Physical characterization of the 16S and 23S ribosomal ribonucleic acids from Escherichia coli

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PHYSICAL CHARACTERIZATION OF THE 16S AND 23S RIBOSOMAL RIBONUCLEIC ACIDS FROM ESCHERICHIA COLI

by

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ABSTRACT

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Physical Characterization of the 16S and 23S Ribosomal Ribonucleic Acids from Escherichia Coli

Director: Walter E. Hill

The 16S and 23S rRNA's from Escherichia coli were isolated in pure homogeneous form and their physical characteristics were studied. The same measurements were made on the samples of the rRNA's whose secondary structure has been eliminated by treatment with formaldehyde.

Precise measurements of the density increment, \( \frac{\Delta \rho}{\Delta c} \), produced values of 0.418 and 0.405 for the native and formaldehyde-treated samples of the 16S rRNA respectively. The corresponding values for the 23S rRNA were 0.456 and 0.438. These figures were coupled with results from sedimentation equilibrium studies to obtain molecular weights of 6.4 x 10^5 for the K salt of the 16S rRNA, and 1.1 x 10^6 for the same salt of the 23S rRNA. The values obtained for the samples treated with formaldehyde were 6.15 x 10^5 and 1.08 x 10^6. The latter results were procured with the intention that they serve as standards for the calibration of a method to measure molecular weights of RNA by polyacrylamide gel electrophoresis. The above molecular weights indicate that both rRNA's are continuous polynucleotide chains of about 1750 residues in length the 16S, and about 3000 residues the 23S.

The extinction coefficient, \( E_{260}^{1mg/ml} \), was 20.7 for the 16S, and 22.3 for the 23S. A value of 28.2 was measured for both RNA's treated with formaldehyde indicating that the extent of the denaturation was similar.

The sedimentation coefficient, \( s_{20,w} \), of 18.4S was measured for the 16S rRNA while the 23S gave a value of 25.0S. The samples treated with formaldehyde produced the figures of 11.0S and 14.1S respectively. The substantial changes in the sedimentation properties of both polymers demonstrate drastic alteration of their structure in the passage from the folded to the fully extended form after the treatment with formaldehyde, the result with the 23S being the more dramatic.

Combining the sedimentation coefficients, \( s_{20,w} \), with the molecular weights for the formaldehyde-treated samples the relationship \( s_{20,w}^0 = 0.0312 M^{0.440} \) was obtained. This equation can be used to obtain approximate molecular weights of RNA by sedimentation analysis of other samples once they are reacted with formaldehyde under similar conditions.
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CHAPTER I

INTRODUCTION

General Features of the Bacterial Ribosomes

The ribosomes are ribonucleoprotein particles found in the extracts of all bacterial cells examined. It is known that these are the sites where the translation step of protein synthesis is accomplished. Because of their complexity, it is surmised that the ribosomes play an active role in the manufacture of proteins from the information encoded in the messenger RNA, but their exact mechanism of operation is yet to be determined.

The ribosomes and their subunits are generally characterized by their sedimentation coefficients. Ribosomes from bacteria are similar in their general properties: all possess the 70S ribosomes dissociable into the 30S and 50S subunits (98). The 70S ribosome of bacterial origin is not as well defined in its physical characteristics as are the subunits. This may be the result of contamination by segments of messenger RNA, unfinished polypeptide chains and protein cofactors, all of which adhere strongly to the particles. There is also some indication that the ribosomes have varying conformations that reflect the states in the process of protein synthesis, and that there is a reversible equilibrium.
between the 70S and the subunits (101). No attempt has yet been made to characterize the 70S particle resulting from the recombination of purified 30S and 50S subunits.

The molecular weight of the 50S component of E. coli is $1.55 \times 10^6$, and that of the 30S particle is $0.9 \times 10^6$ (41). The 50S subunit is constructed of at least 34 individual proteins (104) which comprise about 30% of its mass, the remainder being RNA. Likewise the 30S subunit is approximately 70% RNA and 30% protein, including 21 different proteins (51). The proteins and the RNA of the 30S ribosomal subunits spontaneously recombine in a specific fashion to produce particles which are active in the synthesis of proteins (68, 69). The assembly of the 50S subunit from its components has also been achieved (58), but the process appears to be more intricate than that of the 30S subunit.

When the ribosomal proteins of different bacterial species are analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS), it is found that for each protein of a given molecular weight another protein of about the same weight is found in the ribosomes of all other species, and that these proteins are present in approximately the same number (4, 92). These features were not common with proteins purified from some eukaryotic ribosomes (4). However, in spite of the superficial similarity in size and amount, the primary
structure of the corresponding proteins from various bacterial orders was different, since in the absence of SDS they do not have the same electrophoretic mobility and also when tested serologically, they displayed little or no cross-reactivity (107). Proteins purified within a single taxonomic family show considerable antigenic similarity (75).

Functional homology has been retained at least among the diverse members of the order Eubacteriales. This is evident in the observation that 50S subunits from the genus Bacillus, including Bacillus stearothermophilus (1) and Bacillus subtilis (96), can combine with Escherichia coli 30S subunits to form hybrid ribosomes which are active in protein synthesis. In addition, Nomura et al. (70) have reconstituted functional, hybrid 30S ribosomal subunits from the component proteins and RNA from different eubacteriales, comprising Escherichia coli, Micrococcus lysodeikticus, Asotobacter vinelandii, and Bacillus stearothermophilus. This is feasible even though some of the ribosomal proteins (107) and ribosomal RNA (70, 99) of these organisms are quite dissimilar in their detailed primary structures. The interaction between the RNA and protein components leading to functional aggregation is somewhat specific, since ribosomal RNA (rRNA) isolated from rat or yeast is unable to form complexes with the proteins of the 30S
subunit of Escherichia coli (99). Therefore, although the RNA and protein ingredients of the ribosomes from diverse bacteria had separated considerably in their primary structures during evolution, they have preserved some of the details which allow their functional interaction.

The Bacterial rRNA

The deproteinization of purified bacterial ribosomes yields three discrete RNA constituents. The 23S and the 5S species are derived from the 50S subunit; the 16S is derived from the 30S subunit.

Based on electron micrographs of uranylacetate stained preparations (43), and studies of degradation of intact subunits with exogenous ribonuclease (87), it has been suggested that the RNA is evenly distributed throughout the ribosome. All of the ribosomal RNA components present in growing Escherichia coli are considered to be metabolically stable (32) and density-labeling experiments have shown that there is no exchange of RNA among the available pool of ribosomes in the cell (46, 59). The molecular weights of the 23S, 16S and 5S rRNA's isolated from Escherichia coli are about $1.1 \times 10^6$ (50, 91), $6.4 \times 10^5$ (71) and $4 \times 10^4$ (81) respectively. These weights correspond to polynucleotide chains of approximately 3000 residues for the 23S, 1700 residues
for the 16S and 120 residues for the 5S rRNA. Ribosomal RNA molecules of approximately the same size have been isolated from all the bacteria examined (3, 24, 36, 55, 57, 65, 81, 97).

Detailed inspection has shown that the 23S and the 16S isolated from several genera differ slightly in their sedimentation properties (97) and in their electrophoretic mobility through polyacrylamide gels (55). Evidence has been presented that the 23S and the 16S rRNA's isolated from a given strain of bacteria may each be separated into classes displaying the same electrophoretic mobility in polyacrylamide gels (19, 93); however, it is probable that this heterogeneity only mirrors small differences in the secondary structure of these polymers in solution (20). It is also possible that the variations found in the physical properties of the rRNA molecules from various genera are not due to chain length but rather to conformation differences. A distinction between the two possibilities is seen by comparing the electrophoretic mobilities of the rRNA components once their secondary structure has been irreversibly denatured with formaldehyde (8).

rRNA Integrity and Ribosome Function

The study of photosynthetic bacteria, especially Rhodopseudomonas spheroides, has revealed that instead
of the 23S rRNA, the 50S ribosomal subunit contains two RNA molecules of sedimentation coefficients of about 16S and 15S having molecular weights of $5.3 \times 10^5$ and $4.2 \times 10^5$ respectively (79, 57). It has been suggested that these two separate polymers might be fragments of the 23S rRNA produced during the process of purification. The 23S molecules can be isolated from Rhodopseudomonas spheroides (9, 94), but these are dissociable into the 16S and 15S chains when the scissions hidden by the secondary structure are unmasked by heating or treating with formamide (57, 79). The 23S rRNA exists temporarily, but is cleaved during growth to the two smaller components (57). Lack of intact 23S does not appear to affect the function of these ribosomes. Apparently the two pieces remain associated non-covalently.

Several investigators (14, 21, 33) have studied the effect of ribonuclease treatment on the ability of the ribosomes of Escherichia coli to carry out polypeptide synthesis. They have concluded that as long as the Mg$^{++}$ concentration is sufficiently high to stabilize the digested RNA within the ribosomes, their activity is preserved. It is important to point out that in these investigations only the polyuridilate-directed synthesis of polyphenylalanine was studied, and therefore is not possible to know if the fidelity of translation has been affected.
**Base Composition of the Bacterial rRNA**

Although the genome base composition is extremely variable among bacteria, the data available on the base composition of their respective rRNA's attest to their remarkable similarity (22, 38, 44, 61, 91, 108). Naturally, the DNA segments that represent the rRNA are a minute fraction of the cellular DNA, and therefore contribute little to the base composition of the genome. However, the primary structure of the rRNA from different microorganisms differ greatly (72).

Since functional, hybrid ribosomal subunits may be reconstituted from the 16S rRNA and the 30S proteins of bacteria whose rRNA have negligible portions of their nucleotide sequence in common (70), it is apparent that the secondary and tertiary structure of the rRNA largely determines the nature of its interactions with the ribosomal protein and with the remainder of the rRNA. The RNA sequences not associated with helical regions probably play an important role in the folding of the RNA chain upon itself (tertiary structure) as modification of those bases renders the molecule incapable of combining with the ribosomal proteins required to form an active particle (68).

The 16S and 23S rRNA's of bacteria also contain small quantities of modified nucleosides. No modified nucleosides are found in the 5S rRNA of either bacteria
modified nucleosides found in the rRNA of bacteria investigated so far are methyl-substituted, but no methylated 5-ribosyluridine has yet been isolated (72). The major portion of the studies of methylated nucleosides in rRNA from bacteria have been confined to Escherichia coli. There are two types of methyl-substituted nucleosides in Escherichia coli: those containing methylated bases and the ones having a methyl substitution at the 2'-position of the ribose moiety of the nucleoside. It has been estimated that of the methylated nucleosides of the rRNA of Escherichia coli, 90% are base substituted and the remainder are 2'-methylated. The 16S and 23S rRNA's contain 22 and 23 methyl groups respectively (23, 28). In the rRNA of higher prokaryotes, most of the methyl groups are located in the ribose moiety of the nucleoside (11, 88). The methylated nucleosides in the rRNA of bacteria are not randomly distributed through the length of the polymer but are associated with specific nucleotide sequences (28, 89). It has been reported for the 16S rRNA that the nucleotide sequences in the vicinity of most methylated nucleosides are similar in bacterial species which otherwise differ greatly in their various primary structures (89). The appearance of these characteristics in the rRNA from phylogenetically diverse bacterial species would indicate that the methylated
nucleosides, as well as the nucleotide sequences associated with them, are important in the construction or function of the ribosome, but the nature of the involvement is far from understood. A mutant strain of Escherichia coli resistant to the antibiotic Kasugamycin lacks a methylase specific for 16S rRNA, yet the cells appear to grow normally (39).

**Physical Characterization of the rRNA's**

The molecular weights of the 16S and 23S rRNA's has been determined by several workers, the first of which was Kurland (50), who using a combination of sedimentation-viscosity and sedimentation-light scattering obtained a value of $5.6 \times 10^5$ and $1.1 \times 10^6$ daltons for the 16S and 23S rRNA's respectively. Midgley (62), using end-group analysis, based his calculations on the stoichiometry of the reaction of periodate-oxidized RNA with (carbonyl-$^{14}$C)isonicotinic acid hydrazide obtained $5.3 \times 10^5$ daltons for the 16S and $1.0 \times 10^6$ daltons for the 23S rRNA. Stanley and Bock (91) obtained a value of $5.5 \times 10^5$ daltons for the 16S from sedimentation-viscosity measurements. A value of $1.07 \times 10^6$ daltons for the 23S was obtained using both sedimentation-viscosity and sedimentation-equilibrium. A molecular weight of $6.4 \times 10^5$ was obtained in this laboratory for the 16S.
rRNA by coupling an accurately determined partial specific volume with sedimentation-equilibrium studies (71).

At present only the primary structure of 5S rRNA is completely known (12). Efforts are also being made to sequence the 16S rRNA from E. coli by the lengthy and tedious procedure available. A large portion of the work is now completed (27, 67).

In the study of the sequence of the 5S rRNA from E. coli strain MRE 600, Brownlee et al. (12) have found two types of polymer chains: one of them has a guanine and the other a uridine at position 13 counting from the 5'-end. Since there are several cistrons for each of the rRNA's (48,73), point mutations in one or more of the corresponding DNA segments therefore will give origin to a diverse population of RNA strands.

Several models have been postulated for the secondary and tertiary structure of the 5S rRNA based on the knowledge of its nucleotide sequence and its degree of hydrogen bonding (15, 54, 56, 76). These structures are not completely in accord with the results obtained from the gentle digestion of the polymer in solution by ribonuclease, and the subsequent analysis of the fragments produced (45, 64). From studies of molecular models, it has been suggested that there might not be a unique secondary structure for the 5S rRNA in solution (77).
The partial nucleotide sequence determined for the 16S rRNA (27) is in agreement with infrared spectroscopy in D₂O solutions (37) indicating that about 60% of the bases in the 16S rRNA participate in hydrogen bonding. These two studies suggest a secondary structure for the 16S rRNA in which neighboring nucleotide sequences 10 to 30 nucleotides long are complementary forming a set of hairpin loops. The 16S and the 23S rRNA's have approximately the same percentage of their bases in paired regions. Apparently this secondary structure is maintained whether the rRNA is associated with the ribosomal proteins in the ribosome or as the free polymer in solution (90). It has been observed that the combination of the rRNA with the ribosomal protein lends substantially more stability against denaturation by heat to the paired regions of the rRNA in solution (34, 60). The partial sequence of the 16S rRNA as well as that of the 5S contain several clustered positions that are variable (27, 66, 109). These are probably due to the multiple genes specifying the rRNA as was mentioned above. The primary structure of 16S, as was found for the 5S, appear to contain significant amounts of repetitive sequences (27).

The portions of the 16S rRNA that interact specifically with ribosomal proteins are being investigated by binding ribosomal proteins to purified 16S rRNA, and
then treating the aggregate with ribonuclease. The sections of RNA attached to the protein will not be attacked by the enzyme and later can be isolated and their nucleotide sequence determined (84, 85). In other experiments, fragments of rRNA of known sequence are tested for their ability to bind specific ribosomal proteins (111).

The possibility that certain proteins associated with the 30S ribosomal subunit could be present in less than unit amounts brought considerations that individual ribosomes might be distinguished according to their function (69). However, there is no extensive heterogeneity in the primary structure of rRNA that would suggest a functional differentiation of the ribosomes. As was noted before, minor variations have been observed in the primary structure of the rRNA's which can be attributed to the evolutionary independent changes in their multiple genes. Although it is not yet possible to compare the detailed structures of the 16S and 23S rRNA's from diverse bacteria, the degree of their similarities can be approximately known by DNA-RNA hybridization (95).

Proposed Research

The main purpose of this study was to determine the molecular weights of "native" 16S and 23S rRNA's, and of
the same polymers after their secondary structure had been denatured by treatment with formaldehyde. It was our intent to combine the results of sedimentation equilibrium experiments with those of accurately measured density increments. Of the first importance in our study was the obtaining of intact, homogeneous preparations of the rRNA's. Once these polymers are obtained in pure and stable form, their molecular weights can be measured with a precision of ± 3%. We had three principal reasons for choosing this particular problem, as follows: a) the molecular weights determined to date for the 16S and 23S rRNA's are not of the precision required in present investigations, b) the exact accounting of the ribosomal components (RNA and protein) requires precise molecular weights for the rRNA's, and c) the molecular weights of the rRNA's whose secondary structure was denatured with formaldehyde are needed for the calibration of a method to determine the molecular weights of RNA by polyacrylamide gel electrophoresis. The details of these considerations are given below.

The values obtained previously for the molecular weight of 16S rRNA range from $5.3 \times 10^5$ (62) to $5.6 \times 10^5$ (50, 91). The values for the 23S rRNA varied from $1.0 \times 10^6$ (62) to $1.1 \times 10^6$ (50, 91). These results were given with a precision of about ± 15%. There are
also significant discrepancies in some of the parameters used by the different workers in their calculations.

Recent studies by Kurland et al. (52), Voynow and Kurland (103), and Traut et al. (100), show the possibility of the 30S subparticles of the ribosomes of E. coli being heterogeneous in their protein complement. In other words, of the proteins known to be present in a given population of 30S subparticles not all will be found in each one of the subparticles. Since the weight of the 30S subparticle is known from precise sedimentation equilibrium experiments (41), having an accurate molecular weight for the 16S rRNA could help in clarifying the situation.

As was mentioned earlier, it was reported that the 16S and 23S rRNA's of E. coli could each be separated into several types according to their electrophoretic mobility in polyacrylamide gels (20, 83). Recent studies (20) suggest that this heterogeneity is the result of minor differences in the secondary structure of the RNA polymers in solution.

Recently a method has been proposed for the rapid conformation independent determination of the molecular weights of ribonucleic acids by gel electrophoresis (8). Using this method the secondary structure is destroyed irreversibly by heating at 63° in 1.1 M formaldehyde
before applying the RNA to the gel. A graph of the logarithm of the molecular weight of the RNA versus its relative electrophoretic mobility results in a straight line. The molecular weight for an unknown sample can be obtained from a calibration plot. Although the method does not have the precision of the determinations by sedimentation equilibrium, also a conformation-independent method, the shorter time required for each measurement will be an asset in various applications where values of higher precision are not needed. Other advantages are that a larger number of determinations can be made simultaneously and the equipment is less expensive. For the calibration of this method and the molecular weight of stable RNA preparations whose secondary structure has been eliminated by treatment with formaldehyde are indeed needed.
CHAPTER II

MATERIALS AND METHODS

Growth of Bacteria

Escherichia coli strain MRE 600 lacking the enzyme ribonuclease I (RNase I⁻) was utilized throughout the study. The microorganisms were grown in a fourteen liter Microferm fermenter. The media was composed of the following ingredients.

- Glucose -------------------10.00 grams/liter
- $\text{K}_2\text{HPO}_4$ ------------------- 9.80 grams/liter
- $\text{Na}_2\text{HPO}_4$ ------------------- 7.50 grams/liter
- Yeast Extract --------------- 5.00 grams/liter
- $\text{MgCl}_2$ ------------------- 0.042 grams/liter
- $\text{Na}_3\text{Citrate.3 H}_2\text{O}$ ------- 0.40 grams/liter
- $\text{(NH}_4\text{)}_2\text{SO}_4$ -------------- 0.40 grams/liter
- $\text{Fe (NH}_4\text{)}_2\text{(SO}_4\text{)}_2$ ------- 0.015 grams/liter
- $\text{CaCl}_2$ ------------------- 0.010 grams/liter

The cells were harvested in the middle of the logarithmic growth phase according to a graph of absorbance at 620 nm versus time plotted for each batch of bacteria. They were iced and harvested by centrifugation in a Sharples Supercentrifuge, and the resulting bacterial paste was stored in 100 gram portions in a Revco freezer.
at -70° until needed. Some of the bacteria used in the earlier experiments was supplied by Dr. James Young of the University of Wisconsin.

Isolation of the Ribosomes

A series of buffers at pH 7.4 were used to prepare the ribosomes. Their composition was as follows:

70S Buffer-------------0.07 M KCl
    0.01 M Tris-HCl
    0.01 M MgCl₂

65S Wash Buffer--------0.50 M NH₄Cl
    0.01 M Tris-HCl
    0.01 M MgCl₂

30/50 Buffer----------0.10 M KCl
    0.01 M Tris-HCl
    0.001 M MgCl₂

50S Buffer-----------0.02 M KCl
    0.01 M Tris-HCl
    0.0004 M MgCl₂

30S Buffer----------0.07 M KCl
    0.01 M Tris-HCl
    0.001 M MgCl₂

The procedure employed is similar to that described by Hill et al. (42) where 100 grams of the bacterial paste
were washed by suspending in a portion of 70S buffer and the volume was completed to 300 ml with the same buffer. The cells were sedimented at 12,000 x g for 10 minutes. The procedure above was repeated a second time. After the second wash, the compacted cells were mixed into a slurry with about 40 ml of glass beads of approximately 0.64 mm in diameter and 70S buffer to make a total of 130 ml. The slurry was transferred to the metallic cup of a Gifford-Wood Minimill set at 1.52 mm spacing. The solution was then ground for 30 minutes at 22,000 rpm with the metallic cup surrounded with a salt-brine and ice solution to keep the internal temperature less than 5°C during grinding. After grinding, the slurry was rapidly diluted to 300 ml with cold 70S buffer and centrifuged for 10 minutes at 12,000 x g to remove the glass beads. The supernatant was decanted and then centrifuged for 45 minutes at 48,000 x g to remove the larger cell debris. The supernatant was again decanted and then centrifuged at 250,000 x g for three hours in a Ti 60 rotor on a Beckman L3-50 Ultracentrifuge. The pellet of crude ribosomes was slowly suspended by stirring for 8-12 hours at 4°C in 100 ml 65S buffer. This suspension was centrifuged at 48,000 x g for 45 minutes and then decanted and spun again at 250,000 x g for three hours. The pellet of washed ribosomes was resuspended in 35 ml of 30/50
buffer and either used immediately or stored at -70°C until needed.

Isolation of the Ribosomal Subunits

While in the 30/50 buffer the 70S ribosome dissociated into the 30S and 50S subunits. The two kinds of ribosomal subunits were separated from each other by zonal ultracentrifugation (26) in a Ti 15 zonal rotor with a B-29 insert by using a 10-30% linear sucrose gradient. The gradient (800 ml) was loaded with the aid of an International Gradient Pump into the center of the rotor while it was spinning at 3,000 rpm. The remaining volume of the rotor (~700 ml) was completely filled with a 50% sucrose cushion pumped into the periphery of the rotor with a Gilson Minipuls pump. Approximately 70 ml of the dissociated subunits in 30/50 buffer were applied in a 10-0% inverse gradient to the center of the rotor. The 30/50 buffer was overlayed on the sample through the central conduct and the 50% sucrose cushion was forced out until approximately 150 ml of it remained in the rotor. The rotor was then spun at 120,000 x g for 9.5 hours at 4°C. The rotor was emptied by slowing it to 3,000 rpm and pumping 30/50 buffer into its center to force the gradient out beginning with the 50% sucrose cushion. Fractions of about 10 ml were collected on a Gilson fraction collector. A graph of the absorbance
at 254 mm versus the tube number was obtained from an OmniscrIBE recorder connected to a Chromotronix 220 absorbance detector.

Fractions corresponding to the 50S and 30S subunits were combined. Then sufficient 0.1 M MgCl₂ was added to each of the solutions containing the collected subunits to render them approximately 1.0 M in Mg²⁺, taking into consideration the increase in volume caused by the step that follows. The ribosomal subunits were pelleted by adding 2 volumes of 95% ethanol and centrifuging at 27,000 × g for 15 minutes. The pellets were resuspended in 5-10 ml of the corresponding buffer; the solutions were stored at -76° until needed.

The 30S and 50S subunits were tested for homogeneity on a Beckman Model E Analytical Ultracentrifuge and the pictures of the schlieren patterns were taken with Kodak Ortho Contrast film or on Kodak Metallographic plates.

**Isolation of the Ribosomal RNA**

The method of Stanley and Bock (91), with minor modifications, was used to extract the rRNA. All the operations were carried out at 0-4° except when other temperatures are indicated. Approximately 3 ml of a solution containing 20-30 mg/ml of the corresponding ribosomal subunit were transferred to a Corex glass tube, 0.15 ml of 10% SDS (w/v) was added and the tube was
brought to room temperature (\(\sim 20^\circ\)) and held for 5 minutes and then chilled to 0\(^\circ\) by swirling in an ice bath. Then 0.06 ml of 5\% Macaloid (w/v) were added, followed by 3 ml of phenol equilibrated with RNA buffer (0.05 M Tris-HCl, 0.1 M KCl, pH 7.0). The mixture was shaken gently but thoroughly for 5 minutes in a Vari-Whirl mixer. The phases were separated by centrifuging at 2,800 \(\times\) g for 10 minutes. The upper (aqueous) phase was removed with a Pasteur pipette whose tip had been cut to about 1/3 of its original length. Care was taken not to disturb the interfacial region during the withdrawal of the last portions of the liquid. The aqueous phase was transferred to a clean Corex tube, and Macaloid and buffer-equilibrated phenol were added as described above. The extraction was repeated twice. To the aqueous phase of the last phenol extraction was added 6 ml of 95\% ethanol; it was mixed well and the mixture was centrifuged at 20,000 \(\times\) g for 30 minutes to pellet the RNA. The supernatant was discarded and the rRNA was dissolved in 3 ml buffer and again precipitated with 6 ml of 95\% ethanol and centrifuged at 20,000 \(\times\) g for 30 minutes. The rRNA pellet was dissolved in RNA buffer to give a concentration of 5 to 12 mg/ml. The rRNA that was going to be treated with formaldehyde, as will be described hereafter, was dissolved in phosphate buffer (0.09 M \(\text{Na}_2\text{HPO}_4\), 0.01 M \(\text{NAH}_2\text{PO}_4\), pH 7.7) (7). Once the rRNA was extracted, all
the manipulations and measurements were carried out in a continuous operation. In all steps maximum precautions were taken to avoid contamination of glassware and equipment with ribonuclease.

Zone Velocity

The rRNA extracted from the 50S subparticle was separated into the 5S and 23S components by sedimentation on a 10-30% linear glycerol gradient by a technique adopted from Vedel and D'Aoust (102). In a typical run 0.3 ml of a 10 mg/ml rRNA solution was layered over the 35 ml of gradient contained in each tube. The tubes were centrifuged in a Ti 60 rotor of a Beckman L3-50 Ultracentrifuge at 4° for 3½ hours at 50,000 rpm. The rotor was stopped without braking. The bottom of the tube was punctured and the contents were passed through a Chromotronix 220 Absorbance Detector. The output of the detector was recorded as a function of tube number on an Omniscribe recorder connected to the detector. Fractions of 0.5 ml were collected in a Gilson fraction collector. The 23S rRNA was recovered from its corresponding fraction by addition of 2 volumes of ice cold 95% ethanol and centrifugation at 20,000 x g for 30 minutes. The pelleted RNA was slowly dissolved in the appropriate buffer as described in the preceding section on Isolation of rRNA.
Reaction with Formaldehyde

To denature the secondary structure of the rRNA, about 3 ml of 2-5 mg/ml samples in 1.1 M formaldehyde and 0.09 M Na$_2$HPO$_4$ + 0.01 M NaH$_2$PO$_4$ were heated to 63° for 15 minutes in a closed glass container, cooled rapidly in ice and used immediately (7). The object of the treatment was to modify chemically the amino groups of adenine, guanine and cytosine in such a way that the potential of these groups for forming hydrogen bonds is irreversibly lost (6). Apparently no other group in the polymer is affected. It has been suggested that the formaldehyde reacts with the amino groups of adenine, guanine and cytosine to form Schiff bases (-N=CH$_2$) (30).

Dialysis

Except for the sedimentation velocity measurements, all the samples of rRNA were studied using as reference the dialyzates of their solutions. Approximately 3 ml of the rRNA solution was dialyzed against 1000 ml of the appropriate buffer, described in the section on isolation of rRNA, for 20-24 hours at 4°C, the buffer being changed 3-4 times. The dialysis tubing in this study was ¼ inch Visking variety which was heated for three days in a drying oven at 100°C. This was followed by heating to a temperature of 60-80°C for two hours in ethylpyrocarbonate. The tubing was then placed in a solution of
5.4 \times 10^{-3} \text{ M EDTA}, 1.4 \times 10^{-2} \text{ M NaHCO}_3, 7 \times 10^{-3} \text{ M 2-mercaptoethanol and heated to 60°C for 30 minutes.}

After this treatment, the dialysis tubing was washed by five rinses of tap water followed by extensive rinsing with deionized distilled water, rinsing with distilled deionized water immediately before use.

**Density Increment**

The density increment, \( \left( \frac{\partial \rho}{\partial c} \right)_u \), at constant chemical potential of the diffusible components in the limit \( c \to 0 \) for each of the rRNA species was obtained from density and concentration measurements of a series of solutions. The densities of the dialyzates and of the rRNA solutions were measured with a Precision Density Meter DMA-02C manufactured by Anton Parr (Gratz, Austria) in accord with the design of Kratky et al. (49). The principle of the measurements is the variation of the natural frequency of a hollow oscillator when filled with liquids of different density. In actual measurements, the period \( T \), during a preset number of oscillations is measured by a timer controlled by a quartz crystal. The density of an unknown is measured by reference to a known standard (deionized distilled water in our case). The difference between the density of two samples is given by

\[
\rho_1 - \rho_2 = \frac{1}{A} (T_1^2 - T_2^2)
\]
Where A is an instrument constant obtained from calibration with salt solutions of density previously determined pycnometrically at $4 \pm 0.005^\circ$, the temperature of the experiment. The temperature was controlled by a thermostatic assembly that consisted of a Lauda K4/RD refrigerated circulator in conjunction with a Haake FS pump. The DMA 02C density meter was interfaced with a Wang 600 programmed calculator which controlled the resetting and starting of the measurements through a time delay circuit.

The measurements for our study were made as follows: The instrument was allowed to warm up for about three hours. It was considered stabilized and ready for measurement when no variation greater than $+2 \times 10^{-5}$ sec at a preset count of $1 \times 10^4$ was observed in at least 10 readings in 2 consecutive water samples. Once the instrument was stabilized, the cell was emptied and flushed with two 5 ml portions of absolute ethanol. The cell was air-dried with the pump of the instrument until the readings on the instruments were constant. The sample was then carefully introduced into the cell with a syringe, avoiding the generation of air bubbles. Thirty minutes were allowed for thermal equilibration of the sample before measurements were started. After suitable data were obtained, the sample was withdrawn with a syringe and the cell washed with about 50 ml of distilled deionized water.
With the cell filled with distilled deionized water, 30 minutes were let to elapse for equilibration and then ten readings were taken; these readings were averaged with those taken prior to the sample and this average then used in the calculations of the density of the sample. The value of the density procured for each solution was plotted versus its respective concentration which was determined by dry weight analysis. The best line through the points was determined by the method of least squares in a Wang 600 calculator. The slope of the resulting straight line is the quantity \( \frac{\partial \rho}{\partial C} \) sought. It was used in combination with the results of sedimentation equilibrium experiments to obtain the molecular weights.

Concentration Measurements and Extinction Coefficients

The concentrations by dry weight analysis were obtained by weighing 3-6 ml of the rRNA solutions and dialyzates into separate glass stoppered flasks, lyophilizing for 24-36 hours followed by drying in a Thelco Model 19 vacuum oven at 98-100° to constant weight (41). The volume of the solution was calculated from the weight of the liquid and its previously measured density as stated in the previous section on Density Increment.

The extinction coefficients at 260 nm for 1% solutions of rRNA at room temperature were calculated
from the absorbance and concentrations by dry weights of
diazyed solutions. Subsequently, the concentrations
of the rRNA solutions were routinely determined using
those extinction coefficients and the absorbance of the
solution measured in a Cary Model 15 recording spectro-
photometer.

Sedimentation Properties

To obtain the sedimentation coefficients extrapolated
to zero concentration, a series of sedimentation velocity
experiments were made at different concentrations of the
particular rRNA being studied. These were made in a
Beckman Model E Analytical Ultracentrifuge equipped with
schlieren optics using an AN-D type rotor spun at 60,000
rpm. Five pictures were taken at four or eight minute
intervals, starting 3 minutes after reaching speed. The
pictures were taken on Kodak Metallographic glass plates,
and the peaks on them were measured using a Nikon Model
6C microcomparator.

The sedimentation coefficient, $S$, was calculated
on a Wang 600 calculator from the equation

$$
S = \frac{\ln r_D(t) - \ln r_D(t_o)}{\omega^2 (t - t_o)}
$$

$r_D(t)$ being the position of the peak at time $t$ and $\omega$
is the angular velocity. The resulting values were
corrected to $20^\circ$ in water using the formula
being \( \bar{\nu} \) the partial specific volume of the rRNA, \( \rho \) the density of the solution, and \( \eta \) the viscosity of the solution. The subscript \( T \) is temperature of the experiment, \( w \) stands for water, \( b \) stands for buffer and 20 for 20°C.

A correction for radial dilution was made according to the equation.

\[
C_t = C_0 \left( \frac{r_o}{r_t} \right)^2
\]

where \( C_0 \) is the concentration at time zero, \( r_o \) is the distance from the center of rotation to the miniscus, \( C_t \) is the concentration at time \( t \), and \( r_t \) is the distance from the center of rotation to the schlieren peak. The values for \( r_t \) and \( r_o \) were measured in the third picture in the series as a way of getting an average. The concentration of the rRNA solutions at zero times were calculated from dry weight determinations.

The values for the \( S_{20,w} \) were plotted versus their corresponding concentrations, and the best line through the points was obtained by making a least square fit in a Wang 600 programmable calculator. The extrapolation of the resulting line to zero concentration gave \( S_{20,w}^0 \), the sedimentation coefficient.
The high-speed equilibrium or meniscus depletion technique of Yphantis (110) was used for the sedimentation equilibrium experiments in a Beckman Model E Analytical Ultracentrifuge equipped with Rayleigh interference optics. Three cavities of multichannel cell were filled with 0.10 ml of RNA solutions of concentrations 0.4, 0.5 and 0.6 mg/ml respectively, giving column heights of about 3 mm. The three corresponding reference compartments were filled with 0.11 ml of the respective dialyzates. The cell was spun in an AN-J rotor for 18-24 hours at 6,800 rpm for the 16S rRNA and at 5,200 for the 23S rRNA. The temperature of the run was 4°C. The interference patterns were photographed on Kodak II-6 spectroscopic plates. Displacements were measured for at least five fringes at spacings of 50-100μ in a Nikon Model 6C Microcomparator with a 50 x objective.

The values for the density increment \( \frac{\partial \rho}{\partial c} \) were used in conjunction with the sedimentation equilibrium results to calculate the molecular weights according to the equation

\[
M = \frac{2RT}{\left( \frac{\partial \rho}{\partial c} \right)_{\mu}} \frac{d \ln c}{dr^2}
\]

where \( R \) is the gas constant, \( T \) the temperature of the run, \( c \) the concentration of the RNA salt in the solution,
\[ \omega \text{ the angular velocity and } r \text{ is the distance from the axis of rotation.} \]

Calculations were made using the PDP-10 computer with a program originating from Dr. Robert Dyson of Oregon State University. First the data were smoothed and then used to get the weight-average molecular weights at various points across the solution column.
CHAPTER III

RESULTS

Preparation of the rRNA's

The 16S rRNA extracted from the 30S particle by the SDS-phenol procedure appeared as a single peak in the schlieren pattern when analyzed by sedimentation velocity centrifugation, Plate I(a). At this point, the purity of the material was considered satisfactory for the subsequent studies.

Since the rRNA extracted from the 50S by the same procedure above consists of not only the 23S rRNA but also the 5S rRNA, the latter being an integral part of the subunit, the corresponding sedimentation pattern for this material, Plate I(b), shows a small peak near the meniscus for the 5S rRNA. One can also observe a smaller peak of material sedimenting at 14-16S. This is always present and probably results from the breakage of the 23S molecule into segments of large size during the extraction procedure. The mixture of the rRNA's extracted from the 50S particle was purified in a 10-30% glycerol gradient giving the results shown in Figure 1. In this graph of absorbance at 254 nm versus the tube number, the first peak from the left to right is the
Plate I. Schlieren patterns of rRNA's obtained from sedimentation velocity analysis: a) 16S rRNA as extracted from the isolated 30S ribosomal subunit, b) 23S rRNA as extracted from the isolated 50S ribosomal subunit, c) 23S rRNA of pattern I(b) after purification by zone velocity in a 10-30% glycerol gradient, d) 16S rRNA after treatment with formaldehyde and, e) purified 23S rRNA treated with formaldehyde. The pictures for the formaldehyde-treated samples (d,e) were taken 35 minutes after reaching the speed of 60,000 rpm at 4°C and a phase plate angle of 75°. The remaining pictures (a,b,c) were taken 19 minutes after reaching speed. Sedimentation is from left to right.
closest to the bottom of the centrifuge tube and corresponds to the 23S rRNA; the peak following is that of the breakage products of the 23S and the peak closest to the meniscus represents the 5S rRNA. Plate I(c) is the schlieren pattern of the 23S rRNA collected from the zone velocity purification just described. As with the 16S rRNA, Plate I(a), the sharp peak is an indication of the sample being homogeneous with no small molecular weight contaminants present.

**Treatment with Formaldehyde**

The treatment of purified 16S and 23S rRNA with formaldehyde did not affect the homogeneity of the samples and no small molecular weight contaminants were produced that could be detected by sedimentation velocity centrifugation. The single peaks for the formaldehyde treated samples are shown in Plate I(d) for the 16S, and in Plate I(c) for the 23S rRNA. Any hidden scission in the chain of these polymers will appear in the form of individual peaks after the conversion to the random coil form by the formaldehyde treatment.

**Density Increment**

The densities and concentrations of the different rRNA solutions used in this study are presented in Table I. The density increment at constant chemical
Figure 1. Purification of the 23S rRNA, extracted from the 50S ribosomal subunit, on a 10-30% glycerol gradient.
Table 1
Concentrations and Densities of rRNA Solutions

<table>
<thead>
<tr>
<th></th>
<th>Concentration (mg/ml)</th>
<th>I</th>
<th></th>
<th>Concentration (mg/ml)</th>
<th>II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\rho$ (g/cm$^3$)</td>
<td></td>
<td></td>
<td>$\rho$ (g/cm$^3$)</td>
<td></td>
</tr>
<tr>
<td>16S</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.00750</td>
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<td>1.00750</td>
<td>0.00</td>
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<tr>
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<td>1.00855</td>
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<td>1.00889</td>
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<td>16S-formaldehyde</td>
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<td></td>
</tr>
<tr>
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<td>1.02216</td>
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<td>1.02198</td>
<td>0.00</td>
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</tr>
<tr>
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<td>1.02274</td>
<td>1.41</td>
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<td>2.29</td>
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<td>1.02302</td>
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<tr>
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<td>1.02392</td>
<td>4.34</td>
<td>1.02385</td>
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<td>23S</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>1.00832</td>
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</tr>
<tr>
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<td>1.00841</td>
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<td></td>
<td>1.01119</td>
<td>6.32</td>
<td>1.00958</td>
<td>4.35</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23S-formaldehyde</td>
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<td></td>
</tr>
<tr>
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<td>1.02244</td>
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<td>0.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.02344</td>
<td>2.28</td>
<td>1.02343</td>
<td>3.30</td>
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</tr>
<tr>
<td></td>
<td>1.02395</td>
<td>3.42</td>
<td>1.02485</td>
<td>6.56</td>
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<td>1.02469</td>
<td>5.12</td>
<td></td>
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<tr>
<td></td>
<td>1.02547</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

I and II correspond to buffers of slightly different densities.
potential of the diffusible components, \( \left( \frac{\partial \rho}{\partial c} \right)_\mu \), was taken as the slope of a plot of the density of the rRNA solutions at 4.000 ± 0.005°C versus the rRNA concentration from Table 1. The plot for the 16S rRNA is shown in Figure 2a; the plot corresponding to the formaldehyde-treated sample of the same polymer is depicted in Figure 2b. Figures 3a and 3b correspond respectively to the untreated and formaldehyde-treated sample of the 23S rRNA. The resultant \( \left( \frac{\partial \rho}{\partial c} \right)_\mu \) for the two species of high molecular weight rRNA together with the results of the formaldehyde-treated samples are recorded in Table 4. Were we justified in calculating values for the partial specific volume, \( \bar{v} \), from the expression

\[
\bar{v} = \frac{1}{\rho_o} \left( 1 - \left( \frac{\partial \rho}{\partial c} \right)_\mu \right)
\]  

the results compared to those used by Kurland (50) and Stanley and Bock (91) are shown in Table 2.

**Table 2**

<table>
<thead>
<tr>
<th>Partial Specific Volumes (( \bar{v} )) of the rRNA's</th>
<th>16S rRNA</th>
<th>23S rRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kurland</td>
<td>0.57 ml/g</td>
<td>0.57 ml/g</td>
</tr>
<tr>
<td>Stanley and Bock</td>
<td>0.53</td>
<td>0.53</td>
</tr>
<tr>
<td>This study</td>
<td>0.577</td>
<td>0.540</td>
</tr>
</tbody>
</table>
Figure 2. Plot of density versus concentration for the 16S rRNA at 4°C: a) untreated, and b) formaldehyde-treated. (△) and (•) correspond to buffers of slightly different densities.
Figure 3. Plot of density versus concentration for the 23S rRNA at 4°C: a) untreated, and b) formaldehyde-treated. (▲) and (●) correspond to buffers of slightly different densities.
Extinction Coefficients

The extinction coefficients, $E_{1\text{cm}}^{1\%}$, at 260 nm calculated for the different rRNA species studied (16S and 23S rRNA's, treated and untreated with formaldehyde) are also presented in Table 4. The values obtained for both the 16S and 23S treated with formaldehyde are similar. For comparative purposes, the spectrum from 220-600 nm of the formaldehyde treated and untreated RNA are presented in Figure 4a for the 16S, and in Figure 4b for the 23S. From information obtained elsewhere (17), we have calculated the extinction coefficient of a 1 mg/ml solution of the free four major nucleotides present in the same proportion as in high molecular weight rRNA to be 29.8 whereas the average measured extinction coefficient for the single stranded polynucleotide chain is 28.3. This results in a residual hypochromicity of about 5% which is the same value ascribed by various investigators (29) to the single-stranded base stacking of polynucleotide chains in solution.

Sedimentation Coefficients

The values of $s_{20,w}$ obtained at different concentrations by sedimentation velocity studies are presented in Table 3. These values were plotted for each species of rRNA and extrapolated to infinite dilution as shown in Figure 5. The resultant $s_{20,w}^0$ values are recorded in Table 4.
Figure 4. Spectrum of absorbance of the rRNA's from 220 to 300 nm: a) samples of the same concentration of untreated (I) and formaldehyde-treated (II) 16S rRNA, and b) untreated (III) and formaldehyde-treated (IV) 23S rRNA.
Table 3

Concentrations and $S_{20,w}$ Values for rRNA

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>$S_{20,w}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>16S</strong></td>
<td></td>
</tr>
<tr>
<td>0.41</td>
<td>18.1</td>
</tr>
<tr>
<td>0.86</td>
<td>18.1</td>
</tr>
<tr>
<td>2.70</td>
<td>17.3</td>
</tr>
<tr>
<td>4.55</td>
<td>16.6</td>
</tr>
<tr>
<td>5.78</td>
<td>16.0</td>
</tr>
<tr>
<td><strong>16S-formaldehyde</strong></td>
<td></td>
</tr>
<tr>
<td>0.61</td>
<td>10.8</td>
</tr>
<tr>
<td>1.15</td>
<td>10.3</td>
</tr>
<tr>
<td>2.29</td>
<td>9.4</td>
</tr>
<tr>
<td>3.32</td>
<td>9.0</td>
</tr>
<tr>
<td>4.76</td>
<td>8.2</td>
</tr>
<tr>
<td><strong>23S</strong></td>
<td></td>
</tr>
<tr>
<td>0.65</td>
<td>24.7</td>
</tr>
<tr>
<td>1.20</td>
<td>24.3</td>
</tr>
<tr>
<td>2.50</td>
<td>23.7</td>
</tr>
<tr>
<td>3.88</td>
<td>23.3</td>
</tr>
<tr>
<td>5.39</td>
<td>22.2</td>
</tr>
<tr>
<td><strong>23S-formaldehyde</strong></td>
<td></td>
</tr>
<tr>
<td>1.40</td>
<td>13.3</td>
</tr>
<tr>
<td>2.62</td>
<td>12.8</td>
</tr>
<tr>
<td>4.18</td>
<td>11.6</td>
</tr>
<tr>
<td>6.21</td>
<td>10.8</td>
</tr>
</tbody>
</table>
Figure 5. Extrapolation to infinite dilution of the $s_{20,w}$ values obtained for the 23S rRNA (x), the 16S rRNA (•), the formaldehyde-treated 23S (■), and the formaldehyde-treated 16S rRNA (▲).
Molecular Weights

A typical interference pattern obtained from sedimentation equilibrium studies is pictured in Plate II. A multichannel centerpiece that allows the simultaneous study of three solution-dialyzate pairs was used. This particular measurement corresponds to the 23S rRNA. The pictures of each cavity are actual representations of the concentration of solute (in fringe numbers) versus the radial distance from the meniscus for each solution. The meniscus for each solution is the vertical line to the right of each picture. The centrifugal force is exerted from right to left. The linearity of the fringes for about half of the length of the column in each case indicates the absence of lower molecular weight products.

Combining the results from sedimentation equilibrium with the respective values for the density increment, and using a computer program, the weight-average molecular weights at various points across the solution column were obtained.

In sedimentation equilibrium studies of the 16S it was found that when the sample had been stored frozen at -76°C and later thawed for use, some degradation occurred, giving a much lower molecular weight in the meniscus region. These results were therefore not used, and all the molecular weights were obtained using fresh preparations. Similarly, early samples of the 23S rRNA
Plate II. Interference pattern from sedimentation equilibrium analysis of the 23S rRNA. This picture, obtained after a 7 minute exposure, represent three solution-dialyzate pairs. The velocity used was 5,200 rpm for 26 hours at 4°C. The centrifugal force is exerted from right to left. The concentrations of the rRNA solutions, from left to right, are about 0.4, 0.5 and 0.6 mg/ml respectively.
produced molecular weights in the neighborhood of those reported here, but they increased slowly and continuously toward the bottom of the cell. We ascribe these results to non-equilibrium conditions; but we were not able to determine the exact source of the disturbance. These results were not used, but only those that gave a flat curve across the solution column. The plot of the weight-average molecular weights versus the concentration in fringe numbers for each of the rRNA's studied is presented in Figure 6: a) for the 16S and 23S, and b) for both species after they are treated with formaldehyde. Three distinct preparations of approximately 0.4, 0.5 and 0.6 mg/ml were used in each determination. The values for the weight-average molecular weight obtained are presented in Table 4.

**Degree of Unfolding of the rRNA Chains**

We can obtain an idea of the extent of the unfolding of the rRNA molecules after the treatment with formaldehyde by calculating the axial ratios (a/b) for ellipsoids of revolution from data already tabulated (82). This can be done for both the "native" and formaldehyde treated samples of rRNA from the values previously determined (Table 4) for the molecular weight (M), the partial specific volume (\( \bar{\nu} \)), the density of the dialyzate (\( \rho_o \)), and the sedimentation coefficients (\( S_{20,w}^o \)).
Figure 6. Weight-average molecular weights versus concentration in fringe numbers (j): a) 16S and 23S rRNA's, and b) 16S and 23S rRNA's after treatment with formaldehyde. (•), (x) and (Δ) represent different preparations of concentration about 0.4, 0.5 and 0.6 mg/ml respectively.
### Table 4

Physical Characteristics of the rRNA's

<table>
<thead>
<tr>
<th>rRNA</th>
<th>$(\frac{\Delta \rho}{\Delta C})$</th>
<th>$E_1$ $\text{mg/ml}$ at 260 nm</th>
<th>$S_{20,w}^o$</th>
<th>$M_w$</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S (K salt)</td>
<td>0.418</td>
<td>$20.7 \pm 0.2$</td>
<td>$18.4 \pm 0.2$</td>
<td>$(6.40 \pm 0.19) \times 10^5$</td>
</tr>
<tr>
<td>16S-formaldehyde (Na salt)</td>
<td>0.405</td>
<td>$28.5 \pm 0.3$</td>
<td>$11.0 \pm 0.1$</td>
<td>$(6.15 \pm 0.18) \times 10^5$</td>
</tr>
<tr>
<td>23S (K salt)</td>
<td>0.456</td>
<td>$22.3 \pm 0.2$</td>
<td>$25.0 \pm 0.2$</td>
<td>$(1.10 \pm 0.03) \times 10^6$</td>
</tr>
<tr>
<td>23S-formaldehyde (Na salt)</td>
<td>0.438</td>
<td>$28.0 \pm 0.3$</td>
<td>$14.1 \pm 0.2$</td>
<td>$(1.08 \pm 0.03) \times 10^6$</td>
</tr>
</tbody>
</table>
First, one wants to combine equations

\[ f = \frac{M}{S} \left(1 - \frac{\sqrt[3]{V_{p_q}}}{S}\right), \]  

(3)

\[ R = \left(\frac{3M\bar{v}}{4\pi N}\right)^{1/3} \]  

(4)

and \[ f_o = 6\pi \eta \rho \]  

(5)

into the equation for the frictional ratio

\[ \frac{f}{f_o} = \frac{1}{130.6} \frac{M^{2/3}(1-v_{p_q})}{S_{20,w}(\bar{v})^{1/3}} \]  

(6)

In the equations above, \( f \) is the frictional coefficient of the macromolecule, \( R \) and \( f_o \) are the radius and the frictional coefficient respectively of a sphere of the same volume as the macromolecule, \( \pi \) is 3.1416, and \( \eta \) is the viscosity of the solvent.

The values for the frictional ratios \( f/f_o \) calculated with the equation above are taken to a Table (82) where the corresponding axial ratios for oblate and prolate ellipsoids are compiled. The results are presented in Table 3 for comparison. Although the structure of the rRNA's in solution is almost certainly not an ellipsoid of revolution, this calculation helps us visualize the degree of unfolding of the molecules once their secondary structure is denatured with formaldehyde.
Table 5

<table>
<thead>
<tr>
<th>rRNA</th>
<th>Axial Ratio (a/b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prolate</td>
</tr>
<tr>
<td>16S (K salt)</td>
<td>10.0</td>
</tr>
<tr>
<td>16S-formaldehyde (Na salt)</td>
<td>31.3</td>
</tr>
<tr>
<td>23S (K salt)</td>
<td>15.4</td>
</tr>
<tr>
<td>23S-formaldehyde (Na salt)</td>
<td>52.2</td>
</tr>
</tbody>
</table>

Relationship between \( s_{20,w}^o \) and Molecular Weights for Formaldehyde treated RNA

The reaction with formaldehyde has been used to denature the secondary structure of the RNA converting the polynucleotides into random coils that constitute an homologous family for which a single equation can be obtained relating the logarithm of the sedimentation coefficient, \( s_{20,w}^o \), to that of the molecular weight (7). In the traditional manner, from the determined \( s_{20,w}^o \) and the weight-average molecular weights for the two species of high molecular weight RNA treated with formaldehyde we have derived an equation which should apply to all RNA samples treated in the same way as the ones we have used in these experiments. We used our derived equation,

\[
s_{20,w}^o = 0.312 w^{0.440}
\]  

(7)
to calculate the molecular weights of TMV RNA and R 17 RNA from sedimentation data quoted by Boedtker (7) for the formaldehyde treated samples. The resulting value of $1.67 \times 10^6$ for the TMV RNA and of $1.02 \times 10^6$ for the R 17 RNA are on the average 14% higher than the values given by Boedtker, although they are within the experimental error of her determinations. Also in the derivation of our equation we used our value of $6.15 \times 10^5$ for the molecular weight of the 16S rRNA which is 12% higher than the $5.5 \times 10^5$ used by Boedtker. We have to emphasize that the relation above is valid only when the RNA samples have been treated with formaldehyde in the manner previously described in the Materials and Methods section, and the specific counter-ion concentration, namely $0.09 \text{ M } \text{Na}_2\text{HPO}_4 + 0.01 \text{ M } \text{NaH}_2\text{PO}_4$, is maintained.
CHAPTER IV

DISCUSSION

Earlier physical studies on the 16S and 23S rRNA's from Escherichia coli have produced results that are internally inconsistent and vary significantly from worker to worker. Since the differences in these previous studies appear to be caused by contaminated or polydisperse samples, our main objective was to carry out the planned measurements on pure homogeneous preparations. As can be seen by the reproducibility of the various measurements, we were successful in obtaining suitable preparations in a consistent manner. The comparison of the results obtained in our study with those obtained by other workers can best be made by detailing the different techniques used and the data gathered in each investigation.

In the determination of the molecular weights of the rRNA's, Kurland (50) was unable to obtain monodisperse samples of the polymers which forced him to use two indirect approaches for his calculations. First, he combined the sedimentation coefficients from sedimentation velocity with their respective weight-average molecular weight obtained from light scattering resulting in the empirical equation
\[ s_w = 0.98 \times 10^{-2} M_w \]  

(8)

where \( s_w \) and \( M_w \) are the sedimentation coefficients and the molecular weights determined experimentally. The molecular weights obtained from equation (8) for the 16S and 23S rRNA's were \( 5.6 \times 10^5 \) and \( 1.1 \times 10^6 \) with an error of \( \pm 12\% \). In the second approach, Kurland calculated an average value of \( 2.25 \times 10^6 \) for the shape-volume coefficient using the Scheraga-Mandelkern (86) equation

\[ M_w^{2/3} = \frac{s[\eta]^{1/3} \eta_o N}{\beta (1-\bar{V} \rho)} \]  

(9)

where \([\eta]\) is the intrinsic viscosity of the rRNA solution, \( \eta_o \) is the viscosity of the solvent, \( N \) is Avogadro's number, \( \bar{V} \) is the partial specific volume of the rRNA, \( \rho \) is the density of the solution, and \( s \) and \( M_w \) have the customary meaning. He, then plotted \( \log s_w \) versus \( \log [\eta] \), to obtain values of 0.305 and 0.445 dl/gm for the intrinsic viscosity of the 16S and 23S peaks respectively. Inserting these values back into equation (9), molecular weights of \( 5.6 \times 10^5 \) and \( 1.15 \times 10^6 \) are obtained with an error of \( \pm 15\% \).

Stanley and Bock (91) assumed a partial specific volume of 0.53 ml/gm for the two species of RNA and a shape-volume coefficient \( (B) \) of \( 2.16 \times 10^6 \). These values combined with the sedimentation coefficients \( (s_{20,w}) \) and
the intrinsic viscosities, $[\eta]$, of 0.248 dl/gm for the 16S and 0.379 dl/gm for the 23S in the Scheraga-Mandelkern equation produced molecular weights of $5.5 \times 10^5$ and $1.07 \times 10^6$ for the 16S and 23S species respectively.

As is evident, the values of the partial specific volume ($\bar{\nu}$), the shape-volume coefficient ($\beta$), and the intrinsic viscosities ($[\eta]$) used by Stanley and Bock are considerably lower than the ones used by Kurland (50). However when used in the Scheraga-Mandelkern equation they produced molecular weights which are similar with those reported by Kurland. Stanley and Bock (91) performed a second measurement of the molecular weight of the 23S; using the partial specific volume of 0.53 ml/gm together with the results of sedimentation equilibrium experiments yielded a molecular weight of $1.07 \times 10^6$ for the 23S rRNA. Although there is agreement between the two studies with respect to the molecular weights of the rRNA's, it is obvious that there are major differences in the parameters used to calculate them. These differences can be attributed to the lack of accurate data and/or contaminated samples (71).

Still lower values for the molecular weights of the rRNA's were obtained by Midgley (62). The method used in his determinations was end-group analysis based in the stoichiometry of the reaction of periodate oxidized with (carbonyl-$^{14}$C) isonitonic acid hydrazide. There are
two apparent reasons why the values obtained by Midgley should be smaller. The first is that the molecular weights obtained by Midgley were number-average molecular weights which for a polydisperse sample are always smaller than the weight-average molecular weight as obtained by Kurland (50) and Stanley and Bock (91). The second reason is that the molecular weights measured by Kurland and Stanley and Bock were those of the sodium and potassium salts of the RNA respectively (see Van Holde and Hill, 101) while the weights determined by Midgley correspond to the polynucleotide chain alone. However the difference between the values reported by these workers is significant and would probably indicate a greater degree of degradation in the preparations used by Midgley.

Kurland (50), and Stanley and Bock (91) used one single value of the partial specific volume for both species of rRNA. In our study, for the first time, measurements were made independently for each one of the purified rRNA's. We measured the density increment at constant chemical potential of the diffusible component at $c_1 \to 0$, $(\frac{\partial \rho}{\partial c_1})^0_{\mu_1}$ Table 4. These values can be substituted directly for the term $(1-v_0)$ used by others workers in the sedimentation equilibrium equation (1). With our procedure we have followed the requirements of
the thermodynamic treatment advanced by Casassa and Eisenberg (16). This treatment establishes that since at dialysis equilibrium the chemical potential of the solvent on both sides of the membrane is the same. When the concentrations of the dialyzable component, 2, on each side of the membrane are set equal, the non-diffusible component, 1, must be redefined to account for the excess or deficiency of component 2. To a good approximation, or redefined non-diffusible component would be the sodium or potassium salt of the nucleic acid (19). Our results for the density increment indicate at first approximation a more compact structure in solution for the 16S rRNA than for the 23S rRNA as has been reported by others (37). However, a more precise determination of this characteristic would require a thorough investigation. The considerable difference between or measured individual values for the density increment of the 16S and 23S rRNA's (Table 4 and Table 2) denote that the values for the partial specific volume ($\tilde{\gamma}$) used in previous studies (50, 91) are probably in error. Since for most proteins in water it is possible to calculate an accurate partial specific volume from the amino acid constitution (53), our system with the rRNA's presents an opportunity to investigate the same possibility with nucleic acids, and in case of negative results, acquire
Table 6

Molecular Weights of the 16S and 23S rRNA's by Four Different Methods

<table>
<thead>
<tr>
<th>Method</th>
<th>$M_w \times 10^{-6}$</th>
<th>16S</th>
<th>23S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedimentation-viscosity (50)</td>
<td></td>
<td>0.56 $\pm$ 0.08</td>
<td>1.15 $\pm$ 0.17</td>
</tr>
<tr>
<td>Sedimentation-light scattering (50)</td>
<td></td>
<td>0.56 $\pm$ 0.07</td>
<td>1.10 $\pm$ 0.13</td>
</tr>
<tr>
<td>Sedimentation-viscosity (91)</td>
<td></td>
<td>0.55</td>
<td>1.07</td>
</tr>
<tr>
<td>Periodate-isonicotinic acid hydrazide (62)</td>
<td></td>
<td>0.53 $\pm$ 0.02</td>
<td>1.00 $\pm$ 0.03</td>
</tr>
<tr>
<td>Sedimentation equilibrium (91)</td>
<td>---</td>
<td></td>
<td>1.07</td>
</tr>
<tr>
<td>Sedimentation equilibrium (a)</td>
<td></td>
<td>0.640 $\pm$ 0.019</td>
<td>1.10 $\pm$ 0.03</td>
</tr>
</tbody>
</table>

The numbers in parenthesis correspond to references listed; (a) represents the results of this study.
a fundamental knowledge of the factors (47, 78) that affect the property for this type of biopolymer.

The molecular weight of $6.4 \times 10^5$ determined in the present study for the 16S rRNA is significantly higher than the $(5.3 - 5.6) \times 10^5$ previously reported by Kurland (50), Stanley and Bock (91), and Midgley (62). Our value of $1.1 \times 10^6$ for the molecular weight of the 23S rRNA is similar to the values obtained by Kurland (50) and Stanley and Bock (91) but 10% higher than the value of $1.0 \times 10^6$ given by Midgley (62). We are confident that the homogeneity of our preparations together with the increased precision afforded by our techniques produced results that are of greater reliability. For comparison, these results are presented, together with the techniques used to obtain them, in Table 6. The molecular weights obtained here indicate that there are around 1770 nucleotides in the chain of the 16S rRNA and about 3030 nucleotides in the chain of the 23S rRNA. Using our values for the molecular weights of the K salt of the 16S and 23S rRNA's, we calculated the molecular weight of the polynucleotide chains of "naked" rRNA's to be $5.7 \times 10^5$ and $9.8 \times 10^5$ respectively. These results will help in the exact accounting of the diverse component of the ribosome, each one of which appear to be of the utmost importance for the functioning of the protein synthesizing machinery of the cell (51).
The folding of the RNA is not only determined by the degree of hydrogen bonding between the chains but also by the surrounding cations (30). Consequently it is not surprising that there is not a widely applicable formula relating the sedimentation coefficient to its molecular weight, although several empirical relations have been derived (7, 50) each applying to a particular set of conditions and based on limited number of types of RNA. We have discussed the derivation of the empirical equation used by Kurland (50) in his determination of the molecular weights of the rRNA's. The difficulty in obtaining molecular weights by this method became obvious when it was found that two RNA's differing in molecular weights by a factor of two, had the same sedimentation coefficient when measured under identical conditions (7). It thus became clear that the main chain configuration of a particular RNA depended not only on its overall chain length but also on its specific secondary structure. Hence various samples could not be assumed to compose a homologous series and no one relation between sedimentation coefficient and molecular weight could be presumed to be valid for all ribonucleic acids. The problem of obtaining an accurate molecular weight calibration can be solved by measuring the sedimentation coefficient of the RNA under conditions which eliminate the secondary structure.
Formaldehyde is a very convenient denaturant, because RNA is completely denatured very rapidly at relatively low formaldehyde concentrations (6). Thus the secondary structure can be eliminated without significantly diluting the RNA solution, and physical studies can be carried out in an essentially aqueous medium. Under such conditions, all RNA samples should be homologous and a unique dependence of the sedimentation coefficient on molecular weight should exist. The relationship \( s_{20,\text{w}}^0 = 0.0312 M^{0.440} \) we have derived from the sedimentation values, \( s_{20,\text{w}'}^0 \), and the molecular weights of the 16S and 23S rRNA's treated with formaldehyde should apply to the determination of the molecular weights of RNA's under the same conditions.

Polyacrylamide gel electrophoresis has been used to successfully obtain the molecular weights of polypeptide chains after prior denaturation in 1% sodium dodecyl sulfate at elevated temperatures (105). Gel electrophoresis has also been used to obtain the molecular weights of RNA molecules in conditions where the native conformation remains intact (5, 55). The latter molecular weight determinations have serious limitations since the mobility of the RNA depends not only on its molecular weight but on its molecular volume, and RNA molecules with the same molecular weight have been shown to have different effective volumes (35). Hence it is likely
that the rate of migration of all RNA species will not be proportional to the log of the molecular weight because the former will depend on the extent of the folding or unfolding of the RNA's being examined.

Since it would clearly be useful to have a rapid conformation independent method of obtaining RNA molecular weights, electrophoretic mobilities of RNA were measured after denaturation. Both formaldehyde and urea were used as denaturing agents, but only the former results in fully denatured RNA at room temperature (8). A conformation independent method for the determination of the molecular weights by polyacrylamide gel electrophoresis has been proposed (8) which is both rapid and require very small amounts of sample. It needs to be standardized with the molecular weights of stable and readily available RNA's. For this purpose we have determined the molecular weight by sedimentation equilibrium for the 16S and 23S rRNA's treated with formaldehyde to destroy the secondary structure, the molecular weights are $6.15 \times 10^5$ and $1.08 \times 10^6$ for the 16S and 23S respectively, as with the previous determinations, the measurement of the molecular weights was the result of the combination of the density increment together with the results of sedimentation equilibrium measurements. By having a calibration curve of the logarithms of the molecular weights of different RNA species it is possible
to determine the molecular weights of other RNA's by first treating them with formaldehyde to destroy their secondary structure and then subjecting them to polyacrylamide-gel electrophoresis. Using the same system, their electrophoretic mobility will correspond to a given molecular weight in the graph since this graph has been built with molecular weights and electrophoretic mobilities of known RNA's. Our determinations of the molecular weights of the ribosomal RNA's should provide an accurate calibration for the curves in the determination of the molecular weights of ribonucleic acid by means of polyacrylamide-gel electrophoresis of samples previously treated with formaldehyde.

Ceri and Maeba (18) have reported extensive degradation of the 23S rRNA extracted by the SDS-phenol method from the isolated 50S subunit. This they attributed to the nuclease activity of a protein bound to the rRNA. We have not encountered such difficulties. However, in our preliminary work with the 16S rRNA (71), we found that in all the cases where the sample has been frozen and stored, some degradation occurred. We circumvented this problem by making the measurements on fresh samples and in a continuous operation. Ceri and Maeba (18) stored their samples at -20°C usually as ethanol precipitates.
The values for the sedimentation coefficients extrapolated to infinite dilution, $s_{20,w}^0$, for the samples not treated with formaldehyde are comparable to those obtained by Stanley and Bock (91) but greater than those obtained by Kurland (50) probably indicating that the preparations used in the latter study consisted of less intact molecules. The sedimentation coefficients obtained for the 16S and 23S rRNA's treated with formaldehyde are similar to those reported by Boedtker (7).

In previous studies, the extinction coefficients of the rRNA has been given as a whole and was probably measured from the mixture of all the species as extracted from the 70S ribosome with no distinction made as to the particular specie on which the measurement was performed. We have measured the extinction coefficients: $E_{1\text{cm}}^1\text{mg/ml}$ at 260 nm, for each of the species of high molecular weight rRNA; our results of 20.7 for the 16S and 23.2 for the 23S would indicate a slightly greater hypochromicity and therefore a slightly higher degree of secondary structure for the 16S than for the 23S since both RNA's have approximately the same nucleotide composition (91). Although these results imply that the structural arrangement of the 16S rRNA is somewhat more compact than that of the 23S rRNA, it has been reported that the secondary structure content of both rRNA's is similar
(37). A clarification of these results will require a more detailed study.

The extinction coefficients obtained from the formaldehyde treated samples of both species of rRNA are similar within experimental error. This correlates nicely with the fact that both species of rRNA have similar nucleotide composition (91) and also with the expectation that the reaction is complete (6) or at least occurs to the same extent in both polymers.

In conclusion, we have isolated the high molecular weight rRNA's from Escherichia coli in pure and stable form, and performed physical measurements on them, the results of which we consider the most accurate to date. We surmise that the different values obtained by us are going to be of utility not only to workers in the field of ribosomes but also to those engaged in the study of the properties of nucleic acids in solution.
CHAPTER V

SUMMARY

The 16S and 23S ribosomal RNA's from the bacteria Escherichia coli were isolated in pure homogeneous form and their physical properties were studied. Equivalent measurements were performed on both samples of the native ribosomal RNA and those whose secondary structures has been eliminated by treatment with formaldehyde.

Precise measurements of the density increment, 
\( \left( \frac{\partial \rho}{\partial c} \right)_v \), gave values of 0.418 and 0.405 for the native and formaldehyde-treated samples of the 16S rRNA respectively. The corresponding values for the 23S rRNA were 0.456 and 0.438. These figures were coupled with the results of sedimentation equilibrium experiments to obtain molecular weights of 6.4 \( \times 10^5 \) for the potassium salt of the 16S rRNA, and 1.1 \( \times 10^6 \) for the same salt of the 23S rRNA. The values obtained for the sodium salts of the samples treated with formaldehyde were 6.15 \( \times 10^5 \) for the 16S rRNA and 1.08 \( \times 10^6 \) for the 23S rRNA. The last two results were procured with the intention that they serve as standards for the calibration of a rapid, conformation-independent method to measure molecular weights of RNA by polyacrylamide gel electrophoresis. The molecular
weights presented above, indicate that both the 16S rRNA is a continuous polynucleotide chain of about 1750 residues, and the 23S rRNA about 3000 residues. The density increments suggest a more compact structure for the "native" 16S than for the "native" 23S rRNA in solution.

The extinction coefficient, $E_{260}^{1\text{mg/ml}}$, was 20.7 for the 16S, and 22.3 for the 23S. These values may indicate a slightly higher degree of secondary structure for the 16S than for the 23S, since both rRNA's have approximately the same nucleotide composition. A value of 28.2 was obtained for both rRNA's treated with formaldehyde suggesting that the denaturation was complete or at least occurred to the same extent in the two polymers.

The sedimentation coefficient, $s_{20,w}'$ of 18.4S was obtained for the 16S rRNA while the 23S gave a value of 25.0S. The measurement of the sedimentation coefficients of the samples treated with formaldehyde produced the figures of 11.05 and 14.15 respectively. The substantial changes in the sedimentation properties of both polymers demonstrate the drastic alteration of their structure in the transition from the folded to the fully extended form after treatment with formaldehyde.

After the elimination of the secondary structure, all RNA samples should be homologous giving a unique dependence of the sedimentation coefficient on molecular
weight. Therefore, the sedimentation coefficients, $s_{20,w}'$, were combined with the molecular weights of the formaldehyde-treated samples and the relationship $s_{20,w}^0 = 0.0312 \, M^{0.440}$ was obtained. This equation can be used to calculate approximate molecular weights of RNA by sedimentation analysis of other samples once they are reacted with formaldehyde under identical conditions.
REFERENCES


