coli strain Q13 (Tyr-, Met-, RNase F-, polynucleotide phosphorylase trace) was the bacterial strain used and was kindly supplied by Dr. Walter Gilbert, Biological Laboratories, Harvard University. The bacteria were grown in enriched nutrient broth (1.2% nutrient broth, 0.7% NaCl, 0.1% yeast extract, pH 7.5) at 37°C with aeration. The cells were collected by centrifugation and stored at -70°C until needed. The bacterial paste was suspended in Buffer A, disrupted by a cell mill with glass beads (0.2 mm diameter), diluted with an equal volume of Buffer A, and promptly filtered by suction in order to remove the glass beads. The filtrate was cleared by centrifugation at 15,000 × g for 30 min. The supernate was further centrifuged at 78,000 × g for 7 hours to sediment the crude ribosomes. The upper part of the pellet consisted of DNA and membranes; it was removed by a spatula. The ribosomal pellet was then fractionated into 50 S and 30 S ribosomes by means of zonal sucrose gradient centrifugation, as described previously (14). Purified preparations of 50 S and 30 S ribosomes were stored at -70°C.

Preparation and Assembly of Ribonucleic Acid and Protein Components of Ribosomes—The ribosome suspension at a concentration of 100 to 160 A260 units per ml in 2.2 M LiCl, 4.4 M urea, and 0.001 M dithiothreitol was allowed to stand for 24 hours at 4°C. The white precipitate of RNA-Li salt was collected by centrifugation, as described previously (14). Unlabeled 5 S RNA and tRNA were prepared by the same technique and were used to dilute 32P-labeled RNA to obtain an appropriate specific radioactivity.

Polycrystalline Gel Electrophoresis—Solutions of ribosomal

1 K. Hosokawa, and M. Nomura, unpublished results.
proteins were prepared as follows. To the original and reconstituted ribosomal particles were added 1.2 volumes of 8 M urea, 4 M LiCl-0.002 M dithiothreitol. The mixture was kept at 0° for 24 hours and centrifuged at 8000 rpm for 5 min to sediment the insoluble Li-salt of RNA. The supernatant was dialyzed against 6 M urea-0.002 M sodium acetate-0.006 M β-mercaptoethanol, pH 5.2 for 6 hours and concentrated by dry Sephadex powder to an appropriate volume. The concentration of protein was calculated from the final volume and the starting amount of ribosomes. Usually 6 unit eq (unit equivalent is the amount of ribosomal protein equivalent to 1 A_{460} unit of ribosomes, which is defined as the amount of ribosomes giving A_{460} of 1000 through 1 cm light path when dissolved in 1 ml of Buffer A) of sample were subjected to polyacrylamide gel electrophoresis. The method of a preparation of polyacrylamide gel and the conditions for the electrophoresis were as described previously (14).

Other Procedures for Analysis—Methylated albumin and methylated albumin-Kieselguhr columns were prepared according to the method of Mandell and Huesey (18). The chromatographic analysis of RNA on methylated albumin Kieselguhr columns and methods of analysis of ribonucleoprotein particles and RNA by sucrose gradient centrifugation were described previously (14), except that the condition for centrifugation is specified in each case. Svedberg unit of the sedimentation coefficient of particles was determined as described previously (14).

RESULTS AND DISCUSSION

Ribonucleic Acid and Protein Components of Ribosomes

Fifty S or 30 S ribosomes were fractionated into the protein and ribonucleic acid components by LiCl-urea treatment as described under "Materials and Methods". The RNA fraction, after dialysis to remove Li^+, was analyzed by sucrose gradient centrifugation, as well as by methylated albumin Kieselguhr chromatography. The sedimentation profile of the RNA from 30 S ribosomes showed a faster sedimenting zone of 28 S in addition to the major peak of 23 S RNA and a small peak of 5 S RNA. Twenty-eight S RNA is thought to be a dimer of 23 S RNA, presumably formed during the preparation in a high ionic strength environment (2 M LiCl and 0.3 M KCl). Assuming that the 28 S RNA is a dimer, it can be stoichiometrically converted to 23 S RNA by incubation in Buffer A at 37° for 1 min, though it is not shown here, or by treatment with sodium citrate as presented in Table I. As shown in Fig. 1, RNA prepared from 50 S ribosomes by the LiCl-urea method is eluted from a methylated albumin Kieselguhr column in two distinct peaks corresponding to 5 S RNA and 23 S RNA. The two RNA species exist in equivalent amounts as shown in Table I. The sucrose gradient sedimentation profile of RNA prepared from 30 S ribosomes also showed a faster sedimenting peak of 23 S in addition to the peak of 16 S RNA. The 23 S RNA obtained from 30 S ribosomes is stoichiometrically converted to 16 S RNA by incubation at 37° or treatment with a chelating agent (Table I). Therefore, the 23 S RNA is assumed to be a dimer of 16 S RNA.

Ribosomal RNA thus prepared was used for reassembly experiments and was efficiently incorporated into particles assembled in vitro. Thirty S ribosomes reconstituted with RNA prepared by the LiCl-urea method exhibited more than two-thirds of the original activity (see next section).

The RNA prepared from 50 S ribosomes was treated with RNase and analyzed for protein by polyacrylamide gel electrophoresis. Although no protein band was seen in a gel to which

<table>
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<th>RNA derived</th>
<th>RNA species</th>
<th>Form</th>
<th>Molar ratio of RNA species</th>
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<td></td>
<td>5 S RNA</td>
<td>Monomer&lt;br&gt;</td>
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<tr>
<td><strong>30 S ribosome</strong></td>
<td>16 S RNA</td>
<td>Dimer&lt;br&gt;(23 S)</td>
<td>&lt;0.01</td>
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<tr>
<td></td>
<td></td>
<td>Monomer&lt;br&gt;(16 S)&lt;br&gt;</td>
<td>1.00</td>
</tr>
</tbody>
</table>

* In the presence of the chelating agents, the sedimentation velocities of 23 S RNA and 16 S RNA were retarded by 14 and 9%, respectively. Twenty-three S RNA and 16 S RNA refer to the larger RNA species of 50 S ribosomes and the RNA of 30 S ribosomes, respectively, and are not intended to specify the sedimentation constants observed under these conditions.

* The numbers in parentheses are expressed in the equivalent amounts of monomer.

12 unit eq of the digested RNA were applied (Fig. 2), only one faintly stained band appeared when five times as much RNA digest was used. This indicates that after LiCl-urea treatment virtually all of the protein components of 50 S ribosome were extracted in the soluble fraction.

Assembly of Components of 50 S Ribosomes under Conditions Which Allow Formation of Active 30 S Ribosomes from Their Components

A mixture of 16 S RNA and the protein components prepared by LiCl-urea treatment of 30 S ribosomes can be completely assembled into particles having the same sedimentation velocity as 30 S ribosomes. The reconstituted particles showed 72% of the activity of the original 30 S ribosomes in polyuridylic acid-directed phenylalanine incorporation (Fig. 3B). Mg^{2+} is required for the reconstitution.

In the absence of Mg^{2+}, a broad distribution of particles with a peak at 21 S is produced (Fig. 3A). The 21 S particles had
The RNA component was analyzed by methylated albumin Kieselguhr column chromatography and analyzed as described under “Materials and Methods.” The molar ratio of 5 S RNA to 23 S RNA was calculated from the A260 profile of methylated albumin Kieselguhr column chromatography as described in the legend to Table I.

Therefore, we have systematically examined the effects of physical and chemical factors on the conformation of reassembled particles. As a standard procedure, the assembly of RNA and protein components of 50 S ribosomes was carried out in Buffer E or F at 42° for 15 min as described under “Materials and Methods”. Depending on the purpose of the experiments, each of the conditions was changed independently of the other.

**Effect of Temperature**

At 37° under the standard conditions described above, the functional activity of reconstituted 30 S ribosomes, i.e. the polyuridylic acid-directed phenylalanine incorporation, was 30% of that of the original ribosomes, whereas at 42° 72% of the activity was recovered. The ribonucleoprotein particles reassembled from the components of 50 S ribosome at 37° had a slower sedimentation rate than that of particles produced at 42°. Thus, assembly of the 30 S ribosome, as well as that of the 30 S ribosome is more efficient at 42° than at 37°. At 50° or higher, the 30 S ribosome is inactivated as measured by the polyuridylic acid-dependent incorporation of phenylalanine, and aggregation occurs as evidenced by the increased turbidity of the ribosome solution.

**Effect of Cations**

K+ and NH4+-No significant difference was found between K+ and NH4+ in the assembly of the components of 50 S ribosomes as shown in Table III.

Mg2+-The optimum concentration of Mg++ was determined...
Fig. 2. Polyacrylamide gel electrophoresograms of proteins from ribonucleoprotein particles assembled from 50 S ribosome components. The assembly of ribonucleoprotein particles from the RNA and protein components of 50 S ribosomes was carried out as described in the legend to Table II. The assembly mixture is the same as described in the legend to Table II, except that magnesium acetate, 20 μmoles; and spermine tetrahydrochloride, 5 μmoles per ml were present. The reaction was carried out: a, in the absence of both Mg^++ and polyanines; b, in the presence of Mg^++; and c, in the presence of Mg^++ and spermine tetrahydrochloride. The assembled particles were isolated as described in the legend to Table II. An aliquot of each sample was treated with LiCl-urea to prepare protein samples, as described under "Materials and Methods". RNA precipitates, washed with LiCl-urea, were dissolved in water and then treated with 20 μg per ml of pancreatic RNase at 23°C for 10 min. Both the protein fraction and the RNase digest were dialyzed against 6 M urea-0.006 M β-mercaptoethanol-0.002 M sodium acetate, pH 5.2 and concentrated to an appropriate volume by Sephadex powder. The diagram shows from left to right four pairs of protein samples from a, 23 S particles; b, 37 S particles; c, 45 S particles; and d, control 50 S ribosomes. The left and right gels of each pair represent 6 and 12 unit eq of protein, respectively. The four gels on the right represent four RNase digests. a', b', c', and d' corresponding to particles a, b, c, and 50 S ribosomes (12 unit eq each).

in the reassembly system. The sedimentation constant for each particle was calculated from Fig. 4 and similar experiments, and is summarized in Table IV. The particles assembled in the absence of Mg^++ showed a rather broad boundary with a peak at 23 S (Fig. 4A). In the presence of $3.3 \times 10^{-4}$ M Mg^++, the particles sedimented with a sharper boundary and there was a slight but significant shift of the peak toward the bottom of the tube (Table IV, Experiment 2). As the concentration of Mg^++ was increased, a further shift of the assembled particles to 27 S occurred (Fig. 4B) and another peak appeared at 36 S (Fig. 4C). At $2 \times 10^{-3}$ M Mg^++, only a single species of particles of 37 S was seen (Fig. 4D). In another set of experiments presented in Table IV, Experiment 3, a similar pattern of refolding of the particle is seen. Besides, it was found that the S value of the particles remained at the plateau (39 S) as the concentration of Mg^++ increased from 0.014 M to 0.14 M. From the above results, it appears that two classes of particles are formed. Refolding of 23 S particles to 27 S particles (Class I) takes place as the Mg^++ concentration is gradually increased from zero to $3.3 \times 10^{-4} \text{M}$. Increasing the Mg^++ concentration further converts all of the 27 S particles to particles with a sedimentation constant from 36 S to 37 S (Class II). The results suggest that a change of conformation from a Class I to a Class II particle is discontinuous and a relatively high free energy change may be required for the conversion. It was found by polyacrylamide gel electrophoresis that all of the protein subunits of 50 S ribosomes are associated with the 23 S, Class I particles as well as with the 37 S, Class II particles as shown in Fig. 2, a and b. However, neither of the particles stimulated polyuridylic acid-dependent phenylalanine incorporation.

Effect of Polyamines

Several lines of evidence show that preparations of ribosomes contain polyamines, such as cadaverine, putrescine, spermidine,
and spermine (22-24). Zillig et al. (22) pointed out that, since E. coli cells, as well as those of all other biological sources tested, are relatively rich in free polyamines it is possible that these polyamines replaced Mg++, which is normally bound to ribosomes during isolation of the particles. Spahr (24) demonstrated that 0.4% of the dry weight of E. coli ribosomes consists of the polyamines; putrescine, spermidine, and cadaverine in a 3:2:1 molar ratio.

TABLE III

Effect of NH₄⁺ on assembly of components of 50 S ribosomes

The basic conditions for the ribosomal assembly were described in the legend to Table II, except that 300 pmoles per ml of KCl or K⁺ were added. All other experimental conditions were the same as described in the legend to Table III.

<table>
<thead>
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<th>Monovalent cations</th>
<th>Mg⁺⁺</th>
<th>Particles formed</th>
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<tr>
<td>K⁺</td>
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<td>24 S</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>34 S</td>
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<tr>
<td>NH₄⁺</td>
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<td>25 S</td>
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<tr>
<td></td>
<td>+</td>
<td>34 S</td>
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</tbody>
</table>

Fig. 4. Effect of Mg⁺⁺ and spermine on the assembly of RNA and protein components of the 50 S ribosome. The assembly of RNA and protein components of 50 S ribosomes was carried out as described in the legend to Table II, except that magnesium acetate and spermine tetrahydrochloride were added as indicated in the figure. An aliquot of 0.2 ml of the assembled particles was analyzed by sucrose gradient centrifugation as described in the legend to Table III.

Fully functional 50 S ribosomes can be assembled from 16 S RNA and the 21 protein components (11) in the absence of polyamines. Nevertheless, the following facts made us examine the effect of polyamines on the assembly of 50 S ribosomes. Spermine and spermidine are more effective than Mg⁺⁺ in preventing the unfolding of 50 S ribosomes caused by chelating agents. These polyamines also effectively reform 50 S particles from the dense CsCl core (p = 1.7) (6) and the split proteins derived by CsCl centrifugation of 50 S ribosomes in the presence of sodium citrate (23). As shown in Fig. 4C and summarized in Table IV, Experiment 1, in the presence of 10⁻² m Mg⁺⁺ alone 26 S and 37 S particles were formed. Addition of 4 × 10⁻³ m spermine to this system led to the production of 37 to 42 S particles with the concomitant disappearance of the 26 S peak. Spermidine at the same concentration was also effective in the production of 41 S particles. In contrast, putrescine at 4 × 10⁻³ m had no significant effect on the assembly process. The addition of all three polyamines slightly increased the sedimentation constant of the assembled particles to 41 to 43 S.

The effect of spermine has been studied more in detail. As shown in Fig. 4D in the presence of 2 × 10⁻² m Mg⁺⁺, the assembly mixture gave rise to 37 S particles. Addition of spermine at 10⁻³ m permitted the formation of particles sedimenting in a discrete peak at 40 S, and with 10⁻² m spermine, 45 S particles were formed. The sedimentation constants of particles reassembled under various conditions were calculated from Fig. 4 and other experiments, and summarized in Table IV. Experiment 3 of Table IV lists another series of experiments to test the optimal concentration of spermine. In the presence of 1.4 × 10⁻² m Mg⁺⁺, spermine at 1.8 × 10⁻² m allowed reassembly of 48 S particles from the components of 50 S ribosomes. Higher concentrations of spermine caused aggregation of the particles.

1 K. Hosokawa, to be published elsewhere.

FIG. 3. Reconstitution of 30 S ribosomes from RNA and protein components. RNA and protein components of 30 S ribosomes were prepared as described under "Materials and Methods." A, the assembly mixture contained in a total volume of 1.5 ml: 16 S RNA, 65 A₂₆₀ units; the protein component of 30 S ribosomes, 72 unit eq. Tris-HCl, pH 7.5, 15 pmoles; KCl, 450 pmoles; β-mercaptoethanol, 9 pmoles. Incubation was carried out at 42° for 15 min and then terminated by chilling in an ice water bath. An aliquot of 0.2 ml was mixed with 1 ~1 of 32P-labeled 30 S ribosomes (< 0.01 A₂₆₀ unit), placed on top of a 10 to 30%, linear sucrose gradient made in Buffer C without β-mercaptoethanol and was subjected to centrifugation in a Spinco SW 50.1 rotor at 45,000 rpm for 4.5 hours at 4°. After the centrifugation, the A₂₆₀ unit eq; Tris-HCl, pH 7.5, 15 pmoles; KCl, 450 pmoles; β-mercaptoethanol, 9 pmoles. Incubation was carried out at 4° for 15 min and then terminated by chilling in an ice water bath. An aliquot of 0.2 ml was mixed with 1 ~1 of 32P-labeled 30 S ribosomes (< 0.01 A₂₆₀ unit), placed on top of a 10 to 30% linear sucrose gradient made in Buffer C without β-mercaptoethanol and was subjected to centrifugation in a Spinco SW 50.1 rotor at 45,000 rpm for 4.5 hours at 4°. After the centrifugation, the A₂₆₀ and radioactivity were determined on 3-drop fractions as described under "Materials and Methods." B, the reconstitution mixture contains 30 pmoles of the 21 protein components of 30 S ribosomes and RNA and protein components of 30 S ribosomes during isolation of the particles. Spahr (24) demonstrated that 0.4% of the dry weight of B. coli ribosomes consists of the polyamines; putrescine, spermidine, and cadaverine in a 3:2:1 molar ratio.

Fig. 4. Effect of Mg⁺⁺ and spermine on the assembly of RNA and protein components of the 50 S ribosome. The assembly of RNA and protein components of 50 S ribosomes was carried out as described in the legend to Table II, except that magnesium acetate and spermine tetrahydrochloride were added as indicated in the figure. An aliquot of 0.2 ml of the assembled particles was analyzed by sucrose gradient centrifugation as described in the legend to Table III.

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Addition of polyamines further increased the sedimentation intermediates and the S value increases and plateaus at 37 to 39 S. Instead, particles of 36 S emerge without the formation of a sedimentation constant of the Class I particles does not exceed 27 S. As the concentration of Mg++ increases, the sediments assembled in two distinctive steps which were termed Class I and II particles. In the absence of Mg++, RNA and protein components of 50 S ribosomes assemble into 23 S particles, and as the Mg++ increases to 2 x 10^{-2} M, they are converted to 37 to 40 S particles (Class II particles) produced in the presence of Mg++ and polyamines. Either spermine or spermidine in the absence of Mg++ caused aggregation of the ribonucleoprotein particles and a white turbidity. The 45 S particles assembled in the presence of 2 x 10^{-2} M Mg++ and 10^{-2} M spermine hydrochloride retained all of the protein constituents of 50 S ribosomes, as revealed by polyacrylamide gel electrophoresis and shown in Fig. 2C. The 45 S particles, like the 37 S and 27 S particles, were inactive in polyuridylic acid-directed phenylalanine incorporation (10), the peptidyl transfer reaction (15) and the peptide chain termination reaction (16).

From the experiments described above, it is apparent that 37 S or 40 S particles (Class II particles) produced in the presence of Mg^{2+} could be refolded to a more compact conformation (45 to 48 S) by the addition of spermine.

* S value is not accurate because the particles appeared as a very small shoulder. Parentheses indicate the component appeared in shoulder.

**Effect of Mg^{2+} and polyamines on assembly of ribonucleoprotein particles from RNA and protein components of 50 S ribosome**

The conditions for assembly of ribonucleoprotein particles were as described in the legends to Table II and Fig. 3, except that the concentrations of magnesium acetate, spermine tetrahydrochloride, spermidine trihydrochloride, and putrescine dihydrochloride used are given in the table. The assembled particles were analyzed by sucrose gradient centrifugation as described in the legends to Table II and Fig. 3.

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<th>Experiment</th>
<th>Components derived</th>
<th>Mg**</th>
<th>Additions</th>
<th>Particles reassembled</th>
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<td></td>
<td>None</td>
<td>10^{-1}</td>
<td>27 S + (34 S+)</td>
</tr>
<tr>
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<td>2 x 10^{-2}</td>
<td>(27 S) + 36 S</td>
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<td>37 S</td>
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</tr>
<tr>
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<td>21 S</td>
</tr>
<tr>
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<td></td>
<td>None</td>
<td>2 x 10^{-2}</td>
<td>30 S</td>
</tr>
<tr>
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<td>23 S</td>
</tr>
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<td>1.4 x 10^{-1}</td>
<td>26 S + 37 S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>None</td>
<td>4.3 x 10^{-2}</td>
<td>(27 S) + 40 S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>None</td>
<td>4.3 x 10^{-2}</td>
<td>39 S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>None</td>
<td>1.4 x 10^{-1}</td>
<td>39 S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>None</td>
<td>1.4 x 10^{-1}</td>
<td>42 S</td>
</tr>
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<td></td>
<td></td>
<td>None</td>
<td>1.4 x 10^{-1}</td>
<td>48 S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>None</td>
<td>1.4 x 10^{-1}</td>
<td>48 S</td>
</tr>
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</table>

* S value is not accurate because the particles appeared as a very small shoulder. Parentheses indicate the component appeared in shoulder.

| pH    | Effect of Mg^{2+} and polyamines
|-------|-----------------------------
| 7     | pH in the range from 7 to 8 has little effect on the assembly of the components of 50 S ribosomes. The particles formed at either extremity of the pH range in the absence and presence of Mg^{2+} showed no significant differences in sedimentation behavior. The effect of polyamines at pH 7 and pH 8 were also very similar.

**Order of Addition of Mg^{2+} and Polyamines**

Experiments were performed to determine whether or not the polycations are needed from the beginning of the assembly process or only after the complex of macromolecular constituents is formed.

Fig. 5, A and B shows the sedimentation profiles of the particles assembled when Mg^{2+} was added at 0 and 5 min, respectively, after incubation. If Mg^{2+} is present from time zero, 90% of the assembled particles appear in a 39 S peak, whereas if Mg^{2+} is added after 5 min of incubation a major peak at 24 S with a shoulder at 37 S is seen. In other experiments, Mg^{2+} was added at 1.5 min after incubation. Two-thirds of the particles formed were 37 S and the remaining one-third were 25 S.
These results indicate that Mg++ has to be present in the reaction mixture from the beginning of the assembly of the macromolecules, in order to form Class II particles.

In another set of experiments, as shown in Fig. 5, C and D, the time of addition of spermine and spermidine together with Mg++ was studied. When all three polycations were added at zero time, 47 S particles were formed. If the polycations are introduced to the assembly mixture after 5 min of incubation, the total yield of assembled particles is significantly decreased, as shown by the sedimentation profile (Fig. 5C). This is due to partial aggregation of the particles as evidenced by the sedimentation of ultraviolet-absorbing material to the bottom of the tube. A smaller broad peak of 32 S material appears in addition to the major peak when Mg++ and polyamines are added at 5 min and Mg++ is added only 1.5 min after the start of incubation. A combination of magnesium acetate, 10 μmoles per ml, spermine tetrahydrochloride, 2.1 μmoles per ml, and spermidine trihydrochloride, 2.1 μmoles per ml were added. C, at 5 min; and D, before incubation. The sucrose gradient analysis of the reassembled particles were carried out as described in the legend to Table III.

The presence of 30 S ribosomes in the reconstitution mixture had no effect. In an attempt to find a chemical agent which would lead to the formation of 50 S particles from the 45 to 48 S ones assembled in the presence of Mg++ and polyamine(s), concentrated yeast extract was added to the reconstitution mixture, but it had no effect.

The presence of 30 S ribosomes in the reconstitution mixture for 50 S ribosomes was found to increase the recovery of activity of the reassembled particles in the case of Bacillus stearothermophilus (12) and was absolutely necessary for the functional restoration of the resultant particles in the case of E. coli (13). In contrast, we found that the presence of equimolar amounts of either 30 S or 50 S ribosomes slightly reduced the sedimentation velocity of the resultant particles. Addition of the components of 30 S ribosomes was also ineffective.

### Incorporation of 5 S RNA into Assembled Particles

The RNA preparation for the reassembly of the components of 50 S ribosomes contains both 5 S RNA and 23 S RNA in equivalent amounts. The specificity and stoichiometry of incorporation of 5 S RNA into the reassembled particles was studied and the results are given in Table V. When the 5 S RNA of the RNA mixture was labeled by adding a negligible amount of 32P-labeled 5 S RNA with specific radioactivity, 0.8 cpm of 5 S RNA was found to be incorporated per reassembled particle, as analyzed by sucrose gradient centrifugation. If 1.3 eq of 32P-labeled 5 S RNA are added to the assembly system which way.

#### Table V

**Stoichiometry and specificity of incorporation of 5 S RNA into ribonucleoprotein particles assembled from components of 50 S ribosome.**

The experimental conditions for the assembly of ribonucleoprotein particles from the RNA and protein components of 50 S ribosomes were as described in the legend to Table II, except that the basic reaction mixture contained per ml: RNA component of 50 S ribosomes, 72 A260 units; protein component of 50 S ribosomes, 80 unit eq; KCl, 300 μmoles; magnesium acetate, 20 μmoles; spermine tetrahydrochloride, 1 μ mole; diethanol, 1 μ mole; β-mercaptoethanol, 6 μmoles; and Tris-HCl, pH 7.5, 10 μmoles. 32P-labeled RNA was added as follows: 32P-labeled 5 S RNA, 7.62 × 10^6 cpm, <0.1 A260 unit (Experiment 1), or 3.54 A260 units, 4.3 × 10^6 cpm per A260 unit (Experiment 2); 32P-labeled tRNA (9.2 × 10^6 cpm per A260 unit), 3.54 A260 units (Experiment 3), or 5.12 A260 units (Experiment 4). The system was incubated at 45°C for 30 min. The amounts of 32P-labeled RNA incorporated into the particles was calculated from the optical density and radioactivity profiles obtained after sucrose gradient sedimentation of the particles using the specific radioactivity (cpm per A260 unit) of the added 32P-labeled RNA species. The amounts of 32P-labeled RNA incorporated into the particles was calculated from the optical density and radioactivity profiles obtained after sucrose gradient sedimentation of the particles using the specific radioactivity (cpm per A260 unit) of the added 32P-labeled RNA species.

<table>
<thead>
<tr>
<th>RNA species</th>
<th>Experiment</th>
<th>32P-RNA added</th>
<th>Total amount in the mixture</th>
<th>32P-RNA found</th>
<th>Total amount incorporated</th>
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<tr>
<td>5 S RNA</td>
<td>1</td>
<td>&lt;0.1 molar eq</td>
<td>1 molar eq, 2,376 cpm/A260 unit</td>
<td>0.8 molar eq</td>
<td></td>
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<tr>
<td></td>
<td>2</td>
<td>1.3 molar eq</td>
<td>2.3 molar eq, 3,175 cpm/A260 unit</td>
<td>1.1 molar eq</td>
<td></td>
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<tr>
<td>tRNA</td>
<td>3</td>
<td>1.3 molar eq</td>
<td>Same, &lt;18 cpm/A260 unit</td>
<td>&lt;0.1 molar eq</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2.6 molar eq</td>
<td>Same, &lt;47 cpm/A260 unit</td>
<td>&lt;0.2 molar eq</td>
<td></td>
</tr>
</tbody>
</table>

*a Molar equivalent to 23 S RNA.
*b Counted per min per 1 A260 unit of the reassembled particle.
* Background level.
already contains 1 eq of 5 S RNA (total 2.3 eq) 1.1 eq were incorporated per eq of particles formed. The incorporation of 32P-labeled tRNA into the assembled particles, when added at 1.3 or 2.6 eq, was insignificant.

The results indicate that the incorporation of 5 S RNA into the assembled ribonucleoprotein particles is stoichiometric and highly specific.

The results reported here offer a preliminary step to the systematic study of the mode of assembly, as well as the role in polypeptide synthesis of the large ribosome subunit of mesophilic organisms.

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Assembly of *Escherichia coli* 50 S Ribosomes from Ribonucleic Acid and Protein Components: I. CHEMICAL AND PHYSICAL FACTORS AFFECTING THE CONFORMATION OF ASSEMBLED PARTICLES

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