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MOLECULAR INTERACTIONS BETWEEN

RIBOSOMAL PROTEINS

DISSERTATION

In partial fulfillment of the requirements for the Doctor of Philosophy in the Graduate School of the Ohio State University.

by

Richard L. Coates, Ph.D.

1976

The Ohio State University

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**VITA**

<table>
<thead>
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<th>Year</th>
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"The structure of ribosomes is complex."

K. Nomura

"So it goes."

K. Vonneufut

The ribosome is indeed a complex sub-cellular structure. Ribosomes isolated from *Escherichia coli* can be first dissociated into two subunits, designated as the 30S and 50S subunits. Each subunit is made up of an assembly of specific protein and ribonucleic acid (RNA) molecules. The 30S subunit is composed of 21 distinct proteins and a single segment of RNA with a sedimentation coefficient of 16S; the 50S subunit contains 30 to 35 proteins and two RNA molecules designated as 53 and 23S RNA. (For reviews see Nomura, 1973; Kurland et al., 1972; Wittmann, 1972).

Initial progress was made in elucidating the structure of the 30S subunit by Kizushima and Nomura (1970), who were able to construct an assembly map by reconstituting active ribosomes and partially active ribosomes; their method was to omit certain of the proteins and determine the effect of the omission on the assembly of the subunit. By performing several of these experiments, they were able to determine a rough order of addition and interdependency scheme for many of the 21 proteins. Although this assembly map was
crude and purely two dimensional, it provided a reference point for further work by many groups of investigators.

Since that time, much more has been learned about the proteins by a variety of other techniques. Five of the 21 proteins have been shown to bind to 16S rRNA in the absence of any other components of the system, leading to the conclusion that there are specific binding sites for these five proteins on the rRNA molecule (Schaup et al., 1971; Garrett et al., 1971). Furthermore, the segments of rRNA responsible for the specific binding of these proteins have been isolated by enzymatic digestion of protein-rRNA complexes and sequenced in some cases (Zimmermann et al., 1972; Schaup and Kurland, 1972). Once these five proteins have filled up their sites on the 16S rRNA, the manner in which the rest of the proteins become associated with the nascent ribosome is unknown; presumably protein-protein and protein-rRNA interactions are then responsible for forming the complete ribosomes. At present, there is an attitude which favors solely protein-rRNA interactions made possible by presumed changes in the rRNA structure induced by the binding of the first proteins to cause the formation of new sites for interaction between the rRNA and the later proteins in the assembly sequence (Lutter et al., 1974). This supposition is primarily based on the inability to chemically crosslink more than a few pairs of proteins by treating the intact 30S subunit with bifunctional reagents.
Prior to the present study, no attempt has been reported to demonstrate interactions between ribosomal proteins, or to measure the free energy of such interactions. In this report, evidence will be presented that at least three of the 21 proteins of the 30S subunit participate in protein-protein interactions and that the strength of the interaction measured by the Gibb's free energy of the association is large enough to serve as a driving force for the formation of active ribosomes.
EXPERIMENTAL

Ribosome Preparation

Frozen E. Coli cells were purchased from General Biochemicals, Chagrin Falls Ohio. Late log, high peptone preparations of E. Coli B (ATCC 11303) were used throughout this work. Undissociated ribosomes were obtained from the frozen cells by the procedure employed by Hindennach et al. (1971), with the exception that the thawed cells were disrupted using a French Press at 10,000 to 15,000 psi. A typical isolation consisted of the following steps, all carried out at 4°C: 100 ml of TSM buffer (0.01 M Tris, 0.003 M succinic acid, 0.01 M magnesium chloride, adjusted to pH 8.0 with sodium hydroxide), 5 mg of DNase (Type I from Sigma Chemical Company) and 250 g of frozen cells were homogenized in a Waring Blendor until thawed. The cell suspension was then passed through a pre-cooled French Press while maintaining a pressure of 10,000 to 15,000 psi. Cell debris was removed by centrifugation for 20 min at 30,000 rpm in a Beckman Type 30 rotor. The precipitate was resuspended in TSM to a final volume of 220 ml and recentrifuged at 30,000 rpm for 30 min; since the amount of cell debris per centrifuge tube is greater for the second centrifugation, more time is required to achieve a firm
pellet. The combined supernatants were then centrifuged at 60,000 rpm for 3 hr to obtain the first ribosomal pellet. The clear brown gel of ribosomes was suspended in 100 ml of TS! with the aid of a hand homogenizer to bring the pellet into solution. At this point the solution is less viscous and dense than the first suspension and may be clarified in the SS-34 rotor of a refrigerated Sorvall RCE-2 centrifuge at 20,000 rpm for 10-15 min. The clarified solution is then repelleted, this time at 60,000 rpm for 2 hr. The suspension and repelleting steps are repeated exactly and the ribosomes are next suspended in TS!! buffer containing 0.5 M ammonium chloride at the same pH. This buffer has a higher density such that in order to form a good pellet, the final centrifugation requires 3 hr at 60,000 rpm. This final pellet is resuspended in a low magnesium buffer for separating the subunits; the buffer contains 0.01 M Tris, 0.05 M potassium chloride, 0.3 mM magnesium chloride and 6.0 mM 2-mercaptoethanol, adjusted to pH 7.6 with dilute hydrochloric acid. The solution of ribosomes is diluted until the optical absorbance at 260 nm is 500 for a 1 cm path length. The yield is usually 2.5 to 3.5 g of ribosomes per 250 g of frozen cells. The ribosomes are stored in 20 ml portions at -20°.

The times required for centrifugation in this procedure may be adjusted for different rotors by applying the conversion factors recommended in the instruction sheets.
provided with the Beckman rotors.

**Isolation of the 30S and 50S Subunits**

The procedure of Eickenberry et al. (1970) was modified for use with a Beckman Type Ti-14 rotor. The gradient was generated by the alternate method described by Eickenberry et al. (1970): 45% (w/w) sucrose, buffered with the same low magnesium buffer used for the final suspension of the ribosomes, was added to a stoppered mixing chamber which held 350 ml of buffered 7.4% sucrose. The volume of solution in the mixing chamber was held constant by pumping solution out of the flask at the same rate as the solution enters the flask. When 300 ml of gradient has been delivered to the spinning rotor, the pump is switched to provide a cushion of 45% sucrose. The solution is pumped into the outer edge of the rotor until the top of the gradient emerges from the outlet at the center. At this point the pump is removed from the system and the sample is applied to the top of the gradient with a 50 ml syringe. The sample is then moved into the rotor with 250 ml of buffer containing no sucrose; this transports the sample to a position near the center of the rotor as a lamella between the gradient and the buffer overlay. The rotor is sealed and the chamber is evacuated. The separation is carried out at 48,000 rpm for 3.5 hr if the temperature is less than 14°; above this temperature, the time is reduced to 3 hr. In order to minimize convection, the run is performed at the temperature
of the rotor when the final speed is reached. Rotor unloading is accomplished by pumping crude unbuffered 47% sucrose into the outer edge. Fractions of 10 ml are collected and analyzed by either using an Isco UA-4 absorbance monitor, 2 mm path length at 280 nm or by reading the individual fractions at 260 nm, 1 mm path length. Either method provides enough information to decide which cuts should be made. A single zonal run conducted in this manner yields 100 to 150 mg of purified 30S and 250 to 300 mg of purified 50S subunits from the 600 mg of dissociated ribosomes in the 20 ml starting sample. When twice the volume or twice the concentration of ribosomes was applied, only a 50% increase in 30S ribosomes was obtained. Thus 600 mg seems to be a reasonable limit to the amount that can be processed with this method and rotor. The purified subunits are concentrated for storage by first dialyzing versus T3H buffer in the cold to remove most of the sucrose, followed by the addition of two volumes of cold ethanol (Staehelin et al., 1969). The flocculent precipitate is sedimented for 10 min at 13,000 rpm in the Sorvall CSA rotor, then resuspended in 20-25 ml of T3H buffer and dialyzed to remove the ethanol. The final solution is stored at -20°C. Subunit purity may be analyzed by sedimentation velocity with a zonal center-piece in the analytical ultracentrifuge.

All sucrose solutions used to generate the gradient and cushion are purified to remove possible NAcse contaminations.
tion prior to dilution and addition of the buffer components (Traub and Nomura, 1968). A solution of approximately 50% reagent grade sucrose in water is mixed with 50 g of carboxymethylcellulose in the sodium form for every liter of 50% sucrose. The resin is removed by filtration after stirring 5-10 min, the buffer components are added and the solution is diluted to the desired concentration of sucrose.

**Protein Extraction and Preparation for Chromatography**

The mixture of 30S proteins was extracted from the 16S RNA by the acetic acid procedure of Hardy *et al.* (1969) without modifying their method: one volume of 30S subunits in TSM buffer at 15-100 mg/ml was mixed with one-tenth volume of 1 M magnesium chloride and two volumes of glacial acetic acid. The suspension was stirred in the cold for 45 min and centrifuged to remove the RNA. The precipitate was resuspended in a small amount of fresh solvent and the combined supernatants were prepared for chromatography by dialysis versus the starting buffer for the column described below.

Prior to final equilibration with buffer, the proteins in the 6 M urea buffer were prerduced with 2-mercaptoethanol. The pH of the protein solution was adjusted to pH 8.4 and 2-mercaptoethanol was added to a final concentration of 1%. After stirring for 30 min at 25° the pH was lowered to 5.6, then dialyzed at 4° versus the starting buffer until pH and conductivity of the dialysate were unchanged after
12 hr of dialysis.

**Initial Chromatography**

Again the procedure of Hardy *et al.* (1969) was used, but with modifications to accommodate the amount of protein available. Buffered solutions of urea were prepared by first dissolving reagent grade urea to a concentration of about 8 M; Norit was added to remove color, then the solution was filtered through Whatman No. 1 filter paper. The solution was diluted to 6 M after adding the buffer components (0.05 M sodium phosphate and 0.012 M methylamine hydrochloride, adjusted to pH 5.6) and filtered through a 0.8 μM Millipore filter to remove fine particles of Norit. Prior to use, 50 μl of 2-mercaptoethanol was added for each liter of buffer. Solutions containing this reducing agent were used for periods not exceeding two weeks, then replenished with a like amount of 2-mercaptoethanol or discarded. To minimize the formation of cyanate arising from the decomposition of urea, the buffers were kept in the cold room. Levels of cyanate were monitored by passing a sample of the buffer through the amino acid analyzer. After five months of storage at 4°C, the change in methylamine concentration was less than 1.0 mM and the concentration of ammonium ion was less than 6.0 mM, thus the amount of cyanate formed was below 6.0 mM.

Columns were packed under nitrogen pressure of 5 to 10 psi with cellulose phosphate (*Hannex-P Standard Capacity,
Schwarzmann) which had been washed and equilibrated with
the above 6 M urea buffer. Columns were then equilibrated
by pumping buffer through from the bottom at a flow rate of
approximately 50 ml/hr until pH and conductivity of the
effluent matched that of the buffer going into the column.

A typical resolution of 30S ribosomal proteins consisted of applying 400 mg of protein to a 2.0 x 85 cm column,
followed by one void volume of starting buffer and a linear
gradient of 2 l each of starting buffer and the same buffer
containing 0.6 M sodium chloride. Fractions of 10 ml were
collected and analyzed by following the absorbance at 280
nm with an Isco UA4 absorbance monitor. In addition, every
second or third tube was analyzed by electrophoresis on the
urea gels described below.

This column is usually sufficient to prepare about a
third of the proteins in a pure state; the remainder of the
fractions are contaminated with adjacent fractions and
required further separation methods. Several methods were
used to this end with varying degrees of success.

Rechromatography on Cellulose Phosphate

For this procedure, the guidelines of Hardy et al.
(1969) were followed to select pH and gradient conditions.
A typical separation used a 0.9 x 25 cm column with a 600 ml
gradient covering a range of 0.2 M in sodium chloride. In
this study it was found that better recovery of protein was
obtained by running these columns at 60 ml/hr.
Rechromatography on Carboxymethylcellulose

This method employs the detailed instructions of Kuto et al. (1974). A 0.9 x 25 cm column was packed under nitrogen pressure of 5 psi with washed and equilibrated carboxymethylcellulose (Whatman CIII 32) in a buffer consisting of 6 M urea, 0.05 M sodium acetate and 0.004 M 2-mercaptoethanol, at pH 4.3. The sample was applied to the column and following a wash with the starting buffer, the column was developed with a linear gradient of 300 ml each of the starting buffer and a similar buffer 0.3 M in sodium acetate, pH 4.3; the flow rate was a constant 5 ml/hr.

Rechromatography on Sephadex G-100

Sephadex G-100 was swollen in 15% acetic acid for three days at room temperature and packed into columns 100 cm long with a pressure difference of 25 cm. Two volumes of solvent were passed through the column to equilibrate the gel. The sample was applied in 1 ml or less without markers, since blue dextran and DNP-glycine are relatively insoluble in this solvent. Later separations used columns containing siliconized 6 mm glass beads, allowing the use of Superfine grade of Sephadex G-100 (Sachs and Fainter, 1972).

Preparative Electrophoresis

The analytical slab apparatus described below was used without forming sample wells, but at the same current. The protein mixture was applied in 8 M urea and low ionic

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\[ \text{Rechromatography on Sephadex G-100} \]

Sephadex G-100 was swollen in 15% acetic acid for three days at room temperature and packed into columns 100 cm long with a pressure difference of 25 cm. Two volumes of solvent were passed through the column to equilibrate the gel. The sample was applied in 1 ml or less without markers, since blue dextran and DNP-glycine are relatively insoluble in this solvent. Later separations used columns containing siliconized 6 mm glass beads, allowing the use of Superfine grade of Sephadex G-100 (Sachs and Fainter, 1972).

\[ \text{Preparative Electrophoresis} \]

The analytical slab apparatus described below was used without forming sample wells, but at the same current. The protein mixture was applied in 8 \( \text{M} \) urea and low ionic
strength; sample volume was approximately 1 ml. The electrophoresis was continued until the tracking dye was near the bottom of the gel, then the gel was briefly stained and thoroughly destained with the solutions used for the analytical urea gels. Bands were cut out and homogenized with 6 M CdCl₂ (guanidine hydrochloride), centrifuged to remove acrylamide and the precipitate re-extracted. The combined supernatants were dialyzed versus 15% acetic acid and concentrated by ultrafiltration.

**Analytical Electrophoresis**

Column fractions were analyzed prior to pooling by electrophoresis in the following system: the gel was composed of 9.0% (w/w) acrylamide, 0.8% (w/w) methylene-bis-acrylamide, 8% urea and 1.8% (v/v) glacial acetic acid. To form the gel, 45 ml of this solution was mixed with 1.0 ml of tetramethylethylenediamine and 0.3 ml of 2% ammonium persulfate in water. The gel was either cast in a commercial disc gel apparatus (Bio-rad Laboratories) or in a slab gel apparatus constructed according to the plans given by Reid and Dieleski (1963). Their design was improved by using silicon rubber tubing in place of the agar seals.

Buffer for both the anode and cathode was prepared by dissolving 15.6 g of 3-alanine and 4.0 ml of glacial acetic acid in one liter of water; the pH of this buffer is 4.6. Samples were applied in 50-100 µl for the disc gels and 10-25 µl for the slab gels. Each sample was mixed with 10 µl
of 0.1% Pyronine Y in glycerol as a marker for the buffer front. Electrophoresis was carried out with the anode at the bottom and at a controlled current of 6 mA per disc gel or 120 mA for a slab gel with dimensions 3 x 60 x 120 mm. Gels were electrophoresed until the tracking dye had moved three-fourths of the length of the gel, requiring 3 to 6 hours. The gels were then stained in 0.1% amido-schwarz in 7.5% acetic acid for 1-2 min and destained by diffusion in 7.5% acetic acid. Inclusion of a small amount of anion exchanger, such as Amberlite IRA-400 helped to decrease the time of destaining.

Pooled column fractions and purified proteins were analyzed for molecular weight homogeneity by SDS electrophoresis (Teber and Osborn, 1969). The gel found to give the best separation for ribosomal proteins was the following: 12.5% (w/v) acrylamide, 1.25% (w/v) methylene-big-acrylamide, 0.1% sodium dodecylsulfate (Schwarz-Iann catalogue number 902696) and 0.1 M phosphoric acid adjusted to pH 7.2 with Tris Base (Sigma Chemical Company). Samples were prepared for electrophoresis by dialysis versus 0.1% SDS, 0.1% 2-mercaptoethanol, 0.01 M tris-phosphate at pH 7.2; dialysis was carried out for several days with 3-4 changes per day to ensure complete equilibration with the detergent. Prior to electrophoresis, each sample was mixed with an equal volume of tracking dye solution: 2 ml of glycerol, 1 ml of 0.05% bromophenol blue in water and 1 ml
of 2-mercaptoethanol. Electrophoresis was performed in the same apparatus used for the urea gels; disc gels were run at 8 ma per gel and slab gels were run at 150 ma. Prior to staining, the gel was cut at the tracking dye, then stained in a solution of 0.125 g of Coomassie brilliant blue, 454 ml of 50% methanol and 46 ml of glacial acetic acid. The use of this concentration of dye made it possible to clear the gels of excess dye by soaking the gel overnight with agitation in 7.5% acetic acid and 53% methanol.

Amino Acid Analysis

Lyophilized protein samples were dissolved in constant boiling hydrochloric acid, sealed in evacuated tubes and hydrolyzed for 24 hr at 105°. The hydrolysates were analyzed on a Beckman Model 116 Analyzer.

To facilitate comparisons of two different sets of amino acid molar percentages, the correlation coefficient, r, was calculated according to the following formula:

$$ r = \frac{\sum (X_{ik} \cdot X_{il})}{[(\sum X_{ik}^2)(\sum X_{il}^2)]^{1/2}} $$

(1)

where $X_{ik}$ and $X_{il}$ are the mole percentages of the i amino acids determined or reported. Here k and l can represent any two different proteins or the compositions from two different determinations of the same protein.
**U-Terminal Sequence Analysis**

The detailed procedure of Weiner et al. (1972) was followed exactly without modification. Approximately 4 nmoles of lyophilized protein was used permitting removal of about 0.8 nmoles at each step of the cycle.

**Protocol for the Analytical Ultracentrifuge Experiments**

Optics were aligned for schlieren and Rayleigh interference work by standard procedures (Dyson, 1970; Richards et al., 1971) with the camera focused at the two-thirds plane of the cell. For most experiments an AnD rotor was used, however an AnG rotor was occasionally used with the scanner.

Molecular weights were determined by sedimentation equilibrium employing the high speed method of Yphantis (1964). The majority of the molecular weight determinations were performed using Rayleigh interference optics. Experiments were done using double sector interference cells with interference window holders, sapphire windows and 12 mm Kel-F coated aluminum double sector centerpieces. The use of Teflon strips in place of the usual window cushion (Teller, 1973) generally reduced water blank corrections to less than 5 μ, and corrections were applied to the data only when the deflections were greater than this. Photographic plates were read on an EPOI LP-6 profile projector fitted with a Yikon stage and micrometers.

Determinations using the photoelectric scanner at 280
nm to collect the data produced results which were in agreement with the interference method. Although the ultraviolet procedure yielded molecular weights that were essentially the same as those obtained from interference optics, there was greater scatter in the ln f versus r$^2$ plots. The scanner was not used for mixtures of proteins due to the difference in absorptivities of the proteins used. Attempts were made to use these differences to an advantage by reading at different wavelengths, however when the monochrometer slit was closed off to a position where the band width would be narrow enough to obtain desirable wavelength resolution, the noise level became intolerable.

Sedimentation velocity experiments were performed using the same double sector cells with wide aperture window holders. Data were collected with the photoelectric scanner at 280 nm in all cases.

**Preparation of Protein Samples**

Solutions of protein in 6 M Gdn-HCl were prepared by dissolving lyophilized protein in 6 M Gdn-HCl at 0.1 to 0.4 mg/ml. The solution was dialyzed versus two changes of the same solvent, allowing 12 hr or more for final dialysis equilibrium. The 6 M Gdn-HCl solutions were generally unbuffered and without reducing agent unless the amino acid analysis had indicated the presence of cysteine in the protein. For these proteins, the 6 M Gdn-HCl solutions
contained 0.1 M 2-mercaptoethanol and the pH of the protein-containing solution was adjusted to pH 8.4 for 30 min, then lowered to pH 5.0 prior to dialysis.

To obtain proteins in the "native state", 0.1 to 0.4 mg of lyophilized protein was dissolved in 10 to 50 μl of 6 M Gdn-HCl, containing 0.1 M 2-mercaptoethanol at pH 8.4 if cysteine was present in the protein. To this solution of protein, 0.5 to 1.0 ml of TKK buffer (0.03 M Tris, 0.02 M magnesium chloride and 0.35 M potassium chloride at pH 7.4; 0.1 M 2-mercaptoethanol was added if needed) was added and the resulting mixture was incubated at 37° for 30 to 45 min. Dialysis was carried out at 4° versus TKK buffer with 3 to 5 changes again allowing 12 hr or more for final dialysis equilibrium. Using this procedure, precipitation of protein was minimal, though at higher concentrations of protein than 1.5 mg/ml protein solubility became a problem.

Data reduction for Sedimentation Equilibrium Experiments

Photographic plates were aligned on the microcomparator stage, then the vertical position of three white fringes was measured and averaged. When the average and the central fringe readings were more than 5 μm apart, all three readings were repeated (Aune and Timasheff, 1971). This procedure was applied at 50 to 200 μ intervals from a vertical displacement of 50 μ (relative to the meniscus) to the base of the cell. For all experiments this gave 30 to 40 data points. Experiments using single proteins were plotted on
an ln f versus $r^2$ basis, and the molecular weight was determined from the least squares slope. Partial specific volumes were calculated from the amino acid compositions and the densities were measured on the dialysates pycnometrically. Molecular weight averages were calculated at each point from a program developed in this laboratory (Kar and Aune, 1974). For single proteins, a nonlinear fit was also calculated by a modification of the direct search program (Lohde and Aune, 1974). This fit minimizes the deviation between the experimental points and a theoretical curve described by a single molecular weight and the meniscus concentration of that specie.

**Thin Layer Gel Experiments**

The thin layer gel apparatus used was purchased from Pharmacia Fine Chemicals. Sephadex G-200 Superfine was swollen for three days at room temperature in 6 M Gdn-HCl. Gel layers of 0.4 to 0.6 mm were spread on a 20 x 40 cm glass plate and run at an angle of 10 to 25° at room temperature; elution was carried out until the blue dextran marker reached the lower edge of the plate. A contact replica was made with Whatman 3MM paper and stained with the Coomassie blue stain employed for SDS electrophoresis. The paper was destained by first washing in running water then in methanol-glacial acetic acid-water (50:10:50, v/v). Migrations were measured relative to the blue dextran.
**Determination of Stokes radii by Gel Filtration**

A 1.5 x 90 cm column of Sephadex G-75 in TNE buffer was poured and run with a 25 cm pressure head; buffers contained 0.02% sodium azide to inhibit microbial growth. Flow through the column was controlled by pumping from the bottom with a peristaltic pump at 20 ml/hr. Samples were mixed with blue dextran and DNP-glycine as markers, and contained 20% glycerol to allow the sample to be applied to the top of column without removal of the buffer covering the gel bed. Elution was followed with a UV monitor and elution times were used to calculate the partition coefficient.
RESULTS AND DISCUSSION

Protein Purification

The procedures for isolation of the ribosomes and for separation into 30S and 50S subunits have been taken from the literature; both have given reasonably satisfactory results. Early in the work, the separation of the subunits was attempted by the use of a linear gradient in the zonal rotor. This procedure worked well in about half of the cases in which it was applied; the problems seemed to be instability of the gradient either during generation or loading of the sample, leading to inadequate separations. Once the procedure of Eickenberry et al. (1970) was instituted, the only failures were due to leaks in the rotor seal or when the rotor came to rest before unloading. A typical separation using this nonlinear gradient is shown in Figure 1; assignments and purity estimates are based on analytical zonal determinations. In Figure 1, fraction A contains material of low molecular weight and of low density, thus it is discarded; fraction B is better than 95%; pure 30S subunits; fraction C is a mixture of 30S and 50S subunits and is saved for reprocessing; fraction D is better than 95% pure 50S subunits.
This represents one preparative ultracentrifugation; depicted is the pattern of material absorbing at 280 nm unloaded from a 3.5 hr separation at 48,000 rpm in a Beckman Ti-14 rotor. A "hyperbolic gradient" from 7.4% to 30% sucrose with a cushion of 45% sucrose was used as described by Eickenberry et al. (1970). The fractions marked off on the curve by hatch marks are the following (determined by analytical ultracentrifugation): A is low molecular weight material; B is better than 95% pure 30S subunits; C is a mixture of 30S and 50S subunits; D is better than 95% pure 50S subunits. This represents 660 mg of applied sample, yielding 120 mg of 30S and 275 mg of 50S subunits.
The chromatographic procedure of Hardy et al. (1969) yielded poor results, until modified to include a pre-reduction of the protein mixture. Following adoption of this modification, it was possible to obtain several of the proteins in a pure state with only this step and a subsequent rechromatography on Sephadex C-100 in 15% acetic acid to remove the last traces of contaminating species. Without the prereduction, no fraction contained less than three bands when examined by urea gel electrophoresis. Figure 2 displays a typical separation of the mixture of all 30S proteins.

Rechromatography of a pooled fraction on smaller cellulose phosphate columns was less satisfactory, in that the recovery of the protein applied was at best 75% and often less than 50%. When mixtures of proteins were rechromatographed, pure proteins could only be obtained on the outer edges of the peaks and two cycles of rechromatography were necessary to obtain complete separation. The purification of 35 from fraction F in Figure 2 is depicted in Figure 3. Notice that even with elution at a high pH, portions of the mixture eluted by the gradient contained more than one band, further decreasing the yield of purified protein.

Chromatography on C-100 in 15% acetic acid also had its limitations, mainly as a result of diffusion broadening of the separated peaks. This method is most applicable
Figure 2: Cellulose phosphate chromatography and urea gel electrophoresis of 30S ribosomal proteins. (Details in the text)
Fraction F from Figure 2 was applied to a 0.9 x 25 cm column packed with cellulose phosphate in 6 M urea, 0.05 M sodium phosphate, 0.012 M methylamine hydrochloride and 6.0 ml 2-mercaptoethanol at pH 7.5. After passing through one column volume of buffer, a 600 ml gradient of 0.0 to 0.2 M sodium chloride in buffer was begun. All steps were at 4° with a flow rate of 50 ml/hr; fractions of 10 ml were collected.
when the level of contamination is less than 20% and the difference in molecular weight is at least 50%. The use of glass beads throughout the column with Superfine grade G-100 lessened these requirements since peaks were sharper with the higher flow rate and finer mesh of gel. Again, recovery of protein from the column was less than expected, even when salt was included with the 15% acetic acid solution.

Rechromatography on carboxymethyl cellulose was used to purify S2. Figure 4 indicates that about 60% of the material recovered from the column can be considered to be pure protein, a slight improvement over rechromatography on cellulose phosphate columns. A C18 column was also used in an attempt to resolve S9 and S19 with miserable results: less than 10% of the applied protein was recovered using the pyridine-formate buffer of Hinndenach et al. (1971).

Perhaps the most fruitful method of purification is preparative electrophoresis. Yields are low, but resolution is at a maximum. A note of caution must be added in that proteins purified in this manner may be contaminated with polyacrylamide fragments, as indicated in the discussion of 39.

Criteria of Purity

Proteins were initially judged to be pure when a single band was observed under conditions of urea gel electrophoresis (Figure 5). Several compositions of gels were used
Fraction C from Figure 2 was applied to a 0.9 x 25 cm column packed with carboxymethyl cellulose in 6 M urea, 0.05 M sodium acetate and 4 mL 2-mercaptoethanol at pH 5.6. After passing through one column volume of buffer, a 720 mL gradient was begun 0.05 to 0.30 M in sodium acetate. All steps were at 4°C with a flow rate of 5 ml/hr; fractions of 2.6 ml were collected.
Samples were prepared and electrophoresis as described in the text. Purity is estimated to be better than 95% from the intensity of the stained bands, with the exception of S10 which could only be obtained as a mixture with S5.
before the reported one was adopted. For example, when the concentration of urea in the gel is lowered from 8 M to 6 M, a drastic effect is seen in the pattern of the separations. With the lower concentration of urea, proteins S16 through S21 appear in the tracking dye and no distinction can be made between these species. With 8 M urea, even S16 and S17 mixtures show up as a distinct doublet (see fraction K of Figure 2).

After pools had been made from the initial cellulose phosphate column, aliquots were dialyzed into SDS and then electrophoresed on SDS-acrylamide gels. This aided in protein assignments and also served as a guide in deciding which purification procedure should be next employed. Since all but one ribosomal protein fall into the molecular weight range of 10,000 to 30,000, a gel composition was selected which would give better resolution in this range (Swank and Junkres, 1971) than the more commonly employed system of Weber and Osborn (1969). The use of a gel containing 12.5% acrylamide with 1.25% bis-acrylamide fulfilled this aim and provided a linear relationship between the logarithm of the molecular weight and the mobility of the protein in this gel. Figure 6 demonstrates the linearity obtained in such a plot; the scatter at the low end is reproducible and most probably represents the greater sensitivity of this gel system to deviations in the low molecular weight range (Fish et al., 1970). The resolving power of
Protein standards of known molecular weights in 0.01 M tris-phosphate, 0.1% SDS and 0.1 M 2-mercaptoethanol at pH 7.2 were electrophoresed on a gel containing 12.5% acrylamide, 1.25% bis-acrylamide, 0.1 M tris-phosphate and 0.1% SDS at pH 7.2. The migration distance of the proteins was measured and converted to value, then plotted versus the logarithm of their molecular weight.
of this gel system for ribosomal proteins is demonstrated in Figure 7. The fractions are designated by letters corresponding to the pooled fractions from the cellulose phosphate column. It can be seen that this gel system reveals at least as many components as are found in the urea gel patterns of the same fractions.

When a protein preparation gave single bands in both urea and SDS gels, amino acid analysis was performed as a final test of its identity with previously reported preparations. Table 1 lists the amino acid compositions obtained from the protein preparations used in this study. In order to compare all 16 amino acid percentages of one protein preparation to those of the reported preparations of the 21 purified 30S proteins, correlation coefficients were calculated between the protein under analysis and the reported compositions. The numerical value of the correlation coefficient calculated by equation 1 would in theory vary between 1.0 and -1.0, but in practice is limited to positive values since negative compositions are never obtained. Table 2 lists the correlation coefficients derived from comparisons of all the literature compositions for the 30S proteins; it can be seen that the range is further limited by the gross similarities in the amino acid distributions, such that the numbers fall between 0.7 and 1.0. The main point of Table 2 is that the largest number in each column or row is the diagonal element resulting from
Figure 7: \textit{Ab} elektrophoresis of pooled fractions

Pooled fractions from the initial cellulose phosphate column were dialyzed into 0.01 M tris-phosphate, 0.1% SDS and 0.1% 2-mercaptoethanol, then electrophoresised on the 12.5% acrylamide gel described in the text.
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Number of determinations: 1 10 8 2 1

a 24 hour 6 N HCl hydrolysates at 110°.

b Contaminated with S5.
Table 2

AMINO ACID CORRELATIONS FOR 305 PROTEINS

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Correlations were calculated between the values reported by Craven et al. (1969) and Halschnidt et al. (1970). When a determination was performed on a protein by only one group, those values were used in both column and row calculations; this is the source of the correlations which equal 1.00.
a comparison of the compositions of the same proteins from two different laboratories. It should be noted that the row corresponding to S21 does not fulfill this criterion; this would be expected from a qualitative view of the raw amino acid compositions for the two S21 determinations. The mole percentages are strikingly different for several of the amino acids; these differences are reflected in the correlation coefficient.

Table 3 was prepared to present a picture of the effect of comparing analyses of impure proteins. Here, data were synthesized by assuming various mixtures of the two proteins, S12 and S20, and comparing the synthetic mixture to the reported amino acid compositions for all 21 pure proteins. Even up to 20% contamination by S12, the first two digits of the correlation coefficient are the same when pure S20 is compared to the S12-S20 mixture. This points out that the method is quite applicable as an identification procedure, with indications of purity available in the third and subsequent digits of the correlation coefficient. The most reliable test of purity is still the electrophoretic methods discussed above.

Table 1 presented the amino acid mole percentages determined on the proteins used in this study. Table 4 is a list of the correlations to the previously reported values for all 303 proteins. It is seen that good correlations are found except for the partially purified S10.
Table 3
Amino Acid Correlation Coefficients for Protein Mixtures

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<tr>
<th>Protein</th>
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<th>40</th>
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<th>70</th>
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Amino acid mole percentages were simulated by combining contributions for each amino acid in the amounts designated of the reported values of S12 and S20 from the data of Craven et al. (1969). Comparisons were made to the amino acid compositions for pure proteins S1 through S21 from Craven et al. (1969); since they did not provide data for S13, the data of Bartschmidt et al. (1970) was used for this protein. The largest correlation coefficient in each column is underlined.
Table 4

Correlation Coefficients for Purified 30S Proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>S2</th>
<th>S3</th>
<th>S5</th>
<th>S9</th>
<th>S10</th>
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<tr>
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<td>0.9572</td>
<td>0.9158</td>
<td>0.9664</td>
<td>0.9225</td>
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</tbody>
</table>

\[a\] Calculated from equation 1.
As a further test of identity between the proteins isolated for this study and those reported in the literature, a partial sequence analysis was performed on two proteins, S3 and S9. Using less than 0.2 mg of protein, the first five residues from the amino termini were determined and are reported in Table 5. The analysis of S3 was complicated by the apparent incomplete cyclization and cleavage in the first cycle of the Edman procedure. This produced backgrounds of the previous residues in the subsequent steps, but the major spot in the chromatographic identification corresponded to the previously reported residues (Yamuchi et al., 1973; Wittmann-Liebold, 1973). The sequence determination of S9 proceeded more smoothly and no residues other than ε-dansyl lysine were found in addition to the expected ones.

Thus it has been possible to establish by gel electrophoresis that the proteins isolated are better than 95% pure (with the exception of S10) and can be correlated with the literature references to the same proteins by amino acid analysis, position in urea gels and elution from the phosphocellulose column. Finally, the identity of two proteins has been unambiguously established by partial sequence determination.

**Determination of Molecular Weights**

Initial estimations of molecular weights from SDS electrophoresis were used to establish conditions for the
### Table 5
Partial Amino Acid Sequences

<table>
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<th>Protein</th>
<th>1</th>
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<th>3</th>
<th>4</th>
<th>5</th>
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</thead>
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<tr>
<td>S3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>GLY-GLX-&lt;sup&gt;d&lt;/sup&gt;</td>
<td>VAL-HIS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>GLY-GLX-LYS-VAL-HIS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>GLY-GLX-LYS-VAL-HIS</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ALA-GLU-ASX-GLX-TY&lt;sub&gt;i&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ALA-GLU-ASX-GLX-TY&lt;sub&gt;i&lt;/sub&gt;</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Data from this study  
<sup>b</sup> Data from Yaguchi et al., 1973  
<sup>c</sup> Data from Wittmann-Liebold, 1973  
<sup>d</sup> Sample lost during hydrolysis
determinations of molecular weights using the analytical ultracentrifuge. The purified proteins were analyzed by this method in two solvents: 6 M Gdn-HCl to establish the molecular weight in the absence of possible self-association of the proteins and in Tris buffer to simulate conditions of maximal ribosomal activity (Traub and Komura, 1969). Since differences in the molecular weights were encountered between the methods used, each protein will be discussed separately, with reference to the compilation of molecular weight determinations in Table 6.

Protein S2

Protein S2 is the only example in Table 6 of a ribosomal protein possessing cysteine in its amino acid composition. The sedimentation equilibrium determination of the molecular weight in 6 M Gdn-HCl, 0.1 M 2-mercaptoethanol provided plots of ln f versus r^2 which were linear and yielded molecular weights which were in agreement with the values obtained by SDS electrophoresis. When the sedimentation equilibrium experiments were performed with S2 in Tris buffer containing 0.1 M 2-mercaptoethanol, the ln f versus r^2 plots were curved. Analysis of the tangents at high and low concentrations of protein provided molecular weight averages that qualitatively indicated that the self-association was weak, but that it must be taken into account before attempting an investigation of a mixture of S2 and any other ribosomal protein.
Table 6

Molecular Weight Data\textsuperscript{a}

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sedimentation Equilibrium</th>
<th>SDS Electrophoresis</th>
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<td>T.I.</td>
<td>Cdm-HCl</td>
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<td>S2</td>
<td>25,000±1000 (2)\textsuperscript{b}</td>
<td>26,900± 500 (2)</td>
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<tr>
<td>S3</td>
<td>21,600±1400 (7)</td>
<td>22,600± 500 (5)</td>
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<tr>
<td>S5</td>
<td>17,500±1600 (8)</td>
<td>18,000±2100(13)</td>
</tr>
<tr>
<td>S9</td>
<td>c</td>
<td>17,400± 800(11)\textsuperscript{c}</td>
</tr>
<tr>
<td>S10</td>
<td>c</td>
<td>13,600± 700 (3)\textsuperscript{c}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Number of determinations in parentheses

\textsuperscript{b} Undergoes self-association or covalent dimer present

\textsuperscript{c} Sample contaminated
Figure 8 demonstrates the type of curvature found in the two sedimentation equilibrium experiments performed with S2 in TKB buffer containing 0.1 M 2-mercaptoethanol. By combining the data from determinations in 6 M Gdn-HCl (where no curvature was detected) with the TKB curves, an estimate of $25,100 \pm 1000$ is made for the molecular weight of S2 in TKB buffer.

Protein S3

Protein S3 shows a large discrepancy between the molecular weights estimated by SDS electrophoresis and the values obtained by sedimentation equilibrium in either TKB buffer or 6 M Gdn-HCl. The values obtained thermodynamically in the centrifuge are considered to be the most reliable since no assumptions about shape are required for the determination of molecular weight by this method. The difference between the value from TKB and from 6 M Gdn-HCl is well within the range which can be explained by preferential solvation of the protein in 6 M Gdn-HCl; this increase would correspond to 10 moles of solvent per mole of protein, and values between 0 and 55 moles/mole have been reported in the literature for other proteins (Lee and Timasheff, 1974). The molecular weight determined by SDS electrophoresis is thus anomalously high and could arise from two sources: low level binding of detergent, or existence of a more expanded state in SDS than would be predicted from the behavior of other proteins in this solvent.
Figure 3: Protein 32 in T.K Buffer

52 in T.K buffer, 0.1 M 2-mercaptoethanol at 3^,000 rpm, 10.9°. Column height was 3.3 cm; initial protein concentration was 0.078 mg/ml. Molecular weights were determined from the slopes of the lines drawn through the data points.
Either case would predict that the SDS-S3 complex would move more slowly in an SDS gel than other proteins of the same molecular weight. To decide between the two possible explanations, the binding of SDS to S3 was measured by the method of Steinhardt and Reynolds (1969) and found to be 1.4 ± SDS/g of protein under conditions of electrophoresis. This level of binding is accepted to be the normal level and is the number found for the standards commonly used in the calibration of SDS gels (Reynolds and Tanford, 1970). These observations would indicate that S3 possesses an unusual shape under conditions of denaturation by SDS. To confirm this conclusion, two additional pieces of evidence can be offered: gel chromatography was performed on a column of Sepharose 6B in SDS, using several calibration standards according to the method of Fish et al. (1969; 1970). This method provided a molecular weight of 23,600 ± 1100; this could be explained as either the result of increased SDS binding or an atypical shape for proteins in SDS. Since binding has been measured directly and found to be normal, the conclusion of a more extended structure is supported. A similar technique was applied to the protein in 6 M Gdn-HCl; when the molecular weight was determined by thin layer gel chromatography in 6 M Gdn-HCl on Sephadex G-200, a value of 30,000 ± 4000 was obtained. The high molecular weight in this solvent could only be due to an unusual shape, thus the same conclusion is reached for the
behavior of the protein in both denaturants, namely that S3 has an extended shape beyond that observed for most proteins in these two solvents. In this context, it might be noted that the amino acid composition reveals a high proline content (6.4% from the data of this study and of Kaltschmidt et al., 1970) which could provide the basis for this extended shape.

Another method was developed to quickly screen other ribosomal proteins for similar behavior in SDS electrophoresis. The basis for separation of proteins by such a method is that movement of the protein in the gel will be directly related to charge and inversely related to size or shape. In order for the method to be able to provide reliable molecular weights, all proteins should have an identical ratio of charge to shape. If a series of gels were run with varying pore sizes, the effect of charge to shape ratio would be different for each gel composition. Since it is difficult to exactly determine pore size, an empirical plot of migration as a function of acrylamide concentration was chosen to represent the data. Figure 9 is such a plot, with the least squares slopes and errors indicated in Table 7. It is seen that for each of the standard proteins, the slopes are within experimental error of each other, but that S3 deviates from this constancy of slope. Its deviation is in a direction which would indicate that it possesses the properties described above: atypical shape or lower
Figure 9: Acrylamide Dependence of SDS-Protein Mobilities
(Details in text.)

Table 7
Acrylamide Dependence of SDS-Protein Mobilities

<table>
<thead>
<tr>
<th>Protein</th>
<th>Slope</th>
<th>Number of Points</th>
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</thead>
<tbody>
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<td>-0.0970 ± 0.0091</td>
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</tr>
<tr>
<td>Chymotrypsinogen</td>
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<tr>
<td>α-Lactalbumin</td>
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<tr>
<td>Lysozyme</td>
<td>-0.0990 ± 0.0092</td>
<td>7</td>
</tr>
</tbody>
</table>

(Average of Standards) = -0.09 ± 0.0039

S2  -0.0893 ± 0.0042  3
S3  -0.0991 ± 0.0033  5
S5  -0.0957 ± 0.0315  4
S9  -0.1124 ± 0.0010  3
charge than the standards. In general the method cannot distinguish between the two possibilities, so an outside method such as one of the above applied to S3 would be required to fully characterize any unusual slope in such a plot. In Table 7, proteins S2, S5 and S9 seem to fall more closely in the range of the standards and in Table 6, the difference between the SDS and centrifuge molecular weights is not as great for these proteins as for S3.

It should be noted that two preparations of S3 which had been refolded in T.K buffer and stored at 4°C for periods exceeding two weeks exhibited aggregation when analyzed by sedimentation equilibrium in T.K. Such a condition was not observed with freshly refolded samples of S3, thus all subsequent experiments were performed within three days of refolding the protein. With this precaution employed, no self aggregation was detectable, and all ln f versus r² plots were linear (Figure 10).

**Protein S5**

Protein S5 is more well-behaved, though moderate differences between the molecular weights are noted in Table 6. Although the SDS molecular weight is higher than that determined by sedimentation equilibrium in either T.M or 6 M Gdn-HCl, the deviation is not as great as was the case with S3. A molecular weight was determined by thin layer gel chromatography in 6 M Gdn-HCl and found to be 19,900 ± 2,000. Although the error in this latter measurement is
From the tangent drawn to the plot with a slope of 22.840, the molecular weight of the protein was determined. The molecular weight was 2.4 x 10^4, assuming a protein concentration of 2.0% in 400 mM 3.0 M sucrose buffer.

![Graph showing protein concentration and fringe displacement](image-url)
large, if the average is correct, the conclusion would be that S5 might give an anomalous molecular weight in SDS for the same reason that S3 does, though to a lesser extent. Again, the most reliable determination of the molecular weight is taken to be the value obtained in D.H, since it is confirmed by the determination in 6 M Cdn-HCl and since the ln f versus r² plot for the native determination shows no curvature (Figure 11).

Protein 39

All preparations of 39 used in this work were obtained by preparative electrophoresis. The determinations of the molecular weight in 6 M Cdn-HCl provided linear plots of ln f versus r² in all cases with both interference and ultraviolet optics used to collect the data. These experiments yielded the molecular weight reported in Table 6 of 17,400; this is in fair agreement with the value of 16,100 obtained by SDS electrophoresis. When freshly refolded samples of 39 in \( \text{Tris} \) buffer were subjected to sedimentation equilibrium, inconsistent results were obtained. Most experiments provided linear plots of ln f versus r², but with differing molecular weights. The molecular weights ranged from 13,000 to 18,000 with a few experiments yielding curved plots which indicated a mixture of both ends of this range. No pattern could be detected for this variance and the only explanation which can be offered for this behavior is that the samples used for these determinations were
Figure 11: Protein 35 in T.K buffer

35 in T.K buffer at 40,000 rpm, 3.0°. Column height was 2.5 cm; initial protein concentration was 0.1 mg/ml. The molecular weight from the least squares slope is 16,700.
contaminated, possibly with fragments of polyacrylamide from the preparative electrophoresis. This explanation receives some confirmation from the amino acid analysis in the observation that the peak for ammonia was so large as to be off-scale in both determinations done on this protein. If the supposition that the S9 preparation is contaminated is correct, the molecular weight determined in 6 M Gdn-HCl should be accepted with caution. The molecular weight determined by SDS electrophoresis is however still assumed to be valid; this belief is supported by Table 7 where it is seen that S9 behaves more like the standard proteins than does the deviant S3.

Protein S10

Protein S10 was not completely purified due to the limited quantity available; the preparation used was contaminated only by S5 in an amount estimated to be about 50% S5. The molecular weight determined in 6 M Gdn-HCl is then derived from the tangent to the ln f versus r² curve at low concentrations (Figure 12), to exclude the contribution from S5. Such an extrapolation of data would have been more difficult in T.I buffer, since there was evidence for a complex formation (presumably 1:1 S5 to S10) in the native state. Thus to obtain a native molecular weight, preferential solvation in 6 M Gdn-HCl was assumed to be on the same order of magnitude as has been observed with the other ribosomal proteins to yield an estimate of 13,000.
**Figure 12: S10-S5 Mixture in 6 M Gdn-Cl**

A mixture of S10 and S5 in 6 M Gdn-Cl at 44,000 rpm, 25°. Column height was 4.0 cm; initial protein concentration was 0.25 mg/ml. The molecular weight was obtained from the slope of the line drawn through the data points.
Determination of Frictional Ratios in the Native State

A prerequisite for the observation of any functional characteristics of these proteins is that they are in fact refolded to a somewhat compact shape when treated by the procedures developed in this study. The observation has been made by a number of workers (for a review see Komura, 1973) that even when all the proteins and TNA are separated under denaturing conditions, they may be combined to form active ribosomes. The question arises whether a single protein can by itself attain a true native state when deprived of other components of the system. This question can be approached in a somewhat crude fashion by examining the shape of the refolded protein; this was done through the determination of its frictional ratio and by comparison of the ratio to those of other native proteins. This is the most accessible method of answering the question, since functions of single ribosomal proteins have not been demonstrated in a manner that might be converted to an assay method.

Two techniques were used to determine the frictional ratios given in Table 8: sedimentation velocity and gel permeation chromatography. The frictional ratio may be calculated from the sedimentation coefficient by the following formula:

\[
\frac{f}{f_{\text{min}}} = \frac{(1-\nu p)}{6m^n} \cdot \frac{1}{t_{\text{min}}}
\]
<table>
<thead>
<tr>
<th>Protein</th>
<th>$s_{20,w}$</th>
<th>$f/f_{\text{min}}^b$</th>
<th>$\sigma$</th>
<th>$f/f_{\text{min}}^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>S3</td>
<td>2.28 ± .15 (2)</td>
<td>1.17</td>
<td>0.203 (1)</td>
<td>1.41</td>
</tr>
<tr>
<td>S5</td>
<td>1.49 ± .23 (4)</td>
<td>1.56</td>
<td>0.165 (2)</td>
<td>1.59</td>
</tr>
</tbody>
</table>

a Number of determinations in parenthesis.

b Computed from equation 2.

c Computed from equation 5.
where $M$ is the molecular weight, $\bar{v}$ is the partial specific volume of the protein, $\rho$ is the solvent density, $\eta$ is the solvent viscosity, $N_A$ is Avogadro's number, $s$ is the sedimentation coefficient and $\min r$ is the radius that the protein would have if all the mass were compacted into a perfect sphere. $\min r$ may be calculated from the molecular weight and partial specific volume of the protein:

$$\min r = \left(\frac{3MV}{4\pi N_A}\right)^{\frac{1}{3}}$$

(3)

The frictional ratio may also be calculated from the behavior of the protein on a gel permeation column which has been calibrated with a series of standards of known Stokes radii. The following equation is used to establish the relationship between elution and Stokes radius:

$$R_S = A \text{erfc}^{-1}(\sigma)$$

(4)

where $A$ is a calibration constant for the column used and $\text{erfc}^{-1}(\sigma)$ is the inverse error function complement of the partition coefficient (Ackers, 1967). The Stokes radius is then combined with $\min r$ to obtain the frictional ratio:

$$\frac{f}{f_{\min}} = \frac{s}{\min r}$$

(5)

The two methods should yield the same number, barring concentration dependence in determination of the sedimentation coefficient or interaction between the protein and the gel matrix. Since the sedimentation coefficients were
determined at protein concentrations below 1 mg/ml, concentration effects can be ruled out. Interaction with the gel matrix can also be ruled out since frictional ratios are higher by this method in Table 8. Thus the only basis for the differences in the two methods for these proteins must lie in experimental inaccuracy, most probably the determination of the elution volume.

To extract a meaning for the values of Table 8, typical values of \( f/f_{\text{min}} \) are cited as 1.2 to 1.3 for globular proteins (Tanford, 1961) while a value of 2.35 can be calculated for chymotrypsinogen as a random coil in Gdn-\( \text{HCl} \) (Tanford et al., 1967). Thus it is reasonable to assume that the data for the proteins presented here indicate that under the conditions used for refolding, a native state is attained even in the absence of other components of the ribosomal system. Further support of this statement will be provided in the discussion of the behavior of protein mixtures.

**Behavior of Protein Mixtures**

All sedimentation equilibrium molecular weight determinations using freshly refolded S3 or S5 gave linear plots of \( \ln f \) versus \( r^2 \) and yielded molecular weights that agreed with the determinations of the same proteins in 6 M Gdn-\( \text{HCl} \). This is taken as strict evidence that no self-association was detectable for these proteins in TTR buffer. When sedimentation equilibrium experiments were performed with
plots of ln f versus $r^2$ were curved as would be expected for heterogeneous samples. The presence of a species with molecular weight higher than the individual proteins was indicated upon determination of the slope of the ln f versus $r^2$ curve at high protein concentrations. Figure 13 provides this evidence; if only unassociated S3 and S5 molecules were present, the slope of the tangent to this curve would provide molecular weights in the range of the previously determined molecular weights of these proteins, 17,500 to 21,600. Since the weight average at high concentrations is larger than either of these numbers, a partial association must have taken place between the two proteins. In order to account for such increases in the molecular weights in a quantitative fashion, a method was sought to fit the sedimentation equilibrium data to a reasonable model which would explain this effect.

Calculations to Fixed Protein Data

The basic equation describing the observed concentration distribution in a sedimentation equilibrium experiment is given in equation 6:

$$f_{ij} = f_{aj} \exp (\sum_j a_j \Delta r_i^2/2)$$

(6)

where $f_{ij}$ is the absolute concentration at the radial position $r_i$ due to the jth component; $f_{aj}$ is the meniscus concentration of the sedimenting species j; $\Delta r_i^2$ is the square of the radial position $r_i$ minus the square of the
Figure 13: S3-S5 mixture in TK buffer

A mixture of S3 and S5 in TK buffer at 28,000 rpm, 3.0°C. Column height was 2.7 mm; initial protein concentration was 0.13 mg/ml of each protein. The molecular weights were obtained from the slopes of the lines drawn through the data points.
meniscus radial position $r_a$. The quantity $c_j$ is defined in equation 7:

$$c_j = \frac{M_j(1-\bar{v}\rho)\omega^2}{\gamma^2}$$

Here, $M_j$ is the molecular weight of species $j$; $\bar{v}$ is its partial specific volume; $\rho$ is the density of the solvent; $\omega^2$ is the square of the angular velocity in radians per second; $\gamma$ is the $gas$ constant and $T$ is the temperature in degrees Kelvin. Equation 6 may be rewritten to describe the fringe displacements as measured, by subtracting the meniscus concentration $f_{aj}$ from both sides of the equation to give an expression for $y_{1j}$, the measured displacement at $r_1$ due to the $j$th component. This is done because the total meniscus concentration is usually unknown and all displacements measured by plate reading are relative to an assumed displacement of zero at the meniscus.

$$y_{1j} = f_{aj}[\exp(c_j\Delta r_1^2/2) -1]$$

For a mixture of species with different molecular weights $c_j$, the total displacement at any point, $y_1$, is represented by summing the right hand side of equation 8 over all species:

$$y_1 = \sum_{j=1}^{s} f_{aj}[\exp(c_j r_1^2/2) -1]$$

where $s$ is the total number of sedimenting species in the
system. In this equation all parameters are known with the exception of the individual $f_{a_j}$.

A set of $f_{a_j}$ will yield an estimate of $y_i$ for any point in the cell, through a calculation with the parameters in equation 9. The residual at that point, $\delta_i$, is given as the difference between the observed $y_i$ and the value calculated from the set of $f_{a_j}$:

$$\delta_i = y_i - \sum_{j=1}^{s} f_{a_j} \left[ \exp(\sigma_j \Delta r_i^2 / 2) - 1 \right]$$

(10)

For the set of $f_{a_j}$'s to adequately describe the observed data, all the experimental points should be reasonably approximated by the curve calculated from the $f_{a_j}$. An indication of the fit between theory and experiment is given by an average residual $\bar{\delta}$, defined as:

$$\bar{\delta} = \frac{1}{n-s-1} \sum_{i=1}^{n} |\delta_i|$$

(11)

where $n$ is the number of data points collected. The numerical value of $\bar{\delta}$ should correspond to the expected random error in the data collected; if it is larger than this, the set of $f_{a_j}$'s does not adequately describe the data and new values of $f_{a_j}$ should be sought until $\bar{\delta}$ attains a lower value. The average residual need not be calculated in the manner described by equation 11; for example, the square root of the sum of the squares of $\delta_i$ might be used leading to a slightly different fit. The form presented was chosen...
because it gives equal weight to all points.

Any of several matrix reduction methods failed to yield physically meaningful results due to the near singularity of the matrices derived from the data. Therefore, a direct search method was chosen (Hooke and Jeeves, 1961) to obtain the $f_{aj}$'s. This method proceeds by the following algorithm:

1. Make an initial estimate of the desired $f_{aj}$ and calculate $\bar{R}$.
2. Generate a better value of $\bar{R}$ by adding a change parameter to one of the $f_{aj}$'s.
3. When step (2) fails to decrease $\bar{R}$, make changes in two $f_{aj}$'s at once.
4. When step (3) fails, reduce the size of the change parameters and return to step (2).

This simple method has performed very well in the case of three species with initial estimates of zero in step (1), proceeding to final values of $\bar{R}$ which are on the order of $5 \mu$, the expected error due to plate reading. The method can be programmed into a desk calculator such as the Hewlett-Packard 9810 and has usually reached convergence in 2 to 12 hours, requiring 300 to 2000 calculations of $\bar{R}$. These limits are for the calculator used and result mainly from the time consuming calculations of the many exponentials required for the summation leading to $\bar{R}$. A calculator with a larger number of rapid access storage locations would
require much less time.

Thus, for a sedimentation equilibrium experiment in which a concentration distribution is achieved from a mixture of two proteins that associate to form a 1:1 complex, a description of the amounts of each species may be obtained in terms of the meniscus concentration of each species. These concentrations may be related to an association equilibrium constant; for the reaction:

\[
A + B \xrightarrow{K} C
\]  

the association constant is written in terms of the molar concentrations:

\[
K = \frac{[C]}{[A][B]}
\]  

This expression is easily rewritten in \(\ell/1\) concentrations by dividing the molar concentrations by the molecular weights:

\[
K = \frac{(\frac{c_A}{M_A}) \cdot \frac{c_C}{c_B}}{c_A c_B}
\]

The \(\ell/1\) concentrations are directly related to the fringe displacements through an optical constant \(k\), giving the equilibrium constant as a function of the individual fringe displacements at any point in the cell:

\[
K = \frac{(\frac{f_A}{k \cdot c}) \cdot \frac{f_C}{f_B}}{f_A f_B}
\]
At sedimentation equilibrium, the association equilibrium constant is obeyed at all points in the cell. Thus at the meniscus, where information about the individual species is obtained from the fit, sufficient information is available to determine the thermodynamics of the association.

The fitting procedure provides values of \( f_{aj} \) which are then used to calculate the equilibrium constant. The nature of the fitting procedure ensures that the equilibrium constant for the assumed model is determined from all the data collected. The error in the determination of the equilibrium constant may be assessed from the following considerations. Firstly, it should be determined if each of the \( f_{aj} \) calculated have meaning with respect to their contribution to the total concentration in the cell. For the value of the determined \( f_{aj} \) to be meaningful in a quantitative sense, each must be large enough to contribute to the measured concentration curve at a level larger than the expected error due to plate reading inaccuracies.

For example, if the fitting procedure calculates an \( f_{aj} \) to be \( 10^{-4} \), and by application of equation 6, the concentration at the base of the cell due to species \( j \) is only 0.1, then that \( f_{aj} \) could fall in the range of \( 10^{-\infty} \) to \( 10^{-4} \) with little or no effect on \( \bar{R} \). Thus as an arbitrary limit, any calculation which yielded an \( f_{aj} \) which would give a base concentration of species \( j \) less than 2 \( \bar{R} \) was discarded.

A more quantitative estimation may be obtained for the
error associated with the determination of the $f_{aj}$'s, the association constant and the Gibbs' free energy of the association. The individual $f_{aj}$'s may be used to calculate the total amount of protein, expressed as the initial concentration of all protein, $f_o$, prior to redistribution. The error in $f_o$ should then be the same as $\bar{\Delta}$, the average uncertainty in the $y_i$'s. This can provide a relation between the overall error of the fit, $\bar{\Delta}$, and the individual errors, $\delta f_{aj}$. The expression relating $f_{aj}$ and $f_{oj}$ is the following integral, evaluated from the meniscus to the base of the cell:

$$f_{oj} = \frac{\int_{r_a}^{r_b} f_{aj} \exp(\sigma_j \Delta r^2/2) \, dr}{r_b^2 - r_a^2}$$

(16)

where $r_a$ and $r_b$ are the radial position of the meniscus and the base, respectively. An expression in terms of the errors $\delta f_{oj}$ and $\delta f_{aj}$ is obtained by taking the partial derivative of equation 16:

$$\delta f_{oj} = \frac{\delta f_{aj}}{f_{oj}} \int_{r_a}^{r_b} \exp(\sigma_j \Delta r^2/2) \, dr$$

(17)

If the right hand side of this equation is multiplied and divided by $f_{aj}$ and equation 16 is substituted into the result,
the following simple expression is obtained:

$$\delta f_{o j} = \frac{\delta f_{a j}}{f_{a j}} f_{o j} \quad (18)$$

By recognizing that $\delta f_{o j}$ is approximated as $\bar{K}$, the equation for the individual errors in $f_{a j}$ is found:

$$\delta f_{a j} = \frac{f_{a j}}{f_{o j}} \bar{K} \quad (19)$$

The error in $K$ is obtained from the square root of the sum of the squares of the fractional error in the $f_{a j}$:

$$dK = K \sqrt{\sum_{j=1}^{s} \left(\frac{\delta f_{a j}}{f_{a j}}\right)^2} \quad (20)$$

Dividing through by $K$ gives the error in $\ln K$; this may be used to calculate the error in the Gibbs' free energy:

$$d\Delta G = .5 d\ln K \quad (21)$$

These calculations were then applied to several sets of data for each interaction studied, and the average parameters of $\gamma$ and $\Delta G$ were obtained by weighting the contribution of each determination by the factors $d\gamma$ and $d\Delta G$.

**J2 dimerization and J2-J3 mixtures**

As indicated previously, data from sedimentation equilibrium experiments using pure samples of J2 in 0.1 M buffer
produced plots of \( \ln f \) versus \( r^2 \) which exhibited curvature. The experimental curves were fitted with the direct search program, assuming a model of two species: non-aggregated \( S_2 \) with a molecular weight of 25,000 and a dimer of \( S_2 \) with molecular weight of 50,000. This dimer might be formed either as a non-covalent association of two molecules of \( S_2 \), or through an intermolecular disulfide bond.

Two independent data sets were calculated and produced the result that 13.6% of the mass in the centrifuge cell could be expressed as the dimer, with the rest made up as the monomer. The errors of the fit, \( \bar{\epsilon} \), were 3.9 and 7.7 \( \mu \), well within the range of the expected plate reading errors.

Next, samples of \( S_2 \) and \( S_3 \) in TMK were combined and the resulting mixtures analyzed in the same manner. The species assumed to be present for the direct search fit were monomeric \( S_2 \) with molecular weight 25,000, monomeric \( S_3 \) with molecular weight 21,500 and a dimer with molecular weight either 50,000 (\( S_2-S_2 \)) or 46,600 (\( S_2-S_3 \)). The final results of the fitting procedure were the same for both models, which is not unreasonable, since the two dimer molecular weights are so similar. For four experimental curves, the average residual was 10.7 \( \mu \); while this number is higher than the error found for the \( S_2 \) data in TK, it is still not far from the expected plate reading error.

For all four sets of data, the amount of dimer was low, between 4 and 11% of the protein mass in the cell. Since
this amount of dimer is so close to the amount found in the samples of S2 alone, the conclusion is drawn that any protein-protein interaction between S2 and S3 is weak or non-existent. This conclusion might seem to be contradicted by the observation of Kurland (seminar given at Baylor College of Medicine) that intact ribosomes exposed to chemical crosslinking reagents can produce a cross-linked S2-S3 pair, however the ability to find a cross-linked pair does not necessarily demand that the two proteins be in physical contact with each other. Rather, they may be adjacent to each other on the surface of the ribosome as the result of interactions with other components of the ribosomal system. This argument has been used in a rather unconvincing fashion by Lutter et al. (1974) to explain all the cross-linked pairs as solely the result of protein-DNA interactions with the exclusion of any protein-protein interactions. While this might be the proper explanation for the S2-S3 crosslinked product, the evidence provided below would indicate that such an explanation cannot be universal.

**S3-S5 Interaction**

It has been shown previously in this report that mixtures of S3 and S5 show qualitative evidence of interaction between these two proteins. The preliminary evidence obtained from the tangent to the ln f versus r² plot for this mixture (Figure 13) indicated that the stoichiometry of the complex might be 1:1 S3 to S5, since the molecular
weight average at high concentrations was always less than the molecular weight of the 1:1 dimer. To further test this model in a more quantitative fashion, the direct search program was used to calculate several data sets from sedimentation equilibrium experiments of mixtures of 33 and 35. The results of these fits were very good; the three meniscus concentrations calculated by the direct search program described the experimental curves with values of $\bar{X}$ between 2 and 6 $\mu$. As a further demonstration that the data could not be accounted for by assuming a lack of an interaction, fits were calculated allowing only the two monomeric species. The average residual for these calculations ranged between 20 and 30 $\mu$ and is too large to be explained as simply random data collection error. This is emphasized in Figure 14, where the dashed line describes the fit in terms of two species. The excellent agreement with experimental data in terms of a three species fit is illustrated by the solid line. The largest deviations in this fit are seen near the base; such deviations can be ascribed to inaccuracies of plate reading, since in taking readings where the fringes rise steeply and can often become less distinct, such errors are not unexpected. The systematic deviations from the two species fit in Figure 14 are far beyond the reasonable limits of the method.

Yet another argument in favor of the proposed complex comes from a consideration of the conservation of mass.
Figure 14: S3-S5 Interaction A mixture of S3 and S5 in T1K buffer at 32,000 rpm, 3.0°C. Column height was 1.4 mm; initial protein concentration was 0.066 mg/ml of each protein.
Knowing the meniscus concentration of each species, it is a simple matter to calculate the total amount of each species present in the cell by means of equation 16. The experiments were started with equal weight concentrations of the two proteins. The two species fit in Figure 14 yields a mass ratio of S3 to S5 of 9.35; the mass ratio from all species for the three species fit is 1.04. Similar results were obtained for all other experiments. These results are especially gratifying because the method does not impose this constraint, rather it is derived as a result only by requiring that the three $f_{aj}$ fit the observed data.

S5-S10 Mixtures in TEK

The amount of purified S5-S10 was limited, thus only two determinations were possible in TEK buffer. When the molecular weight averages near the base of the cell were examined, a species was indicated which was higher in molecular weight than either of the two monomeric proteins, but less than the dimeric molecular weight for a 1:1 complex of the two proteins. When the two data sets were subjected to the direct search calculation, invoking a model of these three species, the residual of the final fit in each case was greater than 15 $\mu$. The high residuals could be due to poor data or to an incorrect model. Since the stoichiometric data for the ribosomal proteins (Voynow and Kurland, 1971; Weber, 1972) indicates that one or less copy of each
protein is to be found for each molecule of 16S RNA, the preferred interpretation for the high residual is that the data are poor. A definite choice between the two explanations cannot be made until experiments can be repeated under more favorable conditions.

**S3-S5-S10 Mixtures**

Sedimentation equilibrium experiments were performed with 1:1:1 mixtures of S3, S5 and S10 in T4K buffer and plotted on an ln f versus r^2 basis. Figure 15 is such a plot; once again, a curve was obtained and tangents were drawn to the curve to obtain the limiting molecular weight averages. The figure presented is representative of the four experiments performed with this ternary system: the molecular weight average at low concentrations is indicative of the presence of low amounts of complex species, while the high concentration data indicates that a complex larger than a simple dimer between any two molecules of protein present. An attractive model would be a 1:1:1 complex of S3, S5 and S10. With three proteins in a system such as this, seven possible species would be expected for unitary stoichiometry, namely the three monomeric species, three dimers and a trimer. This is far too complicated a system to resolve from sedimentation equilibrium data; even when the direct search program was modified to make use of the knowledge of how the S3-S5 dimer is formed or by assuming a model with no dimers, the final fit was
Figure 15: 33-35-310 Mixture in T.K. Buffer

A mixture of 33, 35 and 310 in T.K. buffer at 25,000 rpm, 7.0°. Column height was 2.4 mm; initial protein concentration was 0.073 mg/ml of each protein. The molecular weights were obtained from the lines through the data.
dependent on the initial guesses for the $f_{ij}$, or gave results which were physically meaningless. Thus of the methods employed to analyze this mixture, the most useful in this case is the $\ln f$ versus $r^2$ plot. The indication from this plot is consistent with the stoichiometry data in that the molecular weight average at high concentrations approaches that of a $1:1:1$ trimer and does not indicate a higher order complex.

$S_3$ and $S_5$ mixed together have revealed a molecular complex; $S_5$ and $S_{10}$ together also indicate a molecular complex. When all three proteins are mixed, a molecular weight average is observed that is well above any dimer and is consistent with a ternary molecular complex.

**Equilibrium Constants and Free Energy of the Association**

From the three $f_{ij}$ calculated by the direct search fit, an association equilibrium constant was calculated for each of the $S_3-S_5$ sedimentation equilibrium experiments from equation 15. Table 9 presents an average value for each of the nine experiments; these determinations were made using a variety of rotor speeds, column heights and initial loading concentrations to give the average.

Also presented in Table 9 is the average Gibbs' free energy calculated from each of the nine association constants for the $S_3-S_5$ interaction in T:K buffer at 3°.

**Implications for Chromosomal Structure**

In view of the data presented here, it is clear that
TABLE 9

33-35 Complex Formation

Association constant  \[ K = 5.7 \times 10^5 \text{ 1/mole} \]
\[ \pm 1.0 \times 10^5 \]

Free energy of association  \[ \Delta F^\circ = -7.25 \text{ kcal/mole} \]
\[ \pm 0.56 \]
interactions between ribosomal proteins should not neces- 
sarily be eliminated when considering models of ribosome 
structure and assembly. The measured interaction between 
S3 and S5 is considered to be significant for the following 
reasons:

(1) Interaction is always detected immediately after 
mixing the two proteins and demonstrates no short-term time 
dependence which could significantly alter the interpreta-
tion. Although non-specific aggregation is seen in some 
samples of S3, no particular problem is encountered over 
the time course of the experiments carried out here. Thus, 
as indicated by determination of the frictional ratios, the 
state of the protein refolded under the described conditions 
contains sufficient structure that interaction between these 
two proteins does not require the binding energy of further 
components (i.e. A) in order to form a specific complex. 
This interaction was observed whether the proteins were 
refolded separately then mixed or refolded together, in 
concert with the concept that the proteins can find their 
final state independent of other components provided suffi-
cient thermal energy is available.

(2) Since pure isolated S3 and S5 show no self associa-
tion under the same conditions as the mixtures, the inter-
action must be of a heterogeneous nature, as would be found 
in the least complicated model, the S3-S5 dimer. "Goodness 
of fit" alone cannot rule out a higher order complex, but
this would appear to be highly improbable in light of the stoichiometric measurements for these two proteins (Voynow and Kurland, 1971; Weber, 1972). These two proteins, and in fact all 30S proteins, have been reported to be present in amounts that correspond to one or less copy per ribosome.

(3) The value of the equilibrium constant does not vary with total protein concentration or reasonable lengths of time after refolding. This provides the information that all protein molecules are entering into the complex and that the proposed model of one S3 and one S5 combining to form a 1:1 complex is correct. Under the conditions used, a higher order complex such as 1:2 or 2:2 is not consistent with the sedimentation equilibrium data, for it would give rise to a variation in the equilibrium constant of 1-2 orders of magnitude and about 1 order of magnitude in the mass ratio calculated on the assumed model. This indicates that no higher order complex can be detected by the method and that the predominant complex is the postulated 1:1 S3-S5 dimer.

(4) The free energy difference is large and negative, on the order of the difference between the native and denatured states of many other proteins (Brandts, 1964; Aune and Tanford, 1969; Salahuddin and Tanford, 1970). This then represents a moderately strong and specific affinity between two proteins which have been previously implicated to influence each other in binding to a reconstituted ribosome.
(Izushima and Homura, 1970). The fact that this interaction alone is not strong enough to maintain structural integrity, as is seen in the whole ribosome, indicates the possible interdependencies that could exist between the moieties comprising the structure of the ribosome. Evidence is rather compelling that a combination of additional protein-protein and protein-IA interactions does in fact lead to the complex that is known as the ribosome. Direct interactions between single 30S protein and 16S IA have been confirmed for only five proteins; the interactions between the other 16 proteins and the IA have been presumed to occur only when one or more of the five are present. Arguments have been presented that binding of the first five proteins induces conformational changes which uncover new sites on the IA, and that these protein-IA interactions are the only molecular interactions in the 30S ribosome (Lutter et al., 1974). Such a constraining model may be unnecessary if each component of the system participates in one or two of the interactions of the type seen between 33-8S, and such as the ternary complex implied in the 53-85-310 mixtures. A multiplicity of such interactions could provide a system with the degree of structural unity found in whole ribosomes.
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