Isolation and Physical Properties of the Ribosomal Ribonucleic Acid of *Escherichia coli**

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ABSTRACT: Ribosomal ribonucleic acid preparations of exceptional stability were obtained from the ribosomes of *Escherichia coli* by a method of isolation employing phenol, sodium dodecyl sulfate, and a purified hectorite, Macaloid. The sedimentation and viscosity properties of the total ribosomal RNA and of the separated components were extensively investigated and molecular weight determinations were made by sedimentation viscosity, sedimentation equilibrium, and viscosity kinetics at elevated temperatures. Molecular weights of 1.07×10^6 and 0.55×10^6 g/mole were found for the 23 and 16 S components, respectively. The influence of RNA aggregation upon hydrodynamic parameters was evaluated and several methods (organic solvents, reaction with formaldehyde, low ionic strength, and heat) are suggested for the detection of aggregates within RNA preparations. Chromatography of undegraded ribosomal RNA upon DEAE-cellulose was found to be complicated by the formation at equilibrium of a nonelutable complex between the ion exchanger and the polynucleotide. Base composition analyses were performed upon the separated ribosomal RNA components, and slight but significant differences were found between the 23 and 16 S molecules. It is concluded that the ribosomal RNA of *E. coli* is composed of two classes of polyribonucleotide chains, each class being covalently continuous and thus not containing polynucleotide subunits.

Although several studies have appeared in the literature concerning the isolation and physical properties of *E. coli* ribosomal RNA (see, for example, Hall and Doty, 1958, 1959; Littauer and Eisenberg, 1959; Kurland, 1960), none of the RNA preparations investigated was of sufficient stability to warrant carrying out physical measurements which required prolonged periods of observation. Utilizing the negatively charged purified hectorite, Macaloid,[1] we have been able routinely to obtain RNA preparations which have exhibited a freedom from nuclease activity such that their stability has been limited only by the known chemical stability of the phosphodiester bond in polyribonucleotides (Eigner *et al.*, 1961; Ginoza, 1958). It thus became possible to undertake an extensive investigation into the physicochemical properties of such RNA preparations. Many of the results and techniques to be presented will be of value during the characterization of other polyribonucleotides, both natural and synthetic.

During the past few years a number of authors have suggested that ribosomal RNA is formed of "subunits" held together by secondary interactions (Takanami, 1958; Hall and Doty, 1959; Osawa, 1960; Chao, 1961; Aronson and McCarthy, 1961; Otaka *et al.*, 1961). The results reported in this paper are not consistent with this concept. We have found, instead, that the two molecules of ribosomal RNA, the so-called 23 and 16 S RNA, are in fact continuous phosphodiester chains. In addition, we have been able to produce particles which hydrodynamically resemble intact ribosomal RNA chains but which have been subjected under controlled conditions to a limited number of hydrolytic scissions. These modified RNA particles are quite stable under conditions favoring strong secondary interactions; however, they dissociate, revealing fragments of reduced molecular weight, when placed in environments which have often been employed to demonstrate the existence of ribosomal RNA "subunits."

Materials and Methods

Growth and Lysis of Bacteria. Escherichia coli strain W3101, obtained from Dr. J. Lederberg, Stanford University, was grown under forced aeration at 37° in the following medium: Difco Bacto-tryptone, 10 g/liter; dextrose, 5.6 g/liter; Na_2HPO_4, 6 g/liter; KH_2PO_4, 3 g/liter; NaCl, 5 g/liter. Cultures in late log phase were chilled to 0° by the addition of chipped

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[1] Macaloid is a trade name for a purified hectorite (sodium magnesium lithofluorosilicate) obtained from the American Tansul Co., Baroid Division, National Lead Co., 2404 Southwest Freeway, P.O. Box 1675, Houston, Texas 77001. Stock suspensions in dilute buffer were made at 90 to 100° in a high shear mixer.

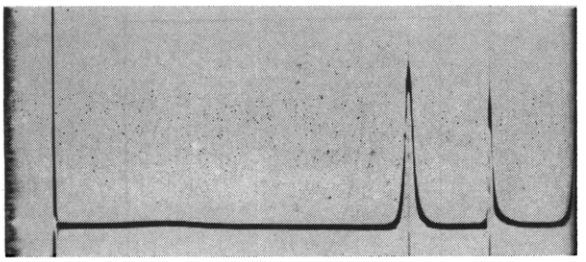

FIGURE 1: A photograph of the schlieren pattern of unfractionated ribosomal RNA in 0.1 M KCl, 0.05 M Tris, *p*H 7. Sedimentation is from left to right. The $s_{20,w}$ of the two main components are 14.5 and 17.4 S. The RNA concentration is 6.7 mg/ml.

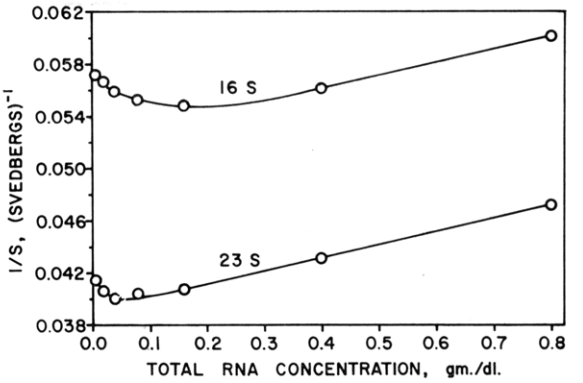

FIGURE 2: The dependence of the sedimentation coefficients of unfractionated ribosomal RNA upon the RNA concentration at 20° in 0.1 M KCl, 0.05 M Tris, *p*H 7. The RNA concentration is expressed as the sum of the two components. The intercepts, $s^0_{20,w}$, are 17.5 and 24.1 S. The significance of the negative slopes at the lower concentrations is not clear.

ice (from distilled water) and harvested by continuous flow centrifugation. The bacterial paste was frozen on solid carbon dioxide and stored at −20°. No differences were found in either the ribosomes or ribosomal RNA prepared from frozen versus fresh bacterial cells.

Thawed bacterial paste was washed twice in ten volumes of 0.01 M Tris, 0.01 M $MgCl_2$, *p*H 7, by centrifugation at 4°. To each kg of washed cells was added 600 ml of the above buffer and 960 ml of 120-μ diameter glass beads. The cells were ruptured by circulating the suspension through an Eppenbach colloid mill (Garver and Epstein, 1959) operated at 10,000 rpm with a gap setting of 0.081 cm. The lysate was maintained between 0 and 8° and complete cell breakage was obtained within 20 minutes.

Isolation of the Ribosomes. All operations were carried out between 0 and 4°. The lysate, freed of glass beads by decantation, was clarified by three successive centrifugations of 20 minutes each at 20,000 rpm in the No. 30 rotor of the Spinco Model L ultracentrifuge. The ribosomes were then sedimented at 30,000 rpm for 4 hours in the No. 30 rotor. Following resuspension in 0.01 M Tris, 0.01 M $MgCl_2$, *p*H 7, and a 20-minute centrifugation at 20,000 rpm in the 40 rotor (sediment discarded), the ribosomes were repelleted at 40,000 rpm for 3 hours in the No. 40 rotor. These final pellets were resuspended at a nucleoprotein concentration of from 15 to 20 mg/ml.

Isolation of the Ribosomal RNA. All operations were carried out between 0 and 4° unless otherwise indicated. The combination of phenol and SDS[2] employed by Hall and Doty (1959), with the modifications noted below, was used for the separation of the RNA from the protein. The nucleoprotein solution was made 0.5% (w/v) in SDS by the addition of 10% SDS. The flask was quickly warmed to 20°, held for 5 minutes, and chilled to 0° by swirling in an ice bucket. The solution was transferred to a separatory funnel containing an equal volume of buffer-equilibrated phenol, and a Macaloid suspension was added to give a final Macaloid concentration of 0.1% (w/v). The mixture was shaken vigorously for 5 minutes and then the phases were separated by centrifugation at 4980 rpm for 20 minutes in the K-6 swinging-bucket rotor of the Spinco Model K centrifuge stopped without braking. The upper (aqueous) phase was removed with a large syringe equipped with a 6-cm square-tipped 12-gauge needle. Extreme care was used to avoid disturbing the interfacial region, and the lower 10% of the aqueous layer was discarded along with the phenol phase. The recovered aqueous phase was returned to a clean separatory funnel, and phenol and Macaloid were added as described above. The extraction was repeated twice more, each time avoiding the interfacial region and each time discarding the 10% of the aqueous phase nearest the phenol.

Dissolved phenol was removed from the final aqueous solution by three extractions with equal volumes of peroxide-free ethyl ether and the residual ether was removed from the RNA solution by bubbling nitrogen gas through the liquid until the odor of ether was no longer discernible. The RNA solution was then dialyzed for 36 hours against a 50-fold volume of 0.1 M KCl, 0.05 M Tris, *p*H 7, changed every 6 hours. This solution was either stored frozen at −20° or lyophilized directly and stored as RNA plus salt powder at −20°. No differences in physical properties were found between fresh RNA, frozen RNA, or lyophilized RNA solutions.

Handling of RNA Solutions. Provided that the procedures given above, from the lysis of the bacteria through the final isolation of the RNA, were carried out in one continuous operation, RNA preparations of high molecular weight were routinely obtained which contained no detectable ribonuclease activity. It was essential for the stability of the RNA during subsequent manipulations, however, that all equipment coming into contact with the RNA solutions was both clean and sterile. Routinely, this involved cleaning glassware

[2] Abbreviations used in this work: SDS, sodium dodecyl sulfate; DMSO, dimethyl sulfoxide.

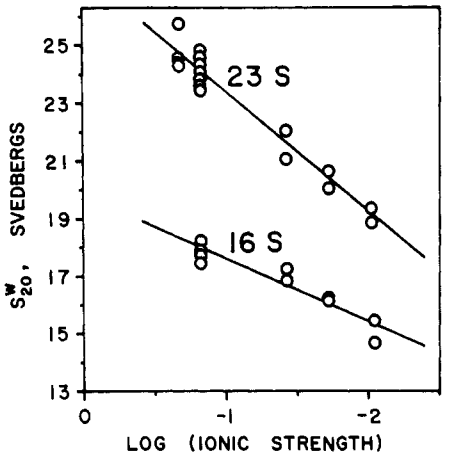

FIGURE 3: The dependence of the sedimentation coefficients of unfractionated ribosomal RNA upon the ionic strength at 20°. The RNA concentration was 40 μg/ml for all analyses.

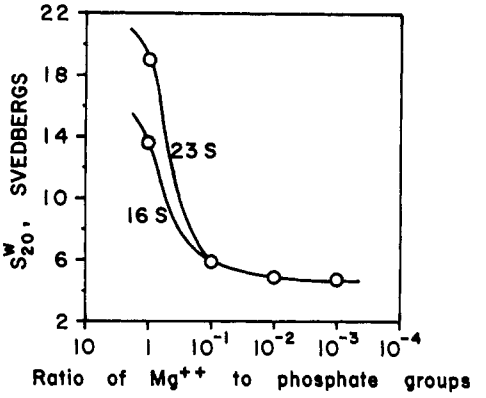

FIGURE 4: The influence of $MgCl_2$ upon the sedimentation coefficients at 20° of the unfractionated ribosomal RNA in 4×10^{-4} M KCl, 2×10^{-4} M Tris, *p*H 7.2. The RNA in this buffer was passed through a column of Dowex A-1 chelating resin in the K^+ form prior to the addition of the requisite magnesium. The RNA concentration was 40 μg/ml for all analyses.

with an acid-chromate cleaning solution, rinsing with distilled water, and drying under vacuum. Plastic materials, such as Kel-F centerpieces for the analytical cells of the Spinco Model E ultracentrifuge, were cleaned at 80° for 3 to 4 minutes in a 0.1% SDS–0.1% Na_2EDTA solution, rinsed with distilled water, and dried under vacuum. Dialysis tubing was boiled for 15 minutes in the SDS-EDTA solution, boiled in distilled water, and rinsed with distilled water.

Sedimentation Measurements. VELOCITY. The Spinco Model E ultracentrifuge was equipped with both phase-plate schlieren and ultraviolet absorption optics. Cell centerpieces were of Kel-F. Sedimentation coefficients measured in either aqueous or organic solvents were corrected to water at 20° assuming a partial specific volume of 0.53 ml/g.

EQUILIBRIUM. Runs were of 18-hours duration in 30-mm epoxy double-sector cells in the 30-mm AN-E rotor operated at 5° in the Spinco Model E ultracentrifuge. Solution column heights were 1 mm (Williams *et al.*, 1958). A speed of 2095 rpm was employed except for an initial period of 1350 seconds at 6166 rpm (Hexner *et al.*, 1961). Schlieren photographs of the refractive index gradients were obtained on Eastman Kodak Kodaline CTC pan plates.

ZONE VELOCITY. Exponential gradients of 5 to 20% sucrose in 0.1 M KCl, 0.05 M Tris, 0.001% SDS, *p*H 6.7, were formed at 4°. The character of the gradient was $c = 23 - 18e^{-v/10}$, where c is the concentration, in per cent, of the sucrose being delivered at a volume equal to v ml. For preparative runs, 6 mg of RNA in 0.6 ml of 0.1 M KCl, 0.05 M Tris, *p*H 7, was layered over each gradient tube, and the tubes were centrifuged in the SW-25 rotor of the Spinco Model L ultracentrifuge at 4° for 20 to 24 hours at 25,000 rpm. The rotor was stabilized by hand during acceleration and deceleration below 5000 rpm and was allowed to stop at the end of the run without braking. The contents of the tubes were analyzed by siphoning the gradients through a 1-mm flow cell mounted in a Beckman DB spectrophotometer operated at 260 mμ. The output of the spectrophotometer was recorded automatically as a function of time. Separated RNA components were recovered from their respective fractions by the addition of one volume of 2-propanol and chilling to −20°.

Viscosity Measurements. Viscosity measurements were performed in a Cannon Ubbelohde semimicro dilution viscometer requiring a minimum of 1 ml of sample and having an outflow time for distilled water at 20° of 317 seconds. Temperature control at 20° was maintained to within ±0.01° in an oil bath regulated by a Sargent S-82055 Model SV Thermonitor. Temperature control at temperatures other than 20° was regulated in a water bath controlled by a LaPine Tempunit to within ±0.1°. Samples were withdrawn from the viscometer following measurements at 20°, and both the concentration and sedimentation coefficients were determined.

Spectrophotometric Measurements. Concentrations were routinely determined by recording the optical density at 20° in a Model 11 or a Model 15 Cary recording spectrophotometer. From the base composition of the RNA and a hyperchromic shift of 40.1 ± 0.2% following alkaline hydrolysis and return to *p*H 7, the extinction of the potassium salt of 1 mg of RNA/ml of 0.1 M KCl, 0.05 M Tris, *p*H 7, was calculated to be 22.3 ± 0.2 at 260 mμ. Ultraviolet absorption measurements at temperatures other than 20°, *i.e.*, melting curves, were determined in a Beckman DB spectrophotometer. The temperature of the solution being analyzed was monitored by a LaPine Type 402 thermister probe connected to a Yellow Spring Model 43-TC Telethermometer.

Base Ratio Determinations. The base compositions

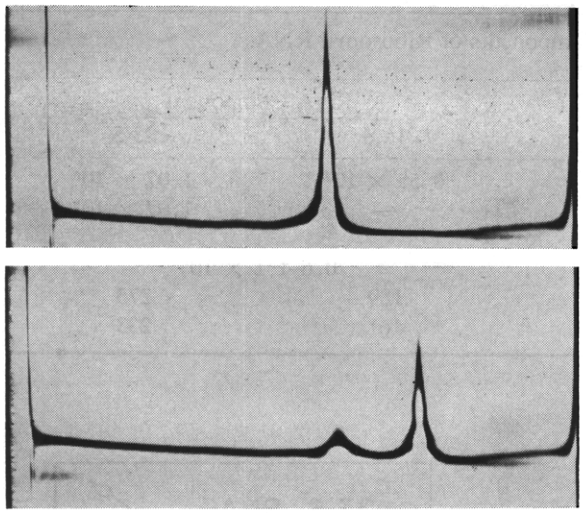

FIGURE 5: Photographs of the schlieren patterns of the components of ribosomal RNA in 0.1 M KCl, 0.05 M Tris, *p*H 7, following one cycle through sucrose gradient zone velocity centrifugation. Sedimentation is from left to right. The upper pattern is of the 16 S fraction. The RNA concentration is 2 mg/ml and the $s_{20,w}$ 14.8 S. The lower pattern is of the 23 S fraction. The RNA concentration is 2 mg/ml and the $s_{20,w}$ equal to 16.6 and 20.7 S. The 23 S fraction was recycled through a second sucrose density gradient centrifugation before use in subsequent experiments.

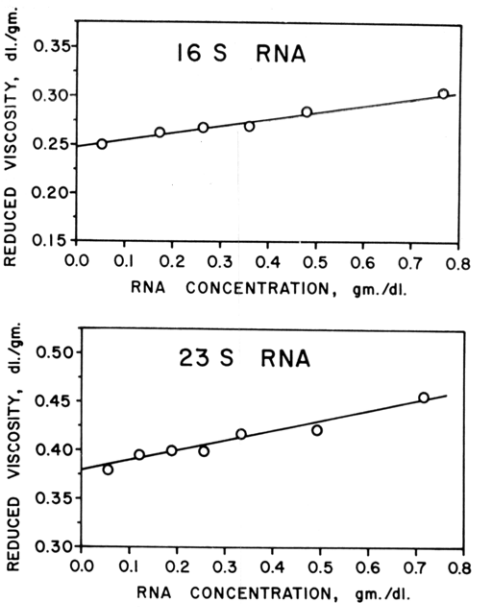

FIGURE 6: The determination of the intrinsic viscosities at 20° in 0.1 M KCl, 0.05 M Tris, *p*H 7, of the separated components of ribosomal RNA. The intrinsic viscosity of the 16 S component was found to be 0.248 dl/g and that of the 23 S component 0.379 dl/g.

of RNA samples were determined after either acid or alkaline hydrolysis. In the case of acid hydrolysis (1 hour, 100°, 1 M HCl) the bases and nucleotides were separated by paper chromatography (HCl-2-propanol-water (Wyatt, 1951)), eluted, and quantitated spectrophotometrically. Following alkaline hydrolysis (18 hours, 37°, 0.3 M KOH) RNA samples were neutralized to between *p*H 4 and 5, diluted 20-fold, and applied to a 225- × 0.9-cm column of Biorad AG 1-X8 resin (−400 mesh, 3.2 meq/g). The nucleotides were eluted at 45° with a 2-liter linear gradient of from 0 to 0.6 M ammonium formate, *p*H 4.00, at 25°.

Results

Sedimentation Properties. The schlieren pattern of the unfractionated ribosomal RNA is depicted in Figure 1. This distribution is characteristic of *E. coli* ribosomal RNA and has been widely quoted in the literature (Littauer and Eisenberg, 1959; Hall and Doty, 1959; Aronson and McCarthy, 1961). The dependence of the sedimentation coefficients upon the total RNA concentration is illustrated in Figure 2 and the effect of ionic strength in Figure 3. The effect of the divalent magnesium cation at low and high ionic strengths is indicated by the data in Figure 4 and Table I, respectively.

The sedimentation coefficients of the two main ribosomal RNA components isolated by zone velocity sedimentation through sucrose gradients were found to agree with those observed in unfractionated material when measured at low concentrations (40 μg/ml). The schlieren patterns of the fractionated material following one cycle through sucrose gradients are illustrated in Figure 5. The 16 S component was employed for further experiments at this stage, but the 23 S material was rerun on a second series of sucrose gradients. Material of greater than 95% purity (as judged by velocity sedimentation) was thus obtained and was employed for subsequent experiments.

TABLE I: Effect of $MgCl_2$ upon the Sedimentation Coefficients of Ribosomal RNA.[a]

$MgCl_2$ Concentration (M)	$s_{20,w}$	
	16 S Component	23 S Component
0.00	18.2	24.2
0.01	21.2	28.2

[a] Sedimentation runs were performed in 0.1 M KCl, 0.05 M Tris, *p*H 7, plus the indicated concentration of $MgCl_2$. The RNA had been pretreated with Dowex A-1 chelating resin and was analyzed at a concentration of 40 μg/ml.

Viscosity Properties. The intrinsic viscosity of unfractionated ribosomal RNA at 20° in 0.1 M KCl,

TABLE II: Molecular Weights and Radii of Gyration of the Components of Ribosomal RNA.

Parameter	Method of Determination	RNA Component 16 S	RNA Component 23 S
Molecular weight, g/mole	S and η	0.55×10^6	1.07×10^6
	Sedimentation equilibrium	—	1.07×10^6
	η kinetics	$0.6\text{–}1.1 \times 10^6$	
Radius of gyration, A	Sedimentation	189	275
	Viscosity	161	233

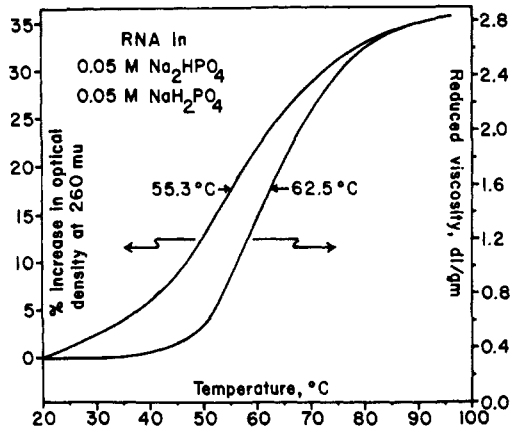

FIGURE 7: The optical density at 260 mμ and reduced viscosity of unfractionated ribosomal RNA as a function of temperature. The temperatures of the midpoints of the two transitions are indicated on the graph. The data for the optical density were obtained at temperature intervals of 2.5° and were corrected for the thermal expansion of the solution. The RNA concentration was 40 μg/ml. The data for the reduced viscosity were obtained at temperature intervals of 5° and were extrapolated to zero time at each temperature by plots of the logarithm of the reduced viscosity versus time. The RNA concentration was 1.9 mg/ml. The experimental points are not indicated since the scatter of each set of data was less than the width of the curves.

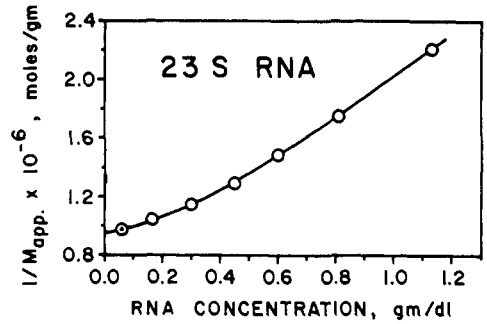

FIGURE 8: The determination by equilibrium centrifugation of the apparent molecular weight of the 23 S component of ribosomal RNA as a function of concentration. The double point at the lowest concentration results from duplicate analyses, one in the presence of a supporting column of silicone oil, and the other in the absence of oil. Following the equilibrium runs, all RNA samples were checked for possible degradation by recovery, dilution, and velocity sedimentation. All samples were found to be monodisperse with sedimentation coefficients of 24 ± 0.5 S. The extrapolation of $1/M_{app}$ to zero concentration yields a molecular weight for the 23 S component of 1.07×10^6 g/mole.

0.05 M Tris, *p*H 7.3, was found to vary between 0.33 and 0.42 dl/g depending upon the history of the sample. Freshly prepared RNA gave the lower value, while heated and cooled material gave high values. The intrinsic viscosities of the separated components of ribosomal RNA were also determined and are represented in Figure 6. It is to be noted that, in all measurements of the intrinsic viscosities, the value of the Huggins constant k in the equation

$$\eta_{sp}/c = [\eta] + k[\eta]^2 c$$

fell between the values of 0.6 and 1.1.

In accord with the results of Spirin and co-workers (Spirin, 1962; Bogdanova *et al.*, 1962; Shakulov *et al.*, 1962), the reduced viscosity of ribosomal RNA was found to be a function of temperature. This variation, along with the variation of optical density, is illustrated in Figure 7.

Molecular Weight Determinations. The values of the sedimentation coefficients ($s^0_{20,w}$) and the intrinsic viscosities of the separated ribosomal RNA components were combined in the Scheraga-Mandelkern equation (Scheraga and Mandelkern, 1953) to yield their hydrodynamic molecular weights. β was assumed to be 2.16×10^6 and $\bar{\nu}$ equal to 0.53 ml/g. The intrinsic viscosities and the sedimentation coefficients were also utilized to calculate the radii of gyration of the two components. These results are summarized in Table II.

The weight average covalent molecular weight of the unfractionated ribosomal RNA was also estimated through a kinetic analysis of viscometric data obtained

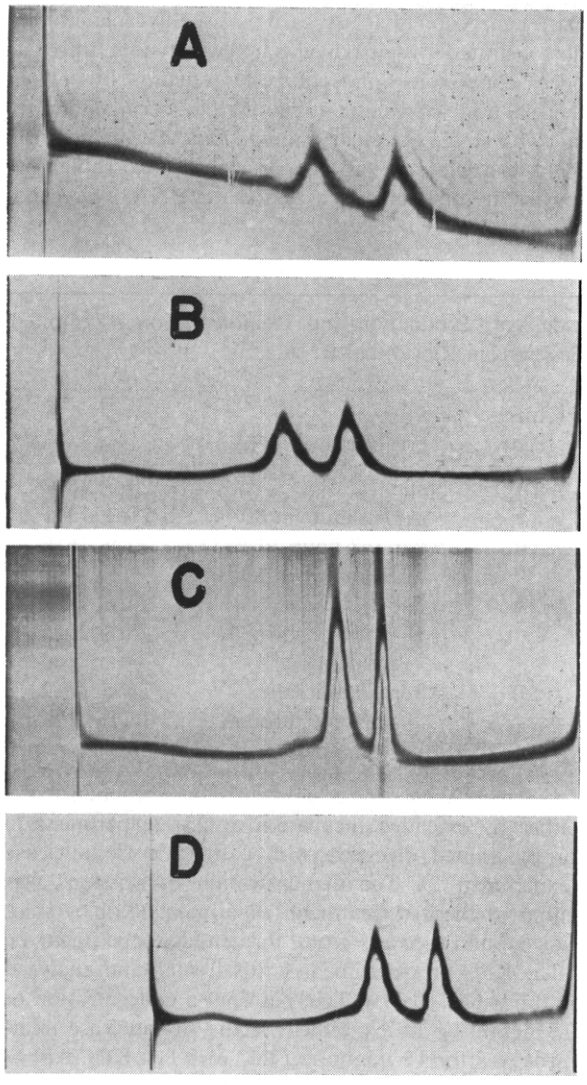

FIGURE 9: Photographs of the schlieren patterns of unfractionated ribosomal RNA in formamide and dimethyl sulfoxide and after return to aqueous medium. Sedimentation is from left to right. (A) Ribosomal RNA in 0.2 M LiCl in formamide; RNA concentration is 2 mg/ml and $s_{20,w}$ 16.5 and 21.1 S. (B) The sample above returned to 0.1 M KCl, 0.0005 M $MgCl_2$, 0.05 M Tris, *p*H 7; RNA concentration is 2 mg/ml and the $s_{20,w}$ equal to 15.9 and 20.3 S. (C) Ribosomal RNA in 0.2 M LiCl in dimethyl sulfoxide; RNA concentration is 3.6 mg/ml and $s_{20,w}$ 13.6 and 16.4 S. (D) The sample above returned to 0.1 M KCl, 0.0005 M $MgCl_2$, 0.05 M Tris, *p*H 7; RNA concentration is 3.4 mg/ml and $s_{20,w}$ 15.4 and 19.3 S.

upon the RNA at 80°. Utilizing the equations for the initial stages of the random degradation of a high molecular weight polymer (see, for example, Tanford, 1961) and making the following assumptions: (a) RNA at 80° in 0.05 M Na_2HPO_4, 0.05 M NaH_2PO_4 is a flexible linear chain, (b) the ratio of the reduced viscosities measured at any two times is equal to the ratio of

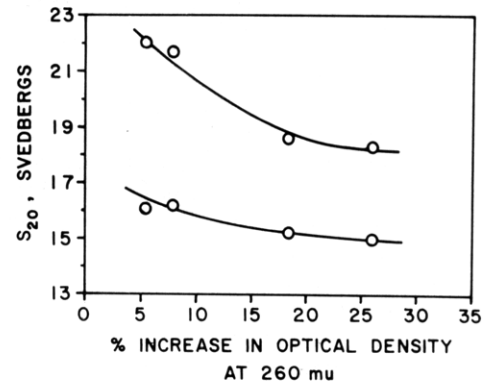

FIGURE 10: The effect of reaction with formaldehyde upon the sedimentation coefficients of unfractionated ribosomal RNA. RNA at a concentration of 0.4 mg/ml was allowed to react at 20° for various times with 1% formaldehyde between *p*H 9.6 and 10.7 in 0.005 M KCl, 0.0025 M potassium phosphate. The reactions were stopped by making them 30 μg/ml in RNA, 1% in formaldehyde, 0.1 M in KCl, 0.05 M in potassium phosphate, *p*H 6.9. The per cent increases in optical density at 260 mμ and the sedimentation coefficients were measured in this solvent. The increases in the optical densities were found to be stable for at least several hours. Complete reaction of the RNA with formaldehyde corresponds to a per cent increase in the optical density at 260 mμ of 32%.

the intrinsic viscosities at the same two times, and (c) that a similar relationship is true for the viscosity average and the weight average molecular weights, the following equation is obtained:

$$-\log \eta_t = -\log \eta_0 + (a) \log \left(1 + \frac{M_0 k t}{2R_w}\right)$$

where η_t is the reduced viscosity at time t, η_0 is the reduced viscosity at time zero, a is a parameter whose value lies between 0.5 and 0.8 for flexible linear chains, M_0 is the initial weight average molecular weight of the polymer undergoing degradation, k is the rate constant for the hydrolyzable bond in the polymer, t is the time of reaction, and R_w is the residue weight of the polymer. Taking 362 g/mole as the value of R_w, 8.6×10^{-8} sec^{-1} as the value of k (Eigner *et al.*, 1961), and 0.5 and 0.8 as the limits of a for a flexible linear chain, it may be calculated that the limits of M_0, the initial average molecular weight of the unfractionated ribosomal RNA, lie between 0.6×10^6 and 1.1×10^6 g/mole. From quantitative zone velocity sedimentation analyses and velocity sedimentation analyses at low concentrations, the mass ratio of 16 S to 23 S RNA in the ribosomal RNA is between 0.67 and 1.0. Taking this ratio to be one, a weight average molecular weight of 0.83×10^6 g/mole may be calculated for the unfractionated ribosomal RNA. The excellent agreement between this calculation and the results of the viscosity kinetics

provides strong evidence that the hydrodynamic and covalent molecular weights of ribosomal RNA are very similar and probably identical.

The weight average molecular weight of the larger of the two main ribosomal RNA components was also measured by the technique of equilibrium sedimentation. This method yielded a value of 1.07×10^6 g/mole (Figure 8), also in agreement with the value derived from sedimentation viscosity measurements.

Aggregation. Once RNA preparations have been obtained free from nuclease activity, aggregation becomes the principal limitation on the accuracy of physical measurements. Both the sedimentation coefficients and the reduced viscosities increase in moderately concentrated RNA solutions following brief exposure to elevated temperatures. This behavior is outlined in Table III, and is undoubtedly due to the formation of intermolecular aggregates during the reestablishment of secondary interactions. It may also be seen that the presence of these aggregates prevents the viscosity at 25° from being a valid measure of the covalent molecular weight of the RNA. Thus Table III indicates that, for a given reduced viscosity, the covalent molecular weight of an RNA preparation may actually vary over at least a factor of two.

TABLE III: Effect of Thermal Treatment upon the Viscosity and Sedimentation Coefficients of Ribosomal RNA.

Minutes at 80°	M_t/M_0[a]	Relative Reduced Viscosity at 25° after Heating[b]	$s_{20,w}$[c] 16 S	23 S
0	1.000	1.000	16.2	20.7
1	0.985	1.216	17.1	21.1
2	0.975	1.213	—	—
14	0.864	1.161	—	—
31	0.725	1.100	—	—
50	0.647	1.090	—	—
78	0.514	1.029	—	—
108	0.423	1.000	—	—
154	0.307	0.920	—	—

[a] The reduced viscosity, measured at 80° at the beginning and at the end of the incubation at 80°, was used to estimate M_t/M_0 from the relationship $M = K[\eta]^{1/a}$ with the assumption that $a = 0.5$. [b] The RNA concentration for the viscosity measurements was 3 mg/ml and the solvent was 0.1 M KCl, 0.05 M Tris, *p*H 7. [c] The RNA concentration for the sedimentation measurements was 9.3 mg/ml and the solvent was 0.1 M KCl, 0.05 M Tris, *p*H 7.

The possibility that undetected aggregation may exist in ribosomal RNA as it is usually isolated was demonstrated by introducing a few scissions into the once continuous phosphodiester chains by either thermal or enzymatic treatment under conditions favoring stable secondary interactions. These modified RNA particles are no longer stable (see Table IV) toward treatments which dissociate RNA aggregates and duplicated, in many respects, the ribosomal RNA preparations which earlier workers have believed to have been composed of "subunits" (Hall and Doty, 1958, 1959; Takanami, 1958; Osawa, 1960; Brown *et al.*, 1960; Chao, 1961; Aronson and McCarthy, 1961; Otaka *et al.*, 1961).

TABLE IV: Production and Demonstration of Limited Scissions in Ribosomal RNA.[a]

Experiment	Treatment of RNA	$s_{20,w}$
1A[b]	None	20.7, 16.2
	+ 1 minute at 80°	21.1, 17.1
1B	After 368 hours at 25°	20.9, 16.6
	+ 1 minute at 80°	15.4[d]
2A[c]	None	24.8, 18.2
	+ Passage through formamide	25.8, 19.5
2B	After ribonuclease	25.5, 18.2
	+ Passage through formamide	15.0[d]

[a] Scissions were introduced into the ribosomal RNA either by extended incubation at 25° (experiment 1) or by limited digestion with pancreatic ribonuclease (experiment 2). The demonstration of scissions was either by thermal treatment (experiment 1) or by solution in, and recovery from, formamide (experiment 2). [b] The RNA in experiment 1 was treated and analyzed in 0.1 M KCl, 0.05 M Tris, *p*H 7, at a concentration of 9.3 mg/ml. [c] In experiment 2, the ribonuclease treatment was for 15 minutes at 20° in 0.1 M KCl, 0.01 M $MgCl_2$, 0.05 M Tris, *p*H 7.3, at an RNA concentration of 1.12 mg/ml and a pancreatic ribonuclease (Pentex 5 × crystallized) concentration of 2.8×10^{-3} μg/ml. Following the digestion, the RNA was reisolated by the SDS–phenol–Macaloid procedure described under Methods. Passage through formamide consisted of precipitation from aqueous medium, solution in formamide for 30 minutes at 25°, precipitation (see legend, Table VI), and solution in 0.1 M KCl, 0.05 M Tris, *p*H 7.3. Sedimentation analyses were carried out in this solvent at a RNA concentration of 40 μg/ml. [d] Broad distribution of sedimentation coefficients.

Several relatively mild procedures were adapted to reveal aggregation when it existed and to provide additional tests of the concept that ribosomal RNA is

TABLE V: Effect of Extended Dialysis upon Ribosomal RNA.[a]

RNA Treatment	$s_{20,w}$
After dialysis without EDTA	24.7, 17.8
+ 5 minutes at 78°	25.0, 19.2
After dialysis with EDTA	23.9, 17.8
+ 5 minutes at 78°	23.7, 18.2

[a] The RNA samples were dialyzed against 200 volumes of 1.25×10^{-2} M KCl, 6.25×10^{-3} M Tris, *p*H 7.2, with and without added 1×10^{-4} M EDTA. Dialyses were continued at 4° for 2 weeks with the dialysis buffers renewed once every 24 hours. Samples were made 0.1 M in KCl and 40 μg/ml in RNA before the sedimentation analyses.

(see Figure 7); further supportive data are given in Table VII. Taken together, these data provide very strong evidence that the two main components of *E. coli* ribosomal RNA are each continuously covalently linked chains.

Interaction with DEAE-Cellulose. Attempts to chromatograph ribosomal RNA upon DEAE-cellulose (Brown Co., 1 meq/g) were unsuccessful. The data presented in Figures 11 and 12 show that this failure was due to the fact that ribosomal RNA only slowly equilibrated with the ion exchanger and that, when equilibrated, formed a nonelutable complex. Additional studies showed that the RNA was not eluted by 1 M KCl at any temperature up to 80°, nor at 25° by 3 M solutions of the most competitive anions.

Base Compositions. The base compositions of the unfractionated ribosomal RNA and of the two separated

TABLE VI: Sedimentation Coefficients of Ribosomal RNA in, and Recovered from, Formamide and Dimethyl Sulfoxide.[a]

RNA Recovered from	Sedimentation Solvent	RNA (mg/ml)	$s_{20,w}$
Aqueous solution	$HCONH_2$ + 0.2 M LiCl	2.0	21.1, 16.5
$HCONH_2$ + 0.2 M LiCl	0.1 M KCl, 0.05 M Tris, *p*H 7	2.0	20.3, 15.9
$HCONH_2$	0.1 M KCl, 0.05 M Tris, *p*H 7	0.04	22.9, 16.8
Aqueous solution	DMSO + 0.2 M LiCl	3.6	16.4, 13.6
DMSO + 0.2 M LiCl	0.1 M KCl, 0.05 M Tris, *p*H 7	3.4	19.3, 15.4
DMSO	0.1 M KCl, 0.05 M Tris, *p*H 7	5.0	15.6, 12.9

[a] RNA samples were dissolved in the organic solvents following precipitation from aqueous solution with ethyl alcohol and washing with absolute ethyl alcohol followed by ethyl ether. Residual ethyl ether was removed under vacuum. Samples were recovered from organic solution by the addition of one volume of ethyl alcohol and two volumes of ethyl ether. The precipitates were washed three times with ethyl alcohol and the residual alcohol was removed under vacuum. The precipitates were then dissolved in the aqueous buffer system. All precipitations of the RNA were at 0°. All sedimentation coefficients have been corrected to water at 20° as the solvent assuming a partial specific volume of 0.53 ml/g.

composed of two classes of continuous chains of molecular weight 1.07×10^6 and 0.55×10^6 g/mole. Long term dialysis, with and without EDTA, was shown to be without effect on the RNA (Table V). RNA when dissolved in either formamide or dimethyl sulfoxide in the presence of a supporting electrolyte exhibited in the ultracentrifuge the usual two components; these were recovered unchanged when returned to an aqueous medium (Table VI and Figure 9). RNA recovered from either of these two organic solvents in the absence of salt also showed the normal distribution of components. Progressive reaction of the bases of the RNA chains with formaldehyde was found only slightly to diminish the sedimentation coefficients, suggesting that the RNA molecules were becoming more extended but not releasing "subunits" of lower molecuar weight (Figure 10). Finally, the effect of heat has already been discussed under the section on viscosity properties

components are summarized in Table VIII. The differences between the 16 and 23 S components are greater than experimental error and are considered significant. It is to be noted, however, that these differences are only significant when the components to be compared are derived from ribosomal RNA preparations obtained from a single bacterial harvest.

Discussion

The results presented in this paper lead to the following concept of *E. coli* ribosomal RNA. The two main components, the 16 and the 23 S RNA's, are separate, noninterconvertible classes and are formed of continuously covalently linked residues. In solution, the chains appear to be coiled or folded particles whose configurations are dependent upon secondary interactions which at moderate temperatures and ionic

TABLE VII: Thermal Expansion of Polyribonucleotides.

Polynucleotide[a]	Buffer[b]	RNA (mg/ml)	Reduced Viscosity (dl/g) Measured at 25°	80°	Ratio 80°/25°
Ribosomal RNA	Phosphate	1.88	0.331	2.65	8.0
Ribosomal RNA	Phosphate	3.03	0.345	2.79	8.1
Ribosomal RNA	Phosphate	3.22	0.348	2.82	8.1
Ribosomal RNA	Phosphate	3.55	0.343	2.88	8.4
Ribosomal RNA	Tris	2.97	0.338	2.40	7.1
Ribosomal RNA	Tris	3.07	0.347	2.42	7.0
s-RNA	Phosphate	8.93	0.090	0.215	2.4
Poly-U	Phosphate	3.73	0.959	0.969	1.0

[a] Polynucleotides: ribosomal RNA, *E. coli* ribosomal RNA; s-RNA, yeast transfer RNA, Lot 2451-D, General Biochemicals; poly-U, polyuridylic acid, Lot 4729, Miles Laboratories ($s_{20,w}$ = 8 S). [b] Buffers: phosphate = 0.05 M NaH_2PO_4, 0.05 M Na_2HPO_4; Tris = 0.1 M KCl, 0.05 M Tris, *p*H 6.7 at 25°.

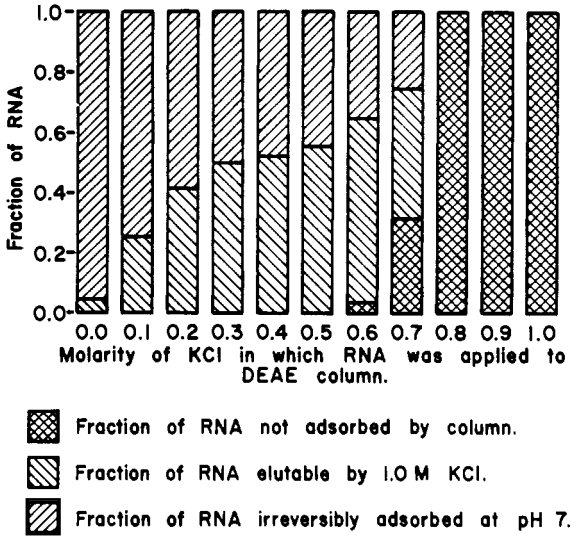

FIGURE 11: The interaction of unfractionated ribosomal RNA with DEAE cellulose at 20° as a function of salt concentration. Eleven DEAE-cellulose (0.9 meq/g) columns (0.7 × 4 cm) were prepared and equilibrated with 0.0, 0.1, 0.2, 0.3, . . . M KCl, 0.001 M Tris, *p*H 6.6. Eleven samples of ribosomal RNA (1 mg each) in 0.0, 0.1, 0.2, 0.3, . . . M KCl, 0.001 M Tris, *p*H 6.6, were introduced into the respective columns. Each column was washed with five bed volumes of the buffer in which the RNA had been applied to the column. The RNA emerging with this wash was labeled "Fraction of RNA not adsorbed by column." Each column was then washed with five bed volumes of 1 M KCl, 0.001 M Tris, *p*H 6.6. The RNA emerging with this wash was labeled "Fraction of RNA elutable by 1.0 M KCl." The RNA not accounted for by the sum of the first two fractions was labeled "Fraction of RNA irreversibly adsorbed at *p*H 7."

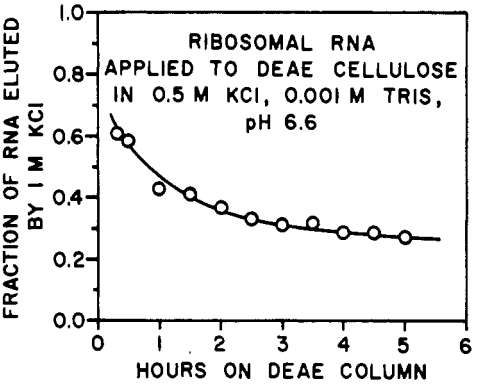

FIGURE 12: The interaction of unfractionated ribosomal RNA with DEAE-cellulose at 20° as a function of time. Eleven columns (0.7 × 4 cm) of DEAE-cellulose (0.9 meq/g) were equilibrated with 0.5 M KCl, 0.001 M Tris, *p*H 6.6. Eleven samples of ribosomal RNA (1 mg each) in 0.5 M KCl, 0.001 M Tris, *p*H 6.6, were introduced into the columns. Each of the columns was washed with five bed volumes of 0.5 M KCl, 0.001 M Tris, *p*H 6.6; no RNA was recovered in these washes. At successive time intervals one of the columns was washed with five bed volumes of 1.0 M KCl, 0.001 M Tris, *p*H 6.6. The fraction of RNA recovered in these washes was labeled "Fraction of RNA eluted by 1 M KCl."

strengths are only slowly equilibrated with their environment. This is indicated by the slowness of the equilibration with DEAE-cellulose and by the fact that the reduced viscosity, measured at 25°, continues to rise for many hours following an exposure to elevated temperatures. The configurations of the two components

TABLE VIII: Base Compositions of Ribosomal RNA.[a]

RNA Preparation[b]	RNA	Method of Hydrolysis	No. of Analyses	Base Composition as Per Cent of Total Bases			
				A	G	U	C
1	16 + 23 S	OH^-	3	25.1	32.4	21.1	21.4
1	16 S	OH^-	2	24.2	32.1	21.3	22.3
1	23 S	OH^-	2	25.5	32.5	21.0	21.0
1	16 + 23 S	H^+	2	25.1	32.3	21.4	21.2
2	16 + 23 S	H^+	2	25.5	31.2	21.1	21.3
3	16 + 23 S	OH^-	2	24.0	33.6	21.3	21.1

[a] The bases and nucleotides were separated, identified, and quantitated as described under Methods. [b] Ribosomal RNA preparations 1, 2, and 3 were obtained from independently grown and harvested cultures of *E. coli*.

when freshly isolated appear to be more compact than might be expected for a purely random coil. This is indicated by the rather high sedimentation coefficients and low intrinsic viscosities and also by the high value of the Huggins constant (0.6 to 1.1 as opposed to a value of approximately 0.35 predicted by a random coil model (Huggins, 1942)). It is quite possible that the configurations observed in solutions of freshly prepared RNA reflect the residual structure of the RNA as it existed in the ribosomes.

Several of the physical characteristics of ribosomal RNA which are measured by hydrodynamic parameters and which have been presented here were found to agree with values previously reported in the literature. This is true for those properties not sensitive to the covalent integrity of the RNA particles. Many of the other results, however, were dependent upon the absence of nuclease contamination and upon the high covalent molecular weight of these *E. coli* ribosomal RNA preparations. It is hoped that some of the techniques which have been reported here will prove useful in studies of conformation and structural integrity of other high molecular weight polyribonucleotides, both natural and synthetic.

References

Aronson, A. I., and McCarthy, B. J. (1961), *Biophys. J. 1*, 215.

Bogdanova, E. S., Gavrilova, L. P., Dvorkin, G. A., Kisselev, N. A., and Spirin, A. S. (1962), *Biokhimiya 27*, 387.

Brown, R. A., Ellem, K. A. O., and Colter, J. C. (1960), *Nature 187*, 509.

Chao, F. (1961), *Biochim. Biophys. Acta 53*, 64.

Eigner, J., Boedtker, H., and Michaels, G. (1961), *Biochim. Biophys. Acta 51*, 165.

Garver, J. C., and Epstein, R. L. (1959), *Appl. Microbiol. 7*, 318.

Ginoza, W. (1958), *Nature 181*, 958.

Hall, B. D., and Doty, P. (1958), Microsomal Particles and Protein Synthesis, New York, Pergamon, p. 27.

Hall, B. D., and Doty, P. (1959), *J. Mol. Biol. 1*, 111.

Hexner, P. E., Radford, L. E., and Beams, J. W. (1961), *Proc. Natl. Acad. Sci. U.S. 47*, 1848.

Huggins, M. L. (1942), *J. Am. Chem. Soc. 64*, 2716.

Kurland, C. G. (1960), *J. Mol. Biol. 2*, 83.

Littauer, U. Z., and Eisenberg, H. (1959), *Biochim. Biophys. Acta 32*, 320.

Osawa, S. (1960), *Biochim. Biophys. Acta 43*, 110.

Otaka, E., Oota, Y., and Osawa, S. (1961), *Nature 191*, 598.

Scheraga, H. A., and Mandelkern, L. (1953), *J. Am. Chem. Soc. 75*, 179.

Shakulov, R. S., Ajtkhozhin, M. A., and Spirin, A. S. (1962), *Biokhimiya 27*, 744.

Spirin, A. S. (1962), Acides Ribonucleiques et Polyphosphates, Structure, Synthèse et Fonctions, Colloques Internationaux du Centre National de la Récherche Scientifique No. 106, Paris, Centre National de la Récherche Scientifique, p. 73.

Takanami, M. (1958), *Biochim. Biophys. Acta 29*, 430.

Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, Wiley.

Williams, J. W., Van Holde, K. E., Baldwin, R. L., and Fugita, H. (1958), *Chem. Rev. 58*, 715.

Wyatt, G. R. (1951), *Biochem. J. 48*, 584.