Isolation and Characterization of Low Molecular Weight Ribonucleic Acid Species from *Bacillus subtilis**

Pierre Morell, Issar Smith, David Dubnau,† and Julius Marmur

ABSTRACT: A procedure is described for the isolation of transfer ribonucleic acid (tRNA) and another low molecular weight RNA species from *Bacillus subtilis*. The latter, as characterized by its chromatographic and sedimentation properties, base ratio, and its association with ribosomes, is similar in many respects to the 5S RNA reported in other species. Purification of both low molecular weight RNA species by column chromatography on Sephadex G-100 and methylated albumin-coated kieselguhr eliminated contamination by ribosomal ribonucleic acid (rRNA) degradation products and made possible RNA–DNA hybridization studies. Such investigations demonstrated that tRNA is complementary to 0.04% and 5S RNA to 0.005% of the *B. subtilis* genome. Competition studies by unlabeled RNA species for deoxyribonucleic acid (DNA) sites occupied by labeled tRNA or 5S RNA demonstrated that the low molecular weight RNA species are distinct from each other and are not products of the random degradation of rRNA.

Hybridization studies of RNA with DNA have indicated that various classes of mRNA and rRNA are complementary to unique regions of the genome (Spiegelman and Hayashi, 1963). Similar studies with tRNA have been reported (Giacomoni and Spiegelman, 1962; Goodman and Rich, 1962), but such investigations are hampered by contamination of tRNA preparations by products of rRNA degradation (Ritossa *et al.*, 1966). In addition, recent reports have described a ribosome-bound, low molecular weight RNA species in a number of organisms (Rosset *et al.*, 1964; Sarkar and Comb, 1965; Galibert *et al.*, 1966; Marcot-Queiroz *et al.*, 1965). This distinct class of RNA, having a sedimentation coefficient of approximately 5 S, has hitherto remained undetected, since the usual techniques for the preparation and identification of tRNA do not clearly reveal its presence (Schleich and Goldstein, 1966). Recently Zehavi-Wilner and Comb (1966) have studied the hybridization of *Escherichia coli* 5S RNA to DNA, showing that this class of RNA is complementary to a restricted portion of the genome.

This report will deal with the isolation and characterization of tRNA and another low molecular weight RNA species from *Bacillus subtilis*. The latter will be referred to as 5S RNA, in accordance with the convention established by Rosset and Monier (1963), although an exact sedimentation coefficient has not yet been established.

Experimental Section

Materials. STRAINS USED. *B. subtilis* A26U⁻, a uracil-requiring derivative of *B. subtilis* 168 obtained from I. Takahashi, was used for the preparation of DNA and for the isolation of RNA. For the preparation of DNA and RNA from *E. coli*, the pyrimidine-requiring mutant ML 63–86 was employed.

MEDIA. *E. coli* and *B. subtilis* cultures used for the preparation of [^{3}H]RNA were grown in uridine-labeling medium (ULM)[1] consisting of minimal salt medium (Anagnostopoulos and Spizizen, 1961) supplemented with 0.2% acid-hydrolyzed casamino acids (Difco, Detroit, Mich.) and 0.5% glucose, sterilized separately. Uridine (Calbiochem, Los Angeles, Calif.) and [5-^{3}H]uridine, 18 c/mmole (Nuclear Chicago Corp., Chicago, Ill.), were added as described in Methods. For ^{32}P labeling of *B. subtilis*, phosphate-labeling medium (PLM), identical with ULM but with 0.025 M Tris, pH 7.0, substituted for phosphate buffer in minimal salts medium, was used. Carrier phosphate and [^{32}P]-Na_3PO_4 (Isoserve Corp., Cambridge, Mass.) were added as described below. V-Y medium (25 g of Difco veal

* From the Departments of Biochemistry and Pathology, Albert Einstein College of Medicine, Yeshiva University, New York, New York. *Received September 26, 1966.* This investigation was supported by research grants from the U. S. Public Health Service (NIH-GM-11946-04), the National Science Foundation (GB-4685), the Atomic Energy Commission (AT-30-1-3311), the U. S. Air Force (R-716-65), and the American Cancer Society (E-380). Financial support for P. M. was from Departmental Biochemistry Training Grant 2-T01-GM00563-06. I. S. was supported by a grant from the National Institutes of Health (CA-06576) awarded to Dr. A. Novikoff. Financial support for D. D. was in part from the Public Health Research Institute of the City of New York and for J. M. from the Health Research Council of the City of New York (I-322).

† Present address: Public Health Research Institute of the City of New York, New York, N. Y.

[1] Abbreviations used in this work: ULM, uridine-labeling medium; PLM, phosphate-labeling medium; and V-Y, veal infusion-yeast extract medium (the composition of these media is described in the text); BSA, bovine serum albumin; MAK, methylated albumin-coated kieselguhr; TCA, trichloroacetic acid; SLS, sodium lauryl sulfate; UMP, GMP, AMP, and CMP, uridine, guanosine, adenosine, and cytosine monophosphates.

infusion broth plus 5 g of Difco yeast extract in 1 l. of water) was used to grow bacteria for the preparation of unlabeled RNA and DNA. Cultures used for the isolation of DNA or RNA were grown at 37° on a rotary shaker.

DNA for hybridization studies with RNA was prepared from late log phase cultures by the method of Marmur (1961), except that after the last chloroform–isoamyl alcohol deproteinization the aqueous phase was shaken with phenol which had been water saturated and neutralized with NaOH. Phenol (Mallinckrodt, St. Louis, Mo.) was redistilled and stored at −10° until use.

Bovine serum albumin (BSA) (fraction V, Armour Pharmaceutical Co., Kankakee, Ill.) was used to prepare methylated albumin (Mandell and Hershey, 1960). Sephadex G-100 (Pharmacia, Piscataway, N. J.) was kept for several days in 1 M NaCl at 4° and washed repeatedly to eliminate fine particles before use. Bentonite was purified as described by Fraenkel-Conrat *et al.* (1961). Electrophoretically purified deoxyribonuclease was obtained from Worthington Biochemical Corp. (Freehold, N. J.). Ribonuclease T_1 (Sankyo Co., Ltd., Tokyo, Japan) and pancreatic ribonuclease (Worthington) were each heated in 0.1 M acetate buffer, pH 5.2, for 15 min at 80° to eliminate possible deoxyribonuclease activity. Absorbancy of nucleic acid preparations was determined in a Zeiss spectrophotometer. Refractive indices were measured in a Bausch and Lomb refractometer. Cell turbidity was measured in a Klett–Summerson colorimeter using a no. 62 filter. One Klett unit corresponds to a viable count of approximately 1.6×10^6 *B. subtilis* cells/ml. Nitrocellulose membrane filters (Millipore HAWP, obtained from Matheson–Higgins, Cambridge, Mass.) were used to trap DNA–RNA hybrids and to collect TCA-precipitable material. These filters were then dried and counted in scintillator containing 0.1 g of *p*-bis-2-(5-phenyloxazolyl)benzene (Pilot Chemicals, Watertown, Mass.) and 4 g of 2,5-diphenyloxazole (Pilot) per l. of toluene. Columns used for the purification of labeled RNA were monitored by counting aliquots of the effluent in Bray's (1960) solution. Radioactivity was determined in a Nuclear-Chicago (Chicago, Ill.) or Ansitron (Wallingford, Conn.) liquid scintillation counter. All glassware was either acid washed or heated to eliminate possible contaminating ribonuclease activity.

Methods. A methylated albumin-coated kieselguhr (MAK) column similar to that described by Mandell and Hershey (1961) was used with slight modification. All NaCl solutions were buffered with 0.025 M Tris, pH 7.0, instead of phosphate, and the column was scaled down to one-half size (8 × 2 cm). Before gradient elution, the column was washed with 100 ml of 0.25 M NaCl. A linear NaCl gradient, prepared by adding 200 ml of 0.25 M NaCl to the mixing chamber and 200 ml of 1.1 M NaCl to the reservoir, was then used to elute RNA from the column. A peristaltic pump (Buchler, Fort Lee, N. J.) was adjusted to deliver the solution from the mixing chamber to the column at a rate of 60 ml/hr. Fractions of 5.5 ml were collected.

Sephadex G-100 columns (1.2 × 115 cm), used to resolve tRNA from 5S RNA, were eluted with 1 M NaCl (Schleich and Goldstein, 1964) by gravity flow at 4°. Columns were repacked when the original flow rate of about 24 ml/hr slowed to 10 ml/hr. Precipitation of RNA eluted from columns, as well as all other RNA precipitations, was accomplished by the addition of two volumes of ethanol at −10° followed by storage at this temperature for at least 12 hr. The precipitate was collected by centrifugation at 27,000*g* at 0° for at least 30 min.

DNA concentrations were determined colorimetrically (Burton (1956) as modified by Giles and Myers (1965)). Concentrations of tRNA and 5S RNA were determined by ultraviolet absorption ($A^{1\%}_{258}$ 215). This extinction value is that used for pure tRNA (Lindahl *et al.*, 1965), and it was assumed that 5S RNA would not differ greatly from this value. The concentration of NaCl in MAK column effluents was determined by measurement of refractive indices.

Density gradient sedimentation of RNA was carried out with linear (15–30%) sucrose gradients prepared in 0.01 M Tris, pH 7.5, 0.01 M EDTA, 0.1 M NaCl, and 0.5% sodium lauryl sulfate (SLS). Centrifugation was at 25,000 rpm for 17 hr at 22° using the SW25 rotor of the Spinco Model L ultracentrifuge. Base ratios of [^{32}P]RNA were determined by high-voltage electrophoresis of alkaline hydrolysates (Sebring and Salzman, 1964).

DNA–RNA hybridization was initially performed by the immobilized DNA membrane filter techniqe (Gillespie and Spiegelman, 1965). Hybridization in solution and trapping of the hybrid on nitrocellulose filters (Nygaard and Hall (1964) as modified by Oishi and Sueoka (1965)) gave the same results with respect to saturation plateaus and background, and this simpler procedure was routinely used. DNA samples in 0.015 M NaCl–0.0015 M sodium citrate were denatured in a boiling water bath for 10 min followed by quick cooling in ice. The denatured *B. subtilis* or *E. coli* DNA (10 μg) was mixed with homologous RNA in 0.5 ml of 0.01 M Tris, pH 7.4, 0.002 M $MgCl_2$, and 0.25 M NaCl containing 30 μg of denatured carrier DNA from the virulent *B. subtilis* phage 2C. The homologous DNA was omitted in control tubes. Annealing was carried out by incubation for 4 hr at 68° in stoppered tubes. The tubes were then transferred to a water bath at 37° and 0.05 ml of a solution containing 40 μg/ml of pancreatic ribonuclease and 100 units/ml of ribonuclease T_1 was added. The enzyme digestion was carried out for 15 min. To each tube 5 ml of 0.5 M KCl in 0.01 M Tris, pH 7.5, was added and the hybrid was collected on filters and washed. The filters were dried for 1 hr at 80° in a vacuum oven and counted in toluene scintillator. In the case of 5S RNA hybridization saturation levels were approximately 110 cpm, of which 60 cpm was found in blanks. In the case of tRNA saturation was at approximately 500 cpm, while appropriate blanks were 100 cpm.

For the preparation of [^{3}H]tRNA and 5S RNA, an overnight culture of *B. subtilis* grown in ULM contain-

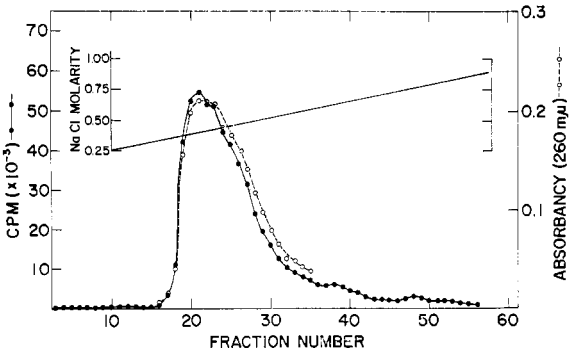

FIGURE 1: Purification of [^{3}H]tRNA by MAK column chromatography. RNA (0.9 mg), isolated from the ribosomal supernatant of disrupted *B. subtilis* cells, was suspended in 20 ml of 0.25 M NaCl buffered with 0.025 M Tris, pH 7.0. This solution was loaded onto a MAK column (2 × 8 cm), washed, and eluted as described in Methods. Radioactivity was determined by counting 50-μl aliquots in Bray's solution.

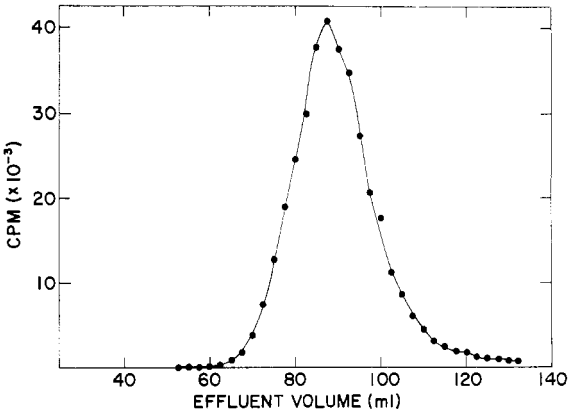

FIGURE 2: Further purification of [^{3}H]tRNA by Sephadex column chromatography. The tRNA recovered from the MAK column effluent (shown in Figure 1) was concentrated by ethanol precipitation and chromatographed on a Sephadex G-100 column (1.2 × 115 cm) using 1 M NaCl as eluent. Radioactivity was determined by counting 30-μl aliquots in Bray's solution.

ing 100 μg/ml of uridine was used. The culture was washed once with ULM and a small innoculum was transferred to 100 ml of fresh ULM containing 5 μg/ml of [^{3}H]uridine (2.44 c/mmole) to give a Klett reading of 4. In order to chase radioactivity from the mRNA after exhaustion of the labeled uridine (Klett reading of 40), the cells were starved for uridine for 30 min; then 200 ml of prewarmed ULM containing 150 μg/ml of unlabeled uridine were added and growth was continued until the Klett reading was again 40. *E. coli* cultures were labeled with [5-^{3}H]uridine by the same procedure.

For ^{32}P labeling of *B. subtilis* an overnight culture was washed and innoculated into 150 ml of PLM containing 0.3 mM [^{32}P]Na_3PO_4 (550 mc/mmole) and 50 μg/ml of unlabeled uridine. Before the end of exponential growth (Klett reading of 100), the cells were harvested, washed, and resuspended in four times the original volume of PLM containing 0.3 M sodium phosphate buffer, pH 7.0, and 50 μg/ml of uridine. The cells were grown for two generations to chase radioactivity out of the mRNA. For the isolation of unlabeled RNA, *B. subtilis* A26U$^-$ was grown in V-Y medium and harvested during exponential growth.

Results

Preparation of Labeled RNA from B. subtilis. After labeling *B. subtilis* with [^{3}H]uridine and chasing as described in Methods, the cultures were quickly chilled in an ice bath and harvested in a refrigerated centrifuge. The cells were washed once with 20 ml of 0.005 M Tris, pH 7.5, and 0.01 M $MgCl_2$ and resuspended in 9 ml of this buffer. The cells were broken in a chilled French pressure cell (American Instrument Co., Silver Springs, Md.) at a pressure of 10 tons/in^2. Deoxyribonuclease and Bentonite were added to a final concentration of 10 μg/ml and 2 mg/ml, respectively. The preparation was centrifuged at 27,000*g* for 15 min at 4° to sediment intact cells and debris. The supernatant was recovered and Bentonite was again added to give a final concentration of 2 mg/ml. This cell extract was centrifuged at 105,000*g* at 4° in the 40 rotor of a Spinco Model L2 ultracentrifuge. The supernatant was used to isolate the tRNA and the ribosomal pellet was saved for the preparation of 5S RNA and, on occasions, rRNA.

Purification of tRNA. The top three-fourths of the supernatant was removed with a Pasteur pipet and to this portion of the supernatant was added Bentonite (2 mg/ml) together with an equal volume of phenol saturated with 0.005 M Tris, pH 7.5, and 0.01 M $MgCl_2$. The mixture was shaken on a Vortex mixer for several minutes and the phenol and aqueous phases were separated by centrifugation at 12,000*g* for 10 min. The aqueous layer was removed and shaken with ether several times to remove phenol. One-tenth volume of 20% potassium acetate and two volumes of cold ethanol were added. The crude tRNA, approximately 0.9 mg, was collected by centrifugation at 30,000*g* for 30 min and chromatographed on a MAK column (Figure 1). The peak fractions from this column were pooled and precipitated with alcohol. The precipitate was suspended in 1.5 ml of 1 M NaCl, loaded on a Sephadex G-100 column, and eluted with 1 M NaCl (Figure 2). The peak fractions were pooled, alcohol precipitated, collected, and stored in 0.1 M sodium acetate, pH 5.2, at −10°.

Purification of 5S RNA. The ribosomal sediment was rinsed twice with 0.1 M sodium acetate, pH 5.2, without disturbing the pellet. The pellet was then resuspended in 10 ml of the same acetate buffer supplemented with 0.5% SLS. This suspension was shaken on a Vortex mixer for several minutes. An equal volume of phenol saturated with acetate buffer was added, and

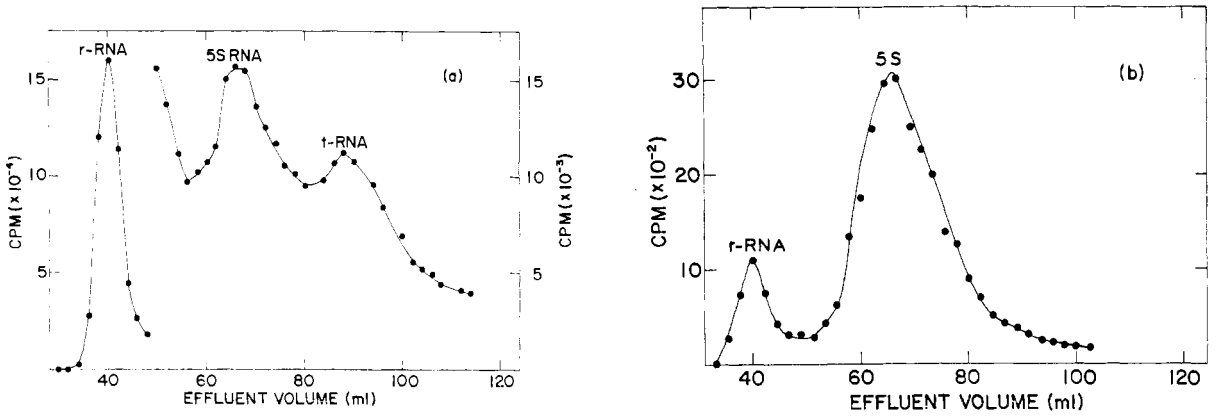

FIGURE 3: Purification of 5S [^{3}H]RNA by Sephadex column chromatography. (a) Chromatography of [^{3}H]RNA from a deproteinized ribosomal pellet, enriched in low molecular weight species by prior 1 M NaCl fractionation. The RNA, representing the total yield from 300 ml of *B. subtilis* cells (Klett 40), was chromatographed on Sephadex G-100 as described in the text. The radioactivity was determined by counting 30 μl of each fraction in 10 ml of Bray's solution. The left ordinate refers to fractions contained in the first 50 ml of effluent, and the right ordinate refers to later fractions. (b) The fractions containing the 5S peak from the Sephadex chromatography illustrated in Figure 3a were pooled, alcohol precipitated, and rechromatographed on the same column.

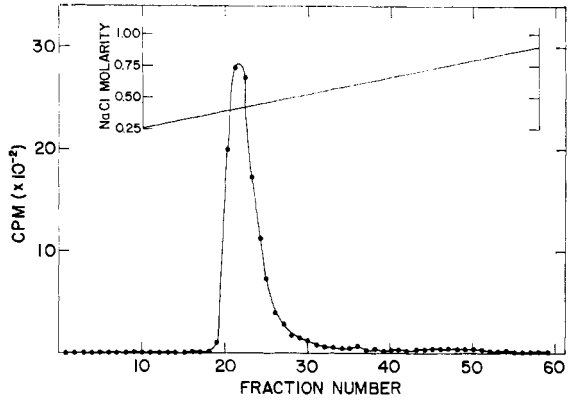

FIGURE 4: Further purification of 5S [^{3}H]RNA by MAK column chromatography. 5S RNA, recovered from the Sephadex column purification step shown in Figure 3, was diluted and chromatographed as described in the text. Radioactivity was determined by counting 50-μl aliquots in Bray's solution.

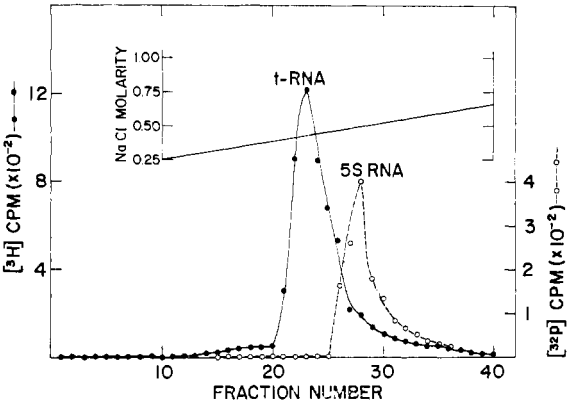

FIGURE 5: Resolution of t- and 5S RNA by means of MAK column chromatography. A mixture of [^{3}H]tRNA (6 μg, 100,000 cpm/μg) and 5S [^{32}P]RNA (1 μg, 100,000 cpm/μg) was chromatographed on a MAK column, as described in Methods. Radioactivity was determined by counting 50-μl aliquots in Bray's solution.

deproteinization and alcohol precipitation of the RNA were carried out as for the supernatant, omitting the ether-extraction step. Ethanol-precipitated RNA isolated from the ribosomal pellet was collected and suspended in 1.5 ml of 1 M NaCl by shaking at room temperature for approximately 1 min. (Care must be taken not to carry over any alcohol as this will interfere with the next step.) The 1 M suspension was left at 0° for several hours. The precipitate which formed (containing the bulk of the high molecular weight RNA) was eliminated by centrifugation at 27,000*g* for 15 min at 0°. The supernatant was chromatographed on a Sephadex G-100 column (Figure 3a). A large ribosomal peak appeared first in the effluent, followed by 5S and tRNA peaks. Larger columns can effect a complete separation of the three peaks, but it proved more convenient to pool the tubes of the 5S peak, precipitate with alcohol, and rechromatograph the RNA on the same column (Figure 3b). The 5S peak fractions from the second Sephadex column were pooled and three volumes of 0.033 M Tris, pH 7.0, was added to bring the NaCl concentration to 0.25 M. This solution was loaded directly onto a MAK column and eluted (Figure 4). The eluent fractions containing the 5S RNA were pooled, precipitated with alcohol, collected, and stored in 0.1 M sodium acetate, pH 5.2, at −10°. The above

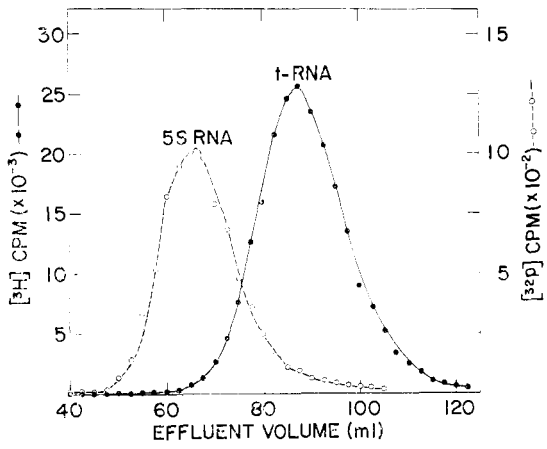

FIGURE 6: Resolution of t- and 5S RNA by means of Sephadex G-100 chromatography. A mixture of [^{3}H]t-RNA (4.5 μg, 100,000 cpm/μg) and 5S [^{32}P]RNA (1.5 μg, 100,000 cpm/μg) was chromatographed on Sephadex G-100 as described in Methods. Fractions (1 ml) were TCA precipitated in the presence of 50 μg of BSA and collected on nitrocellulose filters. Radioactivity was determined in toluene scintillator.

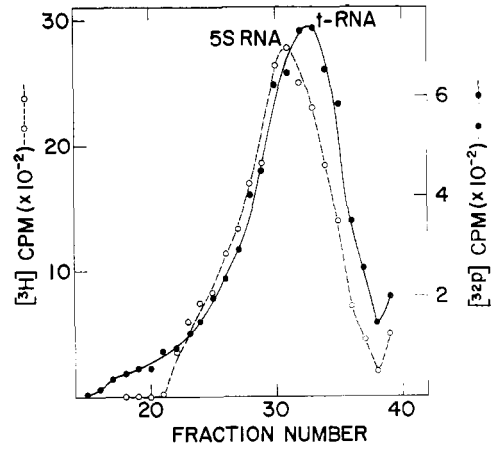

FIGURE 7: Resolution of t- and 5S RNA by means of sucrose density-gradient centrifugation. A mixture of [^{3}H]tRNA (1 μg, 70,000 cpm/μg) and 5S [^{32}P]RNA (0.1 μg, 200,000 cpm/μg) was subjected to sucrose density-gradient centrifugation using conditions described in Methods. Each fraction was precipitated with an equal volume of 10% TCA in the presence of 50 μg of BSA and collected on nitrocellulose filters. Radioactivity was determined in toluene scintillator.

procedure was carried out so that labeling of the cells, preparation of the supernatant and ribosomal pellet fractions, and subsequent deproteinizations and ethanol precipitations were all completed on the same day. The purification procedure can best be interrupted at any of the ethanol precipitation steps. About 150–200 μg of tRNA and 20 μg of 5S RNA were usually obtained from a 300-ml culture with a Klett reading of 40. The procedure can be scaled up as desired. The concentration of cells used during breakage in the French pressure cell and subsequent deproteinization has been increased fourfold, as has the amount of RNA loaded on the Sephadex columns, without affecting the purity of resulting preparations. However, to avoid excessive spreading of the peaks no more than 1 mg of a given species of RNA was loaded on a MAK column.

These procedures were used for the preparation of tRNA and 5S RNA from unlabeled cells as well as from cells labeled with ^{32}P as described in Methods. RNA from [^{3}H]uridine-labeled *E. coli* was also prepared by the same method.

During preparation of low molecular weight RNA, rRNA is a by-product which precipitates when the total RNA from the deproteinized ribosomal pellet is suspended in 1 M NaCl. Unlabeled rRNA for hybridization–competition experiments was prepared in this manner and resolved into 16S and 23S components by repeated sucrose density-gradient fractionation. The ratio of 23S:16S RNA was 2:1 as measured by ultraviolet absorption, indicating that there had been little ribonuclease action during the initial stages of purification.

Resolution of tRNA and 5S RNA. Since tRNA and 5S RNA do not differ greatly in over-all base composition or size (Rosset *et al.*, 1964), various methods of physically separating these species were evaluated. When MAK columns were used analytically to resolve artificial mixtures of 5S [^{32}P]RNA and [^{3}H]tRNA (Figure 5) the separation of the two species was incomplete. When larger amounts of RNA were employed separation was even poorer, since the width of the RNA peaks eluted from the MAK column increased greatly with input and the 5S RNA was eluted at a lower salt concentration. Sephadex G-100 columns have been used for the identification and preparation of 5S RNA from several organisms (Galibert *et al.*, 1965; Schleich and Goldstein, 1966). The ability of such a column to resolve the tRNA and 5S RNA of *B. subtilis* is shown in Figure 6. The resolution of peaks can be improved by increasing the length and width of the column. Repeated chromatography on either MAK or Sephadex columns alone did not reproducibly provide tRNA or 5S RNA of sufficient purity for hybridization studies. Sucrose gradients are of little use in preparative resolution of RNA species although they demonstrate the slight but significantly greater sedimentation velocity of 5S RNA relative to tRNA (Figure 7).

Base Composition. The base analysis of [^{32}P]tRNA and 5S RNA is shown in Table I. The 5S RNA, with a 57.4% guanosine + cytosine (GC) content, is similar to tRNA with a GC content of 58.7%, whereas *B. subtilis* DNA has a 43% GC base composition (Marmur and Doty, 1962). The electrophoresis procedure used (Sebring and Salzman, 1964) did not permit resolution of methylated bases or pseudouridine, reportedly absent in 5S RNA (Rosset *et al.*, 1964). This high GC value for 5S RNA is similar to that reported in other organ-

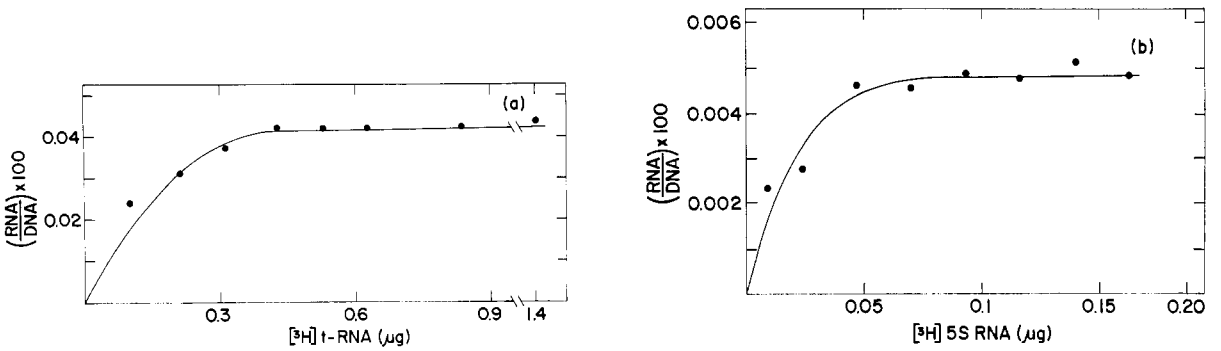

FIGURE 8: Hybridization of *B. subtilis* t- and 5S RNA to DNA. Each experimental point represents the hybridization of 8.75 μg of denatured DNA to the indicated amount of [^{3}H]RNA using the conditions described in Methods. (a) [^{3}H]tRNA (118,000 cpm/μg). (b) 5S [^{3}H]RNA (from the same batch of labeled cells and of the same specific activity as the tRNA).

TABLE 1: Nucleotide Composition (moles/100 moles).[a]

	tRNA	5S RNA
UMP	22.8	19.9
GMP	32.9	30.3
AMP	18.5	22.5
CMP	25.8	27.1

[a] [^{32}P]RNA (30,000 cpm) was hydrolyzed and the nucleotides were separated by high-voltage electrophoresis (Sebring and Salzman, 1964) using yeast tRNA as a carrier. The filter paper was cut in strips and radioactivity was determined in toluene scintillator. Each value is the average of three determinations.

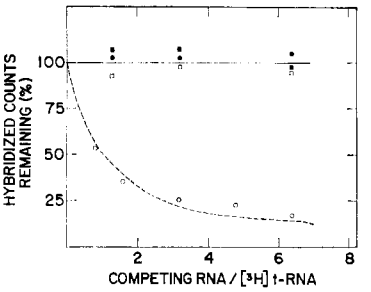

FIGURE 9: Competition of unlabeled *B. subtilis* RNA species with [^{3}H]tRNA hybridized to denatured DNA. Each hybridization tube contained 0.44 μg of [^{3}H]t-RNA (described in Figure 8), 8.75 μg of denatured *B. subtilis* DNA, and competing unlabeled RNA as indicated. ○, tRNA; ●, 5S RNA; □, 16S RNA; and ■, 23S RNA. ———, theoretical curve if unlabeled RNA has no base sequences in common with the labeled species. ---------, theoretical curve if unlabeled RNA is identical in base sequence with the labeled species.

isms (Rosset and Monier, 1963; Galibert *et al.*, 1965).

Hybridization Studies. Hybridization studies with denatured *B. subtilis* DNA were performed using radioactive tRNA and 5S RNA. Saturation curves, showing per cent of the DNA hybridized to RNA as a function of increasing tRNA and 5S RNA input, are shown in Figure 8. The saturation values indicate that 0.04% of the genome of *B. subtilis* is complementary to tRNA, and 0.005% to 5S RNA. Similar values, 0.030–0.045% for tRNA and 0.0040–0.0055% for 5S RNA, were obtained with different RNA and DNA preparations.

Competition studies were performed to determine whether the base sequences of tRNA and 5S RNA are unique. The ability of unlabeled RNA fractions to interfere with hybrid formation between denatured DNA and [^{3}H]tRNA (Figure 9) or 5S [^{32}P]RNA (Figure 10) was tested. Unlabeled 5S, 16S, and 23S RNA did not interfere with the hybridization of [^{3}H]tRNA to DNA. Unlabeled tRNA decreased the amount of radioactive tRNA hybridized to the extent predicted. In the same fashion, unlabeled tRNA, 16S, and 23S RNA did not interfere with the hybridization of 5S [^{32}P]RNA to DNA, although unlabeled 5S RNA reduced the number of counts hybridized as expected.

To show that this method of tRNA preparation is applicable to other microorganisms, [^{3}H]tRNA was isolated from *E. coli*. Hybridization studies with this preparation showed that 0.046% of the *E. coli* genome is complementary to tRNA (Figure 11).

Discussion

Hybridization studies of tRNA and 5S RNA have not been as extensive as those with other classes of RNA because of the stringent requirement for purity of these low molecular weight preparations. This requirement arises because hybridization to the small portion of the genome coding for tRNA or 5S RNA can be completely masked by the presence of RNA species which are complementary to a larger portion of the genome. Contamination of tRNA and 5S RNA by the low mo-

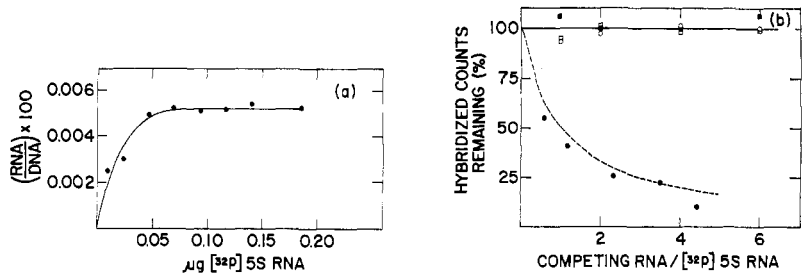

FIGURE 10: Competition of unlabeled *B. subtilis* RNA species with 5S [^{32}P]RNA hybridized to denatured DNA. (a) Hybridization of 8.75 μg of denatured DNA to the indicated amount of 5S [^{32}P]RNA (247,000 cpm/μg), using the conditions described in Methods. (b) Effect of the indicated level of unlabeled RNA upon the hybridization of 8.75 μg of denatured DNA to 0.116 μg of 5S [^{32}P]RNA. Symbols have the same meaning as in Figure 9.

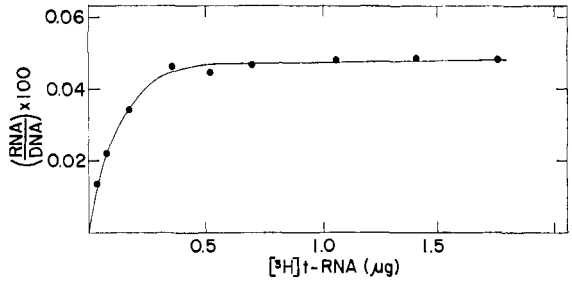

FIGURE 11: Hybridization of *E. coli* tRNA to DNA. Each experimental point represents the hybridization of 8.9 μg of denatured *E. coli* DNA to the indicated amount of [^{3}H]tRNA (92,000 cpm/μg) using the conditions described in Methods.

lecular weight degradation products of rRNA (Aronson and McCarthy, 1961) is probably the major cause of difficulty in such studies (Baguley and Ralph, 1966). Ritossa *et al.* (1966) overcame this problem by use of unlabeled rRNA to supress hybridization of labeled RNA fragments contaminating their tRNA preparation. Our preparations were essentially free of such contamination, as demonstrated by the inability of rRNA to interfere with 5S RNA and tRNA hybridization.

Mechanical disruption of *B. subtilis* with a French pressure cell gave better yields of tRNA and 5S RNA than methods employing organic solvent extraction of whole cells (Monier, 1962; Rammler *et al.*, 1965). This procedure also permitted a partial separation by centrifugation of tRNA from 5S and rRNA. Further purification by both MAK and Sephadex column chromatography was required to reproducibly yield low molecular weight RNA preparations pure by hybridization criteria.

Our value for the portion of the *B. subtilis* genome complementary to tRNA, 0.04%, can be compared to the value 0.07%, reported by Oishi *et al.* (1966), obtained with DNA isolated from exponentially growing cells. This discrepancy is not surprising in light of results which indicate that DNA extracted from *B. subtilis* cells in the exponential phase of growth is enriched in copies of early cistrons (Oishi *et al.*, 1966), which include those coding for tRNA (Dubnau *et al.*, 1965; Oishi *et al.*, 1966). The plateau values reported here were obtained with DNA isolated from cells in the late-log phase of growth at which time many of the chromosomes were probably in the completed state. Our saturation value for tRNA in *E. coli*, 0.046%, is within the range reported by Zehavi-Wilner and Comb (1966) and about twice as high as previously indicated (Giacomoni and Spiegelman, 1962; Goodman and Rich, 1962).

The data for 5S RNA indicate that about 0.005% of the *B. subtilis* genome is complementary to this species of RNA. Competition studies reported here demonstrate that this is a unique species of RNA different in base sequence from tRNA and not a random degradation product of rRNA. These results are essentially similar to those reported in the *E. coli* system (Zehavi-Wilner and Comb, 1966). Similar conclusions have been reached with 5S RNA from mouse Ehrlich ascites tumor cells using the technique of RNA–RNA hybridization (Hayward *et al.*, 1966).

The purification of 5S RNA from *B. subtilis* makes it possible to map its position on the genome by physical means (Dubnau *et al.*, 1965; Oishi and Sueoka, 1965). We have recently found that the structural cistrons controlling the synthesis of t- and rRNA are found in a narrowly confined region of the *B. subtilis* W23 chromosome between the erythromycin resistance and adenine$_6$ markers, and occur in the order 5S, t-, 16S, and 23S RNA. A report on this work will appear shortly.

Acknowledgments

The authors express their thanks to Mr. P. Perlman for technical assistance, to Dr. Bruce Howard for a gift of phage 2C DNA, and to Dr. Ruy Soeiro for assistance in the determinations of RNA base composition.

References

Anagnostopoulos, C., and Spizizen, J. (1961), *J. Bacteriol. 81*, 741.

Aronson, A. I., and McCarthy, B. J. (1961), *Biophys. J. 1*, 215.
Baguley, B. C., and Ralph, R. D. (1966), *Biochem. Biophys. Res. Commun. 22*, 308.
Bray, G. A. (1960), *Anal. Biochem. 1*, 279.
Burton, K. (1956), *Biochem. J. 62*, 315.
Dubnau, D., Smith, I., and Marmur, J. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 724.
Fraenkel-Conrat, H., Singer, B., and Tsugita, A. (1961), *Virology 14*, 54.
Galibert, F., Larsen, C. J., Lelong, J. C., and Boiron, M. (1965), *Nature 207*, 1039.
Galibert, F., Larsen, C. J., Lelong, J. C., and Boiron, M. (1966), *Bull. Soc. Chim. Biol. 48*, 21.
Giacomoni, D., and Spiegelman, S. (1962), *Science 138*, 1328.
Giles, K. W., and Myers, A. (1965), *Nature 206*, 93.
Gillespie, D., and Spiegelman, S. (1965), *J. Mol. Biol. 12*, 829.
Goodman, H. M., and Rich, A. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 2101.
Hayward, R. S., Legault-Demare, J., and Weiss, S. B. (1966), *Federation Proc. 25*, 520.
Lindahl, T., Henley, D. C., and Fresco, J. R. (1965), *J. Am. Chem. Soc. 87*, 4961.
Mandell, J. D., and Hershey, A. D. (1960), *Anal. Biochem. 1*, 66.
Marcot-Queiroz, J., Julien, J., Rosset, R., and Monier, R. (1965), *Bull. Soc. Chim. Biol. 47*, 183.
Marmur, J. (1961), *J. Mol. Biol. 3*, 208.
Marmur, J., and Doty, P. (1962), *J. Mol. Biol. 5*, 109.
Monier, R. (1962), *Bull. Soc. Chim. Biol. 44*, 109.
Nygaard, A. P., and Hall, B. D. (1964), *J. Mol. Biol. 9*, 125.
Oishi, M., Oishi, A., and Sueoka, N. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 1095.
Oishi, M., and Sueoka, N. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 483.
Rammler, D. H., Okabayashi, T., and Delk, A. (1965), *Biochemistry 4*, 1994.
Ritossa, F., Atwood, K., and Spiegelman, S. (1966), *Genetics 54*, 663.
Rosset, R., and Monier, R. (1963), *Biochim. Biophys. Acta 68*, 653.
Rosset, R., Monier, R., and Julien, J. (1964), *Bull. Soc. Chim. Biol. 46*, 87.
Sarkar, N., and Comb, D. G. (1965), *Federation Proc. 24*, 292.
Schleich, T., and Goldstein, J. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 744.
Schleich, T., and Goldstein, J. (1966), *J.Mol. Biol. 15*, 136.
Sebring, E. D., and Salzman, N. P. (1964), *Anal. Biochem. 8*, 126.
Spiegelman, S., and Hayashi, M. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 161.
Zehavi-Wilner, T., and Comb, D. (1966), *J. Mol. Biol. 15*, 435.