Association of 5S Ribonucleic Acid to 50S Ribosomal Subunits of *Escherichia coli* and *Bacillus subtilis**

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ABSTRACT: In confirmation of earlier results with *Escherichia coli* it has been demonstrated that all of the 5S ribonucleic acid (RNA) of *Bacillus subtilis* is accounted for by the presence of one 5S RNA bound to each 50S ribosomal subunit. Isolated 50S subunits from *E. coli* and *B. subtilis* were treated with CsCl, LiCl, or EDTA under a variety of conditions and the particles derived were assayed for the presence or absence of 5S RNA by polyacrylamide gel electrophoresis. Ribonucleoprotein particles, A particles, with a sedimentation coefficient of about 40 S, produced by banding ribosomes in a CsCl gradient in the presence of Mg^{2+}, were deficient in protein but retained all of their 5S RNA. A′ particles, produced by treatment of ribosomes with CsCl at a lower Mg^{2+} concentration or by treatment with LiCl, contained even less protein and were devoid of 5S RNA. It was also possible to remove 5S RNA from 50S ribosomal subunits of *E. coli* by treatment with EDTA. This procedure produced conformational changes of the ribosomes without significant loss of protein. Experiments involving pulse labeling of *E. coli* with [^{3}H]uridine gave indirect evidence for a pool of cold 5S RNA or precursor to 5S RNA. It was also shown that attachment of 5S RNA to ribosomal precursors of the mature 50S subunit occurs at a late stage of ribosome biosynthesis.

The existence of ribosome-bound low molecular weight RNA, distinct from tRNA, was first observed in *Escherichia coli* by Rosset and Monier (1963). This RNA, which has a sedimentation coefficient of about 5 S, has since been identified in a number of organisms (Sarkar and Comb, 1965; Marcot-Queiroz *et al.*, 1965, 1966; Galibert *et al.*, 1965; Brown and Littna, 1966) and its uniqueness was demonstrated by DNA–RNA hybridization (Zehavi-Wilner and Comb, 1966; Morell *et al.*, 1967), RNA–RNA hybridization (Hayward *et al.*, 1966), and oligonucleotide mapping (Forget and Weissman, 1967). In the case of 5S RNA isolated from *E. coli* the entire nucleotide sequence has been elucidated (Brownlee *et al.*, 1967). The association of 5S RNA with the larger of the two ribosomal subunits has been demonstrated for *E. coli* (Rosset *et al.*, 1964) and yeast (Marcot-Queiroz *et al.*, 1965), even at low Mg^{2+} concentrations when no tRNA remains bound. Galibert *et al.* (1966a) have noted that dialysis against buffer lacking Mg^{2+} will remove 5S RNA from ribosomes of KB cells, and Comb and Sarkar (1967) have used EDTA to dissociate 5S RNA from *Blastocladiella emersonii* ribosomes. However, the larger subunit of HeLa cell ribosomes retains 5S RNA even after sedimenting through a sucrose gradient containing 0.01 M EDTA (Knight and Darnell, 1967).

In an attempt to learn more about the structure of ribosomes and possibly obtain a clue as to the biological role of 5S RNA, we have studied various methods of dissociating this RNA species from the larger ribosomal subunit. We have also investigated the presence of 5S RNA in ribonucleoprotein (RNP)[1] particles tentatively identified as precursors to the 50S ribosomal subunit. The systems studied were *E. coli* and *Bacillus subtilis*, the latter because methods have been developed to locate DNA sequences complementary to metabolically stable RNA species in relation to the genetic map of the organism (Oishi and Sueoka, 1965; Dubnau *et al.*, 1965; Smith *et al.*, 1968). Some physical studies of 5S RNA from *B. subtilis* were also undertaken in the hope that some knowledge of its secondary structure might be an aid in determining its mode of attachment to ribosomes.

Experimental Section

Materials. STRAINS USED. *B. subtilis* A26U$^-$, a uracil-requiring derivative of *B. subtilis* 168, obtained from Dr. I. Takahashi, and *E. coli* ML 63-86, a pyrimidine-requiring mutant, were used for the preparation of RNA and ribosomes. For use in experiments involving EDTA treatment, ribosomes were isolated from *E.*

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[1] Abbreviations used: RNP, ribonucleoprotein; V–Y, veal infusion–yeast extract medium (media are described in the text); rRNA, includes 5S, 16S, and 23S ribosomal ribonucleic acid species; SLS, sodium lauryl sulfate; TCA, trichloroacetic acid; TENS, 0.01 M Tris (pH 7.5), 0.01 M EDTA, 0.1 M NaCl, and 0.5% sodium lauryl sulfate.

coli A19, an Hfr, methionine-requiring ribonuclease 1 negative derivative of *E. coli* AB301 (Gesteland, 1966); this strain does not require uracil.

MEDIA. Bacteria used to prepare radioactively labeled RNA or ribosomes were grown in a minimal media (Anagnostopoulos and Spizizen, 1961) containing 0.5% glucose as a carbon source and supplemented as described in Methods. For the growth of unlabeled bacteria, V–Y medium (25 g of Difco (Detroit, Mich.) veal infusion broth plus 5 g of Difco yeast extract in 1 l. of water) was used. Valine assay and leucine assay media were obtained from Difco and amino acids were purchased from Calbiochem (Los Angeles, Calif.). Radioactive materials used included: [5-^{3}H]uridine (14 Ci/mmole) (Nuclear Chicago Corp., Chicago, Ill.), [2-^{14}C]uracil (20 mCi/mmole), L-[1-^{14}C]valine (15 mCi/mmole), and L-[1-^{14}C]leucine (20 mCi/mmole), all obtained from New England Nuclear Corp. (Boston, Mass.).

Polyacrylamide gels were prepared as described by Loening (1967), although materials were not recrystallized since optical purity was not required. The gels, 10% in polyacrylamide and 0.25% in bisacrylamide, 8 cm long and 0.6 cm in diameter, were prepared and run in a buffer consisting of 0.005 M Tris (pH 7.5), 0.025 M sodium acetate, 0.0025 M EDTA, and 0.5% sodium lauryl sulfate (SLS). Other materials included CsCl (Harshaw Chemical, Cleveland, Ohio), LiCl (Fisher, Fairlawn, N. J.), and Whatman 2.4 cm GF/B glass filters. Electrophoretically purified deoxyribonuclease and pancreatic ribonuclease (RNase) were obtained from Worthington Biochemical Corp. (Freehold, N. J.). Other materials and instruments were as described previously (Morell *et al.*, 1967).

Methods. GROWTH AND LABELING OF CELLS. All cultures were grown at 37° with vigorous aeration on a rotary air shaker. Growth was followed by monitoring cell turbidity in a Klett-Summerson colorimeter using a no. 62 filter; one Klett unit corresponds to a viable count of approximately 1.0×10^6 *B. subtilis* cells/ml. Metabolically stable RNA species of *B. subtilis* A26U$^-$ and *E. coli* ML 63–86 were uniformly labeled by growth in minimal medium, containing 0.1% Casamino Acids and 5 μg/ml of [^{3}H]uridine (700 mCi/mmole), until exhaustion of uridine. This was followed by a starvation period and a two-generation chase with cold uridine as previously described (Morell *et al.*, 1967). To prepare *B. subtilis* with [^{14}C]RNA the same procedure was used except that 3 μg/ml of [^{14}C]uracil (20 mCi/mmole) was used during labeling and cold uracil (100 μg/ml) was used during the chase period.

To uniformly label the metabolically stable RNA of *E. coli* A19, the above protocol was slightly modified. Cultures were grown in minimal medium containing 0.1% Casamino Acids and [^{3}H]uridine or [^{14}C]uracil, as above, and 50 μg/ml of L-methionine. When the cell turbidity reached 30 Klett units the culture was centrifuged at room temperature and resuspended in two volumes of the same medium containing 100 μg/ml of unlabeled uridine or uracil. Growth was continued for two generations, and the culture was chilled in ice and harvested. In order to study ribosomal precursor particles, cultures of *E. coli* A19 were pulse labeled with [^{3}H]uridine following a "shift-up." Under such conditions there is a preferential synthesis of metabolically stable RNA (Kjeldgaard, 1960). Cultures were grown in minimal medium supplemented with 50 μg/ml of L-methionine. When a Klett reading of 70 was reached, Casamino Acids were added to a final concentration of 0.3%. Five minutes later [^{3}H]uridine (14 Ci/mmole) was added to a concentration of 0.12 μg/ml and from 1 to 5 min later the culture was poured over an equal volume of crushed ice and harvested.

To prepare ribosomes containing labeled protein from *B. subtilis* A26U$^-$ and *E. coli* ML 63-86, cells were grown from a small innoculum in minimal medium supplemented with 50 μg/ml of uridine, 0.2% valine assay medium, and 5 μg/ml of L-[^{14}C]valine (3 μCi/mmole). Cells were harvested at a Klett reading of about 60. *E. coli* A19 was labeled in the same manner in minimal medium containing 5 μg/ml of L-[^{14}C]leucine (2.5 μCi/mmole) and supplemented with 0.2% leucine assay medium and 50 μg/ml of L-methionine. *B. subtilis* A26U$^-$, used for the preparation of unlabeled RNA, and ribosomes were grown in V–Y medium and harvested in mid log phase.

Ribosomes from *B. subtilis* were prepared as previously described (Morell *et al.*, 1967) except that bentonite was added only once to a concentration of 0.3 mg/ml, after cell disruption in a French pressure cell. Higher concentrations of bentonite lower the yield of ribosomes considerably. Ribosomes from cultures of *E. coli* were prepared in the same manner, except that no bentonite was used. If RNA from the supernatant of a ribosomal pellet was to be examined it was deproteinized with phenol and concentrated by ethanol precipitation as previously described (Morell *et al.*, 1967). In order to prepare ribosomal subunits the ribosomal pellet was suspended at a concentration of 5 mg/ml, with the aid of a Dounce homogenizer, in 0.01 M Tris (pH 7.5), containing 10^{-4} M $MgCl_2$. The 50S and 30S ribosomal subunits were separated on a linear (5–20%) sucrose gradient containing the above buffer. Approximately 1 mg was loaded on each tube of the SW 25.3 rotor and centrifugation was carried out at 25,000 rpm for 12 hr at 4° in a Spinco L2 ultracentrifuge. Following centrifugation, the bottom of each tube was punctured and the gradient solution was collected through a flow cell in the Gilford Model 2000 absorbance recorder. Fractions of 0.5 ml were collected and the 30S and 50S regions were pooled separately. For the preparation of 16S and 23S RNA, the appropriate subunit pool was diluted twofold with 0.01 M Tris (pH 7.5), containing 10^{-2} M $MgCl_2$ and the subunits were pelleted at 100,000*g* for 8 hr at 4°. The pellets were resuspended and deproteinized with phenol and SLS as previously described (Morell *et al.*, 1967). The aqueous layers were ethanol precipitated and the RNA was further purified on linear (15–30%) sucrose gradients prepared in TENS. Centrifugation was for 17 hr at 23° using the SW 25.1 rotor.

In order to prepare ribosomal precursors 200 ml of a culture of *E. coli* A19, pulse labeled with [^{3}H]-uridine as described above, was harvested and washed

once in 0.01 M Tris (pH 7.5), containing 10^{-4} M Mg^{2+}. The cells were resuspended in 6 ml of the same buffer and broken in a French pressure cell. No bentonite was added, for it was found that this caused a preferential loss of precursor particles. The crude extract was clarified by centrifugation in a Sorvall-refrigerated centrifuge at 27,000*g* for 20 min at 4° and the RNP particles were separated on sucrose gradients. Approximately $30A_{260}$ units (0.5–1 ml) of crude extract were loaded on each gradient, using the conditions described above for ribosomal subunit preparation. After collection of the gradients the 40S region was identified, using the absorbance peaks of the 30S and 50S ribosomal subunits as markers, and tubes corresponding to the 40S region were pooled.

For analytical purposes, RNP particles were sedimented through a linear (15–30%) sucrose gradient buffered with 0.01 M Tris (pH 7.5), containing 10^{-3} M $MgCl_2$, in the SW 39 rotor of the Spinco L2 ultracentrifuge. Such gradients were run at 39,000 rpm for from 5 to 6 hr at 4°. To determine radioactivity across the gradient the bottom of each tube was lightly greased and pierced with a size 00 insect pin. Four drop fractions were collected directly onto glass filters, the flow rate being controlled by negative pressure exerted through a syringe (Vinograd, 1963). The filters, sandwiched between two wire screens, were dipped successively into two 5% TCA baths and two 95% ethanol baths, remaining in each tray for about 10 min. Excess liquid was removed on a stainless-steel filter assembly and the remaining solvent was evaporated under a heat lamp. Radioactivity was determined in toluene scintillator. Such gradients were also used to purify small quantities of protein-deficient RNP particles generated by CsCl or LiCl. Four drop fractions were collected in tubes and 10-μl aliquots of each tube were counted in Bray's (1960) scintillator. For assay of the high molecular weight RNA contained in RNP particles linear (15–30%) sucrose gradients, in TENS buffer, were prepared for the SW 39 rotor. Centrifugation was for 6 hr at 24°. The gradient was collected on glass filters, and TCA-precipitable radioactivity was determined as described above.

To prepare RNA from RNP particles for analysis on polyacrylamide gels or sucrose gradients, 1% SLS was used to disaggregate RNA from protein. Samples in 10^{-3} M or higher Mg^{2+} were first ethanol precipitated to remove the magnesium and resuspended in the buffer used to run the gels. Samples for analysis on gels were adjusted to a volume of 0.2 ml containing 10% (w/v) sucrose. A drop of 1% bromophenol blue was added as a marker. The samples were layered on the gels and a current of 7 mA/gel was applied until the dye began to run off, within about 3 hr. Gels were collected in the apparatus described by Maizel (1966) and counted in Bray's (1960) scintillator.

Since radioactivity due to [^{3}H]RNA is quenched by polyacrylamide, the amount of low molecular weight RNA entering the gel relative to the excluded high molecular weight RNA could not be measured directly. In order to avoid determining the extent of ^{3}H quenching by polyacrylamide under a variety of conditions,

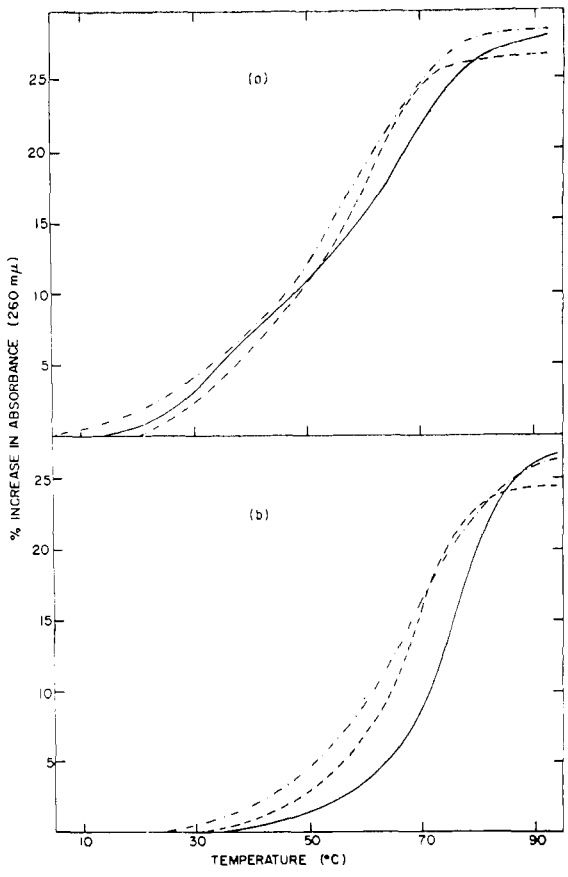

FIGURE 1: Thermal denaturation profiles of 5S, 23S, and tRNA from *B. subtilis* A26U$^-$ in the presence of Mg^{2+} and EDTA. RNA samples were prepared at an initial absorbance of 0.5 (at 260 mμ) and temperature-induced hyperchromicity was determined as described in Methods. The buffers used were: (a) 0.2 M NaCl, 0.001 M EDTA, 0.001 M Tris (pH 7.5), and (b) as in a but contained in addition 0.005 M $MgCl_2$. (——) tRNA, (– – –) 5S RNA, and (–·–·–) 23S RNA.

each set of assays for low molecular weight RNA included one gel containing 50S subunits isolated from cells uniformly labeled with [^{3}H]uridine. The amount of 5S RNA in each sample is expressed as a percentage of the amount of 5S RNA in the standard. This is used as a control for variations due to differences in particular batches of gels and other factors. A correction is made for differences in sample size and, in the figures, input radioactivity of each sample is expressed as a ratio relative to the standard.

To prepare total RNA for assay on Sephadex columns, *B. subtilis* A26U$^-$ cultures, grown in V–Y, were chilled and harvested in mid log phase (Klett reading of 80), and washed and resuspended in 0.01 M Tris (pH 7.5) and 10^{-4} M $MgCl_2$ containing 0.3 mg/ml of bentonite. The cells were broken in a precooled French pressure cell at a pressure of 10 tons/in.^{2}; 10 μg/ml of deoxyribonuclease was added and intact cells and debris were removed by two centrifugations at 27,000*g* for 15 min at 4°. Removal of the unbroken cells was necessary to prevent preferential extraction of low molecular weight RNA during phenol treatment. The clarified crude extract was deproteinized with

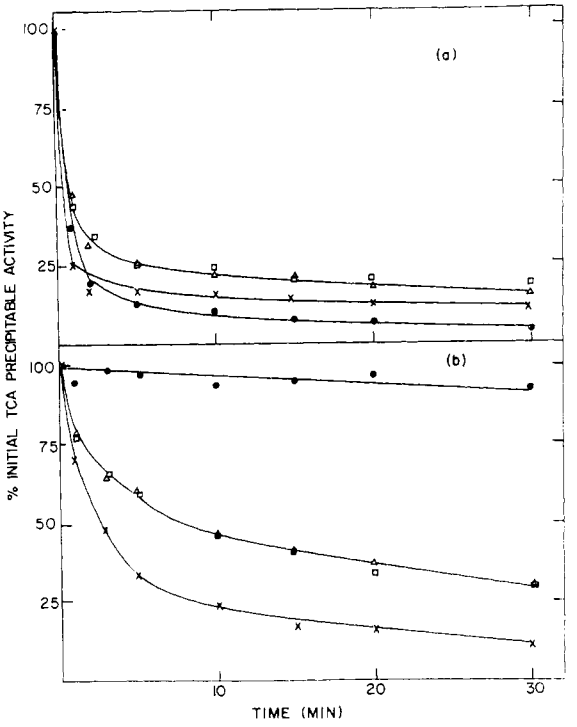

FIGURE 2: Susceptibility of 5S, 16S, 23S, and tRNA of *B. subtilis* A26U⁻ to pancreatic RNase in the presence of Mg^{2+} or EDTA. (a) Three tubes, each containing a total of 0.5 μg of [^{32}P]5S RNA (30,000 cpm/μg) and 2 μg of [^{3}H]RNA (16S, 23S, and tRNA, respectively (120,000 cpm/μg)) in a total volume of 10 ml of 0.01 M Tris (pH 7.5)–0.01 M EDTA were incubated at 30°. The reaction was initiated by the addition of RNase at a level of 0.005 μg/ml and the per cent of TCA-precipitable radioactivity remaining was plotted as a function of time (see Methods). The experimental points for the 5S RNA represent the average of the three determinations. (b) A similar experiment was conducted with 0.01 M $MgCl_2$ substituted for the EDTA and a higher level, 0.1 μg/ml of RNase. (·) tRNA, (x) 5S RNA, (□) 16S RNA, and (△) 23S RNA.

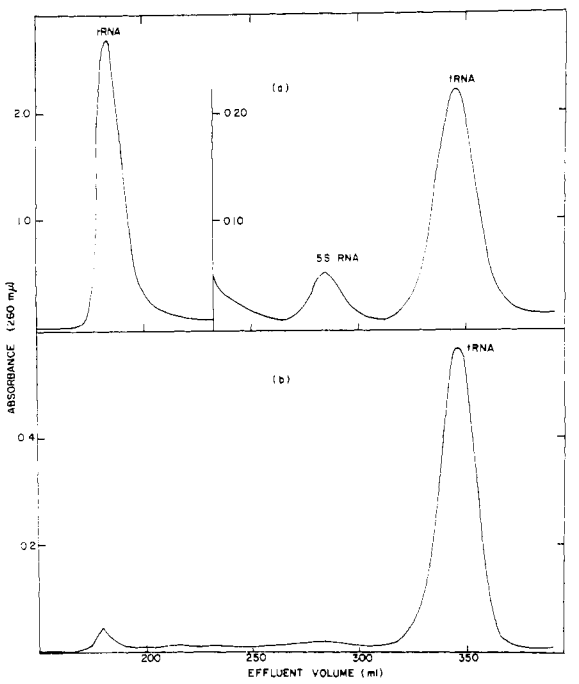

FIGURE 3: Sephadex G-100 column chromatography of RNA isolated from *B. subtilis*. A culture of *B. subtilis* A26U⁻, growing exponentially in V–Y medium, was harvested and a crude extract was prepared. Part of this extract was deproteinized with phenol; the rest was used to prepare a ribosomal supernatant fraction which was also deproteinized with phenol (see Methods for details). (a) Elution pattern of 2.1 mg of nucleic acid from the crude extract. (b) Elution pattern of 0.75 mg of nucleic acid from the ribosomal supernatant fraction.

SLS and phenol (Morell *et al.*, 1967). The RNA in the aqueous phase was concentrated by ethanol precipitation and resuspended in 1 M NaCl. This step, as well as Sephadex chromatography, was carried out at room temperature in order to prevent preferential precipitation of high molecular weight RNA from the NaCl.

To assay for the various RNA species 1 ml of the above preparation, containing approximately 4 mg of RNA, was loaded on a Sephadex G-100 column (200 × 1.8 cm) and eluted with 1 M NaCl at a pressure head of 20 cm (Schleich and Goldstein, 1964). t- and 5S RNA samples used for melting curves were purified by two passages through such a column.

Melting curve determinations were made using a Gilford spectrophotometer with attachments for automatic monitoring of absorbance and temperature. The cuvet chamber was brought to 2° by pumping refrigerated glycerol through thermal spacers and the temperature was then raised at a rate of less than 1° every 2 min using a Haake heating bath and circulator (Cole-Parmer, Chicago, Ill.). Solvents and other conditions are described in the legend to Figure 1.

Susceptibility to digestion by pancreatic RNase was assayed by preparing samples containing both [^{32}P]RNA and [^{3}H]RNA in a total volume of 10 ml and incubating at 30°. The reaction was initiated by addition of pancreatic RNase; 1-ml aliquots were removed to an ice bath at various time intervals and the reaction was stopped by addition of 100 μg of bovine serum albumin and 1 ml of 10% TCA. The precipitate was collected on nitrocellulose filters, washed with 5% TCA, dried, and radioactivity was determined in toluene scintillator. Solvents used and other details are given in the legend to Figure 2. [^{3}H]tRNA and [^{32}P]5S RNA were samples that had been prepared and used previously (Morell *et al.*, 1967). High molecular weight RNAs (16 and 23 S) were prepared from purified 30S and 50S ribosomal subunits isolated from cultures of *B. subtilis* A26U⁻ uniformly labeled with [^{3}H]uridine.

To prepare protein-deficient CsCl core particles, gradients were prepared as described by Meselson *et al.* (1964), except that the sample added to the CsCl consisted of the 50S region taken directly from a sucrose gradient. After centrifugation the bottom of the tube was pierced, as described above, and four drop fractions were collected. Samples in tubes representing the upper and lower parts of the gradient were checked for refractive index and 10-μl aliquots of each tube were counted in Bray's (1960) scintillator. Peak fractions were pooled separately and dialyzed against 0.01 M Tris (pH

7.5)–10^{-3} M $MgCl_2$ before assay on sucrose gradients or polyacrylamide gels. Because of the presence of the sucrose the apparent density, as determined by refractive index, was higher than the true density by 0.02 g cm^{-3} and the reported densities have been so corrected. Some preparations of CsCl core particles (A′ particles) were prepared as described by Lerman *et al.* (1966) except that 0.025 M Tris (pH 7.5) was used instead of phosphate to buffer the CsCl.

To prepare protein-deficient LiCl core particles, 50S subunits were suspended in 2 M LiCl plus 10^{-3} M $MgCl_2$, allowed to stand in an ice bath for several hours, and collected by a 6-hr centrifugation at 150,000*g* at 4° in the L2 Spinco ultracentrifuge (Marcot-Queiroz and Monier, 1966).

To prepare EDTA particles (Gesteland, 1966), 50S subunits were suspended in 0.01 M Tris (pH 7.5) and 10^{-3} M EDTA and immediately sedimented through a linear (15–30%) sucrose gradient in the same buffer. Centrifugation was for 6 hr at 39,000 rpm in the SW 39 rotor at 4°. Fractions were collected as for other gradients.

To measure the amount of protein removed by treatment of ribosomes with CsCl, LiCl, and EDTA, bacteria were labeled with a [^{14}C]amino acid as described above and 50S subunits were isolated. When such particles were sedimented through sucrose gradients in TENS, all of the radioactivity remained at the top while 16S and 23S RNA sedimented. It was therefore assumed that the ratio of radioactivity/A_{260} was proportional to the amount of protein on each ribosome. Such measurements, before and after treatment, were indicative of how much protein had been removed by the salt or EDTA.

The accuracy of such protein determinations is not very great and depends, among other things, on the assumption that the amino acid used is a representative label. This method is useful, however, in estimating the relative amount of protein removed by various procedures using the same initial batch of ribosomes. Nitrocellulose tubes used for CsCl and sucrose gradients were treated with EDTA as described by Meselson *et al.* (1964) and all glassware used in handling RNA or ribosomes was acid cleaned.

Results

Secondary Structure. Figure 1 shows the thermal denaturation curves of t-, 5S, and 23S RNA, both in the presence and absence of Mg^{2+}. It can be seen that within the temperature range used, the extent of hyperchromicity for each type of RNA is similar and not greatly affected by Mg^{2+}. In the presence of Mg^{2+} the secondary structure of all three RNA samples is

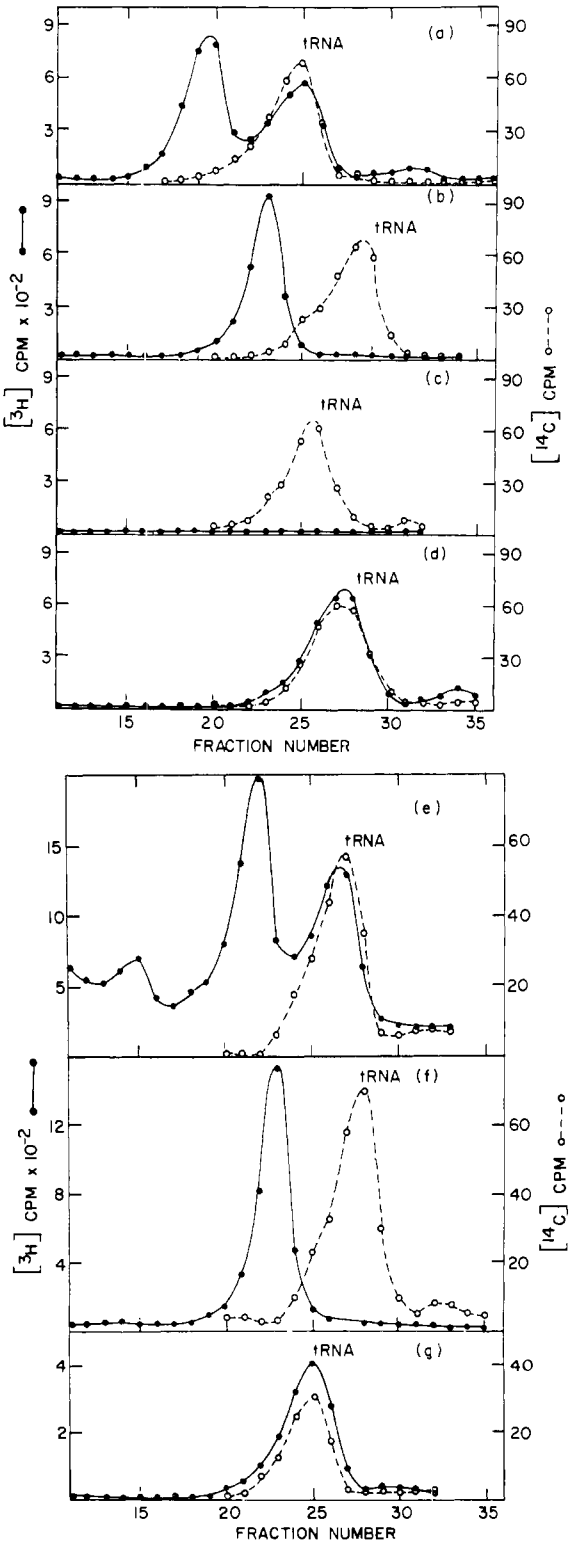

FIGURE 4: Polyacrylamide gel assays of low molecular weight RNA in various ribosomal fractions from *E. coli* and *B. subtilis*. A preparation of [^{3}H]RNA labeled *E. coli* ML 63-86 ribosomes was fractionated on a sucrose gradient as described in Methods. The 4–5S, 30S, and 50S regions of the gradient were pooled separately and then assayed for 5S RNA (see Methods). ^{14}C-labeled tRNA was added as a marker for each gel. (a) Undissociated 70S ribosomes, (b) 50S region of the sucrose gradient, (c) 30S region of the sucrose gradient, and (d) low molecular weight region of the sucrose gradient. To compare the amount of 5S RNA present on different particles a correction must be made for the amount of RNA assayed on each gel. The input of *E. coli* [^{3}H]RNA on gels a, b, c, and d was in the ratio of 1.5:1.0:1.1:0.05, respectively. A similar experiment was performed with ribosomes from *B. subtilis;* (e) undissociated 70S ribosomes; (f) the 50S region of the sucrose gradient; (g) the low molecular weight region of the sucrose gradient. Input of *B. subtilis* [^{3}H]RNA on gels e, f, and g was in the ratio of 1.8:1.0:0.07, respectively.

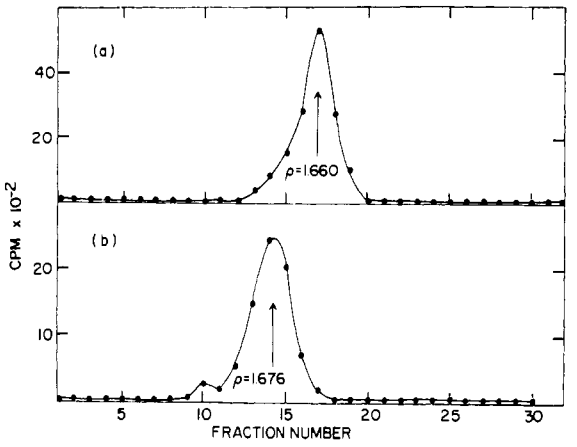

FIGURE 5: CsCl gradients of 50S ribosomal subunits prepared from *E. coli* and *B. subtilis*. (a) [^{3}H]RNA-labeled 50S ribosomal subunits isolated from *E. coli* ML 63-86 were centrifuged in CsCl containing 0.06 M magnesium acetate and fractions were collected as described in Methods. Aliquots (10 μl) were counted in Bray's (1960) solution. (b) Same as a except that [^{3}H]RNA-labeled 50S ribosomal subunits isolated from *B. subtilis* A26U$^-$ were assayed.

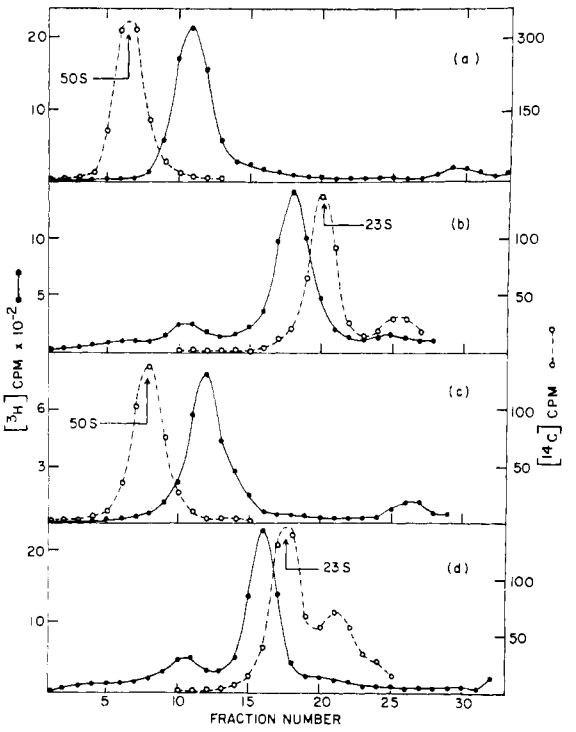

FIGURE 6: Sucrose gradient analysis of RNP particles generated by treatment of 50S ribosomal subunits of *E. coli* and *B. subtilis* with CsCl. (a) [^{3}H]RNA-labeled *E. coli* RNP particles, obtained from the A band of the CsCl gradient shown in Figure 5a, were analyzed on a sucrose gradient with [^{14}C]RNA-labeled 50S subunits of *B. subtilis* as a marker. Centrifugation was for 5 hr using the conditions described in Methods; fractions were collected and TCA-precipitable counts were determined. (b) [^{3}H]RNA-labeled *E. coli* A′ particles, prepared with CsCl by the method of Lerman *et al.* (1966), were analyzed on a sucrose gradient with ^{14}C-labeled RNA of *B. subtilis* as a marker. Centrifugation was for 6 hr using the conditions described in Methods; fractions were collected and TCA-precipitable counts were determined. (c) Same as a except that A-band RNP particles of *B. subtilis* (Figure 5b) were assayed. (d) Same as b except that A′ RNP particles of *B. subtilis* were assayed.

stabilized, as indicated by the narrower temperature range over which the melting occurs and by the displacement of the midpoint of the curves to higher temperatures. The stabilizing effect of Mg^{2+} is most dramatic with tRNA; 5S RNA and 23S RNA probably do not form as stable a structure as the Mg^{2+}–tRNA complex. The above results are supported by studies of susceptibility to pancreatic RNase (Figure 2). In the absence of Mg^{2+} a low level of RNase rapidly degrades 16S, 23S, 5S, and tRNA to TCA-soluble fragments. The presence of Mg^{2+} protects tRNA almost completely from degradation by RNase at enzyme levels which rapidly degrade the various rRNA species to TCA-soluble oligonucleotides.

Amount and Cellular Location of 5S RNA. Chromatography on Sephadex G-100 columns was used to determine the fraction of 5S RNA present in total RNA extracted from *B. subtilis* A26U$^-$. Cells grown in V–Y medium were harvested in mid log phase and bulk RNA was prepared and chromatographed as described in Methods. As shown in Figure 3a, high molecular weight rRNA is eluted in the void volume, followed by 5S and tRNA, all clearly separated from each other. Characterization of RNA present in these peaks has been described previously (Morell *et al.*, 1967). The average of two such elution chromatograms demonstrated that high molecular weight rRNA represents 81.5%, tRNA 16.2%, and 5S RNA 2.3% of the total RNA. Assuming that the total RNA consists of 5S RNA, tRNA, and ribosomal 16S and 23S RNA, it can be calculated that there are 1.15 molecules of 5S RNA present on each ribosome (see Discussion). Since almost no 5S RNA can be detected in the supernatant (Figure 3b), it was assumed that there is one 5S RNA molecule per ribosome. When nucleic acids extracted from a ribosomal pellet were chromatographed, the 5S RNA peak contained 2.3% of the RNA, the tRNA peak 2.0%, and the void volume the rest of the RNA. This corresponds to 1.0 5S RNA molecule/ribosome. This result indicated that if a pool of free 5S RNA exists it represents no more than a few per cent of the total 5S RNA of the cell.

Because of the length of time required for Sephadex G-100 chromatography, further analyses of low molecular weight RNA species were conducted on polyacrylamide gels, using labeled RNA preparations. A series of control experiments was carried out to demonstrate that analysis on either Sephadex G-100 columns or on polyacrylamide gels gave equivalent results.

Localization of 5S RNA on the 50S Subunit. A ribosomal pellet, prepared from *E. coli* ML 63-86 with ^{3}H-labeled RNA, was fractionated on a sucrose gradient and the 50S, 30S, and 4–5S regions of the gradient were pooled separately and then assayed for 5S RNA. Figure 4 demonstrates that all of the 5S RNA present in the original pellet (Figure 4a) is retained in the 50S subunit (Figure 4b) and that none is present in the 30S subunit (Figure 4c). The top of the gradient (Figure 4d) contains only tRNA. A similar result was

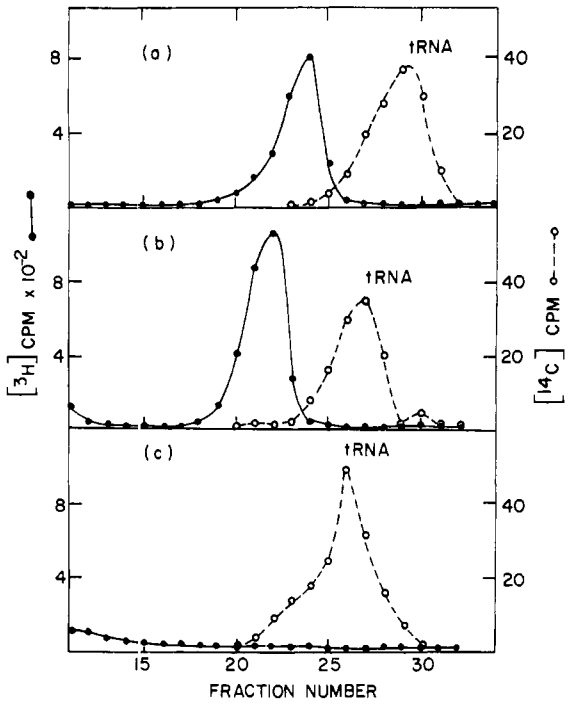

FIGURE 7: Polyacrylamide gel assays for low molecular weight RNA in protein-deficient RNP particles obtained by treatment of 50S ribosomal subunits from *E. coli* ML 63–86 with CsCl. (a) [^{3}H]RNA from 50S subunits. (b) [^{3}H]RNA from the A-band RNP particles formed by centrifugation in CsCl (Figure 5a). (c) [^{3}H]RNA from A′ RNP particles generated by the method of Lerman *et al.* (1966). Before deproteinization the particles were purified on a sucrose gradient to eliminate contaminating A-type particles. Input of *E. coli* [^{3}H]RNA on gels a, b, and c was in the ratio of 1.0:1.4:1.0, respectively. [^{14}C]tRNA was included in each gel as a marker.

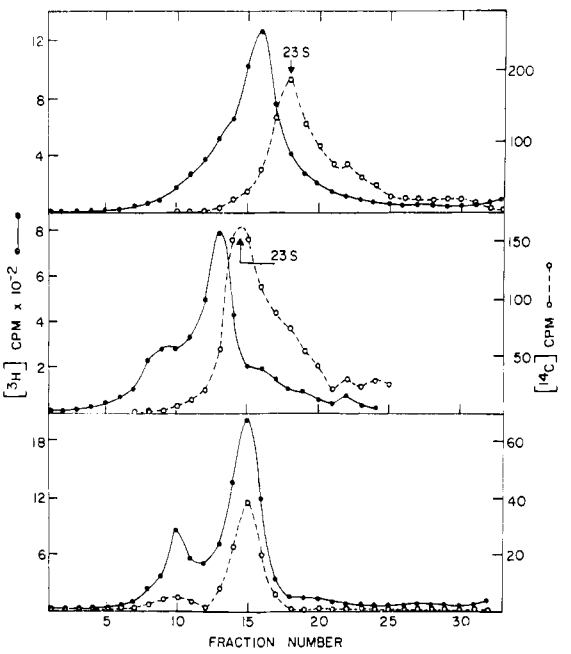

FIGURE 8: Sucrose gradient analysis of RNP particles generated by treatment of 50S ribosomal subunits of *E. coli* and *B. subtilis* with LiCl. (a) (top) [^{3}H]RNA-labeled RNP particles, obtained by LiCl treatment of *E. coli* ML 63–86 50S ribosomal subunits, were sedimented through a sucrose gradient with [^{14}C]RNA of *B. subtilis* as a marker. Centrifugation was for 6 hr using the conditions described in Methods; fractions were collected and TCA-precipitable counts were determined. (b) (middle) Same as a except that LiCl particles obtained by treatment of *B. subtilis* 50S subunits were assayed. (c) (bottom) LiCl particles were prepared from 50S ribosomal subunits of [^{3}H]RNA-labeled *E. coli* ML 63–86 and CsCl generated A′ particles prepared from [^{14}C]protein-labeled *E. coli* ML 63–68 were sedimented through a sucrose gradient. Centrifugation was for 6 hr using the conditions described in Methods; fractions were collected and TCA-precipitable counts were determined.

obtained with ribosomes from *B. subtilis* (Figure 4e–g). The results were not as clear as for the *E. coli* ribosomes because of a high background due to degradation of RNA in the 30S subunit. This degradation represents the breakdown of only a small fraction of 16S RNA; analysis by sucrose density gradient centrifugation showed that almost all of the RNA isolated from the 30S subunits sediments at a sharp peak at 16 S. However, the small amount of RNA that is degraded enters the gel and interferes with the assay. In order to eliminate this problem all further experiments dealing with the location of the 5S RNA species were done utilizing 50S subunits isolated on sucrose gradients. The ribosomal subunits obtained from *E. coli* and *B. subtilis* were treated with CsCl, LiCl, or EDTA and these derived RNP particles were assayed for the presence or absence of 5S RNA.

CsCl Core Particles. The 50S ribosomal subunits isolated from *E. coli* ML 63-86 and *B. subtilis* A26U$^-$ were banded in CsCl as described in Methods. When the concentration of Mg^{2+} is 0.06 M, the 50S ribosomal subunits isolated from either species of bacteria form a single band in the gradient. The density observed for the band of *E. coli* ribosomes (Figure 5a), 1.660 g cm^{-3}, is similar to that reported for protein-deficient CsCl core particles (A-band particles) by Meselson *et al.* (1964). Under these conditions no band of native particles (B band of Meselson *et al.*, 1964) was observed. The lack of the native B band when ribosomal subunits, as opposed to crude extracts, are centrifuged in CsCl has also been observed by Hosokawa *et al.* (1966). Ribosomal subunits from *B. subtilis* banded at a somewhat higher density, 1.676 g cm^{-3} (Figure 5c). Tubes containing the CsCl-generated A particles were pooled and dialyzed for several hours against 0.01 M Tris (pH 7.5), containing 10^{-3} M $MgCl_2$. Particles prepared in this manner were examined on sucrose gradients and their content of low molecular weight RNA was assayed on polyacrylamide gels. The CsCl-generated A particles from both organisms sediment as a homogeneous band at about 40 S (Figure 6a,c). As shown in Figure 7, CsCl-generated A particles from *E. coli* contain all of the 5S RNA originally associated with the 50S subunits. Similar results were obtained with A particles from *B. subtilis*.

Protein assays (see Methods) indicated that the A-band particles contained some 55–60% of the original (before banding) [^{14}C]valine. This is in good agreement with Hosokowa *et al.* (1966) who noted that the A-band particles retained about 60% of the original

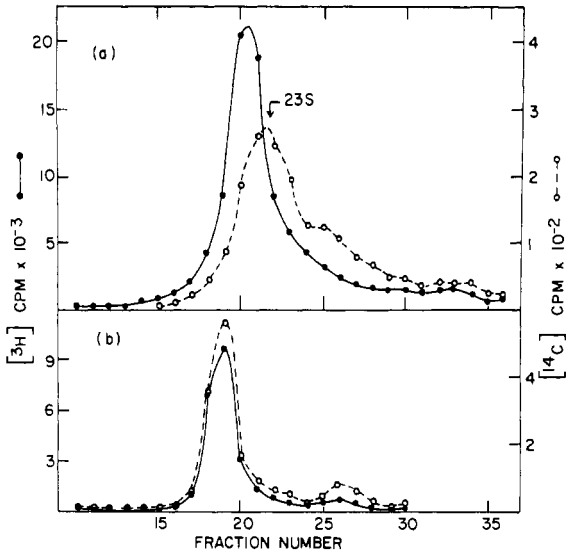

FIGURE 9: Sedimentation of 50S ribosomal subunits of *E. coli* through EDTA-containing sucrose gradients. (a) [^{3}H]-RNA-labeled 50S ribosomal subunits of *E. coli* A19 were sedimented through an EDTA-containing sucrose gradient for 6 hr as described in Methods. [^{14}C]RNA isolated from *B. subtilis* ribosomes was added as a marker. Fractions were collected and TCA-precipitable radioactivity was determined as described in Methods. (b) [^{3}H]RNA-labeled 50S ribosomal subunits of *E. coli* A19 and [^{14}C]protein-labeled 50S ribosomal subunits of *E. coli* A19 were sedimented through an EDTA-containing sucrose gradient as in a.

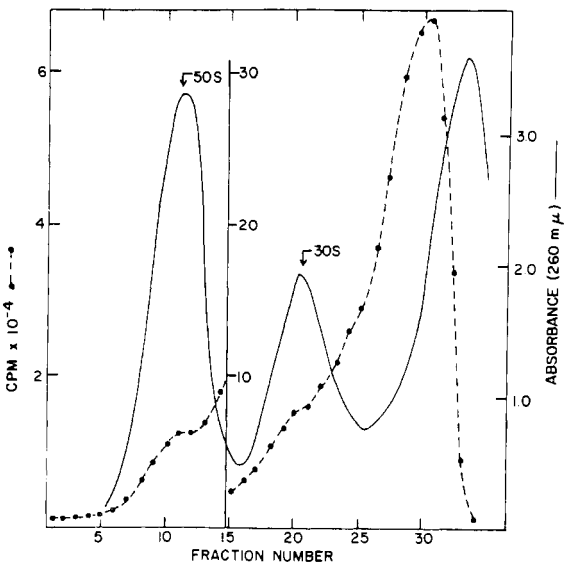

FIGURE 10: Sucrose gradients of crude extract of pulse-labeled *E. coli*. A step-up culture of *E. coli* A19 was pulse labeled with [^{3}H]uridine for 100 sec. A crude extract was prepared and sedimented through a sucrose gradient containing 10^{-4} M Mg^{2+} as described in Methods. Fractions were collected and TCA-precipitable radioactivity in 25-μl aliquots was determined.

^{35}S-labeled protein. Careful chemical assays by Lerman *et al.* (1966) indicate that 80% of the original protein is retained on CsCl-generated A particles. The value from chemical assays is less subject to error and might be more accurate.

Treatment of ribosomes with CsCl at low Mg^{2+} concentrations leads to the production of A′ particles (Spirin *et al.*, 1965), a class of RNP particles containing even less protein than A particles. Preparations of 50S ribosomal subunits from *E. coli* and *B. subtilis* were suspended for several hours in 0.025 M Tris (pH 7.5), containing 2×10^{-3} M Mg^{2+} and 5 M CsCl, then diluted to a density of 1.4 g cm^{-3} and the particles were collected by pelleting in an ultracentrifuge (Lerman *et al.*, 1966). In preparations of A′ particles the bulk of the radioactivity sedimented at about 28 S (Figure 6b,d). Gel assays of such preparations from *E. coli* after purification on sucrose gradients to remove some contaminating A-type particles showed them to be completely devoid of 5S RNA (Figure 7c). Similar results were obtained with A′ particles from *B. subtilis*. Protein assays (see Methods) indicated that the A′ particles contained 35–45% of the original (before banding) [^{14}C]valine. This value is also somewhat lower than the 50% value obtained by chemical analysis (Lerman *et al.*, 1966).

Lithium Chloride Core Particles. Protein-deficient particles can also be generated by treatment of ribosomes with LiCl, as has recently been demonstrated by Marcot-Queiroz and Monier (1966). These authors also reported that the resulting particles were free of 5S RNA. We have confirmed these observations with LiCl core particles formed by treatment of the 50S ribosomal subunits of *E. coli* and *B. subtilis*. Particles formed by such treatment (see Methods) were found to resemble CsCl-generated A′ particles in having a sedimentation coefficient of about 28 S (Figure 8a–c). Such particles, after purification on sucrose gradients to eliminate some contamination by a 40S component, were found to be completely lacking in 5S RNA. When the RNA in the supernatants of the LiCl pellets was assayed on gels, it was found to contain all of the 5S RNA. Protein assays (see Methods) indicated that approximately 35% of the original protein was still associated with the LiCl particles of *E. coli* and *B. subtilis*.

EDTA Particles. Gesteland (1966) demonstrated that *E. coli* ribosomes unfold in the presence of EDTA; the sedimentation coefficient drops from 50 to 21 S with retention of almost all of the protein and RNA originally present. An investigation was conducted to see if such particles contained 5S RNA. When 50S ribosomal subunits from *E. coli* A19 were sedimented through sucrose gradients containing EDTA (see Methods), they sedimented significantly faster than deproteinized RNA isolated from 50S subunits added as a marker (Figure 9a). A preparation of [^{14}C]leucine-labeled 50S subunits was sedimented along with the ^{3}H-labeled subunits. Figure 9b demonstrates that the bulk of the protein and RNA sediment together, indicating that at least 90% of the original protein remains associated with 23S rRNA. In control experiments where labeled ribosomal protein was disaggregated from RNA by treatment with SLS, radioactivity due to protein remained in the top two or three tubes of the gradient. When the RNA from EDTA particles, recovered from a sucrose gradient, was assayed on

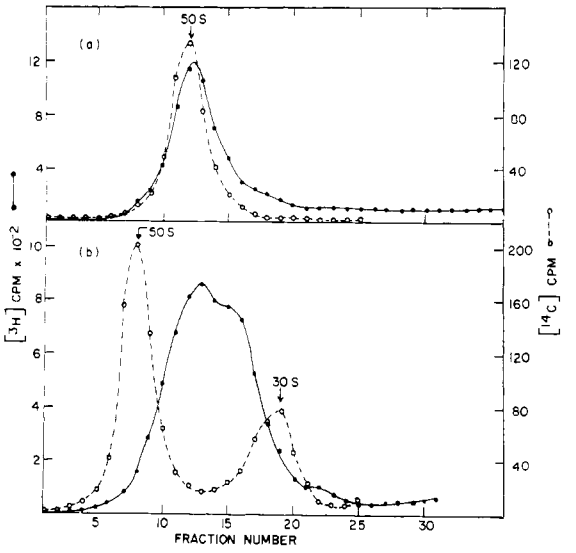

FIGURE 11: Sucrose gradient analysis of RNP particles from pulse-labeled *E. coli* A19. (a) An aliquot of tube 10 of the gradient shown in Figure 10 was run on an analytical sucrose gradient in 10^{-3} M Mg^{2+} as described in Methods. Ribosomal subunits isolated from *E. coli* A19 uniformly labeled with [^{14}C]uracil were added as a marker. (b) Same as a except that tube 16 of the gradient shown in Figure 10 was analyzed with [^{14}C]RNA-labeled 50S ribosomal subunits as a marker.

gels, it was found to be devoid of 5S RNA; the 5S RNA was all recovered from the top of the gradient. Similar experiments were difficult to carry out with *B. subtilis* ribosomes because of nuclease-induced degradation of RNA during the isolation process.

Precursor Particles. It has been noted in several laboratories that RNP particles, precursors to the mature ribosomal subunits, can be detected by sucrose gradient analysis of extracts of pulse-labeled cultures. Roberts *et al.* (1963) have detected a 43S precursor, and Osawa and his collaborators (Osawa, 1965) have

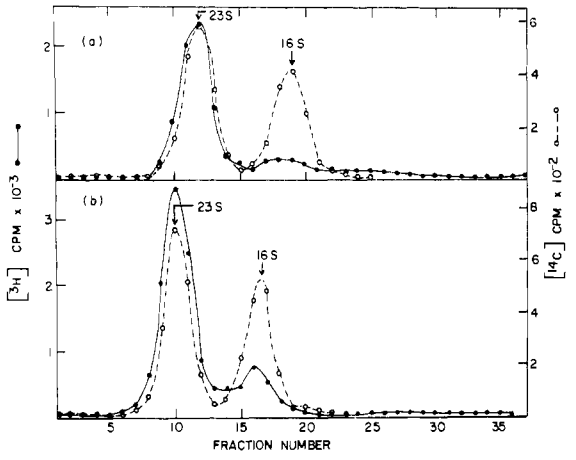

FIGURE 12: Sucrose gradient analysis of RNA in RNP particles from pulse-labeled *E. coli* A19. (a) RNA in particles from tube 10 of the gradient shown in Figure 10 was sedimented through a sucrose gradient containing TENS buffer as described in Methods. The RNA of a ribosomal pellet isolated from *E. coli* A19 uniformly labeled with [^{14}C]uracil was added as a marker. (b) Same as a except that RNA in particles from tube 16 was assayed.

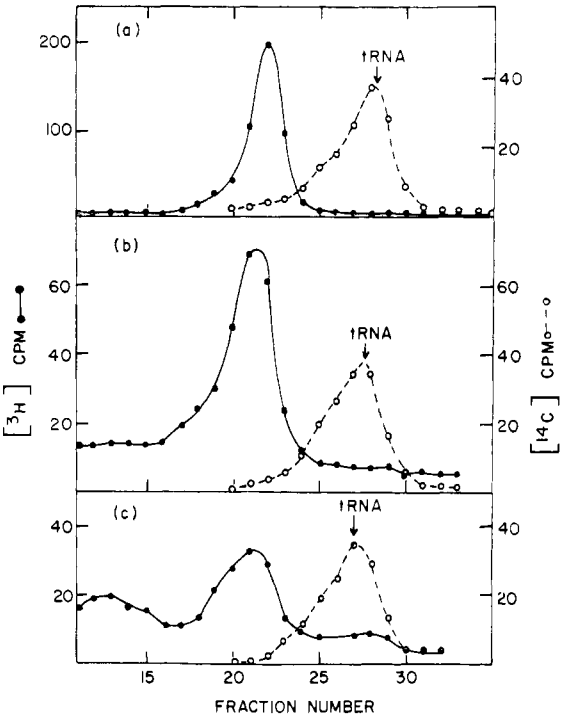

FIGURE 13: Polyacrylamide gel assays for low molecular weight RNA in RNP particles from pulse-labeled *E. coli* A19. (a) [^{3}H]RNA from 50S ribosomal subunits isolated from a uniformly labeled culture of *E. coli* A19. (b) [^{3}H]-RNA from RNP particles of tube 10 of the gradient shown in Figure 10. (c) [^{3}H]RNA from RNP particles of tube 16 of the gradient shown in Figure 10. Input of *E. coli* [^{3}H]RNA on gels a, b, and c was in the ratio of 1.0:1.0:2.0, respectively. [^{14}C]tRNA was included in each gel as a marker.

demonstrated the presence of 38S and 43S RNP particles which they believe to be precursors to the 50S subunits. In our experiments, shift-up cultures of *E. coli* A19 were pulse labeled with [^{3}H]uridine as described in Methods. The cultures were quickly chilled, harvested, and crude extracts were sedimented through sucrose gradients. Figure 10 shows absorbancy and radioactivity profiles of a preparation from a culture pulse labeled for 100 sec. The bulk of the radioactivity is present in the 4–30S region and presumably represents mRNA. Shoulders corresponding to the 30S and 50S regions are also visible. RNP particles in tubes corresponding to the 40S and to the 50S region were examined on sucrose gradients and the RNA they contained was analyzed on sucrose gradients and polyacrylamide gels. Newly synthesized particles isolated from the 50S region cosediment with 50S subunits from uniformly labeled cells (Figure 11a). The high molecular weight RNA isolated from these particles cosediments with the 23S RNA isolated from uniformly labeled cells (Figure 12a). The pulse-labeled RNA particles isolated from the 40S region of the preparative sucrose gradient are heterogeneous and consist of two or more classes of particles with sedimentation coefficients between 35 and 45 S (Figure 11b). Almost all of the radioactivity present in these RNP particles, which shall be referred to as precursor particles, is due to the labeling of 23S RNA (Figure 12b). An RNA component sedimenting

at 17 S can also be distinguished; presumably this is precursor to the 16S RNA of mature 30S ribosomal subunits (Kono and Osawa, 1964).

The low molecular weight RNA present in the precursor and 50S RNP particles was examined on polyacrylamide gels and compared to RNA from 50S subunits prepared from *E. coli* uniformly labeled with [^{3}H]uridine (Figure 13). It can be seen that after a 100-sec pulse there is approximately 35% as much labeled 5S RNA in the initially formed 50S subunits as there is in 50S ribosomal subunits isolated from uniformly labeled preparations. The comparable figure for the precursor particles is about 6%. The rapid appearance of labeled 23S RNA into newly formed 50S ribosomal subunits, relative to the lag in appearance of labeled 5S RNA, is evidence for a pool of 5S RNA, or precursor to 5S RNA, in *E. coli*. This "pool effect" is probably not a product of differential rates of synthesis of 5S and 23S RNA, since Galibert *et al.* (1966b) have shown that in exponentially growing *E. coli* cultures the rates of synthesis of 5S, 16S, and 23S RNA are identical. From the above data it also appears that the bulk of the precursor particles do not contain 5S RNA. The existence of a small class of precursor particles containing 5S RNA has not been excluded.

Preparations from four other *E. coli* A19 cultures, pulsed with [^{3}H]uridine for from 1 to 5 min, were examined using a similar protocol. Sucrose gradient radioactivity profiles of crude extracts showed that, as time of exposure to [^{3}H]uridine was increased, relatively more radioactivity was present in the 50S and 30S regions and less radioactivity was present in the low molecular weight, mRNA region of the sucrose gradient. When the RNA in the 50S ribosomal subunits of a culture pulse labeled for 1 min was examined on polyacrylamide gels, it was found to contain less than 10% of the amount of 5S RNA present in the ribosomes isolated from uniformly labeled *E. coli*. The equivalent figure for 50S ribosomal subunits isolated from a culture pulse labeled for 5 min was 80%. It was noted that with increasing length of radioactive pulse, 5S RNA began to appear on particles in the 40S region of the gradient, although the amount present, as a fraction of total activity, was always much less than in the 50S particles from the same gradient. A culture of *E. coli* A19 was uniformly labeled and chased (see Methods), a crude extract was sedimented through a sucrose gradient, and the particles in the 40S region were examined on sucrose gradients and polyacrylamide gels. The existence of particles sedimenting at about 40 S and containing 5S RNA was observed. It therefore appears that 5S RNA on particles in the 40S region has as precursor 5S RNA which first appears in the 50S particles. This could be due to some breakdown of 50S particles to give 40S particles containing 5S RNA, some other more involved metabolic process, or even a complicated artifact of the isolation procedure.

Discussion

In an effort to learn more about the structure of ribosomes, as a clue toward a further understanding of the mechanism of the complex processes in which these particles participate, a study was conducted of the association of 5S RNA to the 50S subunits. In particular, relatively gentle *in vitro* methods of removing 5S RNA from the ribosomes were studied, in the hope that this approach would eventually lead to reconstitution of biological activity by a procedure in which 5S RNA could be kept as the limiting factor. Certain *in vivo* formed particles were also studied in order to learn something about the biological processes by which 5S RNA enters the ribosomes.

Since Mg^{2+} ion is known to have a highly specific effect on the secondary structure (Monier and Grunberg-Manago, 1962) and ribosome binding properties (Cannon *et al.*, 1963) of tRNA, a preliminary study was conducted of the effects of Mg^{2+} on the structure of purified 5S RNA. Thermal denaturation studies indicated that 5S RNA has approximately the same extent of hyperchromicity as tRNA and the other rRNA species, but that the stabilizing effect of Mg^{2+} is exerted preferentially on tRNA. It was also demonstrated that Mg^{2+} ion preferentially protects tRNA from digestion by pancreatic RNase.

This preferential stabilization of tRNA by Mg^{2+} relative to high molecular weight rRNA has been observed previously when RNase susceptibility and temperature-induced hyperchromicity have been studied (Monier and Grunberg-Manago, 1962; Nishimura and Novelli, 1963). The protection of pancreatic RNase-sensitive sites in tRNA by Mg^{2+} has been attributed to stabilization of double-stranded regions (Litt and Ingram, 1964; Wagner and Ingram, 1966; Bell and Russell, 1967). It appears that 5S RNA resembles the other rRNA species in having a more extended conformation relative to the double-stranded regions induced in tRNA by the presence of Mg^{2+} ion. Since it has recently been shown that all of the Mg^{2+} in ribosomes is bound to rRNA (Goldberg, 1966; Choi and Carr, 1967), the primary effect of EDTA is probably the removal of this RNA bound Mg^{2+}. The electrostatic repulsion between the negatively charged phosphates probably forces the rRNA species into a more extended conformation and may account for the unfolding of ribosomes as well as aid in the dissociation of 5S RNA from the remaining RNP complex.

Analysis of total RNA of *B. subtilis* on Sephadex G-100 columns indicates that there is probably one 5S RNA molecule on each ribosome. Calculations are based on the assumption of a molecular weight of 39,000 for 5S RNA (Brownlee *et al.*, 1967) and the inclusion in each 70S ribosome of one 16S RNA and one 23S RNA molecule of mol wt 0.55 and 1.1×10^6, respectively (Kurland, 1960). Analysis on Sephadex columns demonstrated that the ribosomal supernatant of *B. subtilis* contains no significant amount of 5S RNA, and gel analysis also showed that 30S subunits did not contain 5S RNA. The above results are in agreement with those originally made in the *E. coli* system (Rosset *et al.*, 1964).

Purified 50S ribosomal subunits from *B. subtilis* and *E. coli* were treated by various procedures and the

relationship of 5 S to the RNP moiety was studied. The protein-deficient A-band particles produced by banding of ribosomes in CsCl in the presence of Mg^{2+} (Meselson *et al.*, 1964) are of particular interest because the split proteins can be restored with recovery of biological activity (Staehelin and Meselson, 1966; Hosokowa *et al.*, 1966). When such particles were produced from *E. coli* and *B. subtilis* 50S subunits and assayed for low molecular weight RNA, they were found to contain all of the 5S RNA originally present. Thus, more than 20% of the protein can be removed by this procedure without affecting the binding of 5S RNA to the RNP particles.

By treatment of ribosomes with CsCl under conditions of low Mg^{2+} or by treatment with LiCl, RNP A′ particles with less than 50% of the original protein can be produced. Such particles retain all of their 23S RNA but none of the 5S RNA originally present. It has been reported that split protein can be added back to the CsCl-generated A′ particles with recovery of the original 50S sedimentation coefficient. Particles reconstituted in this manner regain 5–10% of the original biological activity (Spirin *et al.*, 1966). In our hands the A′ particles generated by pelleting from CsCl are contaminated to the extent of approximately 10% by A-type particles, and therefore it is possible that the biological activity reported is due to the reconstitution of the contaminating A particles to their biologically active state. It is also possible that recovery of biological activity is poor because the 5S RNA is limiting, and therefore this system may offer one approach to a study of biological activity of 5S RNA.

The particles generated by sedimentation through a sucrose gradient containing EDTA are of interest because they retain most of the original protein although they have completely lost their 5S RNA. Presumably the 5S RNA is released as the ribosomes unfold, although the 23S RNA still remains associated with the protein. It is possible that a few per cent of the original protein which is released is still specifically bound to the 5S RNA. Biological activity cannot be restored to RNP particles generated in this manner (Gesteland, 1966). During the dialysis of ribosomes against EDTA, an intermediate stage, sedimenting at 36 S, has been observed (Gesteland, 1966) but at the present time no method is available to make quantitative preparations of such an intermediate. It is possible that the loss of 5S RNA is the irreversible step in the unfolding of 50S subunits.

From the data presented in this paper, it appears that the conformation of mature 50S subunits in the presence of Mg^{2+} is such that a considerable amount of protein can be removed, still leaving 5S RNA bound in some manner to the remaining RNP core particle. If more than half of the original protein is removed to form a LiCl core particle or CsCl A′ particle, the 5S RNA is freed from the RNP particle formed. The low molecular weight rRNA can also be released by treatment of the 50S subunits with EDTA, presumably by virtue of the change in conformation undergone by the ribosomes. A system still remains to be developed whereby the biological activity of 5S RNA can be assayed by addition of RNP particles which do not contain this species of RNA.

The biosynthesis of 5S RNA in *E. coli* was investigated by *in vivo* pulse-labeling experiments. It was demonstrated that a pool of 5S RNA, or a precursor to 5S RNA, exists. Since there is little detectable 5S RNA in the ribosomal supernatant of *E. coli* cultures (Cannon and Richards, 1967, and our own unpublished results), it is possible that a pool of high molecular weight precursor to 5S RNA exists. A similar result has been observed in the HeLa cell system; following a radioactive pulse newly formed 5S RNA appears in ribosomes after newly formed high molecular weight rRNA (Knight and Darnell, 1967). This is true in both the nucleus, where ribosomal particles first appear, and in the cytoplasm. However, this lag is accounted for by a pool of 5S RNA, corresponding to about 25% of the total 5S RNA. In our experiments with pulse-labeled *E. coli* cultures it was also demonstrated that, under our isolation conditions, 5S RNA becomes permanently associated with ribosomal precursors at a relatively late stage in the biosynthesis of ribosomes. When 40S precursor particles isolated on sucrose gradients containing 10^{-4} M Mg^{2+} were assayed for the presence of low molecular weight RNA, they were found to contain little or no 5S RNA. It has been shown that transformation from 40S precursor particles to 50S ribosomal subunits involves the addition of protein (Roberts *et al.*, 1963; Otaka *et al.*, 1967). Low molecular weight rRNA could enter at the 40S stage followed by addition of protein preferentially to such particles, or 5S RNA could become attached to a particle complete with respect to protein but lacking 5S RNA. Another explanation that fits this data is the existence of a common precursor for 23S and 5S RNA. If enzymatic cleavage of such a precursor, to yield 5S and 23S RNA occurred at a late stage in the maturation of ribosomal precursor particles a lag in the appearance of 5S RNA relative to that of a high molecular weight rRNA would be observed. The hypothetical precursor could differ in size from mature 23S RNA by only a few per cent and thus would not be observed in our sucrose gradient analysis of rRNA. This process would be analogous to the steps leading to the maturation of 28S rRNA by cleavage of high molecular weight precursors in RNP particles of the HeLa cell nucleolus (Warner and Soeiro, 1967). However, at the present time this hypothesis cannot be easily reconciled with the demonstration, by the technique of DNA–RNA hybridization, that in *B. subtilis* there are approximately twice as many chromosomal cistrons which code for 23S RNA as for 5S RNA (Smith *et al.*, 1968). We cannot differentiate between the various possibilities at the present time.

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