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The Fractionation of Quaternary Ammonium Complexes of Nucleic Acids. Evidence for Heterogeneity of Ribosomal Ribonucleic Acid*

R. J. Young

ABSTRACT: A method for the fractionation of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) as their cetyltrimethylammonium complexes is described. The complexes when precipitated on an inert support are eluted at characteristic sodium chloride concentrations. Low molecular weight RNA, ribosomal ribonucleic acid (rRNA), and DNA are separated from each other by means of a salt gradient. rRNA is separated into two fractions, the relative amounts of which are altered by heating or by repeated freezing and thawing. This separation appears to be a function of the conformation of the RNA rather than of its size. Each of the rRNA fractions is separated into 16S and 23S RNA after centrifugation through a linear sucrose gradient. Differences in the nucleotide frequencies of the rRNA present in each of the fractions are evident after digestion of the fractions with pancreatic ribonuclease or ribonuclease T_1. These results show that the two fractions contain RNA molecules which are chemically distinct and are taken to indicate that heterogeneity exists within the 16S and 23S species of rRNA.

Long-chain aliphatic ammonium compounds combine with polyanions to form water-insoluble complexes which dissolve in salt solution when the salt concentration is above a certain critical level. The critical salt concentration is a function of the nature of the salt, the structure and size of the polyanion, and the structure of the aliphatic ammonium cation. It is constant for a given polyanion when a standard salt and an aliphatic ammonium cation are used. This property of the quaternary ammonium complexes of the polyanions has been extensively used in the isolation and fractionation of acidic polysaccharides (Scott, 1960). Nucleic acids also form complexes with aliphatic quaternary ammonium compounds. The complexes are insoluble in water, but soluble in organic solvents and in strong salt solutions. These properties have been utilized for the isolation and purification of both DNA and RNA, and tRNA has been fractionated as the alkylammonium salt (Kirby, 1964a; Kelmers, 1966; Brown, 1963; Jones, 1963; Burkard *et al.*, 1965; Abel-Sadron *et al.*, 1961).

A technique which has been used in the fractionation of mixtures of acidic polysaccharides relies on the precipitation of the quaternary ammonium complexes of the polyanions on an inert support and the extraction of the precipitated complexes by salt solutions of increasing concentration (Scott, 1960; Antonopoulos *et al.*, 1961, 1965). This method of fractionation depends on differences in the critical salt concentration of

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the quaternary ammonium–polyanion complexes and appears to be quite valid (Scott, 1960). The present communication reports the results of experiments using this technique for the fractionation of nucleic acids.

Materials and Methods

Chemicals. CTAB[1] was recrystallized from acetone–alcohol before use; the same batch was used for a series of experiments as results varied from batch to batch.

Preparation and Operation of the Column. Cellulose powder (Whatman Chromedia CF11) and Kieselguhr (Hyflo-Supercel, Johns-Manville Products Corp.) were stirred with distilled water and the fines removed by decantation. Before being used to prepare a column a thin slurry of the inert support in water was degassed, and the slurry was then poured into a jacketed glass column. The column, which was maintained at 30°, was washed with four to five bed volumes of 1 or 0.1% CTAB (w/v) before use. For most experiments a column of 1-cm diameter was used and good separation was obtained when up to 8 mg of a nucleic acid mixture was placed on a 1 × 35 cm column. Concave gradients were produced by using two cylinders of unequal diameters joined by a piece of capillary tubing (Bock and Ling, 1954). For gradient I, the ratio of the area of the base of the cylinder used as the mixing vessel to the area of the base of the cylinder used as the reservoir was 4. For gradient II, this ratio was 2. The volume of the gradient used varied, but was 1050 ml for a column of size up to 1 × 30 cm. A solution of the nucleic acid in buffer was first degassed and then allowed to drain into the column. Any nucleic acid that had precipitated on the walls of the column was washed into the inert support with a small quantity of 1 M NaCl. When required, low molecular weight RNA was removed by washing with 0.3 M NaCl in 0.05% CTAB before commencing elution with the salt gradient. A flow rate of 0.5–1 ml/min was maintained by a solution metering pump (Beckman Instruments, Inc.). Fractions of 4.5–6 ml were collected and the absorbancy at 260 mμ was determined for alternate tubes. The nucleic acids were recovered as the cetyltrimethylammonium complex by addition of water, or as the sodium salt by precipitation with acetone in the presence of sodium acetate (Kirby, 1965). Recoveries were estimated by measuring the absorbancy at 260 mμ of the combined fractions in each peak. Salts were removed from the recovered RNA by washing with 66% alcohol.

Preparation of the Nucleic Acids. DNA and rRNA from *Escherichia coli* were prepared as described by Kirby (1964b). The DNA preparations showed hyperchromicities of 29–33% when denatured by heating in 0.15 M NaCl–0.015 M trisodium citrate (pH 7.0). *E. coli* rRNA was also prepared by the following procedure. A frozen paste of *E. coli* cells which had been harvested in the exponential phase of growth was stirred with 1% (w/v) sodium triisopropylnaphthalenesulfonate or 1% (w/v) sodium dodecyl sulfate for 2–3 min at room temperature. An equal volume of phenol–*m*-cresol–8-hydroxyquinoline–water (500:70:0.5:55, w/w) was added and stirring was continued for 20–30 min. The aqueous layer was separated by centrifugation at 10,000*g* for 15 min at 4° and extracted twice more for 10 min at room temperature with one-half volume of the phenol–*m*-cresol mixture. Cold ethanol (two volumes) was added to the aqueous layer and after 2–4 hr at −30° the precipitated RNA was collected by centrifugation and washed with cold 3 M sodium acetate and 70% (v/v) aqueous ethanol. The RNA was stored at −30° under 70% (v/v) aqueous ethanol or dissolved in 0.05 M Tris-HCl (pH 7.3)–0.15 M NaCl and stored frozen in batches at −30°. rRNA was also recovered from the aqueous layer by addition of *m*-cresol in the presence of 20% (w/v) sodium benzoate (Kirby, 1965). The precipitate was washed with a water–sodium benzoate–NaCl–*m*-cresol mixture (100:20:3:10, w/w), followed by cold 1 M NaCl, then 70% (v/v) aqueous ethanol, and stored as described above. The RNA preparations contained up to 5% DNA when measured by the diphenylamine procedure (Burton, 1956) and the protein content, measured by the procedure of Lowry *et al.* (1951), was usually less than 1%. The rRNA prepared by the three procedures gave similar profiles when eluted from the inert support. Before use, the physical integrity of each rRNA preparation was checked by sedimentation through a 5–20% linear sucrose gradient.

The 23S and 16S components of *E. coli* rRNA were separated by two centrifugations of the RNA through a linear sucrose gradient (5–20%) prepared in 0.05 M Tris-HCl (pH 7.3)–0.15 M NaCl. The tubes were punctured at the bottom by a hypodermic needle, and fractions of 40–50 drops were collected. The RNA was recovered from the peaks by precipitation with acetone in the presence of sodium acetate (Kirby, 1965).

Yeast rRNA prepared by extraction of yeast ribosomes with the phenol–*m*-cresol mixture was a gift from P. J. Rogers; protein contamination was less than 0.5%. The preparations were stored as for *E. coli* RNA. Highly polymerized yeast RNA and yeast tRNA were obtained from British Drug Houses, Ltd., and Sigma Chemical Co., respectively.

Rat liver rRNA was prepared as described by Kirby (1965). Preparations of nucleic acids containing DNA were obtained by substituting 1% (w/v) sodium triisopropylnaphthalenesulfonate for 0.5% disodium naphthalene-1,5-disulfonate. Calf thymus DNA was obtained from British Drug Houses, Ltd.

Critical Salt Concentrations. Solutions of the polynucleotides (1 mg/ml) in 1% (w/v) CTAB and 2 M NaCl were placed in a water bath at 27° and water was added dropwise until a visible precipitate formed. No change in the end point was obtained when 0.2% CTAB was substituted for water. After 2 hr at 4°, the precipitate was removed by centrifugation and the ultraviolet absorption of the supernatant measured at 260 mμ. The solubility of the cetyltrimethylammonium–

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: CTAB, cetyltrimethylammonium bromide; Ap, Cp, Gp, Up, the (2′)3′-phosphates of adenosine, cytidine, guanosine, and uridine, respectively; G>p, guanosine 2′,3′-cyclic phosphate; ApUp, adenylyl-(3′–5′)-uridyl (2′),3′-phosphate; similarly for other oligonucleotides.

nucleic acid complex in sodium chloride was determined at 27° by suspending the precipitate in 0.05% CTAB; aliquots of 2 M NaCl in 0.05% CTAB were added with vigorous stirring to the suspension until solution of the complex occurred.

Reaction with Formaldehyde. Solutions of the nucleic acids in 0.1 M potassium phosphate buffer (pH 7.2) and 2% formaldehyde were placed in a water bath at 25°. At time intervals the ultraviolet absorption of the solutions at 260 mμ was measured. Blanks consisting of solutions of the nucleic acid in the phosphate buffer and 2% formaldehyde in phosphate buffer were also kept at 25°, and the absorption at 260 mμ of these solutions was subtracted from the readings obtained with the reaction mixture.

Preparation of ^{32}P-Labeled rRNA. E. coli (strain B) was grown in a buffered salts medium supplemented with glucose and Bacto peptone (1.47 g of KCl, 9.36 g of NaCl, 1.07 g of NH_4Cl, 0.71 g of Na_2SO_4, 0.2 g of $MgCl_2 \cdot 6H_2O$, 0.22 g of $CaCl_2$, 0.3 mg of $FeCl_3$, 5 g of glucose, and 3 g of Bacto peptone in 1 l. of 0.1 M Tris-HCl, pH 7.3). After growth commenced 4 mCi of carrier-free ^{32}P-labeled orthophosphoric acid was added and the culture was allowed to grow until the optical density at 660 mμ, measured in a 1-cm cuvet, reached 0.27. The cells were harvested, washed three times with growth medium containing 2 ml of 0.1 M K_2HPO_4/l., resuspended in sufficient growth medium containing 2 ml of 0.1 M K_2HPO_4 to obtain an optical density of 0.25, and aerated at 37° until the optical density reached 0.52. The cells were then harvested and washed with 0.05 M Tris-HCl (pH 7.3)–0.1 M NaCl, and the rRNA was extracted as described above. The ^{32}P-labeled rRNA was incubated with DNase (0.5 mg/ml, Worthington, RNase free) at 0° for 0.5 hr and extracted with aqueous phenol; the aqueous layer was treated with bentonite (3 mg/ml) to remove remaining nucleases. Bentonite was removed by centrifugation for 2 hr at 100,000*g* and the ^{32}P-labeled rRNA was recovered by precipitation with cold ethanol. Fractionation of the rRNA was carried out on a 1 × 23 cm column of Kieselguhr as described above; recovery was 77%. The usual two rRNA peaks with the same specific activity were eluted at 0.7 and 0.76 M NaCl in the ratio of 4:1 together with three smaller peaks (35%) eluted at salt concentrations less than 0.6 M NaCl. Each of the smaller peaks had a different and lower specific activity. Centrifugation of the two major ^{32}P-labeled RNA peaks through a sucrose density gradient showed that each contained 23S and 16S RNA in the ratio of 2:1 with negligible low molecular weight material present. The ^{32}P-labeled RNA was recovered as the sodium salt by precipitation with acetone in the presence of sodium acetate after addition of carrier RNA (0.5–1 mg/ml). Carrier RNA was isolated from *E. coli* in exactly the same manner as the ^{32}P-labeled RNA except that it was not fractionated as the quaternary ammonium salt on a Kieselguhr column.

Base Composition Analysis. Hydrolysis of the ^{32}P-labeled rRNA together with carrier RNA was carried out in 1 M NaOH for 16–18 hr at room temperature. The nucleotides were separated by electrophoresis in 0.05 M ammonium formate (pH 3.3). Autoradiography of the electropherogram showed the presence of a small faint spot slightly ahead of the adenosine phosphate spot, and a similar area slightly behind the guanosine phosphate spot which was split into two corresponding to the 2′- and 3′-phosphates. These were included with the Ap and Gp spots, respectively. The nucleotide spots were cut out, the paper strip was immersed in a toluene base scintillation fluid (138 g of 2,5-diphenyloxazole and 0.173 g of *p*-bis[2-(5-phenyloxazolyl)]-benzene in 3 kg of toluene), and the radioactivity was measured in a Beckman Model LS-200 liquid scintillation spectrometer.

Enzymic Digestion of the Two rRNA Fractions. Each of the ^{32}P-labeled rRNA fractions together with 2.5–3 mg of carrier RNA were digested with pancreatic ribonuclease or ribonuclease T_1 and the oligonucleotides separated as described by Rushizky and Knight (1960a,b) and Rushizky and Sober (1962). The conditions for ribonuclease T_1 digestion were 24 hr at 37° in 0.05 M Tris-HCl (pH 7.3) with 250 units of enzyme (Sankyo Co. Ltd.). The spots, located with the help of the autoradiogram, were cut out and the radioactivity was determined as described above.

Results

Critical Salt Concentration. The measurement of the critical salt concentrations was carried out at 27° by the addition of sodium chloride solution to a suspension of the cetyltrimethylammonium–nucleic acid complexes in 0.05% CTAB. Table I shows the results.

TABLE I: Solubilities of Cetyltrimethylammonium Complexes of Nucleic Acids.

Nucleic Acid	NaCl Concn at Which Complex Ppt (M)	% Nucleic Acid Pptd[a]	Critical Salt Concn (M)
Calf thymus DNA	0.60	98	0.66
Yeast RNA	0.50	89	0.52
Yeast tRNA	0.38	98	0.40

[a] Measured after 2 hr at 4°.

Using a titration technique Barber and Noble (1966) have reported similar results. The solubility of the cetyltrimethylammonium–nucleic acid complexes in sodium chloride solution was also determined by elution, with a linear gradient of sodium chloride solution, of a CTAB-saturated cellulose column on which the nucleic acid had been precipitated. Figure 1 shows the elution profiles obtained.

E. coli DNA was eluted as a single sharp peak at a sodium chloride concentration of 0.75 M (Figure 1a). The sodium chloride concentration at which the cetyl-

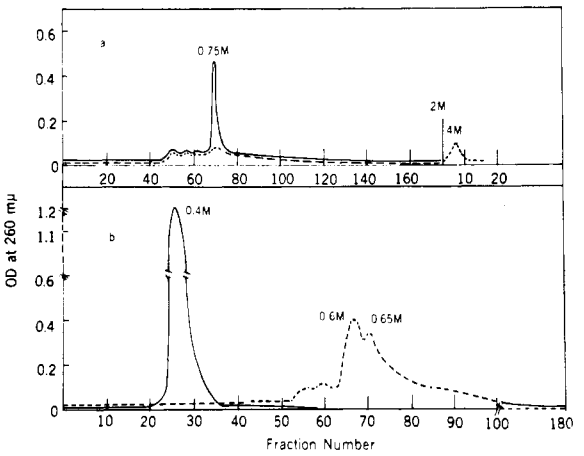

FIGURE 1: Elution profiles of nucleic acids from CTAB-saturated cellulose columns. The nucleic acids (1–5 mg) were added to a 1 × 15 cm cellulose column and eluted with 1000 ml of a linear gradient (0–2 M) of NaCl in 0.05% CTAB. (a) *E. coli* DNA (——); *E. coli* DNA denatured by heating in a water bath at 90° for 10 min and quickly cooled in an ice bath (- - -). Solvent was 0.15 M NaCl–0.015 M trisodium citrate (pH 7.0). (b) Yeast tRNA (——); *E. coli* rRNA (- - -).

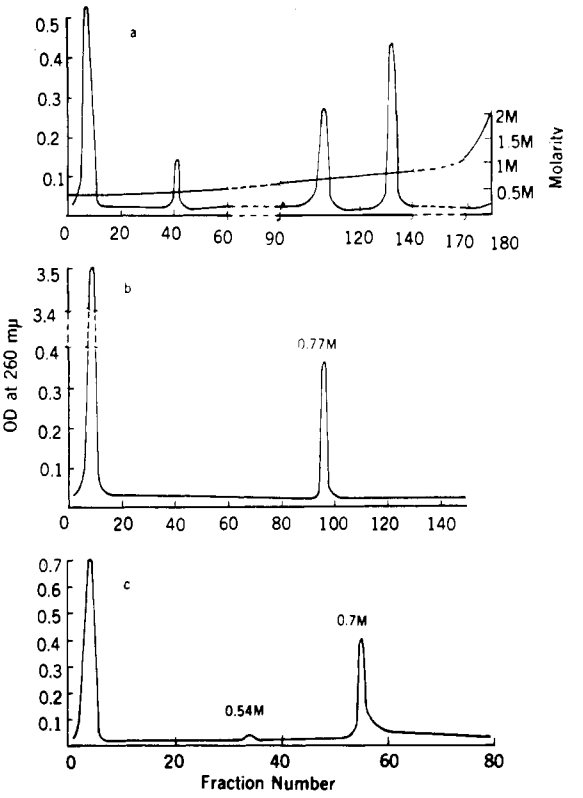

FIGURE 2: Elution profiles of a rat liver nucleic acid preparation from a CTAB-saturated Kieselguhr column. The nucleic acids were obtained by homogenization of rat liver in the presence of 1% sodium triisopropylnaphthalenesulfonate. (a) The nucleic acid preparation (2.5 mg), precipitated onto a 1 × 25 cm column of Kieselguhr, was eluted by means of gradient I (960 ml) with 0.4 M NaCl in 0.05% CTAB in the mixing vessel and 2 M NaCl in 0.05% CTAB in the reservoir. Fractions were 5 ml. (b) Same preparation and elution conditions as part a except that the nucleic acid preparation in 0.05 M Tris-HCl (pH 7.4)–0.15 M NaCl was incubated with ribonuclease (1% by weight) for 20 min at room temperature before addition to the column. Fractions were 6 ml. (c) Same nucleic acid preparation and elution conditions as part a except that the gradient was 510 ml and the eluent was NaCl dissolved in 0.05 M Tris-HCl (pH 7.3) and not in 0.05% CTAB. Fractions were 5.5 ml.

trimethylammonium–DNA complex is eluted from the column varied slightly from batch to batch of CTAB, and with the nature of the inert support, but for a particular batch of CTAB and inert support the sodium chloride concentration remained constant and reproducible. DNA which had been heated at 90° for 10 min and cooled rapidly in an ice bath produced a different elution profile (Figure 1a). A small peak was eluted at 0.75 M sodium chloride, and a second peak, presumed to be denatured DNA, was recovered by washing the column with 4 M sodium chloride.

tRNA was eluted from a CTAB-saturated cellulose column in a single sharp peak at 0.4 M sodium chloride (Figure 1b). This result agrees with the critical solution concentration as determined by titration. There was little change in this value with different batches of CTAB.

A slightly different result was obtained when rRNA was eluted from a CTAB-saturated cellulose column by a linear gradient of sodium chloride solution. The elution diagram is shown in Figure 1b; two major peaks are present, appearing at sodium chloride concentrations of 0.6 and 0.65 M. As with DNA these values changed, varying from 0.5 to 0.70 M and from 0.55 to 0.75 M, respectively, depending on the batch of CTAB and the nature of the inert support used, but remained constant and reproducible for each batch of CTAB and inert support. The rRNAs of yeast and rat liver were also separated into two peaks, the peaks appearing at about the same sodium chloride concentrations as the peaks from the *E. coli* rRNA preparations.

Fractionation of the Nucleic Acids on an Inert Support Saturated with CTAB. The elution diagram obtained by applying a shallow concave gradient to a preparation of rat liver nucleic acid which had been precipitated on a column of Kieselguhr saturated with CTAB is shown in Figure 2a. Figure 2b shows the elution profile of the nucleic acid preparation after incubation with bovine pancreatic ribonuclease. Only one peak was unaltered by ribonuclease digestion. This peak, recovered in 11% yield in both experiments and eluted at 0.77 M sodium chloride, is DNA essentially uncontaminated with RNA. The absence of peaks at 0.49 and 0.66 M sodium chloride after ribonuclease digestion (Figure 2b) shows that RNA was present in these peaks. Degraded RNA appeared after one bed volume; since the gradient commenced at 0.4 M sodium chloride it is possible that the degraded RNA had a lower critical salt concentration than 0.4 M sodium chloride. The sharp peak eluted at 0.77 M sodium chloride contained no RNA as judged by the orcinol reaction. RNA eluted at low salt concentrations gave negative tests with the diphenylamine reagent. A similar result was obtained with crude nucleic acid preparations from *E. coli*. The fractionation procedure is therefore applicable to nu-

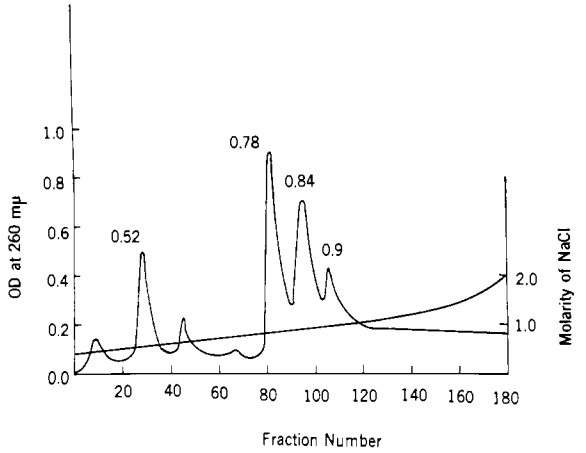

FIGURE 3: Elution profile of *E. coli* rRNA from a CTAB-saturated Kieselguhr column. The rRNA (6.5 mg) was placed on a 1 × 25 cm Kieselguhr column. Elution was carried out using 960 ml of gradient II with 0.4 M NaCl in 0.05 M Tris-HCl (pH 7.3)–0.2% CTAB in the mixing vessel and 2 M NaCl in the same solvent in the reservoir. Fractions were 5 ml.

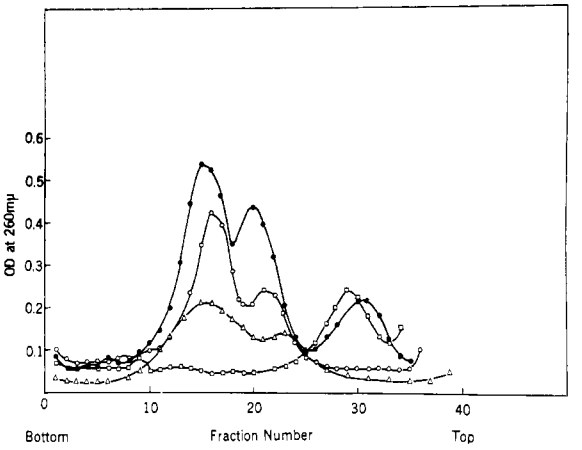

FIGURE 4: Sucrose gradient centrifugation profiles. The material in the peak tubes of the experiment described in Figure 3 was dissolved in 0.05 M Tris-HCl (pH 7.3)–0.15 M NaCl and centrifuged through a linear 5–20% sucrose gradient prepared in the same buffer solution. Centrifugation was at 21,000–23,000 rpm for 16–18 hr in the Spinco Model L2 centrifuge (SW 25.1 rotor) with the temperature setting at 0°. The gradients varied from 25 to 28 ml resulting in a different number of fractions as shown. (●) RNA from *E. coli;* (○) fraction eluted at 0.78 M NaCl; (△) fraction eluted at 0.84 M NaCl; (□) fraction eluted at 0.52 M NaCl.

cleic acid preparations from both bacterial and mammalian sources. Polysaccharides (*e.g.*, glycogen) when present were removed by washing the column with 0.05% CTAB or 0.1 M sodium chloride in 0.05% CTAB before applying the gradient.

Resolution of the rRNA into Two Components and Recovery from the Inert Support. The separation of the rRNA of *E. coli* into two peaks was much improved by the use of shallow concave gradients. Figure 3 shows the elution profile obtained by applying gradient II to a preparation of *E. coli* rRNA that had been added to a CTAB-saturated Kieselguhr column. The small peak eluted at 0.9 M sodium chloride was absent if the RNA preparation was digested with DNase before being applied to the column. Similar elution profiles were obtained with rRNA preparations from yeast or rat liver. Better resolution was obtained with a shallow gradient (gradient I), but the recovery of RNA was lower (see below). There was no change in the elution profile when sodium chloride was replaced by potassium chloride or ammonium chloride in the eluting solution or by the use of a slower flow rate (0.5–0.6 ml/min) when used with gradient II.

The presence of CTAB in the eluting solution of sodium chloride was found to be essential for resolution of the nucleic acid mixture into its components. The concentration of CTAB used could be varied from 0.02 to 0.5% without loss of separation. Figure 2c shows the elution diagram produced by a shallow concave gradient (gradient I) of sodium chloride in 0.05 M Tris-HCl (pH 7.3) when applied to a CTAB-saturated column of Kieselguhr on which a rat liver nucleic acid preparation had been placed. Instead of the four peaks obtained (Figure 2a) when the gradient contained 0.05% CTAB, only two major peaks appeared, indicating that no resolution of the large molecular weight nucleic acid occurs in the absence of CTAB. A similar result was obtained when the eluting gradient was sodium chloride in water.

No difficulty was encountered in the recovery of DNA and tRNA from columns of cellulose or Kieselguhr saturated with CTAB; the recovery was essentially quantitative. However, DNA denatured by heating was recovered in low yields, and recoveries of rRNA from the columns were usually low. rRNA preparations from *E. coli* were recovered in higher yields (up to 75%) than rRNA preparations from yeast or rat liver (less than 40%). Reduction in the flow rate or use of potassium chloride or ammonium chloride as the eluting salt did not affect the recovery, but stepwise elution gave improved recoveries. The poor recovery of rRNA is not due to adsorption of the RNA onto the inert support as solutions of RNA in 0.05 M Tris-HCl (pH 7.3) were recovered in high yields when percolated through columns of cellulose or Kieselguhr. It is also unlikely that insolubility of the quaternary ammonium complex of the rRNA in strong salt solution is the explanation, as suspensions of the cetyltrimethylammonium–rRNA complex in 0.05% CTAB dissolved when sodium chloride was added to 1 M final concentration or to the critical salt concentration of the complex. The recovery is also unrelated to the amount of DNA and protein present in the rRNA preparation.

Nature of the Two Components Produced from rRNA by Gradient Elution. EFFECT OF HEATING AND COOLING AND REPEATED FREEZING AND THAWING. Excess CTAB was removed from aliquots taken from the peak tubes of the major peaks of Figure 3 by extraction with chloroform at 0° or by precipitation with acetone in the presence of sodium acetate. The RNA so obtained was examined by centrifugation through a linear sucrose gradient and the result is shown in Figure 4. It is clear that the rRNA was not resolved into two components according to size as each peak obtained from the column was resolved on sucrose density centrifugation into the

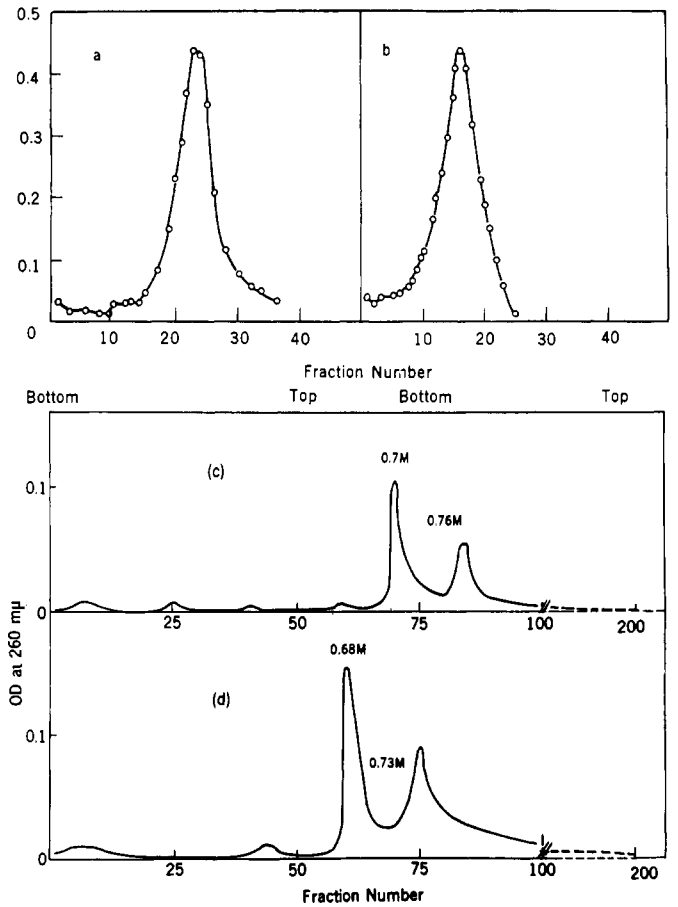

FIGURE 5: Centrifugation studies. (a and b) Centrifugation in a 5–20% sucrose density gradient of 16S and 23S *E. coli* rRNA. Centrifugation was carried out in a Spinco Model L2 centrifuge (SW 25.1 rotor) with the temperature setting at 0°. (a) 16S rRNA, 22,500 rpm for 16 hr. (b) 23S rRNA, 21,000 rpm for 15 hr. (c and d) Elution profiles of the *E. coli* rRNA shown in parts a and b from a CTAB-saturated Kieselguhr column. Columns (1 × 12 cm) of Kieselguhr were used as support for 0.4 mg of 16S RNA and 0.5 mg of 23S RNA. The columns were eluted by means of gradient II (1050 ml) with 0.4 M NaCl in 0.05 M Tris-HCl (pH 7.3)–0.1% CTAB in the mixing vessel and 2 M NaCl in the same solvent in the reservoir. Fractions were 5 ml. (c) 16S r RNA. (d) 23S rRNA.

16S and 23S components. Identical results were obtained when the rRNA preparations from yeast and rat liver were examined in the same way; the peaks eluted from the column produced 19S and 28S components. Further, when the 16S (Figure 5a) and 23S (Figure 5b) components of *E. coli* rRNA were each precipitated on a column of Kieselguhr saturated with CTAB and then eluted with a salt gradient, the rRNAs were each separated into two peaks (Figure 5c,d).

The two RNA peaks eluted at 0.5–0.70 M NaCl and 0.55–0.75 M NaCl were usually equal in size; however, they varied with the RNA preparation. Whenever the RNA peaks were unequal in size, the second peak was more often smaller than the first. The amount of DNA and protein present in the RNA preparations did not affect the relative size of the two peaks as preparations in which the DNA or protein content was 10% gave the same elution profile as RNA preparations which

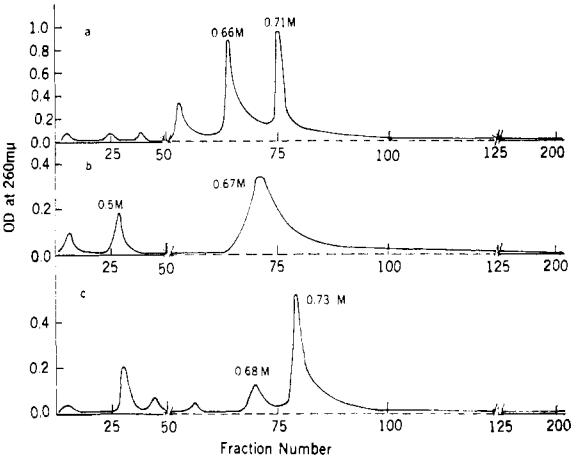

FIGURE 6: Elution profile of *E. coli* rRNA from a CTAB-saturated Kieselguhr column. The RNA preparation (3–5 mg), after stirring with and removal of bentonite, was placed on a 1 × 20 cm Kieselguhr column. The column was eluted with 1050 ml of a concave gradient (gradient II) as described in Figure 5. Fractions were 4.8 ml. (a) *E. coli* RNA. (b) *E.* coli RNA dissolved in 0.01 M Tris-HCl (pH 7.3)–0.015 M NaCl heated to 70–72° for 10 min and quickly cooled in an ice bath. (c) *E. coli* RNA after heating as in part b but allowed to cool slowly (1.5–2 hr) to room temperature in the presence of 0.15 M NaCl.

had been digested with DNase or which contained less than 1% protein. Rather, the relative size of the two rRNA peaks appeared to depend on the manner in which the rRNA preparation had been previously treated. Thus, when the RNA solution (1 mg/ml) in 0.01 M Tris-HCl (pH 7.3)–0.015 M NaCl was heated to 70–72° for 8–10 min and then quickly cooled in an ice bath, the elution profile was greatly changed. Instead of two major peaks being present in equal amounts (Figure 6a), the first peak eluted at 0.67 M NaCl was much greater than the second peak, eluted at 0.71 M NaCl. In some experiments, the second peak was absent after the heat treatment (Figure 6b). However, if instead of rapidly cooling the rRNA solution after heating to 70–72° the sodium chloride concentration was raised to 0.15 M and the solution then allowed to cool slowly to room temperature, the elution profile shown in Figure 6c was obtained. Here the size of the second peak is greater than the first. Sedimentation analysis of the heated RNA preparation (Figure 7) indicated that no degradation had occurred during brief heating to 70–72°; however, a change is evident in the sedimentation profile. Heating the RNA preparation, therefore, alters both the elution and sucrose density sedimentation profiles. Similar alterations have been observed in sedimentation profiles (Kirby, 1965) and elution profiles from columns of methylated albumin Kieselguhr (Sypherd and Fansler, 1967) as a result of heating the rRNA.

Repeated freezing and thawing of an RNA preparation produced no effects as monitored by sucrose density gradient patterns. However, the elution profiles from a Kieselguhr column were altered by such treatment. A solution of rRNA which had not been frozen produced a profile similar to Figure 6a in which the two peaks were about equal in size. With up to 15 cycles of

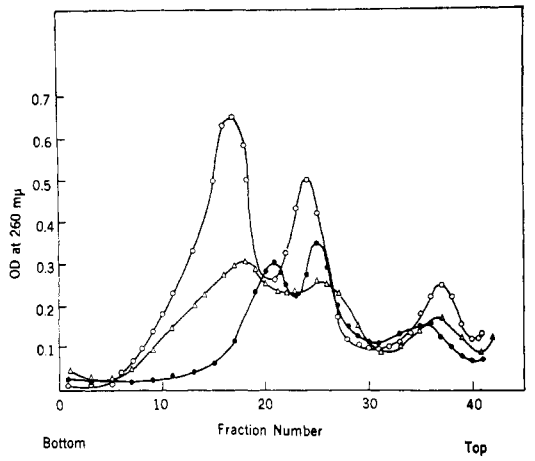

FIGURE 7: Sucrose density gradient centrifugation of *E. coli* RNA preparations. Centrifugation was for 16 hr at 22,000 rpm (SW 25.1 rotor). (○) *E. coli* RNA was layered onto 5–20% sucrose gradient prepared in 0.05 M Tris-HCl (pH 7.3)–0.15 M NaCl. (●) *E. coli* RNA heated to 70–72° for 10 min in 0.01 M Tris-HCl (pH 7.3)–0.015 M NaCl and quickly cooled in an ice bath. The gradient was prepared in the same solvent. (Δ) *E. coli* RNA heated to 70–72° for 10 min in 0.01 M Tris-HCl (pH 7.3)–0.015 M NaCl and allowed to cool slowly to room temperature after addition of NaCl to 0.15 M. The gradient was prepared in 0.01 M Tris-HCl (pH 7.3)–0.15 M NaCl. The RNA was prepared in the presence of bentonite.

slow freezing and thawing, the pattern gradually altered so that the first peak increased in size with a concomitant decrease in size of the second peak; this was accompanied by a lower (5–10%) recovery of material. The change in elution profile is similar to the change produced by heating and cooling.

REACTION OF THE FRACTIONS WITH FORMALDEHYDE. To determine if the two fractions eluted at different salt concentrations differ in secondary structure, the rate of their reaction with formaldehyde, a reagent commonly used to measure the extent of secondary structure in nucleic acids (Doty *et al.*, 1959; Haselkorn and Doty, 1961), was studied. The reaction was followed by measuring the increase in absorbancy at 260 mμ with time. Figure 8a shows the reaction between the two fractions and TMV–RNA (a gift of Dr. H. Fraenkel-Conrat, Virus Laboratory, University of California, Berkeley) with formaldehyde. It can be seen that the material eluted at the lower salt concentration reacted faster with formaldehyde than the material eluted at the higher salt concentration, and both reacted slower than TMV–RNA. After 26 hr, the hyperchromicities were 19, 16, and 26%, respectively, and the ultraviolet absorption maximum showed the expected shift to longer wavelength (Fraenkel-Conrat, 1954).

The reaction between formaldehyde and the fractions eluted at 0.66 and 0.75 M sodium chloride obtained from RNA preparations that had been subjected to heating and quick cooling is shown in Figure 8b. Here the difference in the rate of reaction with formaldehyde is more pronounced, the RNA that had been subjected to rapid cooling being much more reactive. However, the increase in absorbancy at 260 mμ after 26 hr was the same in both cases.

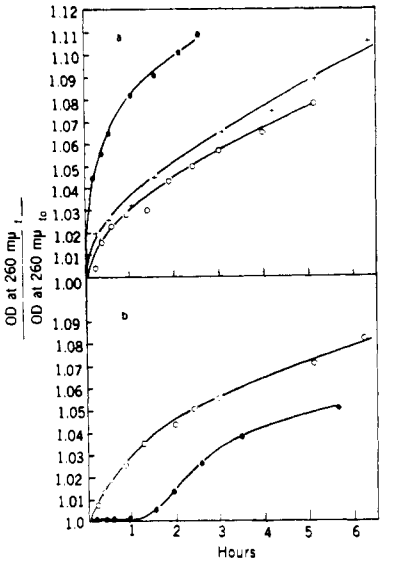

FIGURE 8: Reaction of formaldehyde with *E. coli* rRNA that had been fractionated in a CTAB-saturated Kieselguhr column. (a) (+) RNA eluted at 0.66 M NaCl; (○) RNA eluted at 0.71 M NaCl; (●) TMV–RNA; (b) (○) RNA heated to 70–72° for 10 min in 0.01 M Tris-HCl (pH 7.3)–0.015 M NaCl and quickly cooled in an ice bath, fraction eluted from Kieselguhr column at 0.67 M NaCl; (●) RNA heated to 70–72° for 10 min in 0.01 M Tris-HCl (pH 7.3)–0.015 M NaCl and cooled slowly to room temperature in the presence of 0.15 M NaCl, fraction eluted from Kieselguhr column at 0.73 M NaCl. Time, t; time zero, t_0.

These results indicate that rRNA is resolved by this fractionation procedure into fractions of different secondary structure.

BASE COMPOSITION OF THE TWO PEAKS PRODUCED BY GRADIENT ELUTION. The base composition of the RNA present in each of the two peaks, obtained by gradient elution of the quaternary ammonium salt of *E. coli* rRNA, was determined to ascertain if they were chemically as well as conformationally different. Hydrolysis of ^{32}P-labeled RNA was carried out in 1 M sodium hydroxide and the nucleotides were separated by electrophoresis at pH 3.3. The results are shown in Table II. The base compositions of the two peaks are in close

TABLE II: Base Composition of the Peaks Obtained by Gradient Elution of the Cetyltrimethylammonium Salt of rRNA of *E. coli* from a Kieselguhr Column.

Base	First Peak (0.66 M NaCl)[c] (mole %[a,b])	Second Peak (0.71 M NaCl)[c] (mole %[a,b])
C	21.7	21.9
A	25.3	25.5
G	32.3	32.2
U	20.7	20.5

[a] Average of six determinations. [b] Error less than ±1.5%. [c] Sodium chloride concentration at which peak is eluted from the column.

TABLE III: Oligonucleotides from a Pancreatic Ribonuclease Digest of the Two Peaks.

Composition of Spot[b]	First Peak[a] (% recov)[c]	Second Peak[a] (% recov)[c]
ApApApCp	1.03 ± 0.06	0.87 ± 0.02
(ApApGp)Cp	3.31 ± 0.15	2.57 ± 0.05
(ApGpGp)Cp	1.94 ± 0.12	1.64 ± 0.04
(ApApApGp)Up	0.84 ± 0.06	0.90 ± 0.11
(ApApGpGp)Up	0.69 ± 0.12	0.47 ± 0.01
ApApCp	2.99 ± 0.16	2.87 ± 0.03
ApGpCp, GpApCp	5.86 ± 0.19	5.71 ± 0.09
ApApApUp	1.04 ± 0.23	0.81 ± 0.19
GpGpCp	1.87 ± 0.07	1.98 ± 0.08
(ApApGp)Up	1.86 ± 0.13	1.82 ± 0.08
(ApGpGp)Up	1.65 ± 0.09	1.54 ± 0.13
ApUp	4.97 ± 0.21	5.01 ± 0.05
GpCp	6.23 ± 0.22	6.21 ± 0.11
ApApUp	1.62 ± 0.01	1.62 ± 0.01
ApGpUp, GpApUp	4.24 ± 0.08	3.96 ± 0.16
GpGpUp	2.25 ± 0.14	2.28 ± 0.03
Cp	10.2 ± 0.4	10.1 ± 0.2
ApUp	3.53 ± 0.13	3.39 ± 0.03
GpUp	5.79 ± 0.18	5.60 ± 0.07
Up	9.30 ± 0.16	8.95 ± 0.08
Origin	27.8 ± 1.6	31.2 ± 1.2

[a] Average values from four digests; errors are average deviation from the mean. [b] Composition of spot taken from Rushizky and Knight (1960a,b); brackets signify sequence unknown. Spots with significant differences are in italics. [c] Per cent of total recovered radioactivity.

TABLE IV: Oligonucleotides from a Ribonuclease T_1 Digest.

Composition of Spot[b]	First Peak[a] (% recov)[c]	Second Peak[a] (% recov)[c]
ApGp CpGp	8.22 ± 0.03	7.14 ± 0.03
ApApGp CpCpGp CpApGp ApCpGp	6.67 ± 0.07	6.27 ± 0.01
UpGp	4.62 ± 0.01	4.12 ± 0.07
ApUpGp UpApGp CpUpGp UpCpGp	7.18 ± 0.07	6.42 ± 0.09
(ApApUp)Gp (CpCpUp)Gp (ApCpUp)Gp	4.59 ± 0.11	4.13 ± 0.12
UpUpGp	1.51 ± 0.02	1.31 ± 0.07
(ApUpUp)Gp (CpUpUp)Gp	2.20 ± 0.03	2.03 ± 0.07
UpUpUpGp	0.67 ± 0.03	0.47 ± 0.04
Gp + G>p	10.04 ± 0.11	9.27 ± 0.06

[a] Average of three determinations. Errors are average deviations from the mean. [b] Composition taken from Rushizky and Sober (1962). [c] Per cent of total recovered radioactivity.

agreement with those reported for total rRNA by other workers (Stanley and Bock, 1965; Spahr and Tissières, 1959; Midgley, 1962). It can be seen that there is no difference between the base composition of the two peaks. By this criterion, the population of RNA molecules present in each of the two peaks is chemically homogeneous.

DIGESTION OF THE TWO PEAKS WITH PANCREATIC RIBONUCLEASE OR RIBONUCLEASE T_1. A more sensitive test of the similarity or dissimilarity of RNA molecules is an analysis of the sequence of nucleotides of the RNA. For this purpose the RNA present in the two peaks was digested with pancreatic ribonuclease or with ribonuclease T_1, two enzymes with known specificities. The oligonucleotides present in the digests of the ^{32}P-labeled RNA were separated from each other by the two-dimensional paper method of Rushizky and Knight (1960a,b) and Rushizky and Sober (1962). The position of the ^{32}P-labeled oligonucleotides on the paper was located by the ultraviolet absorption of the oligomers derived from carrier RNA as well as by autoradiography.

The composition of the oligonucleotides present in the two-dimensional chromatogram from a pancreatic ribonuclease digest of ^{32}P-labeled RNA was ascertained by comparison with the published map (Rushizky and Knight, 1960a,b) with which it was completely identical, except for a more extended area at the origin. Table III shows the result obtained. Small but significant differences exist in the frequency of occurrence of three tetranucleotides and one pentanucleotide derived from the rRNA present in the two peaks. Further, the amounts of Up liberated from the two RNA peaks are different, being higher from the peak eluted first. The amount of material at the origin, which represents large oligonucleotides and oligonucleotides rich in purine sequences, is lower for this peak. These results show that the RNAs present in the two peaks are different.

The autoradiograms of the two-dimensional fingerprints of ribonuclease T_1 digests of the two RNA peaks were qualitatively identical; both showed extended and diffuse bands at the origin. They differed from the published map of Rushizky and Sober (1962) in having one fairly strong and four faint spots each well resolved from the other spots in the autoradiogram. The faint spots but not the strong spot could be identified with very faint ultraviolet absorbing areas, derived from carrier RNA, on the chromatogram. The mobilities of

these spots were different from those of Up, Cp, and AP in the same system. Because of the large number of tri-, tetra-, and pentanucleotides which are possible in a ribonuclease T_1 digest of RNA, the resolution is poorer than with a pancreatic ribonuclease digest. For this reason, only the spots whose composition was known, and which were well resolved, were cut out, and the radioactivity was determined. The results are tabulated in Table IV. Differences can be seen in a number of the spots; in addition the amount of guanosine 3′-phosphate and G>p liberated is different from each of the peaks. The result is in agreement with the previous conclusion that the RNAs present in each of the peaks are chemically different.

Discussion

rRNA when fractionated as the cetyltrimethylammonium salt is resolved into two components, each of which contains 16S and 23S RNA. This method of fractionation, therefore, unlike centrifugation through sucrose gradients or chromatography on methylated serum albumin Kieselguhr columns, does not separate rRNA into its components according to size. The two components appear not to be aggregates of 23S and 16S RNA or aggregates between two 16S or two 23S RNA molecules (Marcot-Queiroz and Monier, 1965; Hayes *et al.*, 1966) as conditions required for the formation of these aggregates are not obtained during the fractionation procedure. Rather a separation based on the conformation of the RNA apparently occurs, and the two components represent RNA molecules of different secondary structures. The occurrence of a critical salt concentration is the result of a competition between the organic and inorganic cations for the polyanion (Scott, 1961, 1962); this same competition may account for the fractionation observed.

Analysis of the oligonucleotide frequencies produced by pancreatic ribonuclease or ribonuclease T_1 digestion of the RNA present in the two peaks obtained on fractionation of the quaternary ammonium salt of rRNA shows that the two peaks contain RNA molecules of different nucleotide sequences. Since each of the peaks contain 23S and 16S rRNA, the result implies that heterogeneity exists in the 23S and 16S species of rRNA. This conclusion is contingent on the absence of ^{32}P-labeled mRNA and on the presence in each of the two peaks of the same or similar ratio of 23S and 16S RNA since these components of rRNA have different base compositions (Midgley, 1962), and different nucleotide frequencies following digestion with pancreatic ribonuclease have been found (Aronson, 1962). Errors due to the presence of long-lived ^{32}P-labeled mRNA were eliminated by the use of a steady-state chase (Aronson and Holowezyk, 1965). The base composition analysis (Table II) supports this conclusion. Centrifugation of the RNA present in each of the two peaks through a sucrose density gradient also demonstrated that the 23S and 16S components were present in the ratio of 2:1. Furthermore, differences in the frequencies of oligonucleotides present after pancreatic ribonuclease digestion of 23S and 16S RNA have been found with the dinucleotides ApUp and GpUp and the trinucleotide GpGpCp (Aronson, 1962). The results presented in Table III show that the two peaks do not differ significantly in the frequencies of occurrence of these three oligonucleotides following digestion with pancreatic ribonuclease. Therefore, the difference in the frequency of occurrence of several oligonucleotides following digestion of the two peaks with pancreatic ribonuclease and ribonuclease T_1 indicates that heterogeneity exists within the 23S and 16S species of rRNA.

The demonstration of heterogeneity in 23S and 16S RNA is supported by studies of the end groups present at the 5′ terminus (Takanami, 1967; Nichols and Lane, 1967) and the 3′ terminus (Midgley and McIlreavy, 1967), as well as the alteration of pancreatic ribonuclease digest patterns of rRNA as a result of changes in growth conditions (Aronson and Holowezyk, 1965). The differences in nucleotide sequences between the various species of 23S RNA or between the species of 16S RNA appear to be small. Large differences would be unexpected as multiple species of rRNA probably arose by genetic variation, and hybridization studies indicate that rRNA is conserved in evolution in bacteria (Dubrau *et al.*, 1965; Doi and Igarahi, 1965). Further, Yanofsky and Spiegelman (1962, 1963) and Attardi *et al.* (1965) have demonstrated that the 16S and 23S components of rRNA are coded for by more than one cistron. With the bacterium *E. coli* 1–6 cistrons for the 23S RNA and up to 17 cistrons for the 16S RNA have been suggested. Although these values would represent the upper limit, the results indicate that rRNA is transcribed from more than one region of the genome. The possibility, therefore, exists for the presence of multiple species of 23S and 16S RNA and provides the biological interpretation for the demonstration of chemical heterogeneity in 23S and 16S RNA.

Recent work from a number of laboratories indicates that ribosomal proteins are physically (Traut, 1966; Traub *et al.*, 1966) and chemically heterogeneous (Traut *et al.*, 1967). The possibility therefore exists for multiple species of ribosomes and ribosome subunits arising from interaction between the different rRNAs and ribosomal proteins. Ribosomes are currently thought of as playing a nonspecific role in protein synthesis, but multiple species of ribosomes, if they exist, may indicate a more specific role for ribosomes in the mechanics of translation.

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