Isolation, Characterization, and Stability of the 30S Ribosomal RNA Complex from HeLa Cells†

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ABSTRACT: The HeLa 30S rRNA molecule (historically designated 28S rRNA) can be dissociated into two components, a 7S rRNA and a large rRNA component which we call 29S rRNA. To evaluate conformational differences between the 30S rRNA complex and the isolated 29S rRNA component of the complex, viscosity, sedimentation velocity, circular dichroism, and ultraviolet absorption measurements with the two species were performed. Sedimentation equilibrium studies were also carried out with the 30S rRNA complex. In addition, the kinetics of the reaction which dissociates the 30S rRNA complex were characterized. The removal of glycogen-like molecules by cetyltrimethylammonium bromide precipitation of the rRNA and the preequilibration of rRNA with solvent by Sephadex column chromatography were found to be essential for reproducibility. The $s_{20,w}^0$ values for the 30S rRNA complex and the isolated 29S rRNA were determined from the experimental data obtained at various rRNA concentrations as 29.89 ± 0.40 and 29.09 ± 0.14, respectively. The corresponding intrinsic viscosity values were 74 ± 5 and 67 ± 5 cm^3/g, respectively. The optical properties of the 30S rRNA and 29S rRNA were not significantly different. These results indicate that there is no significant conformational difference between 30S rRNA and 29S rRNA under the conditions studied. We conclude from the sedimentation equilibrium data that the molecular weight of 30S rRNA is 2.1×10^6. From the kinetic data, the 30S rRNA dissociation appears to be an irreversible, cooperative, and ionic strength dependent reaction which at an ionic strength of 0.051 has an activation enthalpy of 123.5 kcal/mol and an activation entropy of 0.32 kcal/(mol deg).

The large subunit of the cytoplasmic ribosomes of HeLa cells contains two rRNA species, 30S and 5S rRNA. The larger species, called 28S rRNA by Pene *et al.*, but here called 30S rRNA for reasons given later in this paper, has been shown to be comprised of a small RNA molecule, designated 7S rRNA, and a large RNA molecule, called heated 28S rRNA by Pene *et al.*, but which we shall call 29S rRNA (Pene *et al.*, 1968). The noncovalently linked complex, 30S rRNA, is stable to phenol extraction at room temperature and an ionic strength, $\Gamma/2$, of 0.05 (Pene *et al.*, 1968). These workers attributed the observed change in sedimentation coefficient which accompanies the release of the 7S rRNA molecule from 30S rRNA to an alteration in the conformation of the 29S component. The existence of analogous 30S rRNA complexes in cytoplasmic ribosomes has also been demonstrated in other eucaryotes including rabbit (King and Gould, 1970; Hunt, 1970), rat (Sy and McCarty, 1970; Plagemann, 1970; Prestayko *et al.*, 1970), mouse (Eliceiri and Green, 1969), hamster (Pene *et al.*, 1968), chick (Pene *et al.*, 1968), sea urchin (Sy and McCarty, 1970, 1971), higher and lower plants (Sy and McCarty, 1970; Payne and Dyer, 1972), and yeast (Udem *et al.*, 1971). No such complexes have been found in bacteria (Pene *et al.*, 1968), mitochondria (Lizardi and Luck, 1971), or chloroplasts (Payne and Dyer, 1972). The 140 rRNA cistrons coding for yeast 5.8S rRNA (the HeLa 7S rRNA analog) are located on at least two chromosomes, with 70% on chromosome I (Cryer *et al.*, 1973; Finkelstein *et al.*, 1972), yet the sequence of the 5.8S rRNA reveals no heterogeneity, except in length at the 5′ end (Rubin, 1974). These findings indicate that the sequence integrity of the 7S rRNA species must be maintained in an evolutionary sense and suggest the 7S rRNA molecule may play a critical role in the function and/or biosynthesis of the ribosome in higher organisms; for example, by serving as a determining factor in the proper conformation of the 29S rRNA with which it is complexed.

It might be possible to understand the function of 7S rRNA in the maintenance of the structural integrity of HeLa ribosomes if the structures of the intact 30S rRNA complex and the separated 29S and 7S rRNA were known. With this overall objective in view, we have measured certain structure-related physical parameters of the 30S rRNA complex and the 29S rRNA component. Also, the kinetics of the thermally induced disruption of the 30S rRNA complex are characterized and interpreted.

Materials and Methods

Cell Growth, Labeling, and Fractionation. Suspension cultures of HeLa S3 cells were maintained at 37° in Joklik-modified Eagle's minimum essential medium (Eagle, 1959) with 5% (v/v) calf serum, except where noted, at concentrations of $2-5 \times 10^5$ cells/ml.

For studies involving ^{32}P labeling, cells were suspended at a cell concentration of $3-4 \times 10^5$ cells/ml in a medium of the following composition; 370 ml of Eagle's medium lacking phosphate; 10 ml of normal Joklik-modified Eagle's minimum essential medium; 20 ml of calf serum (Grand

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Island Biological Company); 4 ml of 1 M Hepes;[1] 4 ml of 10% (w/v) Na_2CO_3 adjusted to pH 7.4. Neutralized $H_3{}^{32}PO_4$, 99% pure (New England Nuclear Corp.), was added to final radioactivity levels of 7–10 μCi/ml and the cultures were harvested after 21–23 hr.

The cultures were fractionated by methods previously described (Penman, 1969). This procedure yields a cytoplasmic fraction and nuclear rinse which were pooled and made to 1% (w/w) in SDS and 0.05 M in EDTA (pH 7.2). This mixture, designated *cytoplasm-SDS,* was then allowed to stand at least 1 hr at room temperature prior to RNA extraction.

Preparation of 30S rRNA. a. SUCROSE GRADIENT FRACTIONATION. The cytoplasm-SDS preparation from 2 l. of HeLa cells at 5×10^5 cells/ml was routinely layered onto six 35-ml linear, 15–30% (w/w) sucrose (Mann Ultra Pure) gradients made in 0.1 M NaCl, 0.01 M EDTA, 0.01 M Tris-Cl, and 0.2% (w/w) SDS (pH 7.4). (The latter buffer is hereafter called NETS buffer.) The sedimentation was performed using an SW 27 rotor in the Beckman L2-65B ultracentrifuge at 24,000 rpm for 16 hr at 18°. The absorbance at 260 nm of each gradient was continuously measured as the gradient was pumped, bottom first, through a flow-through cell mounted in a spectrophotometer. The 30S rRNA in appropriate fractions was precipitated with 2.5 volumes of 95% ethanol at −20°.

b. PHENOL EXTRACTION. Ethanol-precipitated RNA pellets were dissolved in 0.05 M NaOAc, 0.005 M EDTA, and 0.8% (w/w) SDS (pH 5.1), or cytoplasm-SDS preparations were diluted 1:2 with the same pH 5.1 solution to yield final RNA concentrations not greater than 0.5 mg/ml. An equal volume of colorless phenol (Mallinckrodt-liquefied-88% for chromatography) was added, before agitation with a Vortex mixer for 5 min at room temperature. The emulsion was chilled in an ice bath and the phases were separated by centrifugation. The bottom, phenol phase was removed and discarded without disturbing the interface. Another $\frac{7}{8}$ volume of phenol was added and the mixture agitated 3 min as before. After phase separation the top aqueous phase was removed from tube A and added to another tube, B, containing $\frac{5}{8}$ volume of fresh phenol; 0.5 volume of fresh pH 5.1 solution was added to A and then tubes A and B were agitated for 3 min as before followed by phase separation. The aqueous phase in tube A was added to tube B and tube A was discarded. Tube B was agitated 3 min and the phases were separated as before. The aqueous phase from tube B was removed and, after combining it with the previously saved aqueous phase, NaCl was added to a final concentration of 0.1 M and 2.5 volumes of 95% ethanol was added to precipitate the RNA.

c. CTABr PRECIPITATION. RNA pellets obtained from EtOH precipitation were dissolved in 0.025 M Tris-HCl–0.025 M NaCl (pH 8.1) at 2° at an RNA concentration of 1 mg/ml. An adaptation of the procedure of Bellamy and Ralph was carried out as follows (Bellamy and Ralph, 1968). One volume each of 2.5 M K_2HPO_4 (pH 8.0) and 2-methoxyethanol were added to the RNA solution and mixed vigorously for 2–3 min at 2°. The phases were separated by centrifugation at 4000 rpm for 15 min at 4° in an I.E.C. PR-6 centrifuge. The upper aqueous phase was removed and added to 1 volume of 0.2 M NaOAc, and 0.5 volume of 1% (w/v) CTABr at 2°. The temperature had to be kept at 2° or above to keep the CTABr in solution. This solution was mixed well, chilled at 2° for 5 min, and centrifuged 5 min at 9800 rpm at 2° in the Sorvall SS-34 rotor. The RNA pellet was washed by resuspension in 70% (v/v) ethanol containing 0.1 M NaOAc at −20° and centrifuged in the SS-34 rotor as before. This washing procedure was repeated four times. After the last wash, the RNA pellet was dissolved in NETS buffer and reprecipitated with 2.5 volumes of 95% EtOH, for the removal of all the residual CTABr. After 1 hr at −20°, the RNA was collected by centrifugation at 12,000 rpm for 1 hr in the Sorvall SS-34 rotor at 2°. The RNA pellet was dissolved in 0.025 M Tris-Cl–0.25 M NaCl (pH 8.1) at 2° and the above precipitation with CTABr was carried out again, including the five washes and precipitation with 2.5 volumes of 95% EtOH.

d. SEPHADEX G-100 CHROMATOGRAPHY. To control ionic strength, rRNA solutions were routinely filtered through small columns of G-100 Sephadex preequilibrated with solvent of the desired ionic strength and composition. Measurements of sedimentation velocity, viscosity, thermal stability, etc., were always performed the same day as column elution.

Sephadex G-100 beads were routinely swollen by boiling for 5 hr in glass-distilled water to eliminate any contaminating ribonuclease activity. Columns, 15–16 × 0.6 cm, were constructed just prior to use, from a slurry of Sephadex beads which had been washed four times with the buffer desired for the succeeding experiment. Each column was preequlibrated with at least 7 volumes of buffer. Samples having RNA concentrations of up to 3.5 mg/ml and volumes of 0.5–1.0 ml were layered on the columns with two 0.5-ml buffer rinses. Elution was performed at 7–8 ml/hr and 0.5-ml fractions were collected. About 20% dilution of the RNA accompanied this step. The rRNA solutions eluted from Sephadex columns were then usually filtered through 0.22 or 0.45-μ Millipore filters. Each filter was suspended in buffer and heated at 100° for 5 min prior to use. The concentrations of the filtrates were assigned on the basis of the absorbance values at 260 nm of duplicate diluted aliquots. The extinction coefficient was taken to be 23 ml/(mg cm) (Kurland, 1960).

Preparation of 29S rRNA. Sufficient CTABr precipitated 30S rRNA to make a solution of final concentration less than 4 mg/ml was dissolved in 0.025 M acetate buffer (pH 5.1) containing 0.05 M EDTA and 0.5% (w/w) SDS, $\Gamma/2 = 0.051$. This buffer is hereafter referred to as *heating* buffer. The complete dissociation of the 30S rRNA complex to yield a mixture of 29S rRNA and 7S rRNA, designated 29S + 7S rRNA, was achieved by heating this solution for 5 min at 50–51°, followed immediately by chilling at 0°.

The 29S rRNA was then separated from 7S rRNA either by sucrose gradient centrifugation or by chromatography on Sepharose 4B columns. Sucrose gradient separation was performed as indicated above for the isolation of the parent complex; 29S rRNA containing fractions were pooled and stored under ethanol. Just before use the RNA would be dissolved and filtered through Sephadex G-100 as earlier described. Alternatively, Sepharose 4B chromatography of 29S + 7S rRNA was performed on 0.6 × 25 cm columns, before measurement of one of the 29S rRNA physical parameters; the Sepharose 4B being prewashed in the same manner as swollen Sephadex G-100. Elution with L-NETS

[1] Abbreviations used are: CTABr, cetyltrimethylammonium bromide; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; NETS buffer, 0.1 M NaCl, 0.01 M EDTA, 0.01 M Tris-Cl, and 0.2% (w/w) SDS (pH 7.4).

buffer, a buffer having one-tenth the EDTA concentration of NETS buffer, proceeded at 3–4 ml/hr, accompanied by a sample dilution of about 60%. The fractions containing 29S rRNA were filtered through Millipore membranes and the concentration was determined as indicated earlier for the parent complex.

Gel Electrophoresis. Gels, 0.6 × 5.2 cm, containing 10% (w/v) acrylamide and 0.27% (w/v) *N,N*-methylenebisacrylamide in 0.1 M sodium phosphate buffer (pH 7.0), 0.0001 M NaN_3, 0.02 M EDTA (pH 7.0), and 0.1% (w/w) SDS were prepared by standard methods (Maizel, 1969). The gels were preelectrophoresed at 7.7 V/cm for 2 hr. The samples containing 10% sucrose and Bromophenol Blue dye were than layered on each gel, and electrophoresis was carried out for 2.75 hr at 7.7 V/cm while the electrophoresis buffer, 0.1 M sodium phosphate (pH 7.0), 0.02 M EDTA, and 0.1% (w/w) SDS, was circulated between cathode and anode vessels. The top (cathodic) centimeter was discarded from each gel before crushing, anode end first, in a Maizel-type autogeldivider (Savant Co.) and 1-ml fractions were collected (Maizel, 1966); 10 ml of Triton X-100 scintillator fluid (4 parts of toluene scintillator:1 part of Triton X-100) were added to each fraction and counted in a Packard scintillation spectrometer.

Kinetics of Thermal Denaturation. Heating of 30S rRNA for precisely determined time periods at defined temperatures was accomplished by the use of glass melting point capillary tubes. The capillaries were loaded with 40 μl of sample using a microsyringe and placed in a water bath of desired temperature to initiate heating. Thermal equilibrium with the bath was reached in a few seconds as determined by measurement with a thermocouple placed in similar capillaries. Heating was terminated by plunging the capillary into ice–water for 1 min. Aliquots were then removed rapidly for characterization by gel electrophoresis.

Sedimentation Velocity Measurements. Moving boundary sedimentation velocity measurements were performed employing a modified double-sector centerpiece. The aluminum filled Epon centerpiece had two fine V-shaped grooves in the outer sealing surface of the centerpiece; one joining the tops of the two sectors and one joining the bottoms of the two sectors, allowing the simultaneous sedimentation of two samples from the same meniscus position. Routinely 0.40 ml of rRNA solution of one concentration was loaded into one sector and 0.39 ml of rRNA solution of a lower concentration, or buffer alone, was loaded into the other sector. As the rotor accelerated, the transfer of liquid from the former sector to the latter equalized the positions of the menisci in the two sectors. To obtain reproducible s values, it was necessary to soak the centerpiece and quartz windows for 30 min at room temperature in a solution of 1% (w/w) SDS and 0.05 M EDTA (pH 7.4). The velocity sedimentation centrifugations were carried out at 26,000 rpm in an AnD rotor at 20° with the Spinco Model E analytical ultracentrifuge equipped with an electronic speed control. Schlieren records were measured with a Nikon profile projector, Model 6C.

Sedimentation Equilibrium Measurements. Sedimentation equilibrium analyses were performed utilizing the meniscus depletion technique (Yphantis, 1964). The centerpiece and sapphire windows were cleaned and assembled as above. No fluorocarbon "cushion" was used in these experiments. All experiments were performed at 3600 rpm at 20°. Interference fringe patterns were recorded periodically until equilibrium had been reached as judged by the constancy of the interference patterns. The centrifugations extended from 39 hr to as long as 87 hr. The plates were measured with the Nikon projector.

Viscosity measurements were made at 20.00 ± 0.05° in a modified Cannon-Ubbelohde Semi-Micro viscometer which had a flow time for glass distilled water of 295 sec. The viscometer was modified by the addition of a 5-cm long glass tube, 0.55 cm in diameter, to the upper bulb at an angle of about 45°. This modification permitted the removal of bubbles from the meniscus of the SDS containing sample prior to each flow time determination (Ray, 1972).

Temperature Dependency of rRNA Absorbance and Circular Dichroism. A Gilford Model 2000 recording spectrophotometer was used to simultaneously record the temperature and the absorbances of the rRNA solutions as the temperature was raised (or lowered) at a rate of 0.5°/min with a Lauda K-2/R circulating heater. Circular dichroism (CD) spectra of rRNA samples were measured as a function of temperature in a Cary Model 60 spectropolarimeter equipped with a Cary Model 6002 circular dichroism accessory. The CD spectra of samples in a 1-cm path-length cell were recorded from 320 to 220 nm in units of degrees ellipticity, θ. Spectra were scanned at a rate of 2–3 Å/sec, with a constant spectral bandwidth of 15 Å and an instrument time constant of 1 sec.

Results

rRNA Purification. There is no convenient biological assay available for the 30S rRNA molecule. Therefore, the criteria of purity were the magnitudes of the numerical values of the characteristic hydrodynamic parameters, sedimentation coefficient and intrinsic viscosity, and the reproducibility of the values of these parameters from preparation to preparation. The purification scheme consisted of obtaining a cytoplasm–SDS fraction, a sucrose gradient fractionation, phenol extraction, another sucrose gradient fractionation, two cetyltrimethylammonium bromide precipitations, and Sephadex G-100 column chromatography.

Certain features of the purification scheme were particularly important with respect to meeting the purification criteria just mentioned. For example, the insertion of a sucrose gradient fractionation of the cytoplasm–SDS fraction prior to phenol extraction served to remove large quantities of protein, thereby avoiding the generation of thick emulsions at the water–phenol interfaces during the phenol extraction. That the rRNA obtained by the subsequent phenol extraction was essentially free of protein was demonstrated by carrying out the extraction on HeLa cells grown on [^{14}C]leucine. From the results of such an experiment it was concluded that the upper limit on the contamination of the purified RNA by protein was equivalent to 6700 daltons per 30S rRNA complex (Ray, 1972).

From a third sucrose gradient fractionation, not routinely included in the purification scheme, it was evident that the second (post-phenol stage) sucrose gradient fractionation served to remove additional low molecular weight RNA and reduced 18S rRNA to about 0.2% of the total RNA mass.

The utilization of CTABr precipitation was also an essential step in the preparation of 30S rRNA judged pure by the criteria stipulated above. Different batches of 30S rRNA obtained after the two sucrose gradient fractionations and the phenol extraction were found to contain a glycogen-like, high molecular weight material which appeared in the Schlieren patterns of the analytical ultracentrifuge with a hypersharp boundary, see Figure 1. This substance had a

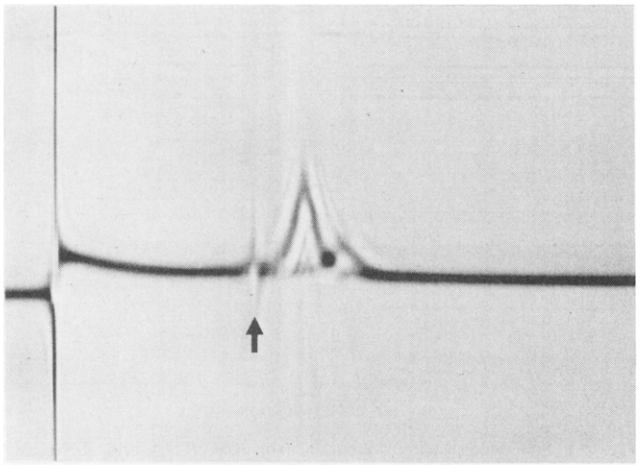

FIGURE 1: Sedimentation of non-CTABr precipitated 29S rRNA. The two 29S rRNA samples, shown here, were prepared from a HeLa cytoplasm–SDS fraction, centrifuged on a sucrose gradient, phenol extracted, refractionated on a sucrose gradient, dissociated from 7S rRNA by heating 5 min at 50° in *heating* buffer, separated from 7S rRNA by fractionation on a third sucrose gradient and preequilibrated with L-NETS by elution through a G-100 Sephadex column. This picture was taken with the analyzer angle set at 65°, 110 min after the rotor had reached a speed of 26,000 rpm at 20°. One sector of the modified double sector centerpiece contained 29S rRNA at a concentration of 1.800 mg/ml while in the other the concentration was 0.817 mg/ml. The small hypersharp peak (see arrow) trailing the two 29S rRNA peaks which appeared in all patterns obtained with non-CTABr treated rRNA, is due to what is thought to be glycogen-like material.

Table I: Recoveries during rRNA Purification.

Purification Step	Yield of 30 S rRNA (mg)	% (w/w) Recovery
Harvest of 250×10^6 cells, sucrose gradients	3.0	63[a]
Standard phenol extraction, sucrose gradients	1.8	60
Two CTABr precipitations	1.4	75
Total recovery	1.4	28

[a] Based on 36×10^{-12} g total of RNA/cell (unpublished results from this laboratory) and 30 S rRNA being 53% of total cell RNA (Soeiro *et al.*, 1968). It has been reported that HeLa cells grown in suspension cultures contain from 20 to 36×10^{-12} g total of RNA/cell (Salzman, 1959).

sedimentation coefficient of about 16S. This contaminant was removed after precipitating the rRNA two times with CTABr as described in Materials and Methods. This material did not significantly affect the reproducibility of the *s* values of the rRNA, but its removal did dramatically improve the reproducibility of the intrinsic viscosity measurement of the 30S rRNA from batch to batch. Before the introduction of CTABr precipitation, intrinsic viscosities from batch to batch ranged from 80 to 164 ml/g.

The recoveries at the various stages of the rRNA purification appear in Table I and an elution profile demonstrating the resolution of the 29S + 7S rRNA mixture subjected to Sepharose 4B chromatography is illustrated in Figure 2.

Thermal Dissociation of 30S rRNA. Upon heating for 5 min at 50° at pH 5.1 in 0.025 M acetate buffer containing 0.005 M EDTA and 0.5% (w/w) SDS, HeLa cell 30S rRNA, labeled *in vivo* with ^{32}P, released 2.3 ± 0.1% of its

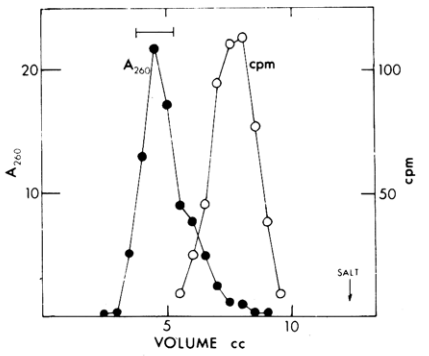

FIGURE 2: Sepharose 4B column chromatography of rRNA. The 0.9-ml sample, in *heating* buffer, contained: 1000 cpm of ^{14}C-labeled 7S rRNA prepared from ^{14}C-labeled 30S rRNA by elution from DEAE-cellulose column in 7 M urea (Ray, 1972), 59.8 A_{260} units of 30S RNA which had been heated 5 min at 50° in *heating* buffer, prior to the addition of 0.03 g of Na_2HPO_4. The column was eluted with L-NETS buffer at 4 ml/hr at room temperature. The radioactivity was determined on aliquots of each fraction, counted in 10 ml of Triton X-100 scintillator. The phosphate was precipitated with $BaCl_2$ to visually locate the salt peak fraction (see arrow).

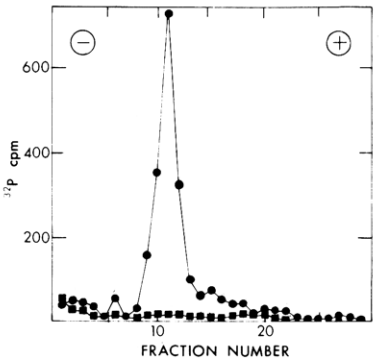

FIGURE 3: Polyacrylamide gel electrophoresis (10%) of heated and unheated 30S rRNA. ^{32}P-labeled 30S rRNA of initial specific activity of 2.3×10^6 cpm/mg was prepared from the cytoplasm–SDS fraction of HeLa cells with two sucrose gradient fractionations, phenol extraction, and two CTABr precipitations. The rRNA was dissolved in *heating* buffer and one portion (50 μg) was heated 5 min at 50° and rapidly chilled in ice. Equal portions of heated (●) and unheated (■) rRNA were subjected to electrophoresis on separate gels and the radioactivity was determined as described in Methods. The high molecular weight component does not migrate into the 10% gel.

radioactivity as 7S rRNA, average of four determinations. Figure 3 portrays the radioactivity pattern of the 10% acrylamide gel electrophoresis separation employed to assess the progress of the dissociation reaction and final yield of 7S rRNA. Over a range of initial 30S rRNA concentrations of 0.1–1.0 mg/ml, there were no significant changes in the per cent of radioactivity appearing as 7S rRNA under these heating conditions.

Assuming that dissociation of the complex is a first-order reaction, the appearance of the 7S rRNA product, P, can be represented by the expression, $P = P_\infty(1 - e^{-kt})$, where P_∞ is the amount of 7S rRNA after the reaction has gone to completion, *i.e.*, at $t \rightarrow \infty$. Since 7S rRNA is 2.3% of the 30S rRNA, the rate constant for the reaction, carried out under a variety of conditions, could be evaluated from plots of log {1 − 43.5[7S (cpm)/30S (cpm)]} *vs.* time. The ratio term in the argument of the logarithm is the quotient of the radioactivity recovered from the electrophoresis gels at the position corresponding to 7S rRNA and the total radioactivity applied to the gel after heating for time, t. Kinetic studies were carried out over the temperature span from

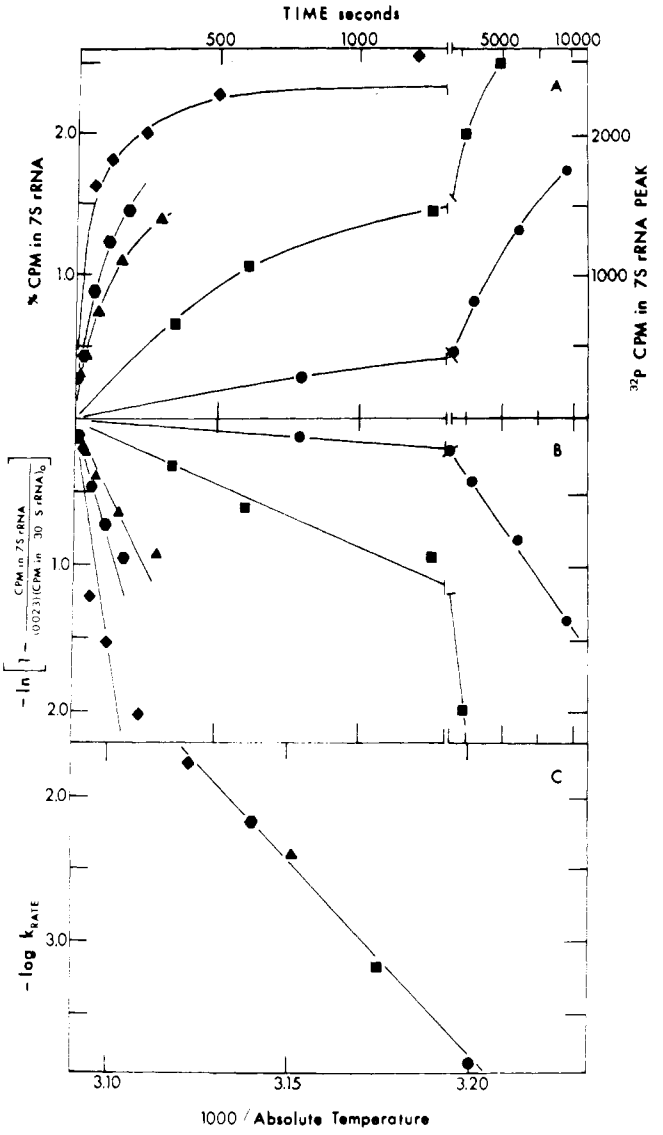

FIGURE 4: The kinetics of 30S rRNA dissociation. ^{32}P-labeled 30S rRNA was prepared from the cytoplasm-SDS fraction of HeLa cells as described in the legend to Figure 3. The ionic strength of the 30S rRNA solutions used for these kinetic experiments was carefully controlled by elution with *heating* buffer through G-100 Sephadex columns. Portions of this 30S rRNA were heated and processed as described in Methods. (●) 39.2°; (■) 42.0°; (▲) 44.4°; (◆) 45.5°; (♦) 47.2°. Direct reaction progress curves, A; linearized progress curves, B; and Arrhenius plot, C. The line in frame C represents the results of a least-squares analysis of the data.

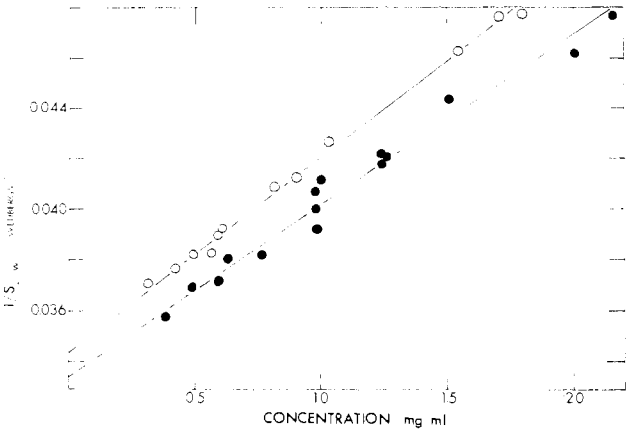

FIGURE 5: Concentration dependence of 30S rRNA and 29S rRNA sedimentation coefficients. (●) 30S rRNA experiments; (○) 29S rRNA experiments. The points include data both from CTABr and non-CTABr precipitated preparations. Sedimentations were performed with L-NETS buffer as solvent ($\Gamma/2 = 0.122$), the samples being preequilibrated by elution with the buffer through G-100 Sephadex columns.

39.2 to 47.2° at a nominal ionic strength of 0.051. The results of these experiments are summarized in Figure 4 in the form of direct progress curves, linearized progress curves in logarithmic form, and an Arrhenius plot. The energy of activation obtained from the slope of the least-squares line of the Arrhenius plot is 124 ± 5 kcal/mol. The absolute reaction rate activation enthalpy was 123.5 kcal/mol and the corresponding free energy, at 40°, and entropy were 22 kcal/mol and 0.32 kcal/(mol deg), respectively.

Several investigators have reported that the ionic strength dependence of thermal melting of the double-stranded helical regions of polynucleotides, at ionic strengths of 0.2 or less, obeys the relationship, $T_m = -k \log (\Gamma/2) + c$, where T_m is the temperature at which one-half of the total double-stranded structure of the sample has been melted but is in equilibrium with the remaining native form, and c is a constant (Schildkraut and Lifson, 1965; Thierr *et al.*, 1971). We undertook studies of the dissociation reaction at various ionic strengths to determine whether this relationship applies to the dissociation of 30S rRNA. The T_m parameter, as it is usually construed, implies the existence of an equilibrium condition. Since this dissociation of the 30S rRNA complex did not appear to be reversible over the wide range of ionic strengths, temperatures, and rRNA concentrations we studied, it is misleading to characterize this thermal dissociation reaction in terms of T_m values. In keeping with the fact that in the case of 30S rRNA we are considering kinetic data, rather than equilibrium data, we constructed a plot, not shown, of the temperature at which the reaction time was 5 min *vs.* $-\log (\Gamma/2)$. The plot was linear over the range of ionic strength examined, 0.022–0.122, and could be described by the relation: $T_{1/2.5\,min} = 70° + 21° \log (\Gamma/2)$. Hence, the dissociation reaction behaves analogously to the thermal melting of helical polynucleotides.

Sedimentation Velocity. The concentration dependency of the sedimentation coefficient of 30S rRNA and 26S rRNA is shown in Figure 5. The results used to construct this figure come from 11 separate centrifugations for each RNA species, using material isolated from six separate batches of HeLa cells. Each $s_{20,w}$ value plotted in Figure 5 was corrected for concentration-dependent sedimentation resulting from radial dilution occurring during the centrifugation. The concentration dependency constant, k, necessary for this correction, was evaluated for the 30S rRNA, and separately for the 29S rRNA, by an iterative process (Ray, 1972). The least-squares $s_{20,w}{}^0$ and k values for 30S rRNA derived from the data of Figure 5 are 29.9 ± 0.4 S and 0.201 ± 0.014 ml/mg, respectively. The corresponding values for so-called 29S rRNA are 29.1 ± 0.14 S and 0.224 ± 0.006 ml/mg. These values apply to the relationship, $1/s_{20,w} = 1/s_{20,w}{}^0(1 + kc_0)$, where c_0 is the initial RNA concentration in mg/ml.

Viscosity Parameters. The results from viscosity experiments with 30S rRNA and 29S rRNA are shown in Figure 6. The 30S rRNA data were obtained in five separate experiments with RNA from three different batches of HeLa cells. The 29S rRNA data are from seven experiments with RNA from four batches of HeLa cells. Extrapolation of the

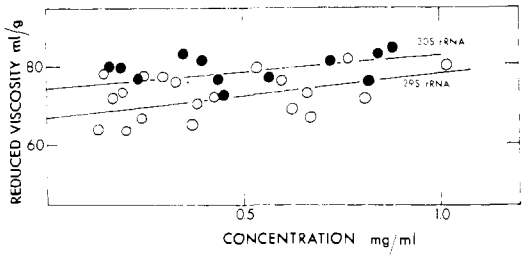

FIGURE 6: The concentration dependence of reduced viscosity of 30S rRNA and 29S rRNA. The rRNA was preequilibrated with L-NETS buffer as in Figure 4. Viscosities were performed with L-NETS buffer as solvent ($\Gamma/2 = 0.122$). (●) 30S rRNA experiments; (○) 29S rRNA experiments. The two CTABr precipitation steps were included in the purification protocol for the RNA used in all experiments.

weighted least-squares line through each set of data points yields an intrinsic viscosity of 74.4 ± 5.4 ml/g for 30S rRNA and of 67.0 ± 5.0 ml/g for 29S rRNA. Data points were weighted inversely with the experimental variance in the corresponding reduced viscosities. The Huggins' constants for 30S rRNA and 29S rRNA are 1.4 ± 1.7 and 2.4 ± 2.2, respectively.

Equilibrium Centrifugation Molecular Weights. The results from six sedimentation equilibrium molecular weight determinations with 30S rRNA at four ionic strengths are presented in Figure 7. Over the ionic strength span of 0.12–0.37 the molecular weights ranged from 1.45×10^6 to 2.08×10^6. Within this span, there was no evidence of variation of apparent molecular weight (determined at increasing r values) with concentration. Only at the highest ionic strength studied, 0.5, did the apparent molecular weight increase with increasing r values, see Figure 7. The apparent molecular weight values *vs.* ionic strength are plotted in the inset of Figure 7.

Effect of Temperature on Spectral Properties. The variation of the absorbance at 260 nm with temperature is shown in Figure 8 for 30S rRNA and for a preheated sample, *i.e.*, an equimolar mixture of 29S rRNA and 7S rRNA. The virtual identity of the two profiles shows that no change in the thermally induced hyperchromism accompanies the release of 7S RNA, implying that no significant fraction of secondary structure is lost irreversibly as the complex dissociates. The cooling curves indicate only slight hysteresis under these conditions. Ultraviolet difference spectra, obtained by pairing 30S rRNA with a preheated portion of the same, also showed no significant differences (Ray, 1972).

The circular dichroism spectra of 30S rRNA and the 29S plus 7S rRNA mixture in heating buffer recorded at various temperatures are shown in Figure 9. As can be seen, there were no significant differences between the CD spectra of the 30S rRNA and the companion mixture of 29S and 7S rRNA at 20°, regardless of whether the mixture was prepared by rapid heating and cooling or by gradual heating and cooling. The CD spectral changes observed at higher temperatures, *e.g.*, 50°, were very similar to reported CD spectral changes associated with decreases in base pairing and base stacking of RNA (Gratzer and Richards, 1971).

RNA Integrity during Physical Studies. The integrity of the rRNA molecules at the end of each sedimentation velocity, viscosity, and sedimentation equilibrium experiment was checked by analysis of a portion taken from the Model E cell or viscometer and layered onto 15–30% (w/w) sucrose gradients in NETS buffer. It was estimated that less

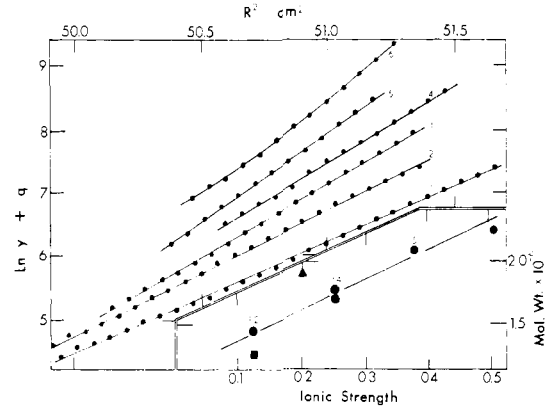

FIGURE 7: Sedimentation equilibrium of 30S. The rRNA was preequilibrated with the appropriate buffer as described in Methods. Centrifugations were performed at 3600 rpm at 20°. The two CTABr precipitation steps were included in the purification protocol for all samples except in experiment 1. To display all data in one figure, the ordinate values shown are equal to $\ln y + q$, where y is the fringe displacement in microns and q was given a different value for each data set. (1) $\Gamma/2 = 0.122$, $q = 0$; (2) $\Gamma/2 = 0.122$, $q = -0.0334$; (3) $\Gamma/2 = 0.252$, $q = 0.4905$; (4) $\Gamma/2 = 0.252$, $q = 1.7494$; (5) $\Gamma/2 = 0.372$, $q = 1.5272$; (6) $\Gamma/2 = 0.500$, $q = 2.7445$. All solutions were 0.01 M Tris-HCl, 0.001 M EDTA, and 0.2% (w/w) SDS (pH 7.4) with enough NaCl added to produce the ionic strength indicated. Inset: Apparent molecular weight of 30S rRNA as a function of ionic strength. (●) 30S rRNA prepared with CTABr precipitation; (■) 30S rRNA prepared without CTABr precipitation; (▲) value obtained for HeLa 30S rRNA in 0.14 M NaCl–0.01 M EDTA (pH 7.0) at 5° (McConkey and Hopkins, 1969).

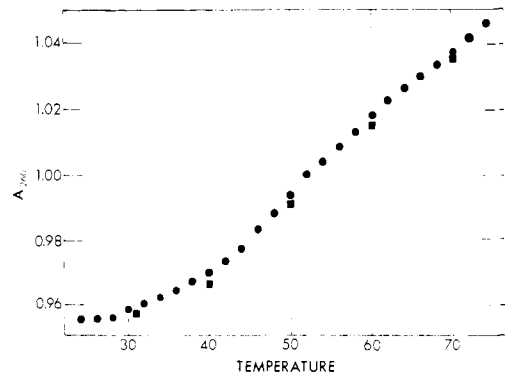

FIGURE 8: Absorbance–temperature profiles of 30S rRNA and 29S + 7S rRNA; 30S rRNA was prepared from the cytoplasm–SDS fraction of HeLa cells with two sucrose gradient fractionations, and phenol extraction. The rRNA was dissolved in *heating* buffer ($\Gamma/2 = 0.051$) and part was heated 5 min at 50° and then rapidly cooled in ice (designated 29S + 7S rRNA). The 30S rRNA and 29S + 7S rRNA solutions were slowly heated and cooled simultaneously in adjacent cuvets and their absorbances at 260 nm recorded as described in Methods. A circle in the figure contains, within its boundaries, absorbance values for both native and dissociated rRNA at that temperature as the temperature of the samples was raised. A square in the figure contains within its boundaries, both absorbance values which were recorded at that temperature as the temperature of the samples was lowered.

than 10% degradation occurred, where degradation was defined as the fractional area of the gradient–absorbance profile representing material sedimenting slower than the 30S rRNA or 29S rRNA peaks.

Discussion

The standard protocol developed for the purification of HeLa 30S rRNA used for the studies reported here includes: the preparation of a cytoplasm–SDS fraction, a sucrose gradient fractionation, phenol extraction, a second su-

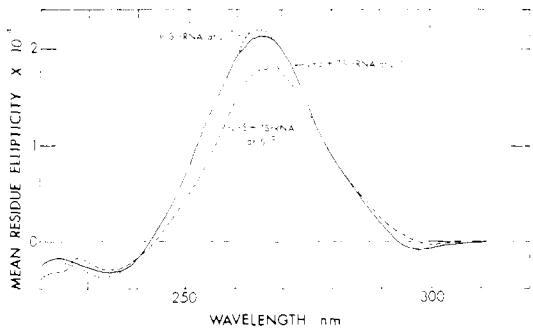

FIGURE 9: Circular dichroism spectra; 30S rRNA was prepared from the cytoplasm-SDS fraction of HeLa cells with two sucrose gradient fractionations, phenol extraction, two CTABr precipitations, preequilibration in buffer by elution through a Sephadex column, and filtration through a Millipore filter as described in Methods. The CD spectrum of one portion was measured at 20° (···). The CD spectrum of this same portion was then measured at 50° (- - -) and, after slowly cooling, it was again measured at 20° (—). The CD spectrum of another portion which had been heated for 5 min at 50° and rapidly cooled was also measured at 20°, essentially the same as that for the slowly cooled material, given by the solid line.

crose gradient fractionation, two cetyltrimethylammonium bromide precipitations, and elution through Sephadex G-100 columns, see Table I. Two of these steps were included after they proved to be essential for obtaining consistent values of kinetic and/or hydrodynamic parameters, namely, the removal of glycogen-like molecules by CTABr precipitation of the rRNA, and the preequilibration of rRNA with the solvent of desired ionic strength and composition by Sephadex column chromatography.

The aqueous phase of phenol extracts of HeLa cell monolayer cultures has been shown to contain significant amounts of polyglucose, 14.6 pg/cell (Graves, 1970). The molecular weight of this HeLa cell polyglucose was estimated to range from 1.4 to 4.6 × 10^6 (Graves, 1970; Sokol *et al.*, 1966) and it was characterized by $s_{20,w}$ values of 31-116 S (Graves, 1967). Without the CTABr precipitation steps, a hypersharp component always appeared in the Schlieren optical patterns of sedimentation velocity measurements of our rRNA preparations, and the 30S rRNA intrinsic viscosity values varied drastically from batch to batch of HeLa cells.

It is well known that the physical properties of a polyelectrolyte such as rRNA are very dependent on the molecule's ionic environment. We observed that the sedimentation coefficients as well as the intrinsic viscosity of the rRNA varied greatly from experiment to experiment unless the rRNA was first equilibrated with an arbitrarily chosen reference solvent by gel filtration.

We shall begin our discussion of the properties and structural aspects of the 30S rRNA molecule by considering the kinetic information concerning the 7S rRNA release reaction. The reaction appears to be first order and irreversible under all the conditions explored to date, see Figure 4. The irreversibility of the reaction is also supported by the fact that reannealing attempts under a limited number of conditions have produced less than 1% yield of a 30S-like complex (Ray, 1972). Reannealing was monitored by measuring the radioactivity carried into the 29–30S region of sucrose gradients after 5.5-hr incubations at 39° of 29S rRNA with radiolabeled 7S rRNA at ionic strengths extending from 0.1 to 1.0. Sy and McCarty have shown that "5.8S" rRNA and 29S rRNA from sea urchin can be reannealed to some extent although with a low degree of specificity, since the reannealed complex had certain physical properties which were significantly different than those of the native sea urchin complex (Sy and McCarty, 1970). King and Gould have obtained similar results with "18S rRNA" and 29S rRNA of rabbit reticulocytes (King and Gould, 1970).

There is no *a priori* reason to suspect that the thermally induced disruption of the 30S rRNA complex should be readily reversible. In fact, in view of the complicated rRNA maturation processes known to occur *in vivo*, it is reasonable to suggest that any other route of complex formation may be unlikely. Specific nucleolar proteins and maturation modifications which are involved in the proper maturation sequence *in vivo* may be absolutely essential to produce the native 30S rRNA complex (Maden, 1971). The formation of active insulin from pro-insulin represents a biological precedent for these types of requirements (Steiner and Clark, 1968). The fact that an exhaustive search has not been made for the appropriate reannealing conditions, of course, must temper any statements made at this point about irreversibility.

From the activation enthalpy of 123.5 kcal/mol for 7S rRNA release, it can be inferred that the activation process involves the disruption of 14 to 16 base pairs per molecule of reactant (Ross, 1971; Krakauer and Sturtevant, 1969). Assuming that activation is essentially a process of melting 15 base pairs, the attendant activation entropy becomes 21 G/base pair, a not unreasonable value. Activation may, and probably does, involve more than base pair disruption. We only wish to emphasize that the activation parameters have respectable magnitudes, consistent with orthodox notions of molecular events such as the melting of one moderately long region or a few short regions of secondary structure.

Moreover, the 7S rRNA release has properties of cooperativity and ionic strength dependence which are typical of the melting out of double-stranded helical regions in polynucleotides. The very small temperature increment which must be traversed in order to increase the amount of 7S rRNA released in 5 min from 0 to 100%, see Figure 4A, is characteristic of a cooperative transition. The temperature at which the reaction half-time, $t_{1/2}$, was equal to 5 min for HeLa 7S rRNA release was found to be proportional to the logarithm of ionic strength. This property is characteristic of strand separation of other double helical polynucleotides (Schildkraut and Lifson, 1965; Thierr *et al.*, 1971; Gruenwedel *et al.*, 1971). Similar conclusions have been made by others with sea urchin 29S rRNA (Sy and McCarty, 1970) and rabbit reticulocyte 30S rRNA (King and Gould, 1970), although they did not report their data in the same form as that used here, *i.e.*, $T_{1/2,\,5\text{ min}}$ *vs.* $-\log(\Gamma/2)$.

We shall now direct our discussion to the results of the velocity sedimentation experiments by first placing them in historical perspective. As mentioned in the introductory comments, the rRNA species from HeLa cells which we have designated 30S rRNA has customarily been called 28S rRNA. Sherrer and Darnell originally approximated this s value on the basis of the relative sedimentation behaviors in sucrose gradients of the HeLa rRNAs and the *Escherichia coli* rRNAs (Sherrer and Darnell, 1962). The s values of these procaryotic rRNAs had been established by boundary sedimentation in the analytical ultracentrifuge at an ionic strength of 0.1 (Kurland, 1960). Apropos to this assignment are the facts that the ionic strength of the Sherrer–Darnell sucrose gradients was apparently only 0.057,

and that the RNA they designated 28S rRNA was isolated by a procedure employing hot phenol, a condition shown later to dissociate the 30S rRNA complex (Pene *et al.*, 1968). Our value of 29.1 ± 0.1S for the sedimentation constant of the *larger* fragment derived from the 30S complex, determined by boundary sedimentation techniques at an ionic strength of 0.12, is thus in reasonable agreement with the estimate of 28S by Scherrer and Darnell.

King and Gould obtained values for rabbit reticulocyte rRNA in 0.1 M KCl–0.01 M Tris (pH 7.6) ($\Gamma/2 = 0.11$) of 29.8S and 29.2S for species corresponding to HeLa cell 30S and 29S rRNA, respectively (King and Gould, 1970). Since the $s_{20,w}^0$ values for rabbit reticulocyte and HeLa rRNA under similar conditions are essentially the same, and since it has been shown that HeLa, rat liver, mouse liver, and rabbit reticulocyte 28S rRNA are inseparable by gel electrophoresis (Loening, 1968), perhaps all larger rRNA components from mammalian cells should be referred to as 29S rRNA and 30S rRNA for the heated and native form, respectively, and indeed we have followed this practice in this report. We also want to emphasize the obvious importance of using properly defined sedimentation coefficients of marker rRNA species when estimating relative sedimentation coefficients and molecular weights of other types of RNA.

Just as there has been ambiguity about the sedimentation coefficient of the rRNA complex in question, there has also been uncertainty about the molecular weight of this species or, depending on the point of view, the nature of the solvated molecular entity to which any given apparent molecular weight should apply. Assuming the integrity of the RNA is maintained during the period of measurement and the absence of any unknown systematic errors, the preferential interaction of any solvent component can be considered the source of apparent molecular weight variability with solvent composition. According to Casassa and Eisenberg, the apparent molecular weight becomes equal to the molecular weight of the species to which the macromolecular solute concentration refers, if (1) the equilibrium centrifugation is performed on an exhaustively dialyzed sample for which the paired reference solvent is the dialysate, and (2) the requisite specific volume is determined from density measurements on the same dialyzed solution and dialysate (Casassa and Eisenberg, 1964).

The 30S rRNA samples used in the present study had, in effect, been exhaustively dialyzed against buffer by Sephadex G-100 column chromatography. To meet the other Casassa–Eisenberg criterion, the appropriate apparent specific volume should have been determined at each ionic strength. Since such measurements were unavailable, it was decided to use the apparent specific volume value of 0.53 ml/g throughout (Petermann and Pavlovec, 1966). This value was obtained with dialyzed non-CTABr precipitated Jensen sarcoma rRNA in 0.1 M NaCl–0.01 M NaOAc (pH 4.6). The observation that the apparent molecular weights increased with increasing ionic strength, Figure 7, is consistent with the view that preferential interaction effects are primarily the result of the polyelectrolyte nature of RNA. If we assume that at the higher ionic strengths, *e.g.*, 0.377–0.5, the equation

$$M_{\text{apparent}} = M - \tfrac{1}{2}ZM_3(1 - \bar{\nu}_3\rho)/(1 - \bar{\nu}_2\rho)$$

becomes applicable to this system (Tanford, 1967) and if we accept the not unreasonable assumption that the charge per nucleotide unit at ionic strengths ranging from 0.05 to 0.4 is between −0.3 and −0.5 (Felsenfeld and Miles, 1967; Ross and Scruggs, 1964; Olivera *et al.*, 1964), then the apparent molecular weight at $\Gamma/2 = 0.377$ is probably within 6–8% of that of the unsolvated sodium ribonucleate. On the basis of this line of reasoning we conclude that the molecular weight of HeLa 30S rRNA is 2.1×10^6. This is in essential agreement with the earlier reported molecular weight value of 1.9×10^6 determined by equilibrium centrifugation (McConkey and Hopkins, 1969). Unfortunately, the data in the inset of Figure 7 do not allow one to draw a definitive conclusion about the limiting molecular weight at high ionic strength. Evaluations at higher ionic strengths than those employed here would be difficult to interpret since, at the highest ionic strength used, the tendency for apparent aggregation was evident. Furthermore, as the salt concentration increases, preferential interactions of specific solvent components generally become a more significant unknown factor.

The molecular weight of 29S rRNA, on the basis of our melting experiments employing ^{32}P-labeled 30S rRNA, is considered to be 2.3% less than that of the parent complex, namely, 2.05×10^6. By the same token, 7S rRNA is assigned a molecular weight of 47,800. The latter value corresponds to 148 nucleotides based on the reported nucleotide composition (Pene *et al.*, 1968). This is very near the value of 150 nucleotides estimated by these same authors from relative gel electrophoretic mobilities.

If one assumes that 30S rRNA and 29S rRNA have the same partial specific volumes and Scheraga–Mandelkern β functions, the molecular weight of the 29S rRNA, calculated from the sedimentation coefficients and intrinsic viscosities reported here, is 91 ± 8% of that of the 30S rRNA complex. The molecular weight of the 29S rRNA obtained by difference from the radiolabeling experiment is within the experimental error of that calculated for 29S rRNA from the Scheraga–Mandelkern relation. There is no compelling reason to believe that the slight difference in molecular weights of 29S rRNA obtained by these two approaches reflects a conformational change which would negate the validity of the above assumption about identity of β factors.

The absence of significant conformational alterations accompanying the release of 7S rRNA is also evident from a consideration of the essentially identical temperature responses of the 260-nm absorption and of the circular dichroism spectra. Undoubtedly some change in secondary and tertiary structure accompanies the quaternary structure change, but this is probably only significant in the product of lesser mass, the 7S rRNA. Since the spectral probes yield information averaged over all the chromophores present, the effect is not detectable.

In summary, we demonstrate that the 30S rRNA complex isolated from HeLa cells is a temperature-labile complex which dissociates, probably irreversibly, with an activation enthalpy of 123.5 kcal/mol and an activation entropy of 0.32 kcal/(mol deg). The magnitude, cooperative nature, and ionic strength dependence of the rate constants are consistent with the picture of the activation step being a process of double strand separation. The molecular weight of the complex is 2.1×10^6 and the complex is hydrodynamically characterized at an ionic strength of 0.12 by a sedimentation coefficient of 29.9 ± 0.4S and an intrinsic viscosity of 74.4 ± 5.4 ml/g. No conformational difference could be detected between the 29S rRNA in the denatured free state and the native 30S complexed state.

References

Bellamy, A. R., and Ralph, R. K. (1968), *Methods Enzymol. 12,* 156–160.

Casassa, E. F., and Eisenberg, H. (1964), *Advan. Protein Chem. 19,* 287.

Cryer, D. R., Goldthwaite, C. D., Zinker, S., Lam, K. B., Storm, E., Hirschberg, R., Blamire, J., Finkelstein, D. B., and Marmur, J. (1973), *Cold Spring Harbor Symp. Quant. Biol. 38,* 17.

Eagle, H. (1959), *Science 130,* 432.

Eliceiri, G. L., and Green, H. (1969), *J. Mol. Biol. 41,* 253.

Felsenfeld, G., and Miles, H. T. (1967), *Annu. Rev. Biochem. 36,* 407.

Finkelstein, D. B., Blamire, J., and Marmur, J. (1972), *Nature (London), New Biol. 240,* 279.

Gratzer, W. B., and Richards, E. C. (1971), *Biopolymers 10,* 2607.

Graves, I. L. (1967), *Biochem. Biophys. Res. Commun. 28,* 875.

Graves, I. L. (1970), *Biopolymers 9,* 11.

Gruenwedel, D. W., Hsu, C. H., and Lu, D. S. (1971), *Biopolymers 10,* 47.

Hunt, J. A. (1970), *Nature (London) 226,* 950.

King, H. W. S., and Gould, H. (1970), *J. Mol. Biol. 51,* 687.

Krakauer, H., and Sturtevant, J. M. (1969), *Biopolymers 7,* 223.

Kurland, C. G. (1960), *J. Mol. Biol. 2,* 834.

Lizardi, P. M., and Luck, D. J. L. (1971), *Nature (London), New Biol. 229,* 140.

Loening, U. E. (1968), *J. Mol. Biol. 38,* 355.

Maden, B. E. H. (1971), *Progr. Biophys. Mol. Biol. 22,* 127.

Maizel, J. V., Jr. (1966), *Science 151,* 251.

Maizel, J. V., Jr. (1969), *in* Fundamental Techniques in Virology, Habel, K., and Salzman, N. P., Ed., New York, N.Y., Academic Press, p 334.

McConkey, D. H., and Hopkins, J. W. (1969), *J. Mol. Biol. 39,* 545.

Olivera, B. M., Baine, P., and Davidson, N. (1964), *Biopolymers 2,* 245.

Payne, P. I., and Dyer, T. A. (1972), *Nature (London), New Biol. 235,* 145.

Pene, J. J., Knight, E., Jr., and Darnell, J. E., Jr. (1968), *J. Mol. Biol. 33,* 609.

Penman, S. (1969), *in* Fundamental Techniques in Virology, Habel, K., and Salzman, N. P., Ed., New York, N.Y., Academic Press, p 37.

Petermann, M. L., and Pavlovec, A. (1966), *Biochim. Biophys. Acta 114,* 264.

Plagemann, P. G. (1970), *Biochim. Biophys. Acta 224,* 451.

Prestayko, A. W., Tonato, M., and Busch, H. T. (1970), *J. Mol. Biol. 47,* 505.

Ray, D. B. (1972), Ph.D. Dissertation, University of Pittsburgh.

Ross, P. D. (1971), *Proced. Nucleic Acid Res. 2,* 417.

Ross, P. D., and Scruggs, R. L. (1964), *Biopolymers 2,* 231.

Rubin, G. M. (1974), *Eur. J. Biochem. 41,* 197.

Salzman, N. P. (1959), *Biochim. Biophys. Acta 31,* 158.

Scherrer, K., and Darnell, J. E. (1962), *Biochem. Biophys. Res. Commun. 7,* 486.

Schildkraut, C., and Lifson, S. (1965), *Biopolymers 3,* 195.

Soeiro, R., Vaughan, M. H., Warner, J. R., and Darnell, J. E., Jr. (1968), *J. Cell. Biol. 39,* 112.

Sokol, F., Graves, I. L., and Ackermann, W. W. (1966), *Biochim. Biophys. Acta 112,* 74.

Steiner, D. F., and Clark, J. L. (1968), *Proc. Nat. Acad. Sci. U.S. 60,* 622.

Sy, J., and McCarty, K. S. (1970), *Biochim. Biophys. Acta 199,* 86.

Sy, J., and McCarty, K. S. (1971), *Biochim. Biophys. Acta 228,* 517.

Tanford, C. (1967), Physical Chemistry of Macromolecules, New York, N.Y., Wiley, p 268.

Thierr, J. C., Dourlent, M., and Leng, M. J. (1971), *J. Mol. Biol. 58,* 815.

Udem, S. A., Kaufman, K., and Warner, J. R. (1971), *J. Bacteriol. 105,* 101.

Yphantis, D. A. (1964), *Biochemistry 3,* 297.