Configurational Changes in Ribosomal RNA as a Function of Ionic Conditions†

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ABSTRACT: The configuration of RNA prepared from 50S ribosomal subunits of *Escherichia coli* has been analyzed by electron microscopy and sedimentation studies as a function of perturbations on the ionic environment. The RNA was extensively depleted of Mg^{2+} with chelating resin at low ionic strength neutral pH. A range of sedimentation values, increasing from 4 S to 32 S, was observed which correlated with an increasingly folded average configuration as visualized by electron microscopy. At pH 3.0 and at low ionic strength, unchelated RNA was rapidly converted to an 18-nm spherical particle sedimenting at 50 S. This conversion was retarded at higher ionic strength. Chelated RNA converted to a more asymmetric 48S particle at pH 3.0. A smooth curve was obtained when the observed sedimentation coefficient was plotted semilogarithmically against the maximum particle diameter measured from the electron micrographs. A close fit to this curve was obtained when these measurements were used in standard sedimentation equations to generate a calculated sedimentation coefficient. This result coupled with the good agreement found with literature values of radius of gyration for 23S RNA affirms the usefulness of this type of electron microscopic measurement in determining parameters related to conformation in solution.

Ribosomal RNA is now generally regarded as consisting of a single polynucleotide chain, about 60% of which is folded (at an ionic strength of about 0.1) (Cotter and Gratzer, 1969a) into many short regions of double helix containing not more than about 20 base pairs each (Cox, 1968). The available spectroscopic evidence indicates that this base pairing persists in the intact ribosome (Cotter et al., 1967; McPhie and Gratzer, 1966; Cotter and Gratzer, 1969b). The extent of base pairing in free RNA in solution is dependent upon the ionic environment and decreases as the ionic strength is lowered. In salt-free solution, electrostatic repulsions between phosphate groups inhibits formation of base pairs, but local order persists as a result of base stacking (Bush and Scheraga, 1967).

It is also known that variations in the ionic environment substantially affect the overall conformation (Colter and Brown, 1956; Littauer and Eisenberg, 1959), as well as secondary structure. Stanley (1963) and Stanley and Bock (1965) showed that complete removal of multivalent ions from rRNA followed by careful adjustment of mono- and divalent cation concentrations resulted in sedimentation rates covering the range of 3.5 S to 28 S for the RNA of the 50S ribosomal subunit of *Escherichia coli*.

Electron microscopy (EM) could be expected to make a contribution to this problem by direct visualization of gross conformational changes. In fact, most of the reported EM studies of rRNA have applied the Kleinschmidt technique (Kleinschmidt and Zahn, 1959) to RNA denatured with urea (Granboulan and Franklin, 1966; Granboulan and Scherrer, 1969; Verma et al., 1970) or with dimethyl sulfoxide (Me_2SO) alone (Nanninga et al., 1972). In addition to these numerous studies of denatured RNA, Littauer et al. (1960), Kisselev et al. (1961), and Matsuura et al. (1970) have reported EM studies of RNA conformation which, however, do not document adequately the integrity or homogeneity of the RNA. The material studied in these cases was apparently prepared without the special precautions now known to be essential for the extraction and handling of intact, stable, and protein-free preparations of rRNA (Stanley, 1963; Stanley and Bock, 1965). Thus, while the behavior of rRNA in solution is now partially documented by sedimentation and spectroscopic studies at neutral pH, this knowledge has not been complemented by electron microscopy. Improved methods of EM specimen preparation (Slayter and Lowey, 1967; Lowey et al., 1969) have been applied here to visualize the detailed nature of the configurational changes produced in rRNA as a function of ionic strength in the range $\mu = 5 \times 10^{-4}$ to $\mu = 1$ and of pH 3.0 and 7.2, which are reflected in the gross variation in sedimentation behavior observed.

Experimental Section

(*a*) *Preparation of 50S Ribosomal Subunits.* Sucrose-washed ribosomal particles were prepared from *E. coli* MRE 600 (late-log phase; obtained as a frozen paste from General Biochemicals, Chagrin Falls, Ohio) essentially by the method of Traub and Nomura (1968). Pili were removed by the method of Brinton (1965) before the cells were broken. After washing with NH_4Cl (Spirin et al., 1963; Hill et al., 1970), the ribosomes were dialyzed into 0.01 *M* Tris (pH 7.4), 10^{-4} *M* $MgCl_2$, 0.06 *M* KCl, and 50S subunits obtained either by double cycles of sucrose gradient separation (Traub and Nomura, 1968) or by differential centrifugation (Tissieres et al., 1959). The final resuspended pellets were stored frozen at −20° in the presence of 10^{-2} *M* $MgCl_2$.

(*b*) *Special Precautions.* The following precautions were observed throughout this work to prevent degradation of ribosomes and rRNA by trace amounts of RNase. Sucrose was RNase-free (Mann). Spinco centrifuge tubes and dialysis tubing were boiled in 0.1% sodium dodecyl sulfate–0.1%

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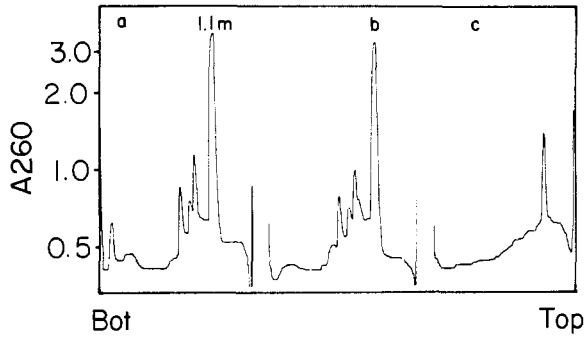

FIGURE 1: Uv profiles of acrylamide–sodium dodecyl sulfate gels of rRNA-23; (a) unchelated; (b) following treatment with Dowex chelating resin; (c) following further sucrose gradient fractionation, dialysis, and lyophilization. 1.1 *M* refers to the molecular weight.

EDTA. Dialysis tubing was handled subsequently only with polyethylene gloves. Macaloid (Stanley, 1963; gift of National Lead Company, Houston, Texas) was included in all RNA dialyses and removed by centrifugation before preparation for EM. All glassware was sterilized because this procedure was found to reduce the activity of pancreatic RNase by a factor of 100. In addition, all glassware used for RNA solutions was cleaned in chromic acid solution. Except where otherwise stated, all operations were carried out at 0–5°.

(*c*) *Extraction of rRNA.* rRNA-23[1] was prepared by sodium dodecyl sulfate–phenol extraction using the procedures described by Stanley (1963). The final solution was dialyzed into 0.1 *M* NH_4OAc (pH 7.2) and stored frozen at −20° in small aliquots at a concentration of 5 mg/ml. Aliquots were thawed only once.

(*d*) *Sucrose Gradient Analysis.* Gradients were 15–30% (w/v) and were run at 58,000*g* in the Spinco SW 25.1 rotor at 5° for the periods indicated in the legend to Figure 2. Relative sedimentation coefficients were based upon simultaneous runs using particles with established sedimentation coefficients, such as 30S and 50S ribosomal subunits. We calculate the maximum deviation in our estimates of sedimentation coefficients to be ±2S units in 50.

(*e*) *Removal of Magnesium.* Dowex A-1 resin, 50–100 mesh, was a product of J. T. Baker Company. The resin was prepared and used as described by Stanley (1963). Solutions to be used in these experiments were treated by stirring with the resin. Portions of frozen RNA preparations were thawed out and dialyzed overnight against several changes of 0.001 *M* NH_4OAc (pH 7.2). An aliquot of 1.0 ml of the dialyzed RNA was passed through a 1 × 10 cm column of Dowex resin at a flow rate of 1 ml/min (to achieve partial chelation) or else was stirred directly with the resin for 2 hr (full chelation). Aliquots of the treated RNA together with portions of untreated RNA were adjusted to the various ionic conditions selected and analyzed by electron microscopy and sucrose gradient sedimentation. In some experiments, adjustment of ionic conditions was carried out immediately after exposure to the chelating resin. In other experiments, the Dowex-treated RNA was frozen directly and examined later, without apparent effect (cf. Rodgers, 1970).

(*f*) *Quantitative Protein Analysis.* Quantitative analysis of the protein content of RNA preparations was carried out using the colorimetric method described by Lowry et al. (1951), with bovine serum albumin as a reference standard. An additional assay for low levels of protein involved running excessive amounts of rRNA on sodium dodecyl sulfate polyacrylamide gels at 10% acrylamide (Weber and Osborn, 1969) after incubation in sodium dodecyl sulfate at 37° to detach any remaining protein, followed by staining with Coomassie Blue (which detects 0.5 μg of protein in a single band).

(*g*) *Gel Electrophoresis of RNA.* Polyacrylamide gel electrophoresis of RNA was carried out on 2.6% acrylamide gels in 0.2% sodium dodecyl sulfate, as described previously (Loening, 1968). Gels were analyzed with a Joyce-Loebl uv scanner.

(*h*) *Preparation for Electron Microscopy.* Ice-cold solutions containing RNA at a concentration of 50 μg/ml and volatile salt at the concentrations indicated in the text and in the plate legends were prepared for electron microscopy by rotary platinum shadow casting, as described elsewhere (Slayter and Lowey, 1967; Lowey et al., 1969). Micrographs were recorded on a Siemens Elmiskop 1A at 25,000× and 50,000×, generally less than 0.5 μm under focus. Magnification calibration was achieved using the 2.49-nm crystal spacing of indanthrene olive (Labaw, 1964). Photographic prints were made from a contact intermediate so that evaporated metal appears light.

Results

(*a*) *Purity and Integrity of rRNA.* The polyacrylamide gel profile in Figure 1a illustrates the nature of the RNA preparations obtained from purified 50S ribosomal subunits. There is contamination of the order of 10% from the fragments arising from breakdown of the intact RNA molecule (Szer, 1969; Cahn et al., 1970) and from the presence of rRNA-16 (from 30S subunits). The preparations are, therefore, more homogeneous than the mixture of rRNA-16 and rRNA-23 species investigated for their sedimentation behavior by Stanley (1963). This gel profile was not affected by treatment with Dowex chelating resin (Figure 1b), by incubation at 37°, or by exposure to pH 3.0. Figure 1c demonstrates the lack of RNase in our dialysis system: this profile was obtained after the 23S peak was removed from a sucrose gradient (run for 24 hr), dialyzed for 24 hr, lyophilized, and analyzed by gel electrophoresis.

The presence of residual ribosomal protein in the RNA preparations was checked as follows. The procedure of Lowry et al. (1951) indicated that the protein content was below the sensitivity level of the test (~0.5% of total protein); 0.5% of total protein represents a possible contaminant of ~3000 or 0.3 mol of the lowest molecular weight protein of the 50S ribosomal subunit (Dzionara et al., 1970) and could conceivably, therefore, be partly responsible for conformational changes. For this reason, further analysis was carried out by electrophoresis in sodium dodecyl sulfate on 10% acrylamide gels (Weber and Osborn, 1969). Analysis of 0.5 mg of RNA in this way yielded a completely blank gel after staining for protein, indicating the presence of less than 0.5 μg of any one protein species. Under these conditions, 0.5 μg represents about 10% of the amount of a protein with molecular weight 10,000 that originally would be associated with the RNA in the 50S subunit (or about 3.5% of a protein with molecular weight 30,000). That is, not more than 10% of any one protein is present in our RNA preparations. Since electrophoresis of ribosomal proteins in one dimension gives rise to multiple bands containing more

[1] Abbreviations used are: rRNA-23, RNA from 50S ribosomal subunit; rRNA-16, RNA from 30S ribosomal subunit.

Table I: Ionic Conditions Used in Comparing Sedimentation Behavior with Electron Microscopic Configuration.

	Stanley[d]					
		$s_{20,w}^0$			s (Sucrose Gradient)	
Ionic Conditions[a]	Salts	D[b]	ND[c]	Salts	D[b]	ND[c]
(1) Low μ	4×10^{-4} *M* KCl 2×10^{-4} *M* Tris	3.66	16.0	5×10^{-4} *M* NH_4OAc	4	15
(2) Low μ + Mg^{2+}	As (1) + 10^{-4} *M* $MgCl_2$	19.0		As (1) + 10^{-4} *M* $MgCl_2$	21	21
(3) Moderate μ	0.1 *M* KCl 0.05 *M* Tris	24.0	25.2	0.1 *M* NH_4OAc	23	23
(4) High μ				1.0 *M* NH_4OAc		26
(5) Moderate μ + Mg^{2+}	As (3) + 10^{-2} *M* $MgCl_2$		28.2	As (3) + 10^{-2} *M* $MgCl_2$	32	28
(6) Low μ, pH 3.0				0.001 *M* NH_4OAc HOAc	48	50

[a] pH is 7.2 except where otherwise stated. [b] D, Dowex-treated RNA. [c] ND, Non-Dowex-treated RNA. [d] Data from Stanley (1963).

than one species, the estimate of 10% represents a maximum.

(*b*) *Configurational Changes.* Each of the five sets of ionic conditions described below resulted in a characteristic EM appearance and sedimentation coefficient for rRNA-23. Ammonium acetate was used for both EM and sedimentation analysis. The low levels of magnesium used could be tolerated in the EM. No difference in sedimentation behavior was found between this buffer system and the nonvolatile Tris-KCl system used by Stanley (1963) (see Table I).

Aliquots of Dowex-treated RNA (see Materials and Methods) together with portions of untreated RNA were adjusted with ammonium acetate, acetic acid, and magnesium chloride. Equilibration at the various ionic conditions was allowed to proceed at 0°; no difference was observed when the temperature was raised to 37°. Sedimentation behavior as a function of ionic conditions is summarized in Table I, and representative sucrose gradient profiles are shown in Figure 2.

$\mu = 5 \times 10^{-4}$, no Mg^{2+}, pH 7.2. At low ionic strength in the absence of added magnesium, unchelated rRNA-23 sediments at 15 S and appears in the EM as an extended, but internally aggregated, particle, as shown in Plate 1d. The fully chelated material sediments at 4 S (Figure 2a) and gives the appearance of being composed mainly of extended strands (Plate 1b), slightly thicker than Me_2SO-treated RNA (Plate 1a). Partially chelated RNA (see Materials and Methods) sedimented at 7 S. It appeared thicker than the 4S species and frequently displayed nodules (Plate 1c).

$\mu = 5 \times 10^{-4}$, 10^{-4} *M* Mg^{2+}, pH 7.2. Chelated rRNA-23 undergoes a major conformational change (4 S to 21 S) when 10^{-4} *M* magnesium is added at low ionic strength. EM photographs (Plate 2a) indicate a configuration considerably more compact than the 4S form. The change in the unchelated RNA is less marked (15 S to 21 S).

$\mu = 0.10$, no Mg^{2+}, pH 7.2. Under these conditions, rRNA-23 sediments at 23 S whether chelated or unchelated. While images of individual 23S particles could not be distinguished from 21S particles, fields viewed simultaneously did appear slightly different in their degree of compactness (Plate 2a and b).

A further small increase in S (to 26 S) was observed when the ammonium acetate concentration was raised to 1 *M*.

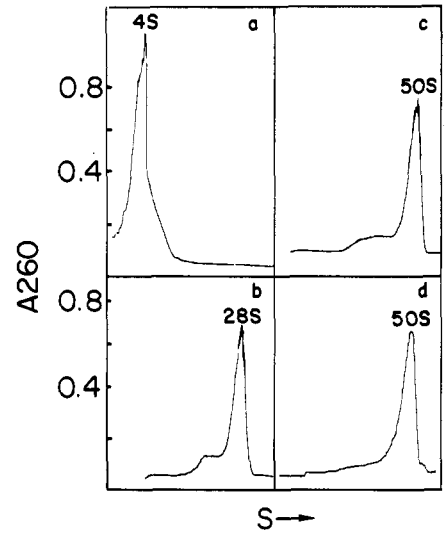

FIGURE 2: Sedimentation profiles of rRNA-23 on 15–30% sucrose gradients under various conditions. Gradients a and b and gradients c and d were run for 24 and 14 hr, respectively, at 58,000*g*. (a) Fully chelated in 5×10^{-4} *M* NH_4OAc, pH 7.2; (b) untreated, in 0.1 *M* NH_4OAc, 10^{-2} *M* $MgCl_2$, pH 7.2; (c) untreated, in 0.001 *M* NH_4OAc, pH 3.0; (d) 50S subunits in 10^{-3} *M* $MgCl_2$, 0.06 *M* KCl, 0.01 *M* Tris-HCl, pH 7.4. Sedimentation is from left to right.

$\mu = 0.10$, 10^{-2} *M* Mg^{2+}, pH 7.2. Nonchelated rRNA-23 sediments at 28 S (Figure 2b) and has a stellate appearance (Plate 2c). Chelated rRNA-23 sediments at 32 S and presents a rounded aspect, clearly more oblate than spherical.

A small amount of RNA sediments slightly slower than the principal peak (Figure 2b). Electrophoretic analysis of the secondary peak indicated that it was enriched in the contaminating fragments present at a low level in the original RNA preparation (Figure 1a).

$\mu = 0.001$, no Mg^{2+}, pH 3.0. When rRNA-23 is adjusted to these conditions it rapidly (in less than 60 min) assumes a compact configuration (Plate 3b) and sediments at 50 S (Figure 2c). This particle is the same shape as, and only slightly smaller than, the original 50S ribosomal subunit (Plate 3a). Chelated rRNA-23 contains a greater proportion of asymmetric particles under these conditions (Plate 3c) and sediments at 48S.

When rRNA-16 was adjusted to pH 3.0, $\mu = 0.001$, it sedimented at 30 S and was found to be 3.0 nm smaller than the 30S ribosomal subunit (Plate 3d). The RNA was

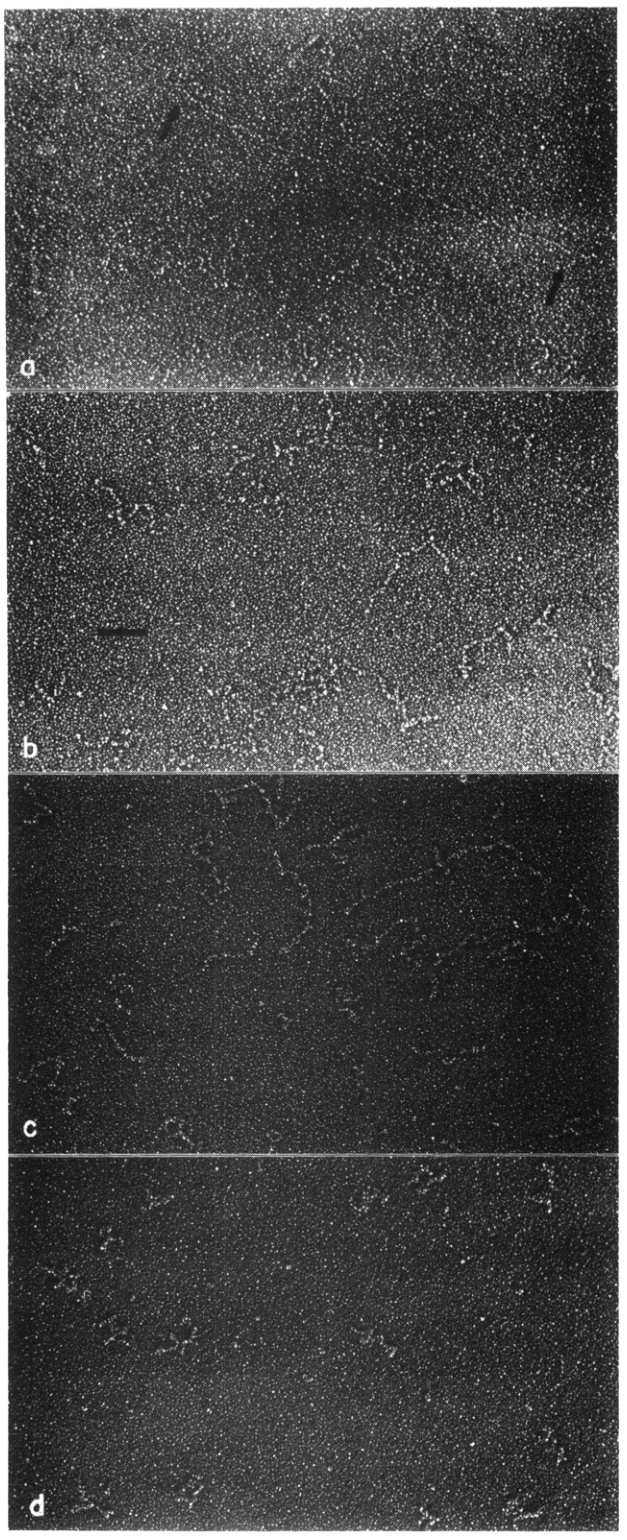

PLATE 1: rRNA-23 in various media: (a) 80% Me_2SO–H_2O; (b) fully chelated, in 5×10^{-4} *M* NH_4OAc (pH 7.2): (c) partially chelated, in 5×10^{-4} *M* NH_4OAc (pH 7.2); (d) unchelated, in 5×10^{-4} *M* NH_4OAc (pH 7.2). Bar indicates 100 nm.

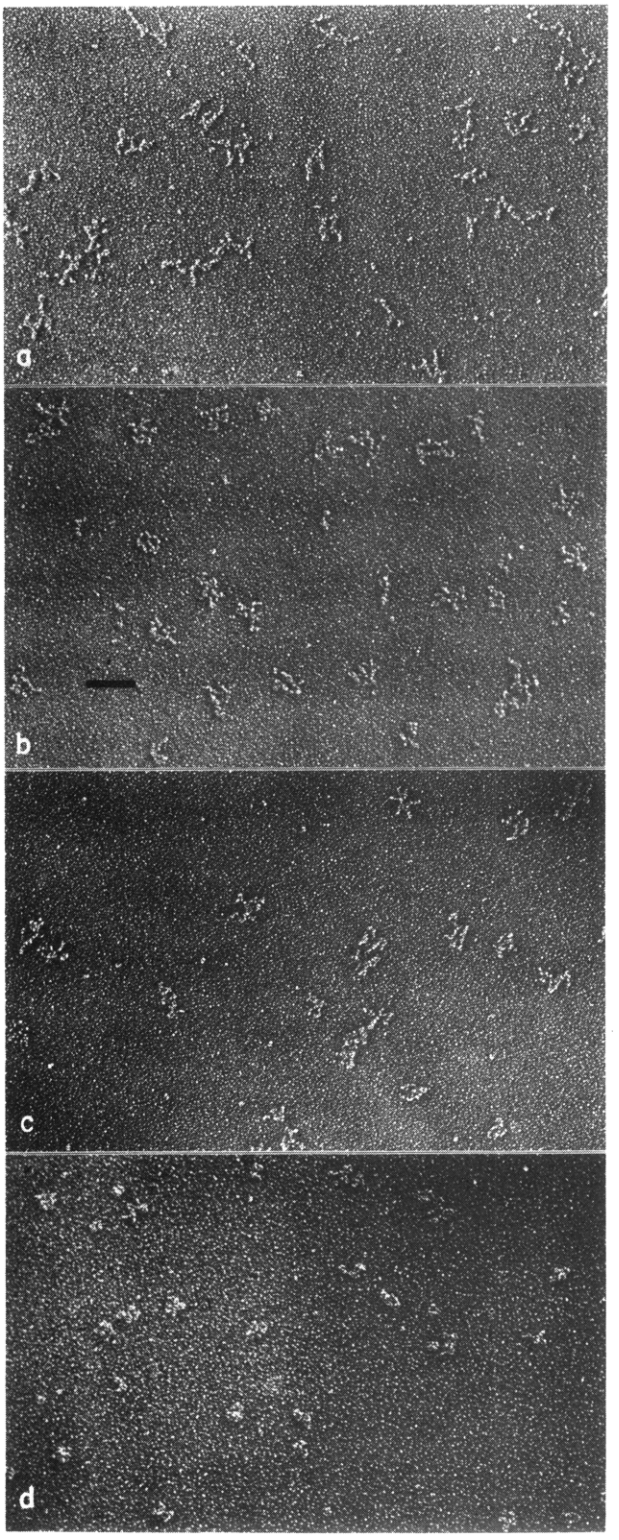

PLATE 2: rRNA-23 in various media: (a) unchelated, in 5×10^{-4} *M* NH_4OAc–10^{-4} *M* $MgCl_2$ (pH 7.2); (b) unchelated, in 0.1 *M* NH_4OAc (pH 7.2); (c) unchelated, in 0.1 *M* NH_4OAc–10^{-2} *M* $MgCl_2$ (pH 7.2); (d) chelated, in 0.1 *M* NH_4OAc–10^{-2} *M* $MgCl_2$ (pH 7.2). Bar indicates 100 nm.

spherical rather than prolate (Plate 3e).

Size distributions of rRNA-23 and rRNA-16 under these conditions are shown in Figure 3.

μ = 0.10, no Mg^{2+}, pH 3.0. Equilibration of rRNA-23 under these conditions was found to be time dependent. One hour after adjustment of ionic conditions to μ = 0.10, pH 3.0 (or after 24 hr of dialysis from μ = 0.10, pH 7.2), the particles shown in Plate 3f were observed. The peak diameter of the component nodules of each clump was found to be about 7.0 nm with approximately 9 nodules per clump. After 24 hr (or 48 hr of dialysis), the clumps were more linear and contained fewer (about six) larger (9.0 nm) nodules.

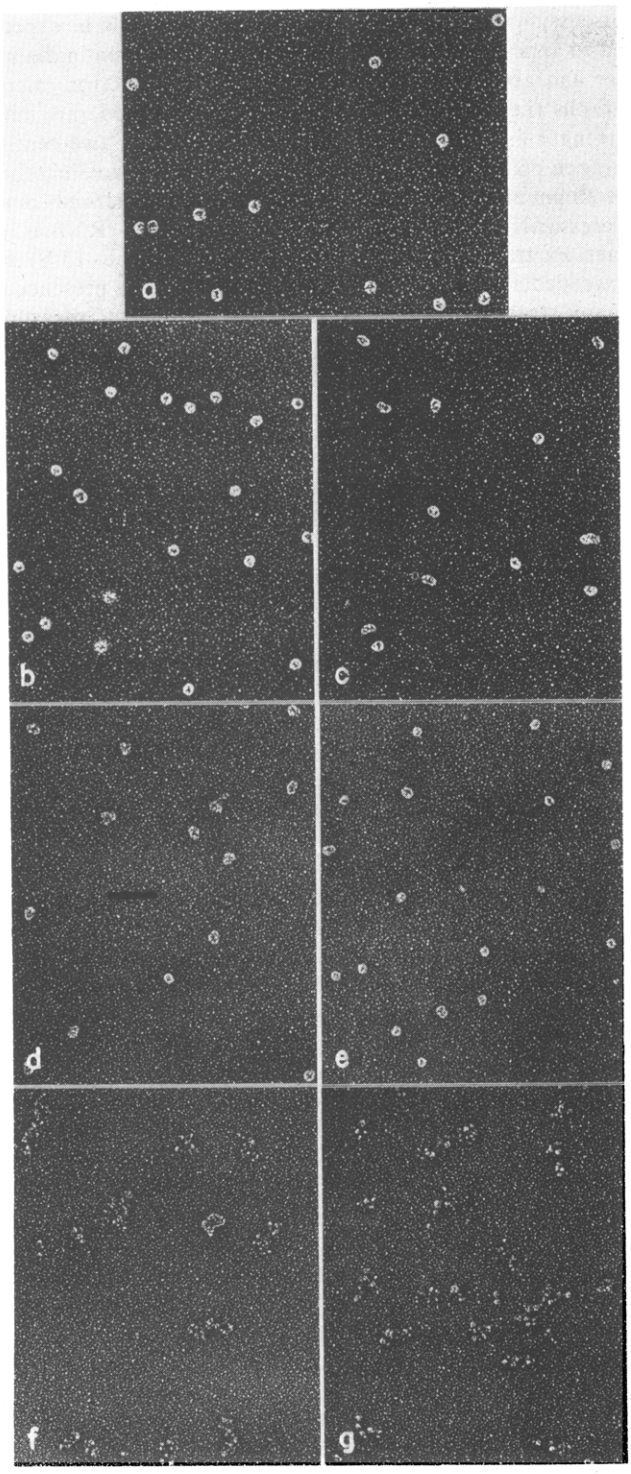

PLATE 3: (a) 50S ribosomal subunits; (b) rRNA-23, nonchelated, pH 3.0, μ = 0.001; (c) rRNA-23, chelated, pH 3.0, μ = 0.001; (d) 30S ribosomal subunits; (e) rRNA-16, nonchelated, pH 3.0, μ = 0.001; (f) rRNA-23, nonchelated, pH 3.0, μ = 0.1 (1 hr); (g) rRNA-23, nonchelated, pH 3.0, μ = 0.1 (24 hr). Bar indicates 100 nm.

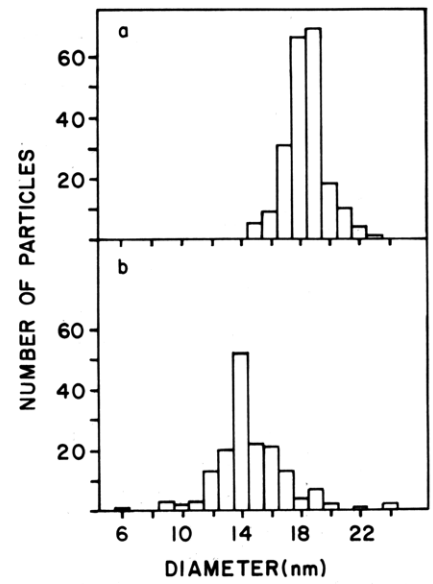

FIGURE 3: Size distribution of rRNA at pH 3.0, μ = 0.001: (a) rRNA-23; (b) rRNA-16. Diameter corrected for metal replica cap.

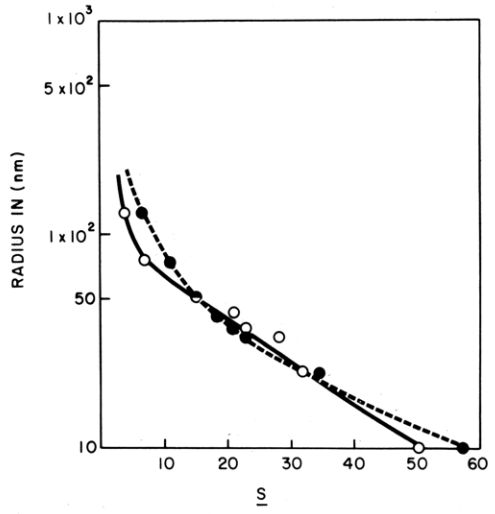

FIGURE 4: Plot measured maximum particle radius vs. observed sedimentation coefficient (——) and calculated sedimentation coefficient (- - - - -). (See text.) The calculated point on the abscissa was based on a sphere model, whereas all other points were based on a rod model.

(*c*) *Quantitative Analysis.* It is clear that condensation of rRNA can be achieved by exposure to (in increasing order of effectiveness) high ionic strength, magnesium ions, and pH 3.0. In order to quantitate the morphological changes with the observed changes in sedimentation, measurements of the maximum dimension (R_{max}) of each whole particle were made. The average values of R_{max} are plotted semilogarithmically vs. the observed S in Figure 4. S was calculated from R_{max} using the relationship $S = k/R_e$ [where R_e is the radius of the equivalent sphere (Tanford, 1961, p 489) and $k = M(1 - \bar{v}\rho)/N6\pi\eta$, assuming $R_e = 0.7\ R_G$, the radius of gyration for a flexible polymer (Tanford, 1961, p 382)]. R_G is calculated from R_{max} using standard equations for a solid rod or sphere (Tanford, 1961, p 306). The fit between the calculated and measured values of S is shown in Figure 4. The partial specific volume, $\bar{v}$, was taken as 0.51 (Stanley, 1963 p 106).

Discussion

(*a*) *Comparison with Solution Studies.* The exact mode of folding of rRNA, either in solution or in the ribosome, is unknown. Models constructed on the basis of the biochemical evidence (Cox, 1968; Spirin and Gavrilova, 1969; Cox and Bonanou, 1969) have assumed that the secondary structure is in the form of hairpin double helical regions distributed more or less uniformly along the polynucleotide chain. Each double-helical region (there may be as many as 50) contains less than 20 base pairs (Cox, 1968), and thus

would not be expected to be more than 6–7 nm long. Timasheff et al. (1961) have presented evidence from small angle X-ray scattering studies that rRNA is composed of rigid rods 5–15 nm long joined by short flexible regions with the rods accounting for about 90% of the entire structure.

The studies summarized above and in previous sections provide clues as to the gross structure of rRNA in solution. The advantage of the EM is that it should be able to provide a direct overall view with resolution down to 2.5 nm. At this level, certain features which have been predicted, such as rods 5–15 nm long, should appear. Also it should be possible to relate the gross configuration observed to solution parameters.

Most previous EM observations on RNA have been carried out in denaturing media, such as urea, in the presence of cytochrome *c* (Granboulan and Franklin, 1966; Granboulan and Scherrer, 1969; Verma et al., 1970) or Me_2SO alone (Nanninga et al., 1972). These methods are convenient for measuring overall length of the resulting straight extended chains, and thus for demonstrating homogeneity or integrity. Such investigations do not, however, provide information about the normal configuration of rRNA in aqueous solution.

The procedure for EM preparation used in our work has been used with success on a variety of molecular species whose configurations so observed were shown to be relevant to the results of solution studies (Hall and Slayter, 1959; Slayter and Lowey, 1967; Lowey et al., 1969; Slayter et al., 1972; Slayter and Codington, 1973). rRNA prepared in this manner has a generally more compact form except at low ionic strength and low magnesium concentration where it assumes a relaxed but extended configuration.

(*b*) *Gross Features.* The sedimentation behavior reported here (Table I) and by Stanley (1963) implied that the compactness of rRNA was directly dependent upon changes in ionic strength and magnesium concentration (together or separately), and upon pH. The configurations observed here in the EM reflect this dependence. For the range of conditions examined, the conformation of the RNA varied between limits identifiable as a thin (1.5 nm), presumably single-stranded rod up to 0.8 μm in length, and a spherical 50S particle with R_{max} = 10 nm. In an effort to relate our EM observations to parallel sedimentation studies, measurements of R_{max} were converted by calculation to S for each of the sets of ionic conditions considered. A plot of R_{max} vs. S_{meas} fitted well with the plot of R_{max} vs. S_{calcd} (Figure 4), given our approximation of no hydration and of a value of 0.7 for the constant of proportionality in the relationship $R_e = 0.7R_G$ (Tanford, 1961, p 382). Given the deviation of the configuration of rRNA from any standard model, such as a single uncharged hydrocarbon polymer chain or rod, the closeness of fit is considered remarkably good.

The radius of gyration, calculated from our measurements at μ approximately 0.1 and pH 7.2, is comparable with the light scattering value of 32 nm given by Timasheff et al. (1958), as well as with the value of 23.3 and 27.5 nm deduced by Stanley (1963) from viscosity and sedimentation data, respectively, at μ = 0.15, pH 7.2.

Thus, observed changes in configuration are consistent with various solution measurements, at least in terms of variations in overall particle size and shape, with environmental changes.

(*c*) *Substructure.* When coated with platinum, a uniform string of hairpin regions (such as is frequently assumed in discussion of the gross structure of rRNA) would be expected to appear in the EM as a rod about 10–20 nm in diameter and about 300–400 nm in length. Our electron micrographs (i.e., 23 S–28 S) (Plate 2) show, at moderate ionic strength and neutral pH, particles with one or two central branch points and many diverging strands (approximately 3 × 20 nm after correction for platinum). These strands must necessarily be folded back on themselves if the rRNA is intact. Particles seen at lower ionic stengths (4 S to 15 S) are extended and composed of fewer branches. The presence of many strands folded in close proximity prevents measurements within 2.5 nm; strands would be expected to be thicker than measured, if anything, due to unassessable corrections.

Our EM observations, under ionic conditions where base pairing is known to be maximized (μ = 0.1), are inconsistent with the concept of a simple string of hairpin regions as a model for the structure of rRNA in solution. The complex tertiary structure observed could be accounted for by the formation of double-helical regions between otherwise widely separated portions of the RNA chain. This would result in continuous branches, or loops, substantially longer than would be expected for individual hairpin regions (Cox, 1968).

This configuration appears to be particularly favored in the presence of magnesium, as would be expected from the results of Fuwa et al. (1960), who showed that metal ions were chiefly responsible for maintenance of tertiary structure in RNA, much as disulfide bonds are responsible for tertiary structure of proteins.

The configurational changes from 4S to the 28S form are also consistent with the findings of Studier (1969) and Uhlenbeck et al. (1973) that double-stranded helices formed from separated portions of the RNA chain are less stable than hairpin loops. This suggests the following interpretation of our EM observations. Residual magnesium and other trace metals may be responsible for the configuration observed in unchelated RNA at low ionic strength, where secondary structure would otherwise be absent. Removal of metal ions with chelating resin has a marked effect on the sedimentation coefficient, probably because of loss of tertiary structure. Restoration of ionic strength with ammonium ions permits the formation of both hairpin loops and double-stranded regions, resulting in the complex tertiary structure observed, which is reflected in the sharp increase in S. Further addition of magnesium permits additional intramolecular interactions which result in a slightly higher sedimentation coefficient.

(*d*) *Effect of Chelation.* Extensive treatment with chelating resin produced, at low ionic strength, the most completely unfolded conformation (4 S; Figure 2a; Plate 1b). This conformation is noticeably different from that in Me_2SO (Plate 1a), both in the thickness (~5 nm) and the length of the molecule (~250 nm). It appears to be more than one polynucleotide chain thick. These observations imply that apparent removal of all divalent cations and most monovalent cations may not be sufficient to disrupt secondary structure altogether (Bush and Scheraga, 1967). Complete suppression of hydrogen bonding is presumed to be responsible for the relaxed conformation observed in Me_2SO (Strauss et al., 1968).

The highest S value observed for Dowex-treated RNA in an environment consisting of moderate concentrations of monovalent (0.1 M) and divalent (10^{-2} M) cations was 32 S, whereas unchelated RNA under these conditions sedi-

mented at 28 S (Table I). One interpretation of this phenomenon is that treatment with the chelating resin removes some pre-existing specific ionic bridge(s) (either a trace metal (Tal, 1969a) or possibly polyamine (Petermann, 1964)). Upon later addition of moderate amounts of magnesium, a different structure, more compact and less specific, results. Thus, removal of the last traces of magnesium (and other metals present in trace amounts) (Fuwa et al., 1960) may eliminate some aspect of secondary or tertiary structure of RNA. Similar phenomena have been noted in ribosomes.

Extensive dialysis against EDTA prevents restoration of the original structure when only magnesium is added back (Gesteland, 1966), but particles sedimenting as ribosomes are obtained when small amounts of nickel and zinc (divalent transition metals) are included (Tal, 1969b).

(*e*) *pH 3.0.* Cox (1966) has reported that base pairing is effectively suppressed at this pH. The net charge on the RNA should also be small. There is, therefore, no reason to expect the RNA to adopt an extended configuration in solution under these conditions (cf. Kisselev et al., 1961). Rather, a conformation thermodynamically more stable would be expected. At pH 3.0, in our hands, this is a compact sphere, a configuration presumably facilitated by the unfolding of base-paired regions and by neutralization of charges at low pH. The appearance of rRNA-23 at pH 3.0, $\mu = 0.001$, is strikingly similar to, but slightly smaller than, the 50S ribosomal subunit at neutral pH (Plate 3a and b). This spherical configuration is probably a reflection of the condition of the RNA itself and is not necessarily related to the RNA conformation in the parent ribosomal subunit. The appearance of rRNA-16 under these conditions is clearly different from that of the prolate, ellipsoidal 30S ribosomal subunit (see also, Hall and Slayter, 1959; Huxley and Zubay, 1960), lending support to this interpretation.

The EM configuration adopted by the Dowex-treated RNA (48S) (Plate 3c) is noticeably different from that of the non-Dowexed RNA (50 S). Also, there is a difference between the response of chelated vs. nonchelated rRNA-23 to moderate ionic strength and magnesium (32 S vs. 28 S), as discussed in the previous section. It is clear, therefore, that the mere addition of magnesium ion to chelated rRNA does not cause the structure to return to its original state.

An approximate calculation distributing the known molecular weight through the measured (EM) volume indicates an effective dry bulk density for the rRNA-23 at pH 3.0 of about 0.25, similar to the value calculated in the same way for the 50S ribosomal subunit. Since the equilibrium sedimentation density value for rRNA-23 obtained in Cs_2SO_4 is 1.663 (Erikson, 1966), the particle is clearly not close-packed. Thus, the 50S sphere could be formed by compaction rather than by rolling from one end, as suggested for the 50S subunit (Hart, 1965).

The fact that rRNA-23 sediments at 50 S, at low ionic strength and pH 3.0, can be attributed to the smaller size of the particle and the concomitant lowering of its frictional coefficient. The increased density of the kinetic unit in the absence of protein may also be a contributing factor. The same considerations apply to similarities between rRNA-16 at pH 3.0 and the parent 30S subunit.

The 50S spherical configuration of rRNA-23 is probably not composed of an aggregate of two or more molecules, due to the uniformity of the particles in size and shape, and also due to the lack of any evidence of aggregation, either by electron microscopy or in the sedimentation pattern. This, coupled with the good correspondence between the measured sedimentation coefficient and the coefficient calculated for a spherical model containing one rRNA-23 molecule, contraindicates aggregation.

Metal replica preparations, such as the ones used here, often present problems in interpretation. For example, in spherical particles generally (including 50S ribosomal subunits and rRNA at pH 3.0), rotary shadow-casting at a low angle of incidence results in a build-up of metal around the particle. The resultant paucity of metal in the center promotes the appearance of a hole. 30S ribosomal subunits collect less metal because of their smaller size and give less appearance of a cavity in the particle. Myosin heads are similar yet (7 nm), but still give a hint of less metal in the center (Slayter and Lowey, 1967). We consider this feature an artifact and do not attach any structural significance to it (cf. Hart, 1965; Nanninga, 1967).

(*f*) *Slow Dialysis to pH 3.0.* The course of the change in configuration of the RNA under these conditions was marked by two factors which distinguished it from the change induced at pH 3 at low ionic strength. Firstly, the process was time-dependent, requiring 24 hr before the maximum degree of condensation was observed (Plate 3f and g). Secondly, condensation was less complete than at low ionic strength. These changes occurred in the presence of substantial amounts of cations which, at neutral pH, are known to stabilize base pairing by neutralization of phosphate charges. A possible interpretation of this phenomenon, especially in relation to the results discussed in the previous section, is that base-paired regions persist at pH 3 and high ionic strength, preventing complete condensation of the molecule. This is consistent with the proposal of Rezvin et al. (1973) that metastable base-paired regions are formed under these conditions.

(*g*) *Conclusion.* The variation in configuration exhibited morphologically by rRNA, as a function of ionic conditions (monovalent and divalent cations) and of pH, indicates, in agreement with previous solution studies, that its tertiary structure cannot accurately be described using standard physicochemical nomenclature (e.g., random coil, rigid rod). Indeed, Watson and Kidson (1969) have summarized the factors which appear to affect RNA tertiary structure: influence of cation concentration upon base stacking, intrachain electrostatic repulsion due to partially charged phosphate groups, and variable extents of base pairing. To these should probably be added the possible formation, at low pH, of metastable base-paired regions involving guanine residues (Rezvin et al., 1973). The results obtained here lead us to conclude that these influences result in several discrete configurations. The first of these is an extended chain, corresponding to a sedimentation range of 4 S to 7 S (neutral pH, low μ, chelated). The second, a compact chain, appears at neutral pH, $\mu = 0.10$ where S varies from 23 to 32, depending upon the divalent cation concentration. These are the conditions of maximum base pairing in solution; our micrographs suggest the possibility that base pairing is not restricted to hairpin loops. The third, a compact 50S sphere, is produced at low pH and low ionic strength. A fourth, consisting mainly of a small number of condensed regions, may also possess the metastable regions proposed by Rezvin et al. (1973).

Thus, conformational changes in "23S" RNA measured by sedimentation can be described morphologically under a variety of ionic conditions, and may be accounted for on the basis of standard mechanisms for structural stabilization.

Also, it seems that RNA should not generally be expected to appear as a long straight rod in electron micrographs, except under certain very unusual and denaturing conditions, and that the conformational state of RNA is exquisitely sensitive to the ionic environment.

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