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STUDIES ON STRUCTURE OF *E. COLI* RIBOSOMES

by

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Dean of Graduate College

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Ames, Iowa

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ABBREVIATIONS AND SYMBOLS

$[\alpha]_\lambda$ = specific rotation at a particular wavelength, $\lambda = \alpha_1$ observed rotation divided by the product of the concentration of solute (g/100 ml) and the length of light path (decimeters)

$\mathring{A}$ = Angstrom = 10^{-8} centimeters

β = hydrodynamic parameter = $\left(\frac{N}{1.62\pi^2 \times 10^4}\right)^{\frac{1}{3}} \frac{\nu^{\frac{1}{3}}}{\left(\frac{f}{f_0}\right)}$

BAG = bentonite-agar-gel

DNase = dexoxyribonuclease

$E_{260}^{0.1\%}$ = extinction coefficient = absorbency of a solution containing 1 mg RNP/ml, in a cell 1 cm in thickness at 260 mμ

E(P) = molar extinction of free nucleic acids or components containing nucleic acid in terms of moles of phosphorus at 260 mμ = average extinction at 260 mμ per mole of nucleotide for free RNA

EDTA = ethylenediamine tetraacetate sodium salt pH 7.4

$\frac{f}{f_0}$ = frictional ratio = ratio of experimentally determined frictional coefficient (f) to the frictional coefficient of a sphere of equivalent volume = 1.0 for an inert sphere and increases the more asymmetrical the particle

g = acceleration due to gravity = 980 cm/sec^2

$[\eta]$ = intrinsic viscosity = reduced viscosity corrected for concentration

M = concentration in moles/liter

mM = concentration in millimoles/liter

M.W. = molecular weight

mg = milligrams

ml = milliliter

mμ = millimicron = 10^{-7} cm

ν = viscosity increment = $\frac{[\eta]}{(1-\bar{V}\rho)}$ = 2.5 for an inert sphere and increases for assymmetrical particles

N = Avagados number = 6.02×10^{23} molecules/mole

ρ = density of solution = density of water at 20° = 1.00 g/ml

PCA = perchloric acid

RNA = ribonucleic acid

r-RNA = ribosomal RNA

RNase = ribonuclease

RNP = ribonucleoprotein

rpm = revolution per minute

S = Svedberg, a unit of sedimentation velocity = 10^{-13} seconds

$S_{20,w}$ = sedimentation coefficient in Svedbergs reduced to viscosity and density of water at 20°

$S^{0}_{20,w}$ = sedimentation constant = sedimentation coefficient corrected for concentration

TCA = trichloroacetic acid

Tris = tris(hydroxymethyl)aminomethane

$\bar{V}$ = partial specific volume in ml/g = increase in volume resulting from the addition of 1 g of RNP to a large volume of solution

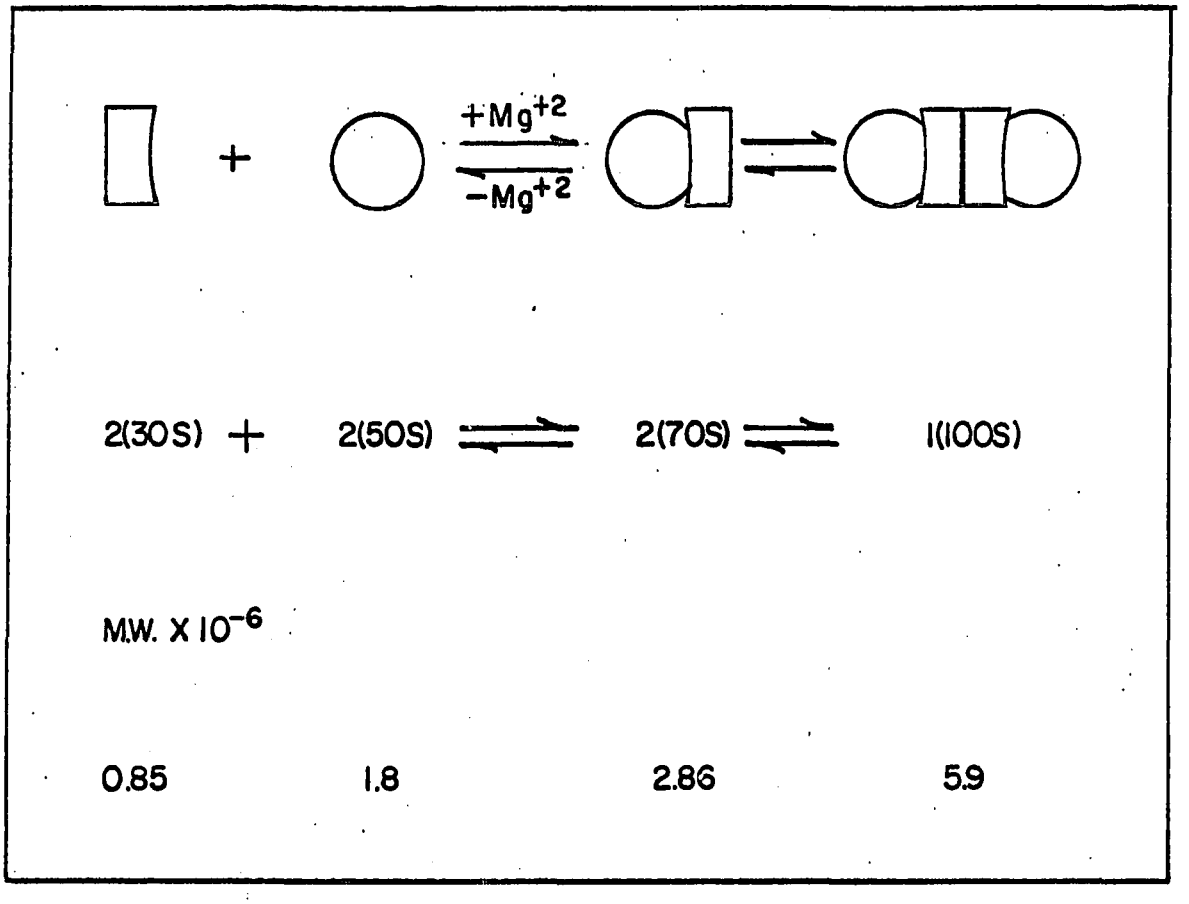
INTRODUCTION

The mechanism by which amino acids in the cell are polymerized into a specific protein involves a small organelle—the ribosome. How the ribosome functions in the biosynthesis of proteins is as yet unknown and the main reason for this is the lack of knowledge concerning the structure of the particle. Studies reported in this dissertation were designed to examine the behavior of the ribosome under artificial conditions in the hope of furthering our knowledge and understanding of the structure of the ribosome.

A review of the physical and chemical properties of the ribosomes from the bacterium E. coli will be presented since these ribosomes were employed in the studies reported.

Ribosomes are ribonucleoprotein particles which are composed of 60-65 percent RNA and 35-40 percent protein (Tissiéres et al., 1959). The structure of the ribosomes as revealed by the work of Tissiéres et al. (1959), Hall and Slayter (1959), and Huxley and Zubay (1960) is schematically represented in Figure 1. A 70S ribosome (molecular weight = 2.86×10^6) is composed of one 30S subunit (molecular weight = 0.85×10^6) and one 50S subunit (molecular weight = 1.8×10^6) with the 100S particle being formed by the dimerization of two 70S ribosomes through the 30S moieties. The stochiometry of the reactions and the molecular weights of various ribosomal particles are shown in the middle and lower rows, respectively,

Figure 1. A schematic of the structure of the ribosomes from *E. coli*


+Mg+2
-Mg+2
2(30S) + 2(50S) 2(70S) 1(100S)
M.W. X 10-6
0.85
1.8
2.86
5.9

in Figure 1. Magnesium reversibly effects the association of the ribosomes with all four components being observed at the appropriate concentration; in 10^{-1} mM Mg^{+2} only 30S and 50S subunits are observed while increasing the Mg^{+2} concentration to 10 mM results in the formation of 70S and 100S particles. The 50S subunit when observed in the electron microscope either combined with the 30S in the 70S ribosome or free in solution is nearly spherical in shape with a diameter of about 160-180 Å. However, the 30S is more asymmetrical and when observed combined with the 50S appears as a more flattened structure having dimensions of about 70 x 170 Å with the smaller dimension lying along the major axis of the 70S particle. When the 30S is observed free in solution, the subunit assumes many different shapes, triangular, trapozoidal, crescent, etc.

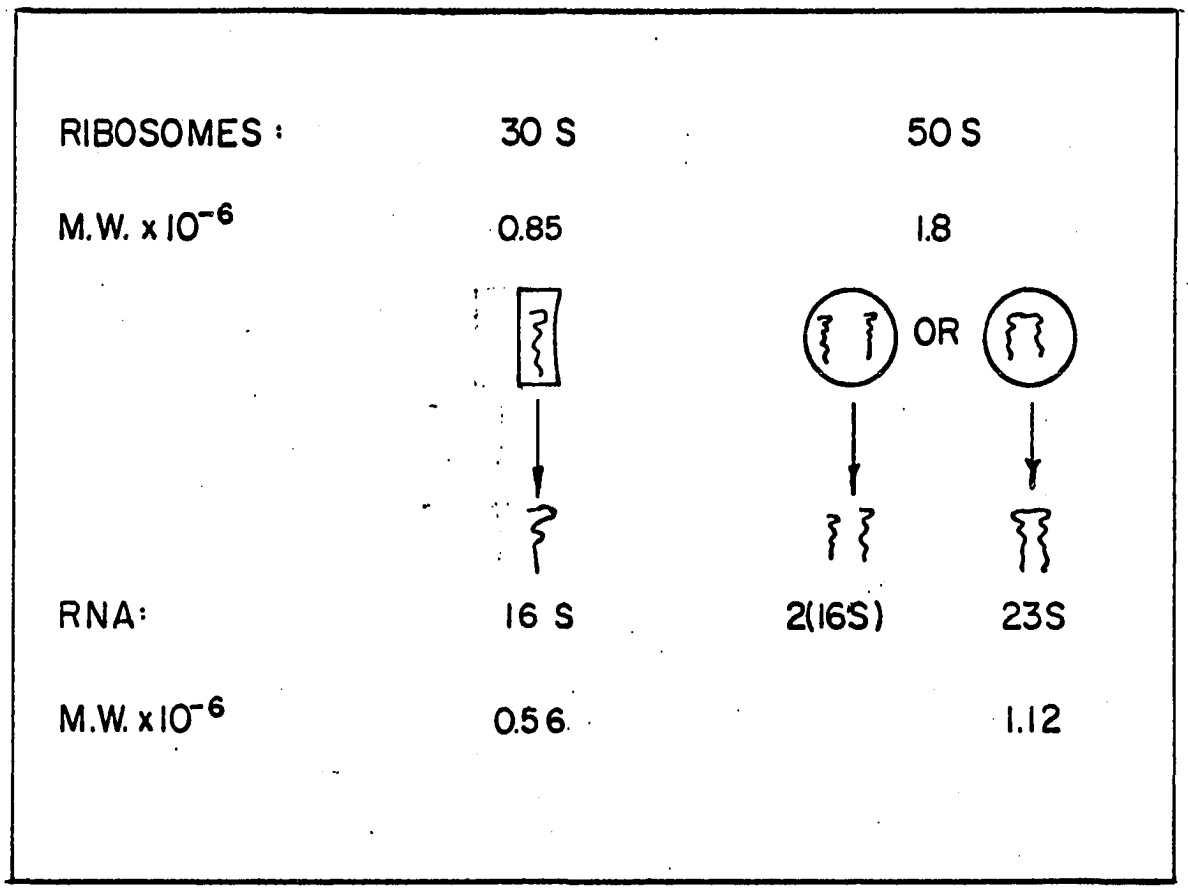
In some laboratories, cell-free extracts of E. coli prepared in Mg^{+2} sufficient to stabilize the 100S particle contained in addition to the four main components mentioned, an 85S particle and a small amount of a 20S component (Bolton et al., 1959). The 85S particle is presumed to be a 70S ribosome which is sedimenting faster because of a change in shape and/or hydration while examination of the 20S material revealed two components, one rich in RNA and the other rich in protein (Roberts, 1960).

Kurland (1960) extracted the RNA from the 70S ribosome and the purified subunits and showed the 30S particle

contained one molecule of 16S RNA (molecular weight = 0.56 × 10^6) while most 50S subunits contained one molecule of 23S RNA (molecular weight = 1.12 × 10^6)(Figure 2). However, a few 50S particles contained two molecules of 16S RNA. Green and Hall (1961) examined the 50S ribosomes and found those 50S subunits that did not form 70S ribosomes in 10 mM magnesium contained 16S RNA while the 50S subunits derived from the dissociation of the 70S particle contained 23S RNA.

The structure of free ribosomal RNA has been studied extensively by a number of physical methods and the results of the investigations by Littauer (1961), Cox and Littauer (1962), Kisselev et al. (1961), Spirin (1960 and 1961), Hall and Doty (1959), Doty et al. (1959), Fresco et al. (1960), Spirin and Milman (1960), Boedtker et al. (1962), and Stanley and Bock (1965) can be summarized as follows: each RNA molecule (16S or 23S) exists as a single continuous polynucleotide chain with a number of helical regions which are formed when the polynucleotide chain loops back on itself. These double stranded regions were originally thought to be stabilized entirely by hydrogen bonds between base pairs, adenine-uracil and guanine-cytosine, but more recent experiments have suggested interactions in addition to hydrogen bonds may be important in maintaining the double stranded regions (Tinco et al., 1963; Fasman et al., 1964). The structure of RNA (secondary and tertiary) under a specific ionic condition and temperature is the result of the interplay among molecular

Figure 2. An illustration of the type of RNA extracted from the 30S and 50S ribosomes


RIBOSOMES :
30 S
50 S
M.W. x 10⁻⁶
0.85
1.8
OR
RNA:
16 S
2(16S)
23S
M.W. x 10⁻⁶
0.56
1.12

forces—the most important of which are the hydrogen bonds between bases and the electrostatic repulsion force between unshielded phosphate groups of the RNA chain. Thus in low ionic strength solutions (water) the double stranded regions are destabilized at room temperature and RNA exists as a long single stranded polynucleotide chain while at a higher ionic strength (about 10^{-1} M salt) where the salt shields the negative changes on the phosphate groups, the helical regions are stabilized and the RNA assumes a highly compact coil conformation. Spirin's laboratory has indicated that in the intermediate ranges of ionic strength the RNA forms a rod-shaped structure in which the double stranded regions are ordered, but other laboratories (Hall and Doty, 1959) contend the RNA exists as a more highly expanded coil which lacks a definite tertiary structure in these solutions.

Much less is known about the structure of the RNA in the ribosome. Hyperchromicity studies with E. coli ribosomes (Schlessinger, 1960; Zubay and Wilkins, 1960; Bonhoeffer and Schachman, 1960) and x-ray diffraction studies of wet ribosomal gels and dry specimens compared with free RNA and protein (Zubay and Wilkins, 1960; Klug et al., 1961; Langridge and Holmes, 1962; Langridge, 1963) indicated that the RNA, as it exists in the ribosome, has essentially the same secondary structure as the RNA extracted from the ribosome in high ionic strength solutions (about 10^{-1} M salt)(i.e., about 80 percent of the nucleotides in the RNA chain are organized into regions

of helical structure).

The proteins of E. coli ribosomes extracted with 67 percent acetic acid, showed about twenty bands on starch-gel electrophoresis and an estimate of the average molecular weight from N-terminal analysis gave a value of 25-30,000 (Waller and Harris, 1961; Haruna, 1963; Waller, 1963; Waller, 1964). More recently disc-gel electrophoresis of protein prepared by treating ribosomes with 8 M urea and 4 M LiCl revealed 21 bands, 17 basic and 4 acidic, in the protein of the 50S and 13 bands, 11 basic and 2 acidic, from the protein of the 30S subunit with only one band common to both subunits (Cox and Flaks, 1964). Thus, the protein of the ribosome appears to be composed of a very heterogeneous class of low molecular weight subunits with most of the subunits being basic proteins (28 out of 34). However, discussions with other scientists, recently, have suggested that the heterogeniety of ribosomal protein may not be as great as indicated by electrophoretic analysis.

Positive staining of the nucleic acid component of the ribosome with uranyl acetate and examination in the electron-microscope showed the RNA and protein were mutually interwoven rather than the nucleic acid component being located inside a protein shell—a structure common to the small spherical viruses (Huxley and Zubay, 1960).

Studies designed to learn more about the interaction of the RNA and protein or about the organization of these

components in the ribosome have been hampered by the instability of the particles (Spirin, 1964). This instability is due to an RNase bound to the 30S subunit (Tal and Elson, 1963) that degrades the ribosomal RNA when the effective magnesium concentration is reduced much below 10^{-1} mM (Bolton et al., 1958; Tissiéres et al., 1959).

Recently, evidence from several laboratories has suggested the RNase may not be a structural part of the ribosome, but is adsorbed to the ribosomal surface during the preparative procedure. The evidence for this consists of: (1) the RNase is released into the medium when E. coli cells are converted to spheroplasts (Neu and Heppel, 1964), and (2) RNase can be washed from the ribosome by treating the particles with 0.5 M ammonium chloride and these RNase-deficient ribosomes are active in in vitro protein synthesis (Stanley and Bock, 1964).

Protection against degradation of nucleoprotein particles by RNase can be achieved by adding bentonite, a nuclease inhibitor to the solutions (Brownhill et al., 1959; Frankel-Conrat et al., 1961). Bentonite is a naturally occurring aluminum silicate clay with cation exchange properties (Hendricks, 1945; Barr and Guth, 1951). The empirical formula for montmorillorite, chief component of bentonite, is $MgAl_5Si_{12}O_{36}H_6$ where Mg^{+2} is the exchangeable cation (Hendricks, 1945). Structurally bentonite consists of molecular sheets of the alumino-silicate polymer that have an

excess negative charge because of the replacement of Al^{+3} for Si^{+4} in the structural framework (Hendricks, 1945). The excess negative charge of the molecular sheets is balanced by external and exchangeable cations such as Mg^{+2} or Na^{+} (Hendricks, 1945). Recently, Keller et al. (1964) extended the cation exchange studies to include the basic protein pancreatic RNase and found a binding order as follows, RNase > Mg^{+2} > Na^{+} or K^{+}. Therefore, the inactivation of RNase by bentonite appears to be due to the adsorption of the nuclease onto the surface of the clay.

In the hope of learning more about the structure of the ribosome, we have studied the effects of removing magnesium from the nucleoprotein particles under conditions where little, if any, damage to the ribosomal RNA occurred. Degradation of nucleoprotein particles by the ribosomal RNase was prevented by adding bentonite to the solutions and performing all operations in the cold. Evidence was obtained for the polyelectrolyte nature of the ribosome structure.

Since we were using bentonite in ribosomal solutions and in the isolation of RNA, we decided to study the properties of the clay to better define conditions under which bentonite could be used in nucleoprotein solutions. The properties of bentonite are reported in the latter part of the thesis.

METHODS AND MATERIALS

Preparations of Ribosomes

E. coli strain B grown in Penassay Broth at 37° under forced aeration and harvested during exponential growth or purchased as a frozen paste from Grain Processing Corp., Muscatine, Iowa was used in these studies. The purified ribosomes were prepared essentially by the method of Tissiéres *et al.* (1959) which will be described briefly. Cell-free extracts were prepared by grinding the cells by hand in a cold mortar for 5-10 minutes with 2.5 parts (wt./wt.) of alumina powder (bacterological grade A-305, Alcoa Chemicals) and extracting the paste with three volumes of 10^{-1} mM or 10 mM magnesium acetate in 1 mM Tris-HCl [Tris (hydroxymethyl)-aminomethane-hydrochloric acid] buffer pH = 7.4 containing 2 µg of DNase/ml. All manipulations were carried out at cold room temperature (4-6°C). The mixture of the alumina and broken cells was centrifuged at 12,000 g for 25 minutes, giving a sediment composed of alumina, some unbroken cells and large cell debris. The supernatant was clarified further by another centrifugation. From this, the ribosomes were isolated by centrifuging at 105,000 g in the Spinco Model L or L-2 for 3-5 hours. The golden-brown pellet obtained was suspended in buffered solution containing either 10^{-1} mM or 10 mM magnesium acetate using a stirring rod and the solution clarified by centrifuging at 12,000 g for 10 minutes. The

supernatant then represented a crude ribosomal preparation and further purification was obtained by repelleting the ribosomes several times. The ribosomal solutions were either used immediately or frozen at -20° for future experiments. Complete dissociation of the 70S ribosomes to their 30S and 50S subunits was accomplished either by dialysis of ribosomal solutions against buffer containing 10^{-1} mM Mg^{+2} or by employing such a solution in the preparation of the extract or in the purification procedure. Purified 50S ribosomes were obtained by differential centrifugation of solutions containing the dissociated ribosomes (Tissiéres et al., 1959). The concentration of ribosomes was estimated from the absorbance at 260 mμ using $E^{0.1\%}_{260} = 16$, an average value for ribosomes taken from Tissiéres et al. (1959).

Sucrose Gradient Centrifugation

A modification of the sucrose gradient technique of Britten and Roberts (1960) was used. About 1-2.5 mg of RNP-particles in a ml or less was layered on a 25 ml, 5-20 percent linear gradient of sucrose. The sucrose solutions were prepared in 1 mM Tris-HCl pH 7.4 containing the same concentration of Mg^{+2} as the nucleoprotein sample. After centrifuging for five hours in the cold (5-8°C) at 25,000 rpm in the SW 25.1 rotor of the Spinco Model L ultracentrifuge, the bottom of the tube was punctured and one ml fractions were collected. The A_{260} profile (A_{260} versus fraction number) was determined

after diluting the samples to 3 ml with the appropriate buffered solution.

Isolation of RNA

The RNA was extracted from RNP-particles by the phenol-sodium dodecyl sulfate method of Kurland (1960). An appropriate amount of a stock suspension of bentonite was added to RNP solutions to give a concentration of 0.5 mg/ml and the solutions were shaken at room temperature for a few (2 to 5) minutes with 0.5 percent sodium dodecyl sulfate. A volume of the SDS treated RNP solutions (RNP concentration = 2-4 mg/ml) was shaken with an equal volume of 80 percent phenol at room temperature for 15 minutes. Prior to use liquified phenol was distilled under vacuum to eliminate any oxidation products and then 80 percent phenol (v/v) was prepared by mixing appropriate amounts of liquid phenol and a solution buffered to pH 7.4 with 1 mM Tris-HCl containing 10 mM magnesium. The mixture was centrifuged at about 5000 g in the Serval SS1 for a few minutes and the aqueous phase was pipetted free of the denser phenol phase. This operation was repeated two or three times more with smaller volumes of phenol. Bentonite was added to the RNP solution at a concentration of 0.5 mg/ml before each subsequent extraction with phenol.

Two volumes of cold 95 percent ethanol were added to the final aqueous phase and potassium acetate was added to make

the solution about 0.2 M. After at least 4 hours at -20° the flocculent precipitate was centrifuged down and suspended in 10 mM magnesium acetate, 0.15 M sodium chloride and 1 mM Tris-HCl pH 7.4. The precipitation was repeated two more times. The final solution was adjusted to a concentration of about 2-3 mg/ml and analysed in the ultracentrifuge. RNA concentrations were estimated from the absorbance at 260 mμ and using $E_{260}^{0.1\%} = 25$.

Processing of Bentonite

The bentonite was processed according to the procedure of Frankel-Conrat et al. (1961). Two grams of commercial bentonite were suspended in 40 ml of distilled water and the suspension was graded for particle size by differential centrifugation as follows. The suspension was centrifuged at 1000 g for 15 minutes in the Serval SS1 and the sediment was discarded. Then the supernatant was recentrifuged at 10,000 g in the SS1 for 20 minutes and the supernatant containing fine particles was decanted. The sediment from the 10,000 g centrifugation was resuspended in 0.1 M EDTA (Na salt) pH 7.4 and after a 48-hour incubation at room temperature the suspension was again centrifuged differentially. Finally the sediment from the 10,000 g centrifugation was suspended in 10^{-1} mM magnesium, 1 mM Tris-HCl pH 7.4 and the suspension was dialyzed against this solution for 24-48 hours. The magnesium concentration in which the bentonite was suspended was changed

by centrifuging the bentonite from 10^{-1} mM magnesium and resuspending in the appropriate solution. Dry weights were determined on stock solutions by evaporating the water from aliquots in a 110°F oven until constant weights were obtained. Bentonite was added to ribosomal solutions to give a concentration of 0.5 mg/ml unless otherwise stated in the text.

Preparation of Bentonite-Agar Gel

The bentonite-agar gel was prepared either by autoclaving 5 percent Bacto-agar for 20 minutes (150°), removing from the autoclave and immediately adding solid bentonite (not processed) to give 5 percent bentonite or by autoclaving 10 percent Bacto-agar, removing from the autoclave and immediately adding an equal volume of well-suspended 10 percent bentonite in water. In either method after adding the bentonite the suspension was then placed in ice and stirred until the gel formed. This was to insure the uniform suspension of the bentonite in the gel. The gel, which now appeared grey due to the bentonite, was cut into small pieces and forced through a 60-mesh screen. Fine particles were washed away by collecting the gel particles on an 80-mesh screen and flushing several volumes of distilled water over them. The gel was then treated 24 hours or longer with 0.5 M EDTA (sodium salt) pH 7.4 to produce sodium bentonite. Finally, the EDTA was washed away with distilled water and columns of the particles were packed by gravity in the cold room.

Several hundred ml of 10 mM magnesium in 1 mM Tris-HCl pH 7.4 were passed through the column to form magnesium bentonite. The usual column size was 10 cm high and 1.6 cm in diameter.

Physical Methods

Ultracentrifuge assays were done with a Spinco Model E equipped with schlerien and ultraviolet optical systems. The films obtained with the ultraviolet optics were analyzed for their optical density with the Beckman Model RB Analytrol densitometer equipped with a film adaptor. Sedimentation coefficients were corrected to the viscosity and density of water at 20°. They were reported in Svedberg units.

Relative amounts of components were determined by measuring the optical density contributed by each component on the films from the ultraviolet optics or by measuring the area under the various peaks on the schlieren pattern. The area under the boundary on the schlieren pattern was magnified several times by projection of the pattern through an enlarger and the boundary was traced onto paper. A plannimeter was used to quantitate the areas under the individual peaks. The areas and optical densities were corrected for radial dilution.

The viscosity of nucleoprotein solutions was measured with an Ubbelohde viscometer, with a solvent flow time of 580 sec at 5°. Measurements were made in a refrigerated water bath at 5° ± 0.03°. Viscosity of RNA solutions was measured with an Ostwald type viscometer with a flow time for water of

60 sec at 25°.

Absorption measurements were made with a Cary 15 or a Beckman DU spectrophotometer thermostated to the temperature indicated. Ultraviolet optical rotatory dispersion traces were made with a Jasco Model ORD/UV-5 automatic recording instrument.

Chemical Methods

Samples to be analyzed for RNA and protein were acidified with cold TCA, to a final concentration of 7 percent. Precipitates were collected after an hour incubation in the cold (4-6°C) and washed several times with cold 7 percent TCA. The nucleic acid was extracted from the precipitate with 7 percent TCA at 95-99° for 10 minutes and the concentration of RNA in the extract was estimated by the orcinol method of Mejbaum (1939) with E. coli ribosomal RNA as a standard. The hot TCA insoluble pellet (protein fraction) was dissolved in 0.2 N sodium hydroxide and the concentration of protein was determined by the method of Lowry et al. (1951) with bovine albumin as a standard.

Ribonuclease content of RNP samples was estimated by measuring the amount of acid soluble material produced by the action of the enzyme on yeast RNA as described by Neu and Heppel (1964). The enzyme was prepared by incubating ribosomal solutions (concentration = 0.03 - 0.25 mg/ml) at 37° for 40 minutes in the presence of 10^{-2} M EDTA and 10^{-1} M phosphate

pH 7.4. This treatment results in the activation of the ribosomal bound RNase and subsequent degradation of the ribosome. An aliquot (0.05 - 0.4 ml) of the enzyme preparation was added to 0.6 ml of a solution containing yeast RNA (2 mg/ml), EDTA (10^{-2} M EDTA) and phosphate buffer (10^{-1} M) pH 7.4. The reaction was stopped after 60 minutes at 37° by the addition of 0.25 ml of cold 0.75 percent uranyl acetate in 25 percent PCA (perchloric acid). After 10-30 minutes in ice the precipitate was packed down by centrifuging in the International PR-2 at 2000 rpm for 10 minutes at 4-6°. An aliquot (0.5 ml) of the clear supernatant was added to 2.5 ml of water and the absorbance was determined at 260 and 310 mμ. Each sample had its own blank which consisted of everything mentioned above, but the enzyme was added after the precipitation of the RNA. The results were reported as percent of the activity measured in the original ribosomes.

Nucleoprotein samples to be analyzed for phosphorus content were digested by adding 7.5 N PCA and heating for 1.5 minutes over an open flame. The digested samples were then diluted with distilled water and the phosphorus content was estimated by the method of King (1932).

Reagents and Special Chemicals

Pancreatic RNase and DNase were obtained from Worthington Biochemical Corporation as the crystalline enzymes. Yeast RNA (20-30,000 molecular weight) was obtained from CALBIOCHEM and

was a B grade preparation. Distilled water was passed through a Barnstead Still Model BD5 to eliminate divalent cations. Bentonite was obtained from Fisher Scientific Co. and was U.S.P. grade. Penassay broth and Bacto-agar were obtained from Difco Laboratories. All other reagents used were either primary standard or analytical grade reagents.

RESULTS

The Effects of Removing Mg^{+2} from Ribosomes

Changes in sedimentation patterns

The removal of Mg^{+2} from ribosomes (concentration = 2.5 mg/ml) by dialysis against 5 mM EDTA pH 7.4 in the cold (4-6°C) and in the presence of bentonite at a concentration of 0.5 mg/ml resulted in changes in the sedimentation pattern of the ribosomes (Figure 3). Dialysis of a mixture of 30S (31S) and 50S (47S) ribosomes (Figure 3a) for one hour against EDTA led to the appearance of new components sedimenting at 20S, 28S and 37S with a reduction in the area under the 50S (45S) boundary (Figure 3b). Presumably, one of the slower sedimenting components represented the 30S ribosomes. After one and a half hours of dialysis against EDTA, the 50S ribosomal boundary could not be seen in the ultracentrifuge pattern while the area under the 20S boundary had increased (Figure 3c). Continued dialysis resulted in the disappearance of the 28S and 37S components with an additional increase in the area under the slow boundary (Figure 3d). The sedimentation coefficient of the slow components decreased from about 20S to 12S as the dialysis against EDTA was increased from 1 to 2.5 hours (Figures 3b and 3e). Little, if any, mass was lost as measured by the area under the schlieren patterns on treating the ribosomes with EDTA so that both the 30S and 50S ribosomes were converted to the 12-15 components. In some experiments a

Figure 3. Removal of Mg^{+2} from a mixture of 30S and 50S ribosomes

Ribosomes dialyzed against 5 mM EDTA for:

3a. 0.0 hours, starting material at 10^{-1} mM Mg^{+2}, $S_{20,w}$ = 31.1 and 46.6S

3b. 1.0 hours, $S_{20,w}$ = 20.0, 28.2, 36.5 and 45.4S

3c. 1.5 hours, $S_{20,w}$ = 18.7, 27.8 and 35.2S

3d. 2.0 hours, $S_{20,w}$ = 14.3 and 15.2S

3e. 2.5 hours, $S_{20,w}$ = 12.1S

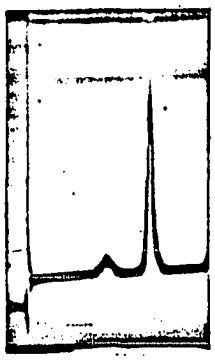
Sedimentation from left to right. Concentration of ribosomes = 2.5 mg/ml. Pictures taken at 45 minutes for a-c and at 53 and 81 minutes for d and e, respectively, after reaching 31,410 rpm. BAR angle 35°.

Figure 4. Removal of Mg^{+2} from 50S ribosomes

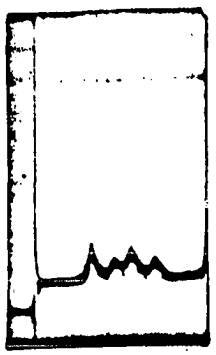
4a. Purified 50S ribosomes at 10^{-1} mM Mg^{+2}, $S_{20,w}$ = 46.4S

4b. 10 mM EDTA added to a sample like 4a, $S_{20,w}$ = 20.0, 34.1 and 44.5S

Concentration of ribosomes = 2.5 mg/ml. Pictures taken 25 and 30 minutes for a and b, respectively, after reaching 31,410 rpm. BAR angle 35°.



3a



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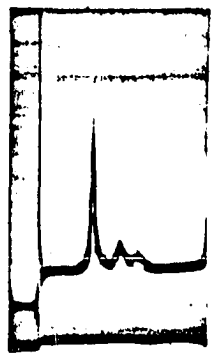

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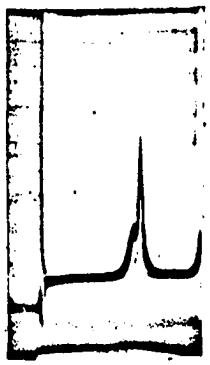

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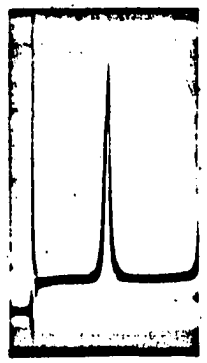
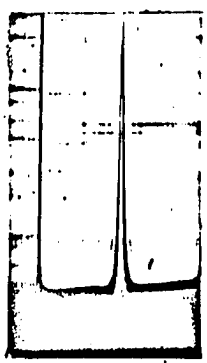


3e



4a

4b

small amount of material sedimenting slowly (4-5S) was observed in the ultracentrifuge patterns in addition to the components mentioned. Other laboratories (Elson, 1961; Cammack and Wade, 1965) have reported a similar release of 4-5S material from ribosomes on varying the ratio of Na^{+} to Ca^{+2} or Mg^{+2} in the solutions.

The ratio of 30S and 50S ribosomal subunits in this experiment was somewhat lower than is usually encountered, most probably because of incomplete pelleting of the 30S subunit during the isolation of ribosomes from bacterial extracts at 10^{-1} mM Mg^{+2}.

Almost identical sedimentation patterns were obtained when EDTA was added at concentrations of 3-10 mM to mixtures of 30S and 50S ribosomes at 2.5 mg/ml and when ribosomes were dialyzed for long periods of time against buffered solutions containing very low levels of Mg^{+} (i.e., 10^{-4} to 10^{-5} mM).

The removal of Mg^{+2} from purified 50S (46S) ribosomes (Figure 4a) by adding 10 mM EDTA led to the appearance of 20S and 34S components as well as 50S particles in the same solution when the sample was examined in the ultracentrifuge within half an hour (Figure 4b). Therefore, the 37S component observed in the previous experiment appeared to be derived from the 50S ribosome and, presumably, represented an intermediate in the conversion of the 50S to the 12-20S component. Since the 28S component seen in the previous experiment was not observed in this experiment with purified 50S ribosomes,

it appeared the 28S component may have represented 30S ribosomes that had been affected little by treatment with EDTA.

Experiments at low ribosome concentrations

There are two major difficulties encountered in interpreting ultracentrifugal data at ribosome concentrations of 2.5 mg/ml (previous experiments) especially under conditions where the RNP-particles are highly charged (i.e., in EDTA): (1) the contribution of the primary charge effect to the decrease in sedimentation rates is unknown, and (2) the increased John-Ogston effect makes relative concentrations in multi-component systems uncertain. Experience had indicated the determination of sedimentation coefficients at 60-90 µg/ml employing the ultraviolet optics of the ultracentrifuge yielded essentially the same value obtained by the extrapolation of sedimentation coefficients from higher concentrations to infinite dilution (i.e., zero concentration). Thus, the sedimentation values obtained at these low ribosome concentrations are corrected for primary charge effects and relative concentrations of components are much more certain.

Therefore, the effect of time of dialysis against EDTA on sedimentation constants and relative amounts of components was examined in detail at ribosome concentrations of 60-90 µg/ml. All operations were conducted at 4-6°C including the ultracentrifugal assays.

These studies indicated that the 30S and 50S ribosomal

subunits were converted on treatment with EDTA to two components sedimenting in the range 20-23S and 33-43S (Table 1a, Experiments 1 and 2). As the dialysis against EDTA was increased, a greater proportion of the material sedimented at 20-23S until eventually only this boundary could be seen in the ultracentrifuge pattern (Table 1a, Experiment 2). The sedimentation constant of the faster component depended on the time of dialysis against EDTA with the value gradually decreasing from 50S to 33S while the sedimentation rate of the slow component changed from 30S to about 20-23S.

These studies largely corrected for the technical effects mentioned earlier; therefore, it was clear that removal of Mg^{+2} from ribosomes resulted in changes in the sedimentation constants of the nucleoprotein particles.

Reversible and non-reversible changes in the sedimentation rates of ribosomes

The sedimentation rates of EDTA-treated ribosomes increased on addition of Mg^{+2}, but not always to the original values. Table 1b and 1c show the results of restoring the levels of Mg^{+2} to 10^{-1} mM and 10 mM, respectively, in the EDTA-treated ribosome preparation described in Table 1a, Experiment 2. The figures in parentheses in the last column of Table 1a, Experiment 1 show the same thing (10^{-1} mM Mg^{+2}) for this sample. Since the relative amounts of the two components at 10^{-1} mM Mg^{+2} were essentially the same as in the corresponding samples at low Mg^{+2} (i.e., in EDTA), it appeared

Table 1a. Variation of sedimentation constants and relative amounts of components with the time of dialysis against 5 mM EDTA

Experiment 1					
Time of dialysis against EDTA, minutes	0	30	60	90	
Sedimentation constants, Svedbergs	31.8 50.6	31.8 47.8	30.2 42.9	23.0 33.2	(31.2) (50.4)
Relative amounts (slow:fast)	1:4	1:2	1:1	3.5:1	(3:1)
Experiment 2					
Time of dialysis against EDTA, minutes	0	15	30	60	120
Sedimentation constants, Svedbergs	27.4 47.9	28.8 49.4	20.0 42.5	31.0 51.0	20.9
Relative amounts (slow:fast)	1:2.5	1:1.8	1:1	1.8:1	Single component

Table 1b. Effect of restoring Mg^{+2} to 10^{-1} mM on sedimentation patterns of EDTA-treated ribosomes

Time of dialysis against EDTA, minutes	0	15	30	60	120
Sedimentation constants, Svedbergs	27.4 47.9	33.0 52.0	34.0 52.3	31.0 51.0	27.2
Relative amounts (slow:fast)	1:2.5	1:2.1	1:1.6	1.8:1	Single component

Table 1c. Effect of increasing the Mg^{+2} concentration to 10 mM on sedimentation patterns of EDTA-treated ribosomes

Time of dialysis against EDTA, minutes	0	15	30	60	120
Sedimentation constants, Svedbergs	66.5	34.1 64.5	37.2 60.4	33.0 53.2	34.6
Relative amounts (slow:fast)	Single component	1:7	1:4	1:1.4	Single component

the components derived from the 50S ribosomes sedimenting at 33-43S in EDTA now again sedimented at about 50S on restoration of the Mg^{+2} to 10^{-1} mM while the sedimentation constant of the slower component (20-23S) increased to about 30S (27-34S)(Table 1b).

After the removal of bentonite from nucleoprotein solutions, the level of Mg^{+2} in the solutions was increased by dialysis to 10 mM. The bentonite was removed because the RNP-particles would have been adsorbed by the clay at the higher Mg^{+2} concentration (Table 3). At 10 mM Mg^{+2}, some 70S particles were formed in those samples which showed little effect of Mg^{+2} ion removal, but in samples where significant changes had occurred, no 70S particles were formed (Table 1c). It appeared, however, the higher Mg^{+2} concentration had caused the shift of some mass from the slow to the fast boundary in the sample at the later times (compare data from Tables 1b

and 1c). This transfer of mass may represent reformation of the 50S structure at the higher Mg^{+2} concentrations or may be the result of aggregation of the slower sedimenting particles. Aggregation of 30S ribosomes to yield 60S particles at 10 mM Mg^{+2} was reported previously by Cannon et al. (1963). At still higher concentrations of Mg^{+2}, 100 mM, the 30S nucleoprotein particles prepared from a mixture of 30S and 50S ribosomes by treatment with EDTA (like the 120 minute sample in Table 1) were insoluble and precipitated from solution. The 30S particles (prepared in a similar manner) were precipitated from solution at 10 mM Mg^{+2} at higher concentrations of RNP-particles (2.5 mg/ml).

These experiments showed that two types of changes occurred in the 50S ribosomes on treatment with EDTA; (1) a smaller decrease in sedimentation constant to about 33-43S that could be reversed with Mg^{+2} (0.1-10 mM) to 50S and (2) a larger decrease in sedimentation constant to 20S that was increased to about 30S instead of 50S on restoration of Mg^{+2}. As more Mg^{+2} was removed from the ribosomes, a greater proportion of the mass was observed sedimenting at 30S (at higher Mg^{+2}) until only this component could be seen in the ultracentrifuge pattern. The behavior of the 30S ribosomal subunit on treatment with EDTA is more difficult to determine because the 50S particle yields a component sedimenting at about 30S on removal of Mg^{+2}. However, since there is no apparent loss of mass in these experiments and all the

nucleoprotein mass can be found sedimenting about 20S in EDTA and 30S on restoration of Mg^{+2} (see 2-hour sample, Table 1a, Experiment 2 and Table 1b), it appeared the 30S ribosome may have undergone a reversible decrease in sedimentation constant from 30S to 20S on removal of Mg^{+2}. This conclusion has been confirmed by more recent experiments with purified 30S ribosomal subunits (Shechter and Horowitz, 1965). The 30S particles prepared from purified 50S ribosomes or a mixture of 30S and 50S ribosomes by treating the ribosomes with EDTA will be referred to as 30*S particles in order to avoid these particles being confused with the 30S ribosomal subunits.

Results of increasing the ionic strength with salts

The sedimentation coefficients of the EDTA-treated ribosomes were increased by the addition of salts to the nucleoprotein solutions with the magnitude of the increase depending on the concentration of the salt. Addition of solid KCl at a concentration of 0.19 M to a nucleoprotein solution at low Mg^{+2} containing 18S and 29S components (Figure 5a) caused the sedimentation coefficients of the components (concentration = 2.5 mg/ml) to increase to 28S and 46S, respectively (Figure 5b). At a lower salt concentration (0.07 M), the sedimentation rates were increased to only 20S and 41S, respectively (Figure 5c). The behavior of the components was different at the lower salt concentration (0.07 M) for the sedimentation rate of the fast component (29S) was

Figure 5. Addition of potassium chloride to nucleoprotein solutions at low Mg^{+2}

Potassium chloride added to a final concentration of:

5a. 0.0, starting material in EDTA, $S_{20,w}$ = 18.0 and 28.7S

5b. 0.19 M, $S_{20,w}$ = 27.8 and 46.3S

5c. 0.07 M, $S_{20,w}$ = 20.2 and 40.7S

Sedimentation left to right. Concentration of RNP particles = 2.5 mg/ml. Pictures taken 21 minutes after reaching 31,410 rpm. BAR angle 30°.

Figure 6. Removal of Mg^{+2} from ammonium chloride treated ribosomes

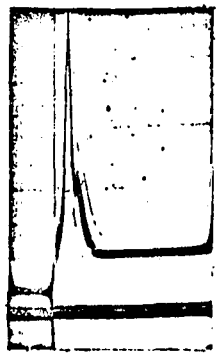
6a. Lower pattern, RNP-particles pelleted from a solution like the upper pattern and resuspended in 1 mM Tris-HCl pH 7.4 (no Mg^{+2}), $S_{20,w}$ = 18.2 and 30.2S

Upper pattern, ribosomes after an overnight incubation at 4-6° in 2 mM Mg^{+2}, 0.5 M NH_4Cl, $S_{20,w}$ = 24.2, 32.7, 47.3 and 63.2S

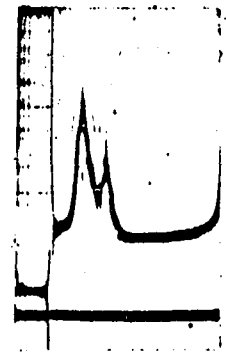
6b. Lower pattern, same solution as (6a) lower pattern incubated at 4-6° overnight, $S_{20,w}$ = 13.9 and 29.8S

Upper pattern, RNP-particles pelleted from solution like lower pattern and resuspended in 0.5 M NH_4Cl, $S_{20,w}$ = 24.6 and 41.8S

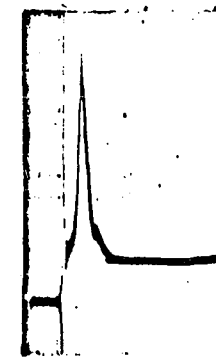
Concentration of RNP-particles = 2.5 mg/ml. Pictures taken 37 minutes after reaching 31,410 rpm. BAR angle 45°.



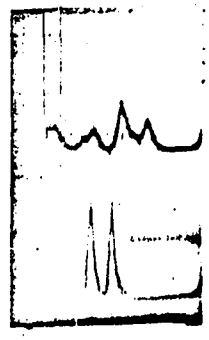
5a



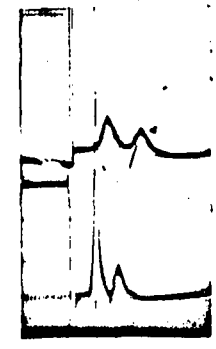
5b



5c



6a



6b

increased to 41S, while the sedimentation rate of the slow component was increased only slightly (18S to 20S) by the addition of the salt. The reason for the different effect of K^+ on the two components is not clear. EDTA-treated ribosomes used in this experiment were prepared by dialyzing a mixture of 30S and 50S ribosomes for about 3 hours against EDTA.

The addition of NaCl up to 0.13 M to a solution of 30*S (28S) particles (prepared from purified 50S ribosomes) at 10^{-1} mM Mg^{+2} increased the sedimentation coefficient (concentration = 2.5 mg/ml) steadily to 32.5S. However, at 0.26 M NaCl the nucleoprotein particles were unstable and a broad slower sedimenting boundary was observed in the ultracentrifuge pattern.

Thus, increasing the ionic strength in solutions of EDTA-treated ribosomes by the addition of KCl or NaCl gave the same results as restoring the Mg^{+2} levels reported previously (Table 1). A smaller decrease in the sedimentation rate of the 50S ribosomes (29S) was reversed to about 50S (46S) at the higher salt concentrations (0.2 M). The larger decrease in sedimentation rate of the 50S (18S) was not reversed to 50S by salt, but the sedimentation rate of the component was increased to about 30S (28-33S). Since no mass was lost in the experiments, the 30S ribosomal subunit, again, appeared to undergo a reversible decrease in sedimentation rate on the removal of Mg^{+2}.

Experiments with ammonium chloride treated ribosomes

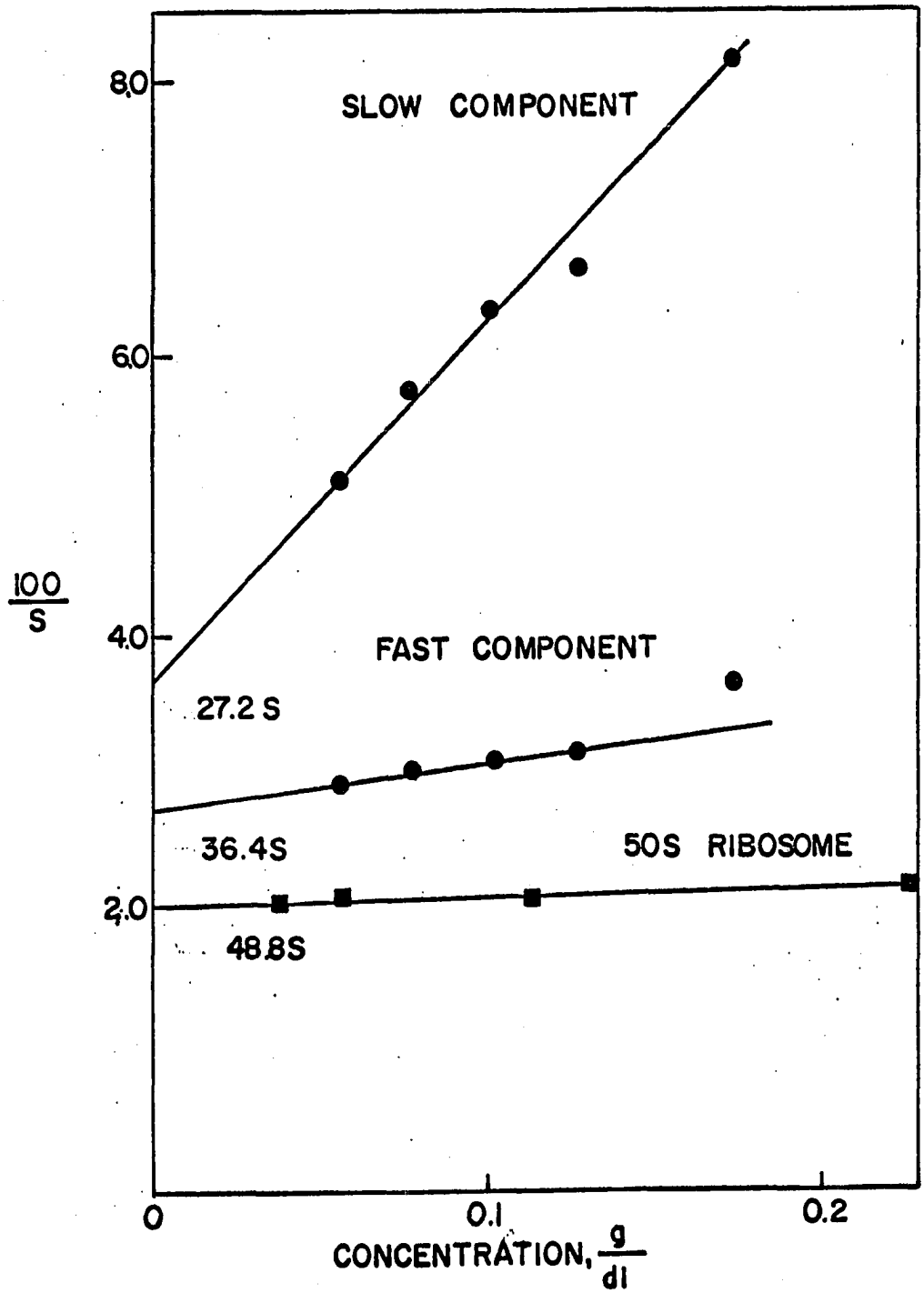
Transferring ribosomes that had been washed free of the latent RNase after treatment with 0.5 M NH_4Cl (Stanley and Bock, 1964) into buffered solution without Mg^{+2} resulted in decreased sedimentation rates of the 30S and 50S ribosomal subunits to 12-15S and 25-30S (concentration = 2.5 mg/ml), respectively (Spirin et al., 1963). Restoration of the Mg^{+2} concentration or increasing the ionic strength of the solutions, Spirin et al. (1963) claimed, led to the appearance of 30S and 50S components in the usual ratio. Thus, it appeared they had not observed the non-reversible conversion of the 50S ribosomal subunit to the 30*S component on removal of Mg^{+2}. Therefore, the sedimentation experiments with NH_4Cl treated ribosomes were repeated in order to better contrast and compare results from the two laboratories.

Ribosomes pelleted from an extract prepared in 10 mM Mg^{+2} were suspended in buffered solution containing 2 mM Mg^{+2}, 0.5 NH_4Cl and incubated overnight at cold room temperatures (4-6°C). Examination of the nucleoprotein solution in the ultracentrifuge after the overnight incubation showed typical 70S (63S) and 50S (47S) ribosomal boundaries (Figure 6a, upper pattern). However, the 30S boundary was heterogeneous and contained two components sedimenting at 24S and 33S. A considerable amount of material that sedimented slowly (4-5S) was released from the ribosomes by treatment with NH_4Cl and material sedimenting between the 50S and 70S boundaries could

be seen in the ultracentrifuge pattern (Figure 6a, upper pattern). These ribosomes were pelleted from solution and resuspended in 1 mM Tris-HCl pH 7.4 without Mg^{+2}. Analysis of the nucleoprotein solution in the ultracentrifuge within two hours showed two components in about equal amounts sedimenting at 18S and 30S (Figure 6a, lower pattern). Ultracentrifugal analysis of the same sample after an overnight incubation at cold room temperatures (4-6°C) showed a preponderance of the slower component (Figure 6b, lower pattern); thus the faster component was being converted with time to the slower component. Restoration of the ionic strength in the nucleoprotein solution by pelleting the RNP-particles from the Tris buffer and resuspending the particles in Tris with 0.5 M NH_4Cl caused the sedimentation rates of the two components to increase to 25 and 42S without a significant change in the relative amounts of the components (Figure 6b, upper pattern). The extrapolation of the sedimentation coefficients of the two components at low Mg^{+2} (designated slow and fast) to infinite dilution gave values of 27.2S and 36.4S (Figure 7). The 50S ribosomes at 2 mM Mg^{+2}, 0.5 M NH_4Cl exhibited a small dependence of sedimentation on concentration with the value at infinite dilution being 48.8S.

Thus, in our hands the NH_4Cl treated ribosomes behaved like untreated ribosomes on removal of Mg^{+2} showing two transformations of the 50S ribosomes; a smaller decrease in the sedimentation constant (36S) that was reversed to about 50S on

Figure 7. Concentration dependence of sedimentation for ammonium chloride treated RNP-particles in 1 mM Tris-HCl pH 7.4


8.0
6.0
4.0
2.0
100/S
SLOW COMPONENT
FAST COMPONENT
27.2 S
36.4S
50S RIBOSOME
48.8S
0
0.1
0.2
CONCENTRATION, g/dl

restoration of the ionic strength and a larger decrease in the sedimentation constant (27S) that could not be reversed to 50S by restoring the ionic strength of the nucleoprotein solutions.

Properties of the 30*S Particles

Inspection of the Svedberg equation (Schachman, 1959) reveals, $S \propto \frac{M}{f}$. Therefore, a decrease in sedimentation constant (S) of the ribosomes on the removal of Mg^{+2} could be the result of a dissociation of the 30S and 50S ribosomes into lower molecular weight (M) subunits or due to an increase in the frictional coefficient (f) of the ribosomal subunits, or a combination of the two. An increase in the frictional coefficient of a molecule can be due to a change in shape, an increase in the volume of the particle without a change in shape, an increase in the hydration (tightly bound water) of the molecule, or any combination of these changes in molecular structure. In order to avoid confusion, the 30S particles prepared from the purified 50S ribosomes or mixtures of 30S and 50S ribosomes by removal of Mg^{+2} will be referred to as the 30*S particles. The properties of 30*S particles were examined to determine the structural change (or changes) responsible for the decreased sedimentation rates of the ribosomes.

RNA isolated from the particles

RNA isolated by the phenol-sodium dodecyl sulfate method

from the 30*S particles prepared from a mixture of 30S and 50S ribosomes contained 16S and 23S RNA components (Figure 8b) in about the same ratio as the RNA components isolated from the original ribosomes (Figure 8a). Isolation of RNA from 30*S particles prepared from purified 50S ribosomes gave mainly 23S RNA (Figure 8c, lower pattern), like the 50S ribosomes. A similar result was obtained when the conversion of 50S ribosomal subunits to the 30*S particles was only partially complete (i.e., 50 percent complete) (Figure 8c, upper pattern). The solvent for the ultracentrifugal analysis of the RNA was 1 mM Tris-HCl pH 7.4, 10 mM $MgAc_2$, 0.15 M NaCl.

Yields of RNA obtained by the phenol-sodium dodecyl sulfate method were variable and ranged from 50-90 percent. Therefore, the RNA was prepared by a modified sodium dodecyl sulfate method (Kurland, 1960) that examines essentially the entire RNA population. RNA prepared by this method from the 30*S particles derived from purified 50S ribosomes again showed that these particles contained 23S RNA, while the 30*S particles obtained from mixtures of 30S and 50S ribosomes contained 16S and 23S RNA in the same ratio as the original ribosomes. Thus, the results with the phenol-sodium dodecyl sulfate method were not due to preferential loss during the preparative procedure of degraded RNA.

Since the 30*S particles prepared from purified 50S ribosomes contained 23S RNA and 30*S particles prepared from mixtures of 30S and 50S ribosomes contained 16S and 23S RNA in

Figure 8. RNA isolated from nucleoprotein particles

RNA from:

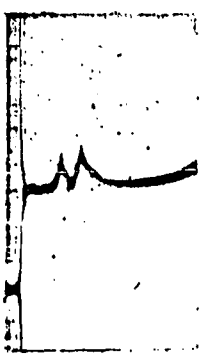
8a. A mixture of 30S and 50S ribosomes, $S_{20,w}$ = 15.1 and 23.0S

8b. 30*S particles prepared from a mixture of 30S and 50S ribosomes, $S_{20,w}$ = 15.5S and 22.1S

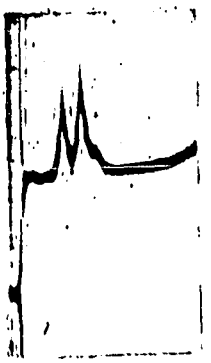
8c. Lower pattern, 30*S particles derived from purified 50S ribosomes, $S_{20,w}$ = 24.8S

Upper pattern, a nucleoprotein solution in which about half of the 50S ribosomes had been converted to the 30*S particles, $S_{20,w}$ = 23.3S

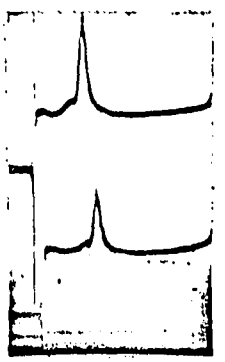
Sedimentation from left to right, concentration of RNA = 2-3 mg/ml. Pictures taken 15 minutes after reaching 50,740 rpm. BAR angle 30°, 25° and 43°, respectively, for a, b, and c.



8a



8b



8c

the same ratio as the original ribosomes, it appeared the 30S ribosomal subunit after treatment with EDTA still contained 16S RNA. The isolation of the usual 16S and 23S RNA components from EDTA-treated ribosomes, however, did not eliminate the possibility that the RNA was damaged by the latent RNase on removal of Mg^{+2} from the ribosomes. Single breaks in the polynucleotide chain in double stranded regions would not necessarily split the RNA molecules into smaller fragments since the fragments would be held together by the forces stabilizing the helical regions. Bogdanova et al. (1962) developed a method for testing for such hidden damage in the RNA molecules that is based on the rise in reduced viscosity on heating RNA solutions. If the RNA molecules are intact (undamaged), a several-fold increase in the reduced viscosity occurs on heating the solution to 70°C; however, if the RNA molecules are damaged, then the reduced viscosity rises only slightly or not at all (Bogdanova et al., 1962).

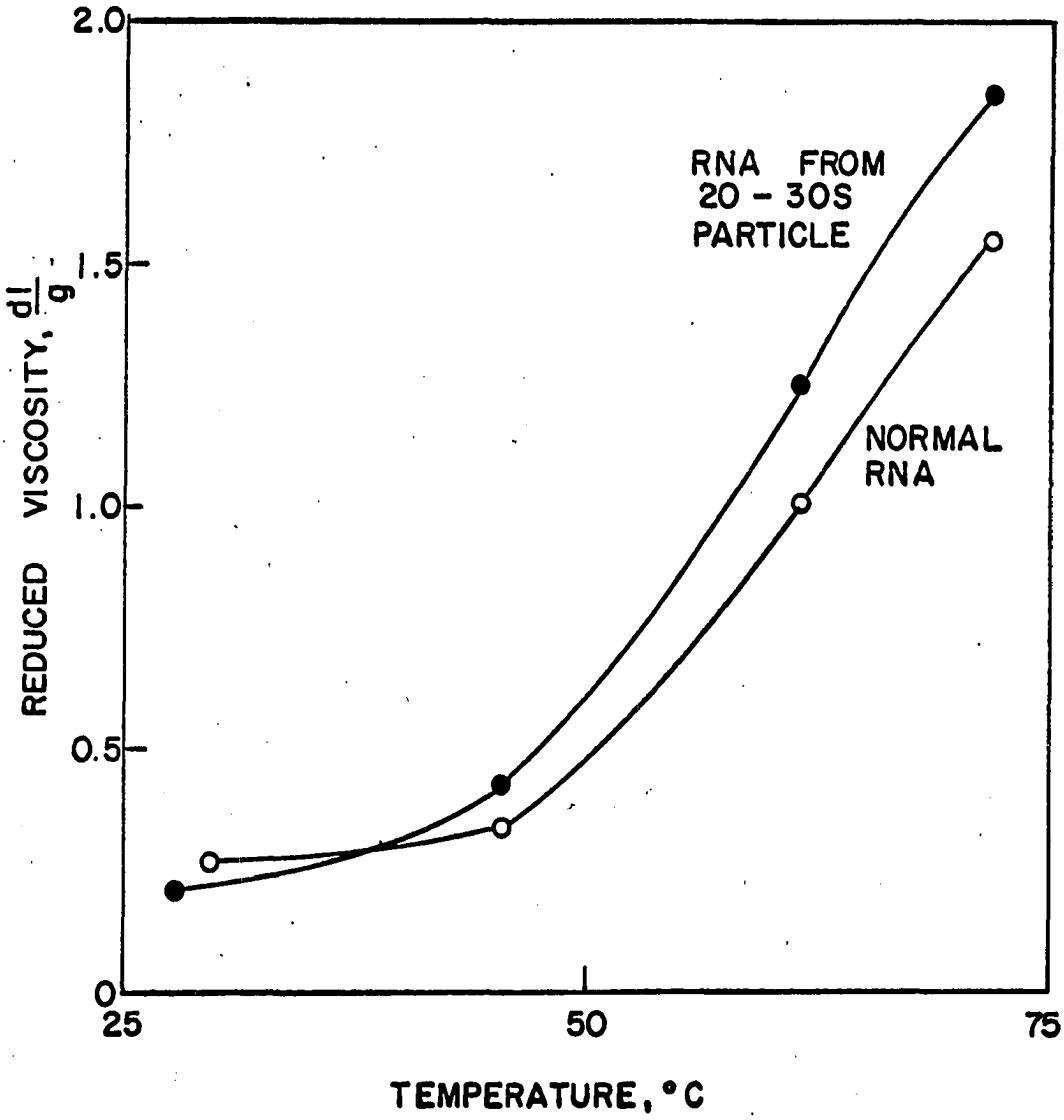
Viscosity measurements were carried out on RNA extracted by the sodium dodecyl sulfate method (Shakulov et al., 1962) from 30*S particles prepared from a mixture of 30S and 50S ribosomes and the original ribosomes (**Figure** 9). The RNA extracted from the 30*S particles exhibited a ninefold increase in the reduced viscosity while the RNA from the original ribosomes gave a sixfold increase in reduced viscosity as the temperature of the solution was increased from about 27°C to 72°C. Thus, it appeared the RNA molecules from

Figure 9. Reduced viscosity temperature profile of RNA

RNA from:

(0-0) Mixture of 30S and 50S ribosomes

(●-●) 30*S particles prepared from the mixture of 30S and 50S ribosomes


2.0
1.5
1.0
0.5
0
REDUCED VISCOSITY, dl/g
RNA FROM
20 - 30S
PARTICLE
NORMAL
RNA
25
50
75
TEMPERATURE, °C

the 30*S particles had been damaged little, if any, by the latent ribosomal RNase during the treatment of ribosomes with EDTA. The RNA concentration was 0.17 g/dl and the solvent was 1 mM Tris-HCl pH 7.4, 10^{-1} mM magnesium acetate, 0.15 M NaCl, 0.5 percent sodium dodecyl sulfate.

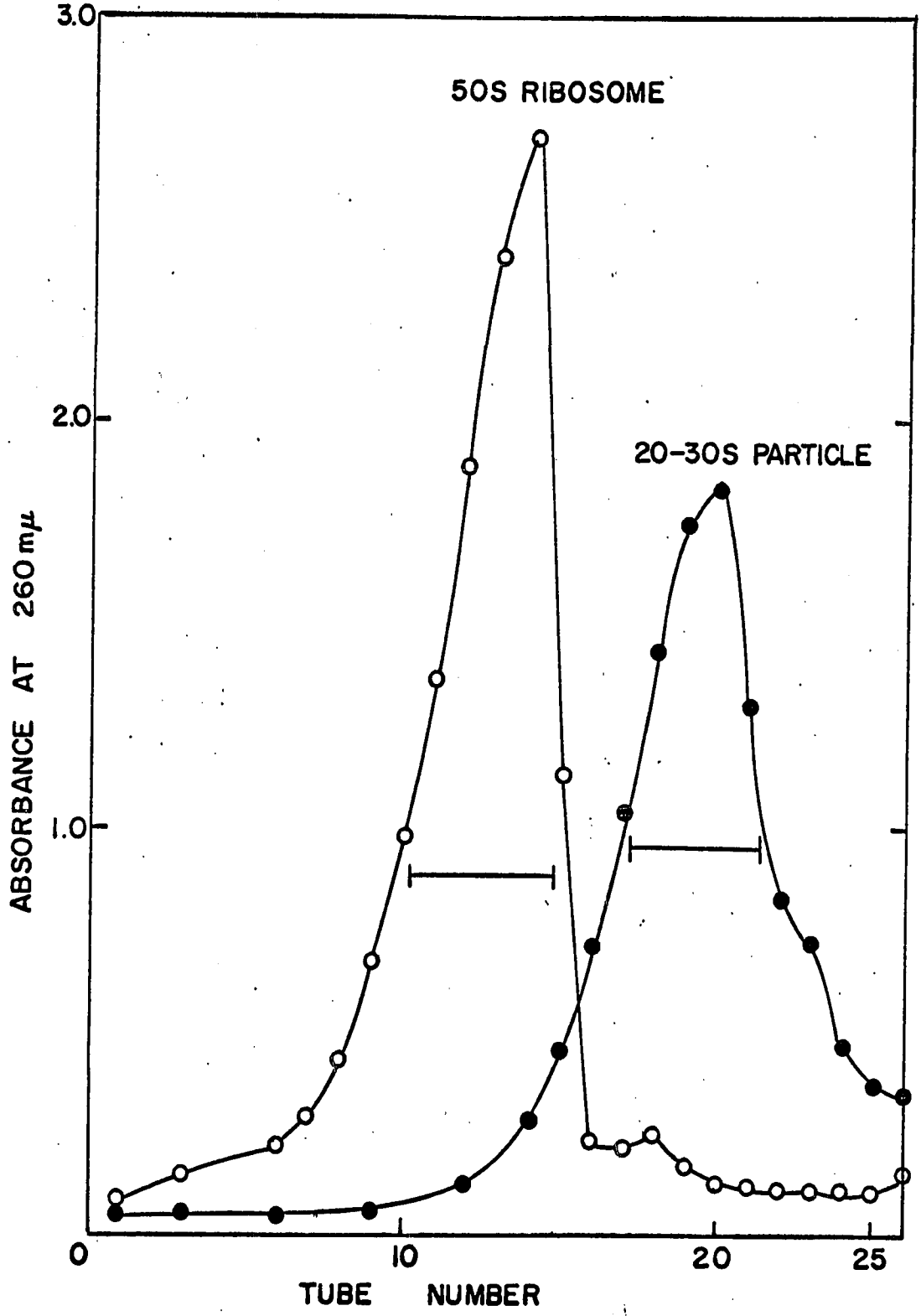
Relative amounts of RNA and protein

The 30*S particles prepared from purified 50S ribosomes contained essentially the same relative amounts of RNA and protein as the original 50S ribosomes (Table 2). Purification of the nucleoprotein particles was accomplished by centrifugation through a density gradient of sucrose. Typical sucrose gradient experiments for purified 50S ribosomes and the 30*S particles prepared from purified 50S ribosomes are shown in Figure 10 which is a composite of the two separate runs. Both the nucleoprotein samples and the sucrose gradients contained 10^{-1} mM Mg^{+2} and 2.6 mg of ribosomes were layered on the gradients. The material in tubes above the horizontal bars (indicated on the graphs) were pooled for RNA and protein analysis. Results from two separate experiments shown in Table 2 indicated the 30*S particles contained essentially the same amounts of RNA and protein as the 50S ribosomes, 65 percent RNA and 35 percent protein. In certain experiments, however, the 30*S particles had slightly less protein than the 50S ribosomes (Table 2, Experiment 2). Essentially the same results were obtained when analyses were carried out at low

Figure 10. Sucrose gradient centrifugation of RNP-particles

(0-0) Purified 50S ribosomes

(●-●) 30*S particles derived from purified 50S ribosomes


3.0
2.0
1.0
0
50S RIBOSOME
20-30S PARTICLE
ABSORBANCE AT 260 mμ
10
20
25
TUBE NUMBER

Mg^{+2} (5×10^{-5} mM) on 30*S particles prepared from mixtures of 30S and 50S ribosomes by dialyzing against 5×10^{-5} mM Mg^{+2} for a number of days.

Table 2. Relative amounts of RNA and protein in various particles

Experiment number	Component	Percent RNA	Percent protein
1	50S ribosome	65	35
	30*S particle	65	35
	30*S particle	65	35
2	50S ribosome	64	36
	30*S particle	68	32

Dependence of sedimentation constant on Mg^{+2} concentration

In order to better characterize the dependence of sedimentation on Mg^{+2} for the 30*S particles, the sedimentation coefficients of the particles were extrapolated from higher concentrations to infinite dilution at various Mg^{+2} concentrations (Figures 11 and 12). Mixtures of 30S and 50S ribosomes (concentration = 3.5 mg/ml) were dialyzed 5 days against buffered solutions containing 10^{-4} to 10^{-5} mM Mg^{+2}. Periodically (about every 24 hours) during the 5 days aliquots of the nucleoprotein solutions were analyzed in the ultracentrifuge. Little, if any, change in the sedimentation patterns occurred

Figure 11. Mg^{+2} dependence of sedimentation for the slow component

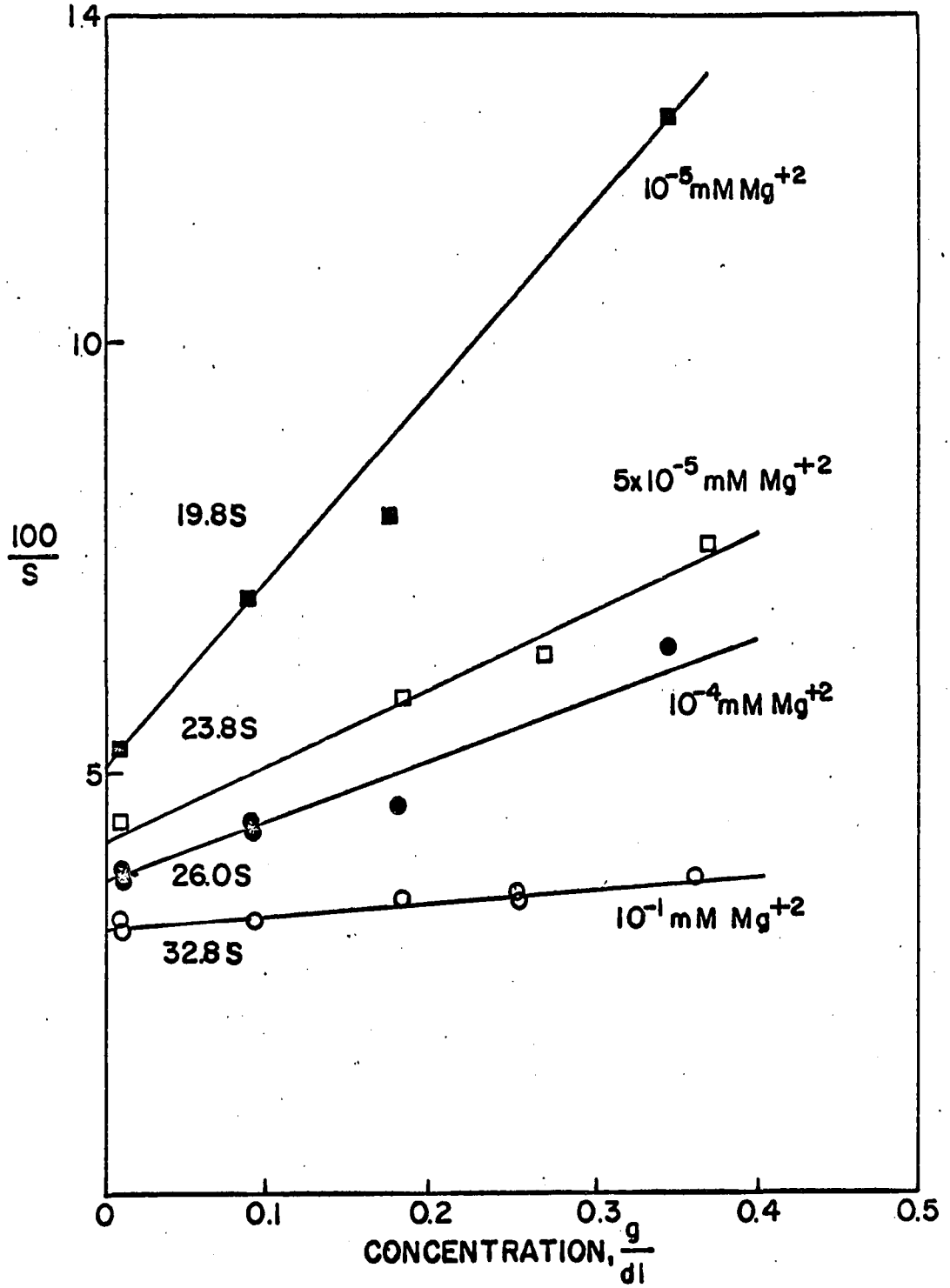
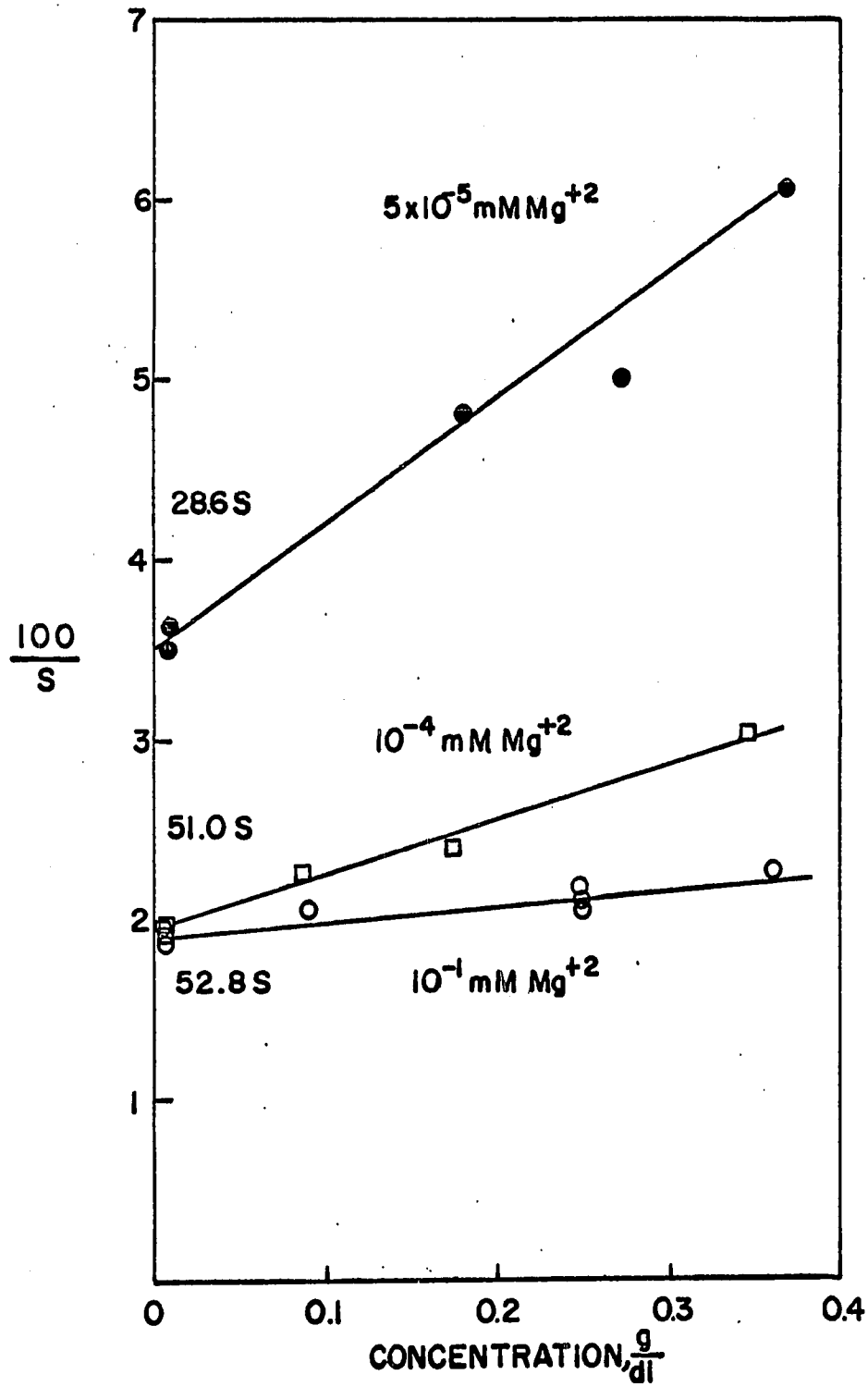

1.4
1.0
5
100/S
19.8S
23.8S
26.0S
32.8S
10^-5 mM Mg^+2
5x10^-5 mM Mg^+2
10^-4 mM Mg^+2
10^-1 mM Mg^+2
0
0.1
0.2
0.3
0.4
0.5
CONCENTRATION, g/dl

Figure 12. Mg^{+2} dependence of sedimentation for the fast component


7
6
5
4
3
2
1
100/S
5x10⁻⁵mM Mg⁺²
28.6 S
10⁻⁴ mM Mg⁺²
51.0 S
52.8 S
10⁻¹ mM Mg⁺²
0
0.1
0.2
0.3
0.4
CONCENTRATION, g/dl

between the fourth and fifth days and the buffered solutions outside the dialysis bags were used for dilution of the nucleoprotein samples. The bentonite concentration was 0.7 mg/ml in these experiments. All operations were conducted in the cold including the sedimentation analyses which were made at 4-8°C.

Two boundaries could be seen in the ultracentrifuge pattern in most experiments; one sedimenting at 8-16S and the other sedimenting at 17-33S. The sedimentation constant (sedimentation coefficient extrapolated to infinite dilution) of the slower boundary decreased steadily from 30S at 10^{-1} mM Mg^{+2} to 20S at 10^{-5} mM Mg^{+2} with values determined at 10^{-4} mM and 5×10^{-5} mM Mg^{+2} of 26S and 24S, respectively (Figure 11). Restoration of the Mg^{+2} to a concentration of 10^{-1} mM increased the sedimentation constant of the boundary to 33S. Since no mass was lost on removal of Mg^{+2}, the sedimentation constant of the 30*S particles derived from both the 30S and 50S ribosomal subunits depended on the Mg^{+2} concentration.

A single boundary was observed in the ultracentrifuge pattern of the sample at 10^{-5} mM Mg^{+2} while at 10^{-4} mM and 5×10^{-5} mM Mg^{+2} a faster sedimenting boundary was also observed. The sedimentation constant of the faster boundary decreased from 50S at 10^{-1} mM Mg^{+2} to 29S at 5×10^{-5} mM Mg^{+2} with the value determined at 10^{-4} mM Mg^{+2} being 51S (Figure 12). These sedimentation constants were compared with the value of 53S determined for the 50S ribosomal subunit at 10^{-1}

mM Mg^{+2} because, presumably, the faster components would reverse to 50S on restoration of Mg^{+2}. Results reported earlier (Table 1) had indicated components derived from the 50S ribosomal subunit sedimenting in the range 33-43S at infinite dilution were reversed to 50S by Mg^{+2}.

Variation of the intrinsic viscosity with Mg^{+2} concentration

Viscosity measurements are very sensitive to changes in molecular weight and frictional coefficient of molecules. Therefore, the viscosity of the 30*S particles was examined at different Mg^{+2} concentrations. The extrapolation of the reduced viscosity of the 30*S particles prepared from purified 50S ribosomes to infinite dilution at low Mg^{+2} (i.e., in 5 mM EDTA) and at 10^{-1} mM Mg^{+2} showed the intrinsic viscosity of the particles depended on the Mg^{+2} concentration (Figure 13). Flow time measurements were made at 5°C and sedimentation analysis of samples were made at 4-8°C. The intrinsic viscosity values are shown on the graph under the lines near the ordinate while the sedimentation constant of the particles under the given ionic condition is shown in the parentheses.

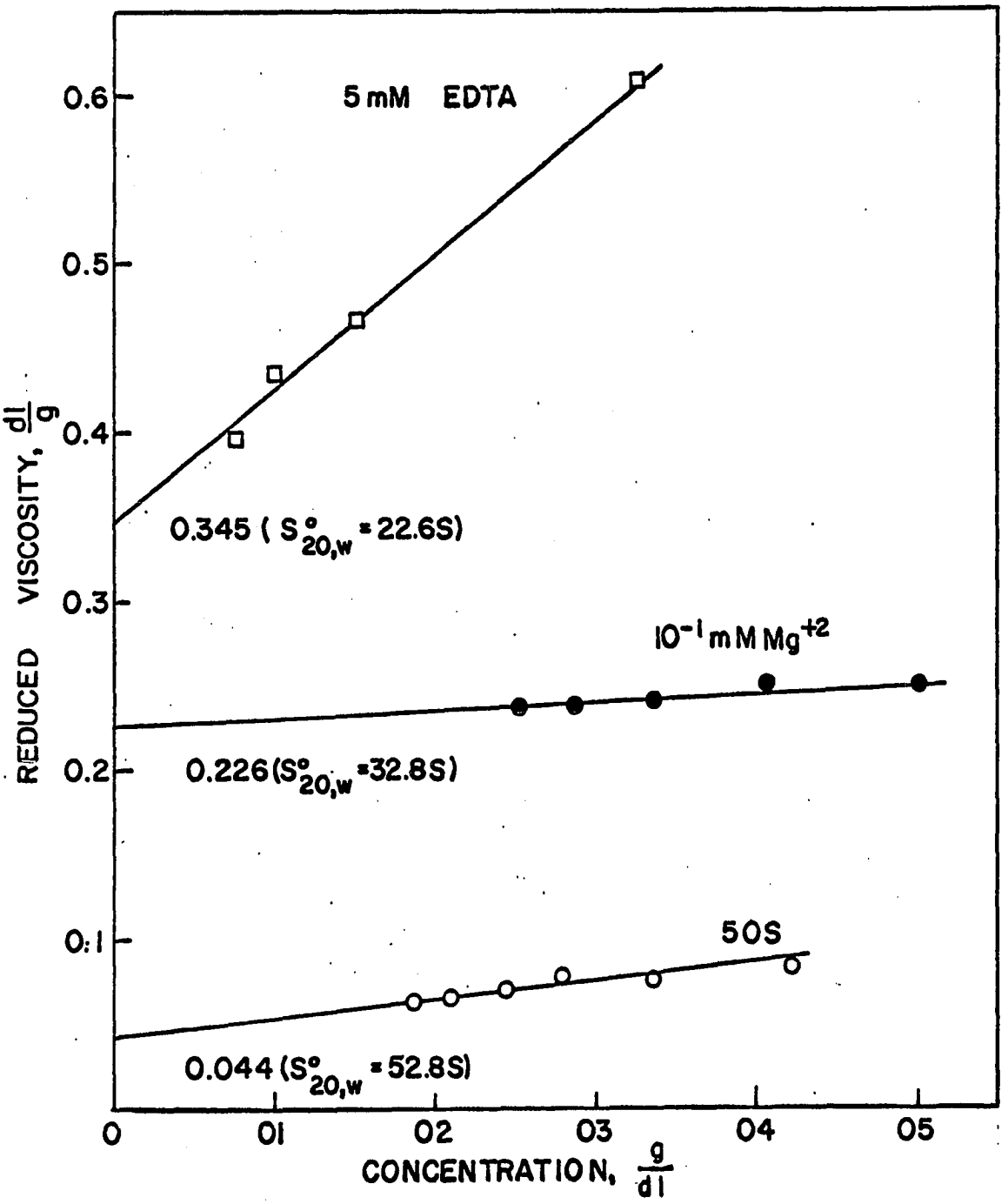
An eightfold increase in the intrinsic viscosity occurred when purified 50S ribosomes were converted to the 30*S particles and the flow times were measured in the presence of EDTA (Figure 13, compare lines labeled 50S and 5 mM EDTA). Restoration of Mg^{+2} in another 30*S sample caused the intrinsic viscosity of the particles to decrease from 0.35 dl/g at low

Figure 13. Dependence of the reduced viscosity on concentration

(0-0) 50S ribosomes at 10^{-1} mM magnesium

(●-●) 30*S particles derived from purified 50S ribosomes at 10^{-1} mM magnesium

(□-□) 30*S particles derived from purified 50S ribosomes in EDTA


5 mM EDTA
0.345 (S°20,w = 22.6S)
10⁻¹ mM Mg⁺²
0.226 (S°20,w = 32.8S)
50S
0.044 (S°20,w = 52.8S)
REDUCED VISCOSITY, dl/g
CONCENTRATION, g/dl
0.6
0.5
0.4
0.3
0.2
0.1
0
01
02
03
04
05

Mg^{+2} (i.e., in EDTA) to 0.23 dl/g at 10^{-1} mM Mg^{+2} (Figure 13, compare lines labeled 5 mM EDTA and 10^{-1} mM Mg^{+2}). The intrinsic viscosity of purified 50S ribosomes at 10^{-1} mM Mg^{+2} was determined to be 0.04 dl/g, a value that compared favorably with the 0.05 dl/g reported by Tissiéres et al. (1959) for 50S particles. At the same time that the intrinsic viscosity increased, the sedimentation constant of the particles decreased. Thus, both $S^{0}_{20,w}$ and [η] of the nucleoprotein particles were shown to be dependent on the Mg^{+2} concentration of the solution.

Sensitivity to degradation by RNase

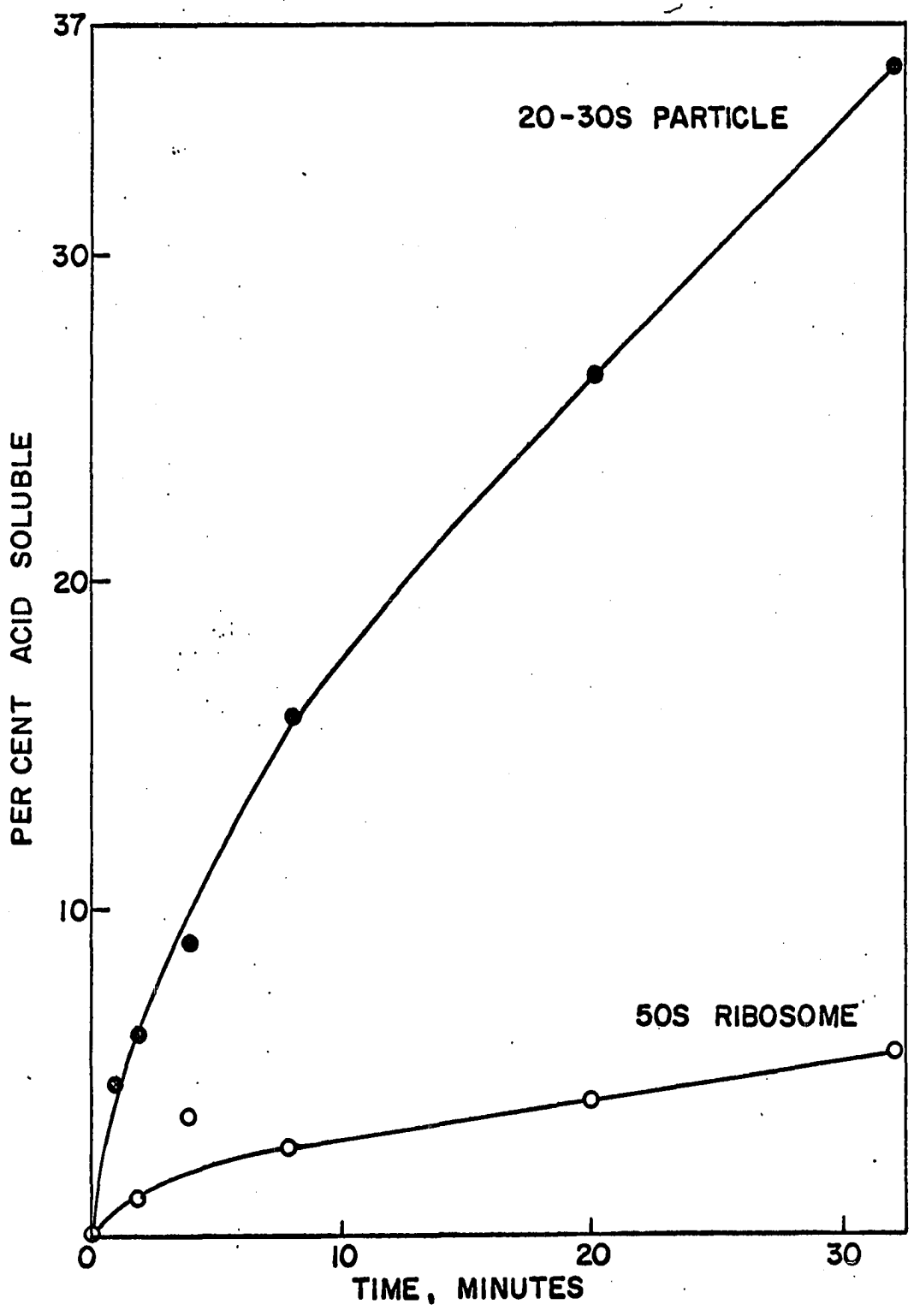
The 30*S particles prepared from purified 50S ribosomes were very sensitive to RNase (1 μg/ml) degradation with a little more than a third of the nucleic acid in the particles made acid soluble within half an hour at 4°C (Figure 14, curve labeled 20-30S particles). Under the same conditions the RNA in the 50S ribosomes was relatively resistant to degradation by RNase with only about 5 percent of the nucleic acid of the particles made acid soluble. Both the 30*S particles and the 50S ribosomes were in 1 mM Tris-HCl pH 7.4, 10^{-1} mM Mg^{+2}. Similar studies at room temperature showed about 50 percent of the nucleic acid of the 30*S particles was made acid soluble within 5 minutes, while little degradation of the 50S ribosomes had occurred during this period of time. Thus, the RNA in the 30*S particles at 10^{-1} mM Mg^{+2} was several times more

Figure 14. Sensitivity of nucleoprotein particles to degradation by RNase

Release of acid soluble material on incubation with RNase for:

(0-0) Purified 50S ribosomes

(●-●) 30*S particle derived from purified 50S ribosomes


37
30
20
10
0
10
20
30
PER CENT ACID SOLUBLE
TIME, MINUTES
20-30S PARTICLE
50S RIBOSOME

sensitive to RNase degradation than the RNA in the 50S ribosomes.

Spectral characteristics

The ultraviolet absorption spectra of the 30*S particles prepared from a mixture of 30S and 50S ribosomes and the original ribosomes were identical; the curve for the 30*S particles is shown in Figure 15. Concentration of the particles was 24 μg/ml and the particles were in 1 mM Tris-HCl pH 7.4, 5×10^{-1} mM magnesium acetate. The absorption spectrum of the nucleoprotein particles agreed well with that published by Tissiéres et al. (1959) for ribosomal particles at 5×10^{-1} mM Mg^{+2}.

Unfortunately, the absorption of EDTA in the region 240-280 mμ (region of characteristic absorption of ribosomes) was dependent on the Mg^{+2} concentration (Figure 16). This made the interpretation of changes in the absorbance of ribosomes on treatment with EDTA more difficult. The absorption spectrum of 10 mM EDTA pH 7.4 in 10 mM Mg^{+2} (Figure 16, curve labeled Mg-EDTA) was shifted to lower wavelengths compared with EDTA in 10^{-1} mM Mg^{+2} (Figure 16, curve labeled EDTA). At Mg^{+2} concentrations higher than 10 mM, the absorption spectrum of EDTA was the same as for the 10 mM Mg^{+2} experiment.

Removal of Mg^{+2} from purified 50S ribosomes (concentration = 0.06 mg/ml) by the addition of 5 mM EDTA at 4°C without bentonite in the ribosome solution, resulted in a slight

Figure 15. Absorption spectrum of the 30*S particles prepared from a mixture of 30S and 50S ribosomes

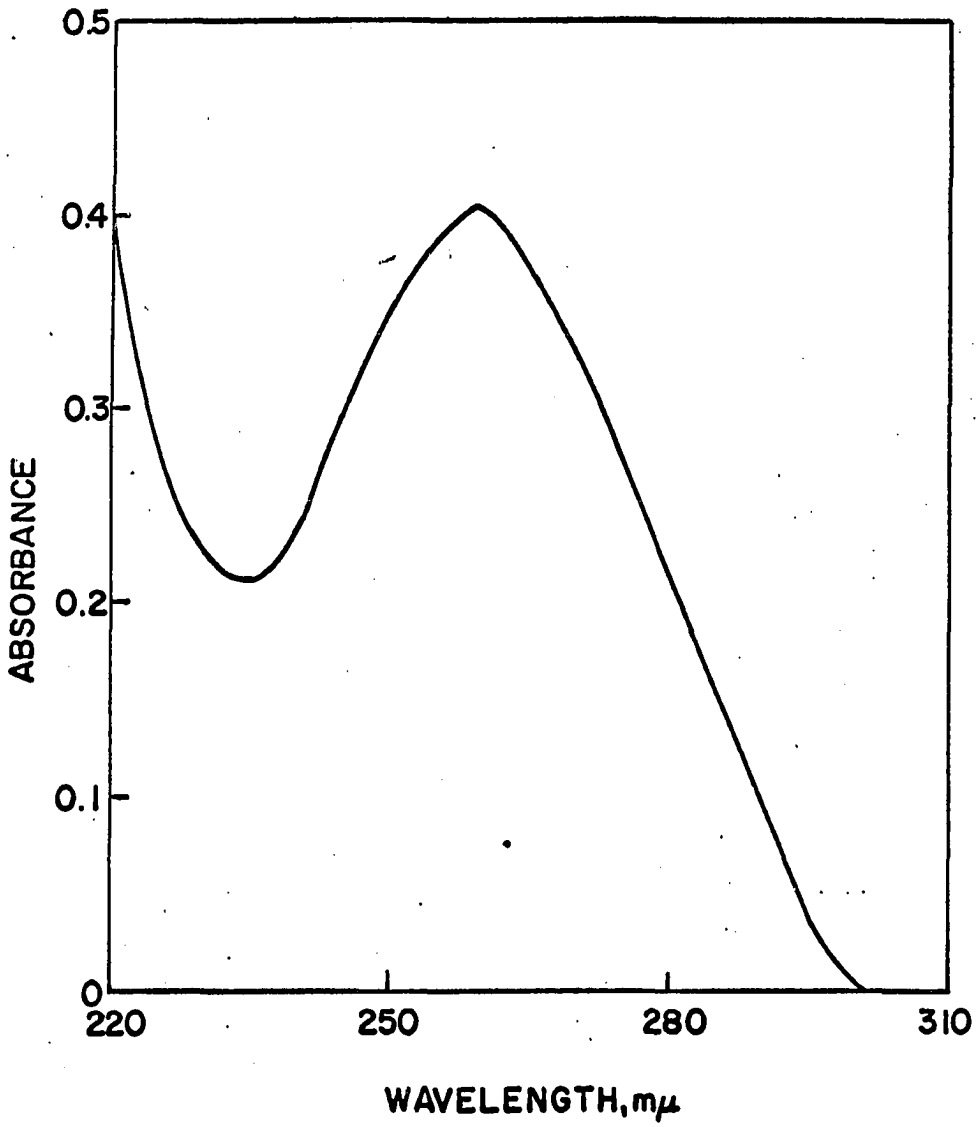

0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE
220
250
280
310
WAVELENGTH, mμ

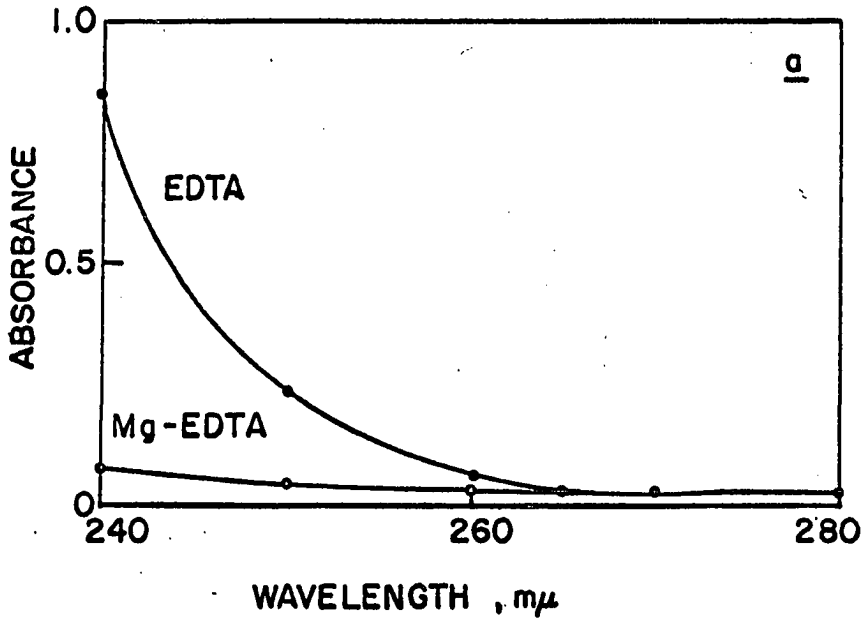
Figure 16. Absorption spectrum of EDTA

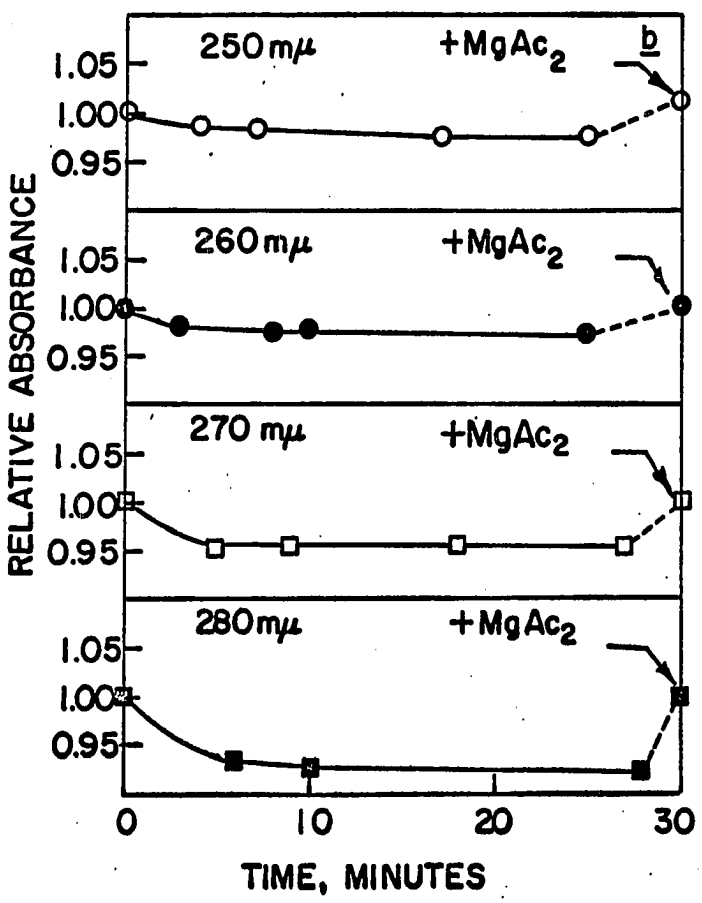
10 mM EDTA in 1 mM Tris-HCl pH 7.4 at:

(●-●) 10 mM magnesium acetate

(0-0) 10^{-1} mM magnesium acetate

Figure 17. Effect of removing magnesium from ribosomes on the absorbance


1.0
0.5
0
ABSORBANCE
a
EDTA
Mg-EDTA
240
260
280
WAVELENGTH , mμ


250 mμ
+MgAc2
b
260 mμ
+MgAc2
270 mμ
+MgAc2
280 mμ
+MgAc2
1.05
1.00
0.95
RELATIVE ABSORBANCE
0
10
20
30
TIME, MINUTES

decrease in the absorbance of the nucleoprotein solution at various wavelengths in the region 250-280 mμ. The majority of the decrease occurred within a few minutes after the addition of EDTA (Figure 17). After 30 minutes 5 mM Mg^{+2} was added to the nucleoprotein solution and the absorbance rose immediately at each wavelength to the initial value (Figure 17, point marked with arrow $+Mg^{+2}$). Ultracentrifugal analysis of the nucleoprotein solution showed the 50S ribosomes had been completely converted to the 30*S (33S) particles during the incubation with EDTA.

The small decrease in absorbance of ribosomal solutions at each wavelength could be explained by the dependence of the extinction of EDTA on the concentration of Mg^{+2}. However, this explanation is obviously not entirely correct since the decrease in absorbance was greatest at the longer wavelengths where the absorption of EDTA is relatively independent of Mg^{+2} concentration.

Because of the difficulty in interpreting the changes in absorbance of ribosomal solutions on the addition of EDTA, the molar extinction in terms of phosphorus [E(P)] at 260 mμ was measured for 30*S particles and ribosomes. Both the 30*S particles prepared from purified 50S ribosomes and the 50S ribosomes used in this experiment had been purified by centrifugation through a density gradient of sucrose. The E(P)'s of the 30*S particles and the 50S ribosomes were calculated from phosphorus and absorbance measurements to be 7870 and 7420 liter/mole P × cm, respectively. Therefore, it appeared the

average extinction per mole of nucleotide (i.e., mole of phosphorus) at 260 mμ was slightly greater in the 30*S particles than in the 50S ribosomes.

Ultraviolet ORD-curve

The ultraviolet ORD-curve of the 30*S particles prepared from purified 50S ribosomes showed a broad peak at 280 mμ, cross-over at 265 mμ and a rather sharp trough at 250 mμ: $[\alpha]_{280}$ = +2800 and $[\alpha]_{250}$ = -4000 (Figure 18, curve labeled 20-30S particles). Concentrations of RNP-particles were adjusted to give $A_{260} \approx 1.0$ for the runs and the particles were in 1 mM Tris-HCl pH 7.4, 10^{-1} mM magnesium acetate. The ORD-curve of the 50S ribosomes under identical conditions was qualitatively the same as for the 30*S particles, but the magnitude of the specific rotation at the peak and trough were different: $[\alpha]_{280}$ = +2500 and $[\alpha]_{250}$ = -4750. Thus, the specific rotation of the 30*S particles was 12 percent more positive at the peak (280 mμ) and 19 percent more positive at the trough (250 mμ) than the specific rotation of the 50S ribosomes.

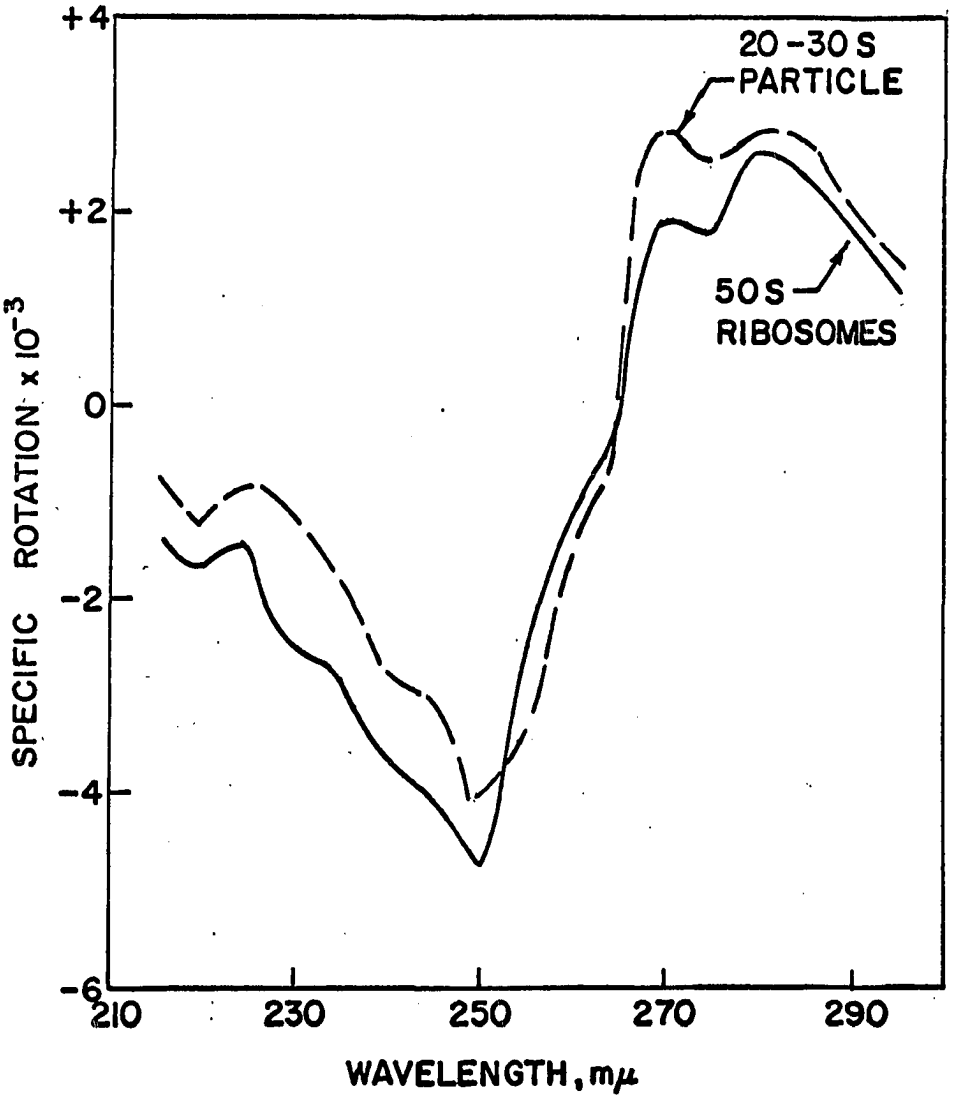
Interaction of Bentonite with Various Components

Since bentonite was used in studies with ribosomes and in the isolation of RNA, some of the properties of the clay were examined in order to better define conditions under which bentonite could be used in nucleoprotein solutions. The

Figure 18. The ultraviolet ORD trace of nucleoprotein particles

(——) 50S ribosomes

(---) 30*S particles prepared from purified 50S ribosomes


+4
+2
0
-2
-4
-6
SPECIFIC ROTATION x 10^{-3}
210
230
250
270
290
WAVELENGTH, mμ
20-30 S
PARTICLE
50 S
RIBOSOMES

properties studied included inhibition of RNase and interaction of bentonite with ribosomes and ribosomal RNA.

Inhibition of RNase

The ability of bentonite to protect E. coli ribosomal RNA from degradation by pancreatic RNase was examined using the ultracentrifuge. Protection of the high molecular weight state of RNA at pH 7.4 was studied at two bentonite concentrations (0.5 and 3 mg/ml) under three different ionic conditions, 5 mM EDTA and 10^{-1} mM and 10 mM Mg^{+2}. The ionic conditions were chosen because in the studies reported, bentonite was used in solutions at low and high Mg^{+2} concentrations to protect RNP-particles and RNA from degradation by nucleases. RNA was exposed to the action of 3.3 µg/ml of RNase in the presence of bentonite for one hour at room temperature. At the end of this time the Mg^{+2} concentration in the 5 mM EDTA and 10^{-1} mM Mg^{+2} samples was increased to 10 mM by the addition of an appropriate amount of a stock magnesium acetate solution. The ultracentrifuge patterns of the samples treated with RNase were compared with the pattern of RNA not treated with RNase (Figure 19). Stock bentonite suspensions used for the 5 mM EDTA and 10^{-1} mM Mg^{+2} experiments were in 10^{-1} mM Mg^{+2} while those for the 10 mM Mg^{+2} experiments were in 10 mM Mg^{+2}. The E. coli ribosomal RNA preparation used showed two main boundaries in the ultracentrifuge sedimenting at 19S and 26S (Figure 19a). Both bentonite concentrations (0.5 and 3 mg/ml)

Figure 19. Inhibition of RNase by bentonite

Bentonite concentration was 0.5 mg/ml (upper pattern) and 3 mg/ml (lower pattern). RNA at:

19a. 10 mM Mg^{+2} without RNase or bentonite control sample, $S_{20,w}$ = 19.4 and 26.4S

19b. 5 mM EDTA

Lower pattern, $S_{20,w}$ = 15.3 and 21.2S

Upper pattern, $S_{20,w}$ = 15.5 and 21.4S

19c. 10^{-1} mM Mg^{+2}

Lower pattern, $S_{20,w}$ = 14.5 and 25.2S

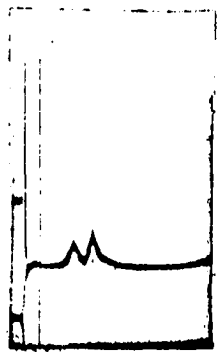
Upper pattern, no characteristic S

19d. 10 mM Mg^{+2}

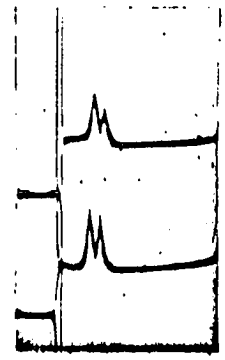
Lower pattern, $S_{20,w}$ = 20.2 and 25.8S

Upper pattern, $S_{20,w}$ = 18.7 and 27.4S

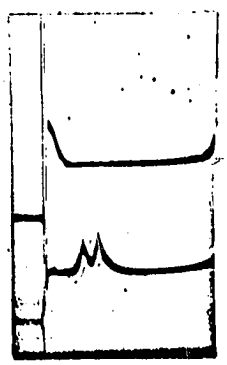
Sedimentation from left to right. RNA concentration = 2.3 mg/ml. Pictures taken 13 minutes after reaching 50,740 rpm. BAR angle 50°.



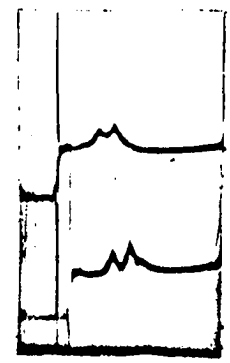
19a



19b



19c



19d

were sufficient to preserve the high molecular weight of the ribosomal RNA on treatment with RNase in 5 mM EDTA (Figure 19b). A shift of mass occurred, however, from the faster to the slower boundary on treatment with RNase and the sedimentation coefficients of the components were slightly lower than in the control sample (compare Figures 19a and 19b). Bentonite at 3 mg/ml afforded protection for the RNA at 10^{-1} mM and 10 mM Mg^{+2} (Figures 19c and 19d, lower patterns). However, material sedimenting between the two main components was observed in the presence of 0.5 mg/ml of bentonite at 10 mM Mg^{+2} (Figure 19d, upper pattern) and the RNA was degraded to slow sedimenting material in the 10^{-1} mM Mg^{+2} sample (Figure 19c, upper pattern).

Therefore, a concentration of bentonite of 3 mg/ml was sufficient to protect the high molecular weight of ribosomal RNA from degradation by 3 µg/ml of RNase at pH 7.4. At lower concentrations of bentonite (0.5 mg/ml), it appeared the protection afforded by bentonite was dependent on the Mg^{+2} concentration.

Binding of purified ribosomes to bentonite

Bentonite was used in experiments reported earlier (Figures 3 and 4, and Table 1) to protect nucleoprotein particles from possible degradation by the latent ribosomal RNase. Therefore, the binding of ribosomes to bentonite was examined at different Mg^{+2} concentrations with various ratios of

ribosomes and bentonite.

The bentonite and ribosome solutions at the appropriate Mg^{+2} concentration were mixed and incubated for one hour and then the bentonite was removed by centrifuging the solutions in the Serval SS1 for one hour at 12,000 g. All operations were performed at cold room temperatures (4-6°C). The amount of ribosomes bound to the bentonite was estimated by determining the concentration of ribosomes in the supernatant after removal of the bentonite and substracting this from the concentration of ribosomes in a control sample that had no bentonite. Ribosome concentrations were estimated from the $\Delta A(A_{260} - A_{310})$ of the solutions using $E_{260}^{0.1\%} = 16$.

Results from a typical experiment showing the Mg^{+2} dependence of ribosomal binding to bentonite is shown in Table 3. The ribosome concentration was 5.9 mg/ml and

Table 3. Dependence of ribosome binding to bentonite on concentration of Mg^{+2}

Magnesium acetate concentration mM	mg of ribosomes bound/ml	mg of ribosomes eluted/ml
0.1	0.0	-
1.0	0.2	0.1
10.0	4.1	2.4

bentonite was present at 3.1 mg/ml. There were no ribosomes bound to the bentonite at 10^{-1} mM Mg^{+2} and only a small fraction of the total (3 percent) was bound at 1 mM Mg^{+2}; 0.2 mg bound out of a possible 5.9 mg. When the Mg^{+2} concentration was increased to 10 mM Mg^{+2}, however, about 70 percent of the nucleoprotein mass available (4.1 mg out of 5.9 mg) was bound. Therefore, the binding of ribosomes to bentonite depended on the Mg^{+2} concentration.

To determine if the binding phenomenon was reversible, the bentonite pellet obtained from the binding experiment was resuspended in a volume of 10^{-1} mM Mg^{+2} equal to the initial volume. After incubation of the suspension overnight at 4-6°C, the bentonite was pelleted down and the concentration of the RNP-particles in the supernatant was determined. It was found that some of the bound ultraviolet absorbing material was released by this procedure; 2.4 mg of 4.1 mg bound at 10 mM Mg^{+2} (Table 3). This represented about 60 percent of the total ribosomes originally bound.

The interaction of ribosomes with bentonite was examined in more detail at 10^{-1} mM and 10 mM Mg^{+2} because these concentrations of Mg^{+2} are the most frequently used in research with E. coli ribosomes. Binding and elution procedures were the same as those described in the previous experiment. The ribosomes and bentonite solutions used in these studies were in the appropriate Mg^{+2} concentration (i.e., either 10^{-1} mM or 10 mM Mg^{+2}, depending on the experiment).

Table 4. Effect of bentonite concentration on adsorption of ribosomes[a] at 10 mM Mg^{+2}

Concentration of bentonite mg/ml	mg of ribosomes bound/ml	mg of ribosomes eluted/ml
0.5	2.6	2.1
2.0	2.9	1.9
4.0	4.2	2.6
8.8	4.2	1.5

[a]Concentration of ribosomes was 4.5 mg/ml.

The effect of increasing the level of bentonite on the amount of ribosomes bound at 10 mM Mg^{+2} is shown in Table 4. As the bentonite concentration was increased from 0.5 mg/ml to 4.0 mg/ml, the amount of ribosomes bound to the clay increased. A further increase in the bentonite level (8.8 mg/ml) did not cause any additional binding. The ribosome concentration was 4.5 mg/ml; thus it appeared that a small fraction of the ribosomes did not bind to the clay at 10 mM Mg^{+2}. The reason for this is not clear. Analysis of the supernatant from the binding experiment (Table 4, line 3) in the ultracentrifuge indicated that the concentration of ribosomes calculated from absorbance measurements was essentially correct since only a small amount of mass could be seen in the ultracentrifuge pattern (Figure 20, upper pattern). Absorbance measurements

Figure 20. Schlieren pattern of ribosomes from a bentonite experiment

Lower pattern, ribosomes eluted from the bentonite, $S_{20,w}$ = 31.4, 48.2 and 65.4S

Upper pattern, supernatant above the bentonite pellet in the binding experiment

Sedimentation from left to right. Picture taken 10 minutes after reaching 31,410 rpm. BAR angle 45°.

Figure 21. Effect of adding bentonite to ribosomal extracts

21a. Cell free extract prepared in 10 mM Mg^{+2}

Lower pattern, control, no bentonite

Upper pattern, bentonite concentration = 4 mg/ml

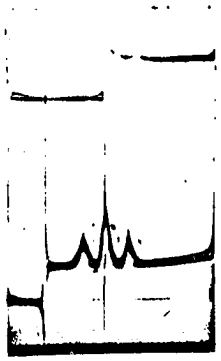
Picture was taken 38 minutes after reaching 31,410 rpm. BAR angle of 65°.

21b. Cell free extract prepared in 10^{-1} mM Mg^{+2}

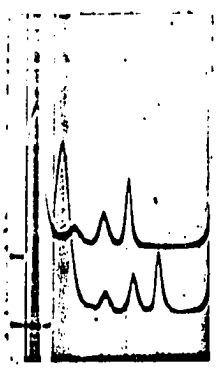
Lower pattern, bentonite concentration = 3 mg/ml

Upper pattern, control, no bentonite

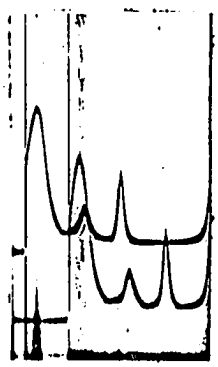
Picture was taken 59 minutes after reaching 31,410 rpm. BAR angle of 65°.



20



21a



21b

had indicated the concentration of the sample should have been 0.3 mg/ml.

Decreasing the concentration of Mg^{+2} in the bentonite suspension to 10^{-1} mM Mg^{+2} resulted in the release of variable amounts of nucleoprotein particles from the clay (1.5-2.6 mg) (Table 4). Examination of the material released from the bentonite (Table 4, line 3) in the ultracentrifuge showed typical boundaries for 30S (31S), 50S (48S) and 70S (65S) ribosomes (Figure 20, lower pattern). Presumably, the 70S particles were released directly from the bentonite rather than being formed from 30S and 50S subunits in the solution because the bentonite pellet had been resuspended in 10^{-1} mM Mg^{+2}. The effect of increasing the ribosome concentration on the amount of ribosomes bound to a fixed concentration of bentonite (2.9 mg/ml) at 10 mM Mg^{+2} is shown in Table 5. As the concentration of ribosomes was increased from 2.3 mg/ml to

Table 5. Effect of ribosome concentration on binding to bentonite[a] at 10 mM Mg^{+2}

Concentration of ribosomes mg/ml	mg of ribosomes bound/ml	mg of ribosomes eluted/ml
2.3	2.1	1.2
4.3	3.6	2.5
8.6	4.5	2.7
11.6	5.3	3.4

[a]Concentration of bentonite was 2.9 mg/ml.

11.6 mg/ml, the amount bound to the bentonite increased steadily. From these results, therefore, little could be said about the maximum amount of ribosomes bound per mg of bentonite. However, it was apparent one mg of bentonite could bind about 2 mg of ribosomes (Table 5, last line) and that perhaps at higher ratios of ribosomes to bentonite, even more RNP-particles would be bound to the clay. From 60-70 percent of the RNP-particles bound were released from the bentonite by decreasing the concentration of Mg^{+2} to 10^{-1} mM in the suspension. The yield was increased to about 80 percent by dialyzing the bentonite suspension overnight against 10^{-1} mM Mg^{+2}.

Similar binding experiments at 10^{-1} mM Mg^{+2} again showed that bentonite had little affinity for ribosomes at this Mg^{+2} concentration. The effect of increasing the level of ribosomes at a fixed concentration of bentonite (3.2 mg/ml) on the amount of ribosomes bound at 10^{-1} mM Mg^{+2} is shown in Table 6. A rather constant, but small amount of RNP-particles were bound to the bentonite at all concentrations of ribosomes. At the lowest concentration of ribosomes used (0.1 mg/ml), all of the ribosomes were adsorbed by the bentonite (Table 6, line 1). Thus, even at 10^{-1} mM Mg^{+2} in dilute solutions of ribosomes, the concentration of bentonite that can be used is limited. Experience has indicated a bentonite concentration of 0.5 mg/ml removes few RNP-particles from dilute solutions (concentration = 0.06-0.09 mg/ml) at 10^{-1} mM Mg^{+2}.

Table 6. Effect of ribosome concentration on binding to bentonite[a] at 10^{-1} mM Mg^{+2}

Concentration of ribosomes mg/ml	mg of ribosomes bound/ml
0.1	0.1
2.5	0.1
4.9	0.3
9.7	0.3

[a]Concentration of bentonite was 3.2 mg/ml.

When the ribosome concentration was fixed at 4.9 mg/ml and the bentonite concentration was increased from 0.5 mg/ml to 9.3 mg/ml, 0.2-0.3 mg of ribosomes were bound per ml over all concentrations of bentonite at 10^{-1} mM Mg^{+2}.

Use of bentonite in cell extracts

There are two reasons for wanting bentonite in cellular extracts; (1) to protect the nucleoprotein particles against nuclease damage, and (2) to remove extraneous proteins from solution that might otherwise bind to the ribosomes. Therefore, the use of bentonite in extracts was explored.

When bentonite was added to E. coli extracts prepared in 10 mM Mg^{+2} and 10^{-1} mM Mg^{+2} to give concentrations of 4 mg/ml and 3 mg/ml, respectively, and the extracts examined in the ultracentrifuge, typical boundaries could be seen for soluble

protein, 30S and 50S subunits (10^{-1} mM Mg^{+2}) (Figure 21a, upper pattern) and 70S ribosomes (10 mM Mg^{+2}) (Figure 21b, lower pattern). The corresponding extracts without bentonite are shown in Figures 21a, lower pattern (10 mM Mg^{+2}) and 21b, upper pattern (10^{-1} mM Mg^{+2}). Comparison of areas under the boundaries for the extracts with and without bentonite revealed few, if any, ribosomes were adsorbed by the bentonite at either 10^{-1} mM or 10 mM Mg^{+2}. The soluble protein peak was reduced slightly in the extract at 10 mM Mg^{+2}, but a similar effect was not evident in the extract at 10^{-1} mM Mg^{+2}.

The amount of bentonite (4 mg/ml) added to the extract at 10 mM Mg^{+2} would have been sufficient, with purified ribosomes, to bind all the RNP-particles in the solution. Therefore, it appeared that the soluble proteins and the ribosomes may be competing for the bentonite and the proteins have a greater affinity for the clay. Another possibility is that the effective concentration of Mg^{+2} in the extract was lower than 10 mM because some of the Mg^{+2} was bound by soluble proteins and other components in the solution.

Binding of ribosomal RNA to bentonite

The mechanism for the binding of ribosomes to bentonite was of interest so the affinity of the clay for the nucleic acid component of the ribosomes was examined. Procedures for these studies were the same as those used previously for ribosomes. Results from a typical experiment are shown in

Table 7. Binding of r-RNA[a] to bentonite at 10 mM Mg^{+2}

Concentration of bentonite mg/ml	mg of RNA bound/ml
0.5	0.1
3.0	0.1
9.9	0.0

[a]Concentration of RNA was 2 mg/ml.

Table 7. The concentration of RNA was fixed at 2 mg/ml and the ratio of bentonite to RNA was varied by increasing the concentration of bentonite from 0.5 mg/ml to 9 mg/ml. Bentonite had little affinity for the ribosomal RNA at 10 mM Mg^{+2} since little, if any, of the RNA was adsorbed by the clay. Therefore, it appeared the binding of ribosomes to bentonite was due to the interaction of the protein of the ribosomes with the clay.

Chromatography of ribosomes on bentonite-agar gel

The adsorption of ribosomes to bentonite at 10 mM Mg^{+2} and the partial reversal of the binding when the concentration of Mg^{+2} in the clay suspension was reduced to 10^{-1} mM Mg^{+2} was interesting because of the possibility of employing bentonite as an ion exchange or adsorbent for chromatography of ribosomes. Free bentonite could not be used directly as an adsorbent because the flow of eluent through columns of the

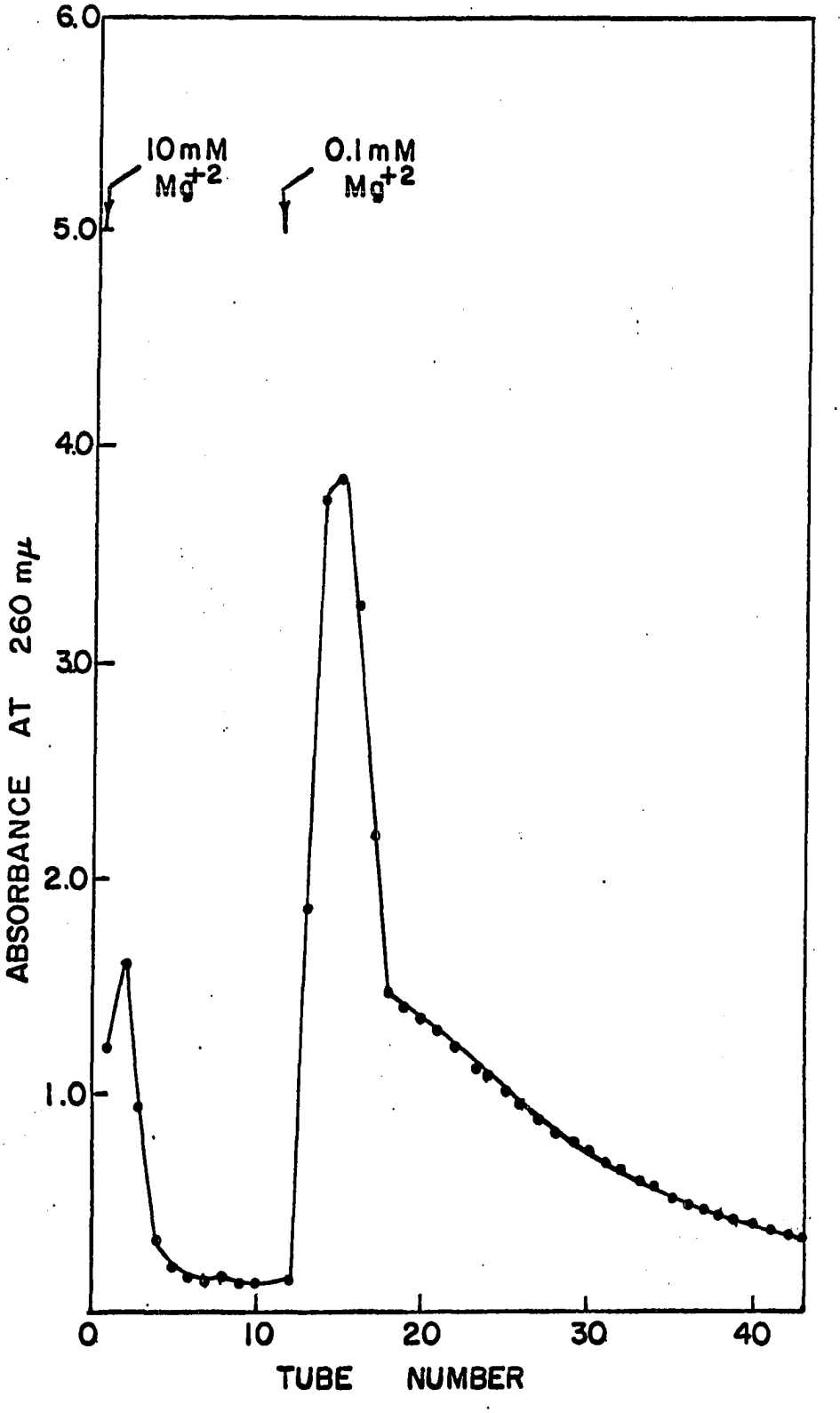
clay particles was too slow (i.e., essentially zero). Therefore, a chromatographic material was prepared by trapping bentonite in agar gel (BAG) and the interaction of ribosomes with the BAG was studied. All operations in these experiments were conducted at 4-6°C.

Results from an experiment demonstrating the Mg^{+2} dependency of ribosomal binding to the BAG are shown in Figure 22. The BAG column had been equilibrated with 10 mM Mg^{+2} before the ribosomal sample was introduced. Twenty-five mg of ribosomes (in one ml) at 10^{-1} mM Mg^{+2} were layered on a column 1.5 cm in diameter and 10 cm high; the sample was washed into the column with 4-5 ml of 10 mM Mg^{+2}. After incubating the sample for an hour on the column, the eluting buffer containing 10 mM Mg^{+2} was started. Some A_{260} absorbing material did not bind to the BAG and passed through with the solvent front, but the majority of the material was adsorbed to the BAG at 10 mM Mg^{+2}. Changing the Mg^{+2} concentration in the eluting buffer to 10^{-1} mM resulted in an immediate release of material from the column (Mg^{+2} change indicated by arrow in Figure 22). Recovery of A_{260} absorbing material from the BAG column was usually better than 90 percent.

Ultracentrifugal analysis using the ultraviolet optics of material in the peak tubes at 10^{-1} mM Mg^{+2} (Figure 22, Tube 15) and 10 mM Mg^{+2} (Tube 2) showed single main components sedimenting at 51.7S and 78.2S, respectively. The original ribosome solution at 10^{-1} mM Mg^{+2} contained 30S (31.8S) and

Figure 22. The Mg^{+2} dependence of ribosomal binding to BAG

Column was 1.5 cm in diameter and 10 cm high. Flow rate = 1 ml/min. and 9 ml collected per tube.


6.0
5.0
4.0
3.0
2.0
1.0
10mM
Mg+2
0.1mM
Mg+2
ABSORBANCE AT 260 mμ
0
10
20
30
40
TUBE NUMBER

50S (50.6S) subunits in a ratio of 1:8 (enriched in 50S ribosomes). Therefore, the interaction of the 50S ribosomal subunit with the BAG had not changed the sedimentation constant of the subunit. Precipitation of the material from various fractions with 7 percent TCA and analysis of the precipitate for the relative amounts of RNA and protein showed the 50S subunits released from the BAG at 10^{-1} mM Mg^{+2} contained 66-72 percent RNA (Table 8, tubes 15, 25 and 43). The ribosomes used in this experiment had been washed only one time and apparently were contaminated with soluble proteins because analysis of the original ribosome solution gave 44 percent RNA. Some of the extraneous proteins did not bind to the BAG since analysis of material that passed through with the solvent front at 10 mM Mg^{+2} gave 20-27 percent RNA (Table 8, Tubes 1 and 2). Ribosomes washed by centrifugation through

Table 8. Relative amounts of RNA and protein in various fractions from BAG column experiment[a]

Tube number	Percent RNA	Percent protein
1	20	80
2	27	73
15	66	34
25	68	32
43	72	28
Original ribosomes	44	56

[a]Figure 22.

a density gradient of sucrose when analyzed for RNA and protein gave 65 percent RNA (Table 2). Therefore, the RNP-particles eluted from the BAG column at 10^{-1} mM Mg^{+2} were slightly richer in RNA than washed ribosomes. Inspection of the results in Table 8 suggested the particles that interacted strongest with the BAG (eluted later) lost more protein to the BAG because the percent RNA increased steadily from 66 in the early fractions (Tube 15) to 72 in the later fractions (Tube 43).

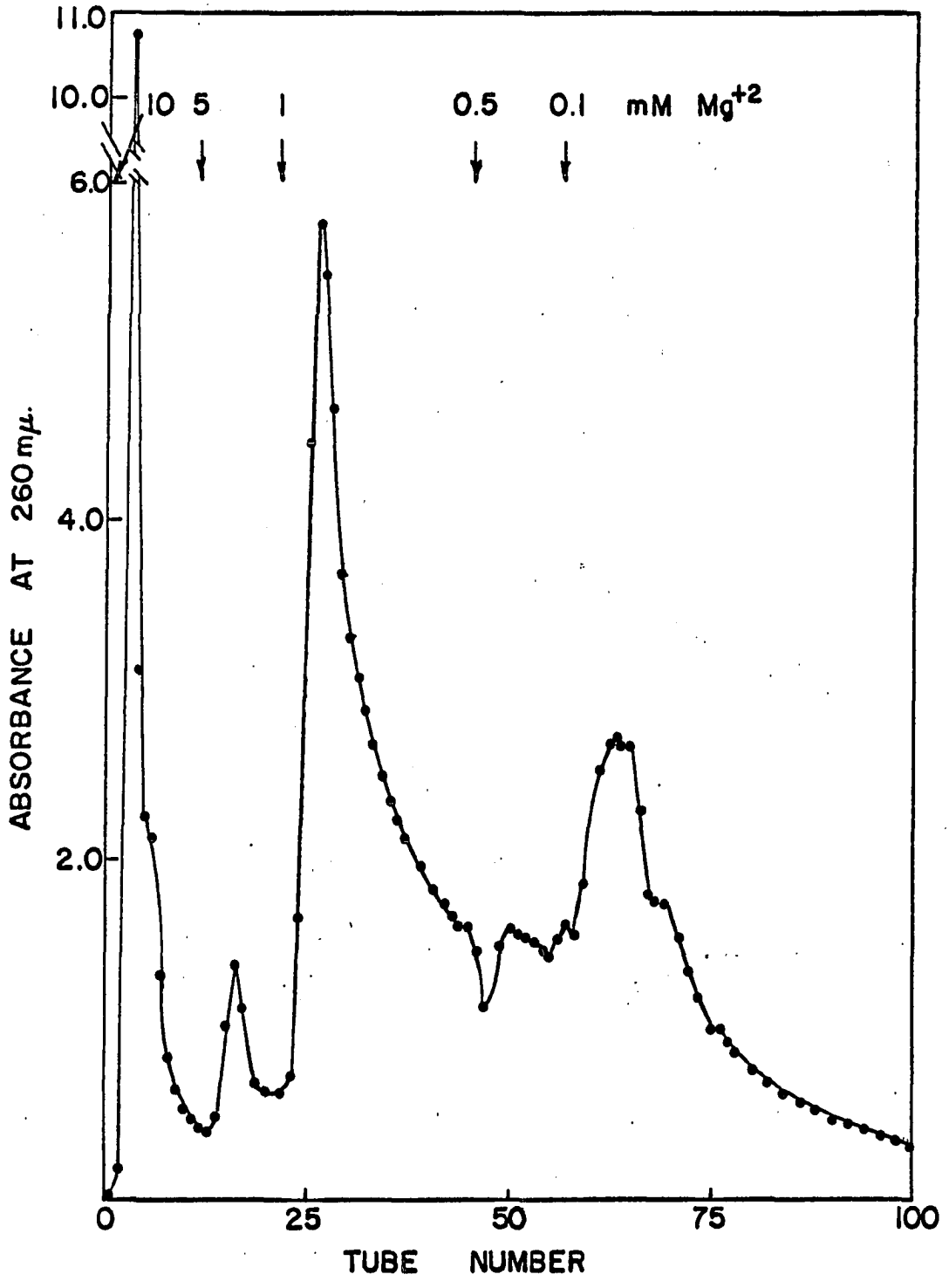
Two main fractions of nucleoprotein particles were obtained in addition to material not bound if the Mg^{+2} concentration in the eluting buffer was decreased from 10 mM to 10^{-1} mM in steps of 5, 1 and 5 × 10^{-1} mM Mg^{+2} (Figure 23). Fifty-five mg of ribosomes (in 0.5 ml) at 10^{-1} mM Mg^{+2} were layered on a BAG column that had been equilibrated with 10 mM Mg^{+2}. The sample was washed into the column with 3-4 ml of 10 mM Mg^{+2} and after incubating the sample on the column for half an hour, the eluting buffer containing 10 mM Mg^{+2} was started. Changes in Mg^{+2} concentration in the eluting buffer were made as indicated on the graph. One fraction was eluted at 1 mM Mg^{+2} and the other at 10^{-1} mM Mg^{+2}. For reasons that are unclear, sometimes the first fraction was eluted at 5 mM Mg^{+2} instead of 1 mM Mg^{+2}. The second peak (eluted at 10^{-1} mM Mg^{+2}) on some occasions appeared heterogeneous containing at least two components.

For characterization of the nucleoprotein particles from

the experiment shown in Figure 23, the peak tubes at 10 mM Mg^{+2} (Tubes 3 and 4), at 1 mM Mg^{+2} (Tubes 25-28), and at 10^{-1} mM Mg^{+2} (Tubes 62-65) were pooled. The fraction eluted at 1 mM Mg^{+2} after dialysis for 24 hours against 10^{-1} mM Mg^{+2} to dissociate any aggregates showed two components, on ultracentrifugal analysis (ultraviolet optics) sedimenting at 30.1S and 52.8S in a ratio of 1:2, respectively. A similar experiment with the fraction eluted at 10^{-1} mM Mg^{+2} showed 32.8S and 55.6S components in a ratio respectively of 1:5.3. The original ribosome solution at 10^{-1} mM Mg^{+2} contained 30S (27.8S) and 50S (51.5S) subunits in a ratio of 1:3. Thus, the interaction of the 30S and 50S ribosomal subunits with BAG had not changed the sedimentation rates of the particles. It appeared from the relative ratios of 30S:50S in the two fractions that some fractionation of the subunits may have occurred on chromatography. A mixture of the two fractions giving a relative ratio of 30S to 50S of 1:3 (like the original ribosomes) was dialyzed 24 hours against 10 mM Mg^{+2} to determine if the subunits would associate to form 70S particles. The original ribosomes formed a single component sedimenting at 71.1S (70S ribosome) at 10 mM Mg^{+2}, while analysis of the solution containing the 30S and 50S particles from the BAG column showed two components sedimenting at 27.4S and 56.0S in a ratio of 1:5.2, respectively. Since the starting ratio of 30S (30-33S) to 50S (53-56S) in the nucleoprotein sample was 1:3 and no mass (based on A_{260}) was lost on increasing the

Figure 23. Elution of ribosomes from BAG

Column was 1.5 cm in diameter and 10 cm high. Flow rate = 0.5 ml/min. and 4.8 ml collected per tube.


11.0
10.0
6.0
4.0
2.0
10
5
1
0.5
0.1
mM Mg+2
ABSORBANCE AT 260 mμ.
0
25
50
75
100
TUBE NUMBER

Mg^{+2} concentration to 10 mM, it appeared that some of the 30S particles aggregated forming a particle sedimenting about 56S at the higher Mg^{+2}. Therefore, the 30S and 50S subunits appeared to be damaged on interaction with BAG since the subunits did not associate to form 70S particles at 10 mM Mg^{+2}.

Analysis of the nucleoprotein particles from the two fractions for RNA and protein indicated the particles contained 74-75 percent RNA while the original ribosome solution contained 49 percent RNA (Table 9). The ribosomes used in this experiment again had been washed only one time and were

Table 9. Relative amounts of RNA and protein in various fractions from BAG column experiment[a]

Fraction	Percent RNA	Percent protein
Did not bind at 10 mM Mg^{+2}	39	61
Eluted at 1 mM Mg^{+2}	75	25
Eluted at 10^{-1} mM Mg^{+2}	74	24
Original ribosomes	49	51

[a]Figure 23.

contaminated with soluble protein. Material that did not bind to the BAG column at 10 mM Mg^{+2} was poorer in RNA than the original ribosomes, yielding 39 percent RNA. Thus, some of

the protein contaminating the ribosome solution did not bind to the BAG at 10 mM Mg^{+2} and passed through with the solvent front.

It again appeared from the relative amounts of RNA and protein that the interaction of ribosomal subunits with BAG had resulted in loss of protein from the ribosomes to the BAG. However, the RNA estimates in this experiment may be high because of contamination of the nucleoprotein samples by fine particles of agar; the ratio of $\frac{A_{660}}{A_{600}}$ for the orcinol analysis of ribose (RNA) (Mejbaum, 1939) was 2.4 where usually it is 2.25.

The latent ribosomal RNase was originally thought to be a structural part of the 30S ribosome, but recent evidence (see Introduction) has indicated the RNase may be adsorbed to the ribosome surface during the preparation of ribosomes. Thus, it was of interest to determine the RNase activity of the subunits after chromatography on BAG. Results of the RNase assay of various fractions is shown in Table 10. The subunits

Table 10. RNase activity of nucleoprotein samples from BAG column experiment[a]

Fraction	RNase activity, percent of original
Did not bind at 10 mM Mg^{+2}	72
Eluted at 1 mM Mg^{+2}	36
Eluted at 10^{-1} mM Mg^{+2}	45

[a]Figure 23.

after elution from the BAG exhibited about a third to a half of the RNase activity of the original subunits. Particles that did not bind to the BAG column showed a slight reduction in RNase activity. Therefore, the 30S subunits on interaction with BAG appeared to lose some of the bound RNase to the BAG.

DISCUSSION

Behavior of Ribosomes at Low Mg^{+2}

The removal of Mg^{+2} from mixtures of 30S and 50S ribosomes resulted in decreased sedimentation rates of the ribosomal subunits. Two transformations in the 50S ribosomal subunits were observed on treatment with EDTA; (1) a decrease in sedimentation constant (sedimentation coefficient corrected for concentration) from 50S to 33-43S that was reversed to about 50S by Mg^{+2} (0.1-10 mM), and (2) a larger decrease in sedimentation constant to 20S that was increased to about 30S (30*S) instead of 50S on restoration of Mg^{+2}. As the time of dialysis against EDTA was increased, more of the 30*S component was produced until only this component could be seen in the ultracentrifuge pattern. The sedimentation constant of the 30S ribosomal subunits appeared to decrease to 20S on treatment with EDTA and to reverse to approximately 30S (30*S) on restoration of Mg^{+2}. Some of the results presented for discussion were previously published (Weller and Horowitz, 1964).

Spirin et al. (1963) reported the removal of Mg^{+2} from ribosomes produced components sedimenting at 12-15S and 25-30S (concentration = 2.5 mg/ml). Increasing the ionic strength or restoration of the Mg^{+2} to 10 mM in the nucleoprotein solution led to the appearance of 30S and 50S components in the usual ratio of 1:2 (30S:50S) (Spirin et al., 1963). These

observations appeared to differ from those obtained by treating ribosomes with EDTA in this laboratory. The ribosomes used by Spirin et al. (1963) had been pre-treated with 0.5 M NH_4Cl to remove the latent RNase (Stanley and Bock, 1964). Therefore, the experiments with NH_4Cl treated ribosomes were repeated in this laboratory in order to better compare results. Attempts to reproduce the results of Spirin et al. (1963) were only partially successful since the 50S ribosomal subunits were converted non-reversibly after longer times at low Mg^{+2} to a component sedimenting about 30S (at higher ionic strength), like the 30*S component. Spirin (1965) has since indicated the non-reversible conversion of 50S ribosomes to a component sedimenting about 30S on removal of Mg^{+2} was observed in his laboratory. Other workers have reported similar conversion of 50S ribosomes to 30S particles on decreasing the Mg^{+2} concentration (Rodgers, 1964) or increasing the ratio of Na^{+} to Mg^{+2} (Cammack and Wade, 1965) in ribosomal solutions.

The properties of the 30*S particles were examined in an attempt to learn more about the structural changes responsible for the decreased sedimentation rates of the ribosomes at low Mg^{+2}. Extraction of RNA from 30*S particles prepared from purified 50S ribosomes by the phenol-SDS or the SDS method showed that the particles contained 23S RNA, like the 50S ribosomes. The 30S ribosomes after treatment with EDTA appeared to contain 16S RNA, like the original 30S ribosomal

subunits, since 16S and 23S RNA in the same ratio as the RNA components in the original ribosomes was extracted from 30*S particles prepared from a mixture of 30S and 50S ribosomes.

Isolation of the usual 16S and 23S RNA components from EDTA-treated ribosomes meant the decreased sedimentation rates of the 30S and 50S ribosomal subunits at low Mg^{+2} were not the result of dissociation of the particles into a lower molecular weight nucleoprotein subunits because each RNA molecule (16S and 23S) is a single continuous polynucleotide chain (see Introduction) and dissociation would have split the polynucleotide chains into slower sedimenting (lower molecular weight) fragments. This conclusion differed from those reached by some other laboratories. These laboratories reported that the removal of Mg^{+2} from the 40S and 60S ribosomal subunits of Pea (T'so, 1958; Bayley, 1964), Yeast (Chao, 1957), and Jensen sarcoma (Petermann and Hamilton, 1961) resulted in the non-reversible dissociation of the subunits into 25-30S particles that constituted one-sixth the molecular weight of the 80S ribosome. Thus, the 40S and 60S subunits were thought to be composed of 2 and 4 of these 25-30S particles, respectively. It has since been suggested these observations were the result of degradation of the subunits by RNase (Petermann, 1965). Since no apparent damage to the ribosomal RNA occurred on treating the ribosomes of E. coli with EDTA in our laboratory, this could explain the difference in interpretation.

It was possible that some of the low molecular weight protein subunits had dissociated from the ribosomes on removal of Mg^{+2} and had been adsorbed to the bentonite or precipitated from solution. However, chemical estimates of the relative amounts of RNA and protein in the 30*S particles prepared from purified 50S ribosomes showed the particles had essentially the same composition as the 50S ribosomes, 65 percent RNA and 35 percent protein. However, since RNP-particles are known to bind many extraneous proteins (Petermann and Hamilton, 1961), it is possible that treatment with EDTA dissociated some of the protein subunits from the ribosomes and these were then non-specifically adsorbed to the 30*S nucleoprotein particles. Recently, disc-gel electrophoresis of the basic protein fraction from the 50S ribosomes and the 30*S particles prepared from purified 50S ribosomes suggested the loss of a specific protein subunit from the ribosomes may have occurred during the treatment with EDTA since on some occasions a protein band was absent from the pattern of the 30*S protein (Shechter, 1965). However, as mentioned in the Introduction, each band obtained on disc-gel electrophoresis of ribosomal protein may not correspond to a different protein. Thus, the significance of the missing band is not clear. Experiments are under way in this laboratory to determine if some of the protein of the 30*S particles can be removed by rather mild treatments such as electrophoresis.

Since the decreased sedimentation rates of the 30S and

50S ribosomes on the removal of Mg^{+2} were not the result of dissociation into lower molecular weight subunits, then the changes must have been due to increases in the frictional coefficient of the particles. Both sedimentation and viscosity are very sensitive to changes in the frictional coefficient of molecules. Therefore, these parameters were determined for the RNP-particles at different Mg^{+2} concentrations to determine if the frictional coefficient of the particles varied with the amount of Mg^{+2} removed from the nucleoprotein structure. The sedimentation constant of 30*S particles prepared by dialyzing a mixture of 30S and 50S ribosomal subunits for 5 days against buffered solution containing 10^{-4} to 10^{-5} mM Mg^{+2} changed steadily from 26S at 10^{-4} mM to 20S at 10^{-5} mM while restoration of the Mg^{+2} to 10^{-1} mM in the solutions increased the sedimentation constant to 33S. Thus, the sedimentation constant of the 30*S particles derived from 50S and 30S ribosomal subunits depended on the Mg^{+2} concentration with values ranging from 20S at 10^{-5} mM Mg^{+2} to 33S at 10^{-1} mM Mg^{+2}.

Determination of the intrinsic viscosity of 30*S particles prepared from purified 50S ribosomes showed the viscosity of the particles varied with Mg^{+2} concentration; at low Mg^{+2} (i.e., in EDTA) the intrinsic viscosity was 0.35 dl/g while at 10^{-1} mM Mg^{+2} the value determined was 0.23 dl/g (Table 11). The intrinsic viscosity of purified 50S ribosomes was determined to be 0.04 dl/g.

Therefore, the frictional coefficient of the nucleoprotein particles depended on the Mg^{+2} concentration (or the amount of Mg^{+2} removed from the RNP-particles) since at the same time that the sedimentation constant of the RNP-particles decreased, a concurrent increase in the intrinsic viscosity of the particles occurred.

Calculation of the molecular weight of nucleoprotein particles was made from $S^0_{20,w}$ and $[\eta]$ data using the Scheraga-Mandelkern equation (1953)

$$M = \frac{(1.47\times10^{13})(S^0_{20,w})^{\frac{3}{2}}[\eta]^{\frac{1}{2}}}{(\beta)^{\frac{3}{2}}(1-\bar{V}\rho)^{\frac{3}{2}}} .$$

A value of 2.12×10^6 was assumed for the hydrodynamic parameter, β; this is the value for an inert sphere. The calculated molecular weight, however, should be within 10 percent of the actual value because β is relatively independent of the shape of the particles. At 10^{-1} mM Mg^{+2} the calculated molecular weight of the 30*S component was 2×10^6 which was essentially the same as the molecular weight calculated for the 50S ribosomes, 1.8×10^6 (Table 11). The calculated molecular weight for 50S ribosomes agreed well with the value reported by Tissiéres et al. (1959) for the 50S particles. However, for reasons that are not clear, the calculated molecular weight of the 30*S particles at low Mg^{+2} was 1.4×10^6, a value about 20 percent lower than the

Table 11. Physical constants and molecular weights of RNP-particles

Component	Ionic condition	$S^0_{20,w}$	[η], dl/g	M x 10^{-6}[a]
50S	10^{-1} mM Mg^{+2}	52.8	0.044	1.8
30*S	10^{-1} mM Mg^{+2}	32.8	0.226	2.0
30*S	low Mg^{+2} (i.e., in EDTA)	22.6	0.345	1.4

[a]Calculation made using $\bar{V}$ = 0.64 ml/g (Tissiéres et al., 1959).

molecular weight of the 50S ribosomes.

The calculated molecular weight for the 30*S particles derived from 50S subunits, 1.4 - 2.0 x 10^6, indicated the decreased sedimentation rate of the 50S ribosomes on removal of Mg^{+2} was not due to dissociation of the particles into nucleoprotein subunits of half the molecular weight of the 50S ribosomes.

Spirin et al. (1963) shadowed the 30S and 50S ribosomal subunits with platinum at low Mg^{+2} (12-15S and 25-30S components) and observed rods up to a 1000 Å long and 30-40 Å in diameter in the electron-microscope. Attempts in our laboratory to observe the EDTA-treated ribosomes in the electron-miscroscope by negatively staining the particles with phosphotungstic acid (PTA) were unsuccessful.

Recently, Petermann and Pavlovec (1965) reported large

reversible changes in the conformation of the 60S ribosomal subunit from rat liver on the removal of Mg^{+2}. Thus, the flexible nature of the larger ribosomal subunit (50S or 60S) may be a general property of the RNP-particle and might be important in the function of the subunit in the biosynthesis of proteins. The smaller subunit (40S) from rat liver ribosomes was affected much less by the removal of Mg^{+2} than the larger subunit (Petermann and Pavlovec, 1965), like the 30S ribosomal subunit of E. coli. Therefore, the behavior of the large and small ribosomal subunits on removal of Mg^{+2} appear to be different and this difference may reflect the different functions of the subunits in the biosynthesis of proteins.

If the removal of Mg^{+2} from ribosomes resulted in a less compact folding of the RNA in the nucleoprotein particles, then the RNA in the 30*S particles should be more readily degraded by RNase than the RNA in the ribosomes. The 30*S particles prepared from purified 50S ribosomes were several times more sensitive to degradation by RNase than the 50S ribosomes. About one-third of the nucleic acid of the 30*S particles was made acid soluble at 10^{-1} mM Mg^{+2} by 1 µg/ml of of RNase after half an hour at 4°C. During the same time only 5 percent of the nucleic acid of the 50S ribosomes was made acid soluble. Thus, these results were consistent with the hypothesis that removal of Mg^{+2} from ribosomes caused an unfolding of the compact nucleoprotein particles.

Did the change in the structure of the ribosomes on removal of Mg^{+2} involve changes in the secondary structure of the ribosomal RNA? The answer to this question was found by comparing the extinction at 260 mμ of the EDTA-treated ribosomes and the untreated ribosomes. Changes in the degree of secondary structure of RNA will result in changes in the extinction at 260 mμ of RNA containing components. The extinction of the 30*S particles prepared from purified 50S ribosomes or from mixtures of 30S and 50S ribosomes at 260 mμ was essentially the same as the original ribosomes, about 7500 liter/mole P x cm and $E_{260}^{0.1\%} = 16$ (Tissiéres et al., 1959). Therefore, the unfolding of the compact nucleoprotein structure on treating the ribosomes with EDTA produced little, if any, change in the secondary structure of the RNA in the particles. Since changes in optical rotation have been correlated with changes in the degree of secondary structure of RNA (Littauer, 1961), the fact that the ultraviolet ORD traces of EDTA-treated ribosomes and untreated ribosomes were essentially the same supported the conclusion that the secondary structure of the RNA was unchanged by treating the ribosomes with EDTA. However, conclusions about the degree of secondary structure of RNA from the ORD curve of ribosomes are rather uncertain because the particles are composed of RNA and protein and both presumably contribute to the optical rotation.

Thus, the removal of Mg^{+2} from the 30S and 50S ribosomal subunits resulted in the unfolding of the compact RNP-structure

probably because of the uncovering of negatively charged phosphate groups of the ribosomal RNA. The electrostatic repulsion force between the phosphate groups could have caused the separation of adjacent regions of the folded RNA chain without altering the secondary structure of the RNA.

Many of the properties of 30*S particles derived from the 50S ribosomes were similar to properties of the unusual RNP-particles that accumulate in E. coli cells when the bacteria are grown in the presence of chloramphenicol (Nomura and Watson, 1959) or puromycin (Dagley et al., 1963). There are two such particles; one contains 16S RNA and sediments at about 18S, the other contains 23S RNA and sediments at 25S (Osawa, 1965). These particles are richer in RNA than ribosomes (70-75 percent RNA) and are considered to be immature ribosomes requiring the addition of protein to become mature particles (Osawa, 1965). The chloramphenicol (CM) particles are the best characterized of the apparent ribosomal precursor particles; therefore, a brief comparison of the properties of these particles and the 30*S particles will be made. Data quoted for the CM-particles was taken from the papers of Nomura and Watson (1959) and Kurland et al., (1962). Both the CM-particles and the 30*S particles behaved like polyelectrolytes with the hydrodynamic parameters $S^{0}_{20,w}$ and $[\eta]$ showing a marked dependency on the ionic strength or Mg^{+2} concentration of the solution. The CM-particles and 30*S particels were found to be very sensitive to degradation by RNase in contrast

to ribosomes which were relatively resistant to attack by the nuclease. The 30*S particles were stable up to 0.13 M Na^+ while the CM-particles were found to be stable only in counter ion concentrations of less than 10^{-2} M Na^+. Any quantitative differences in properties of the particles (CM and 30*S) may be due to the fact that the 30*S particles have the usual amount of protein for ribosomes (i.e., 35 percent) while the CM-particles are poorer in protein (i.e., 20-25 percent).

Interaction of Bentonite with Various Components

A concentration of bentonite of 3 mg/ml was sufficient to protect the high molecular weight state of E. coli ribosomal RNA from 3 µg/ml of RNase at low Mg^{+2} (i.e., 5 mM EDTA) and at 10^{-1} mM and 10 mM Mg^{+2}, pH 7.4. However, at a lower concentration of bentonite (0.5 mg/ml) the inhibition of RNase by the clay appeared to be dependent on the Mg^{+2} concentration with adequate protection afforded at 5 mM EDTA and 10 mM Mg^{+2}, while at 10^{-1} mM Mg^{+2} the RNA was degraded by the nuclease. Keller et al. (1964) found that bentonite inhibits RNase by adsorbing the enzyme to the clay surface with the affinity of bentonite for various components decreasing in the following order; RNase (pancreatic) > Mg^{+2} > Na^+ or K^+. Therefore, bentonite should be a better inhibitor of RNase in 5 mM EDTA (sodium salt) than in 10^{-1} to 10 mM Mg^{+2} because some of the Mg^{+2} bound to the clay has been replaced by Na^+ and RNase can displace Na^+ more easily than Mg^{+2} from the surface of the

clay. The enhanced protection afforded the RNA at 10 mM Mg^{+2} compared to 10^{-1} mM Mg^{+2} probably was due to the effect of Mg^{+2} on the structure of the RNA or on the activity of the RNase.

Littauer and Sela (1962) showed a bentonite concentration of 3.5 mg/ml was required to protect E. coli ribosomal RNA (in 0.2 M NaCl) from degradation by 1 µg/ml of pancreatic RNase.

Our studies extended the observations of Littauer and Sela (1962) in several ways: (1) the ability of bentonite to protect against RNase degradation of RNA containing components was examined under ionic conditions commonly employed in the preparation of ribosomes and the isolation of RNA, (2) the inhibition of RNase by the clay was shown to be dependent on the Mg^{+2} concentration, and (3) concentrations of bentonite required to protect RNA containing components from degradation by RNase were re-evaluated.

Bentonite was used in the studies with ribosomes so the interaction of ribosomes with the clay was examined. A Mg^{+2} dependent binding of the ribosomes to bentonite was observed. Few ribosomes were bound at 10^{-1} mM Mg^{+2} (0.03-0.06 mg/mg bentonite) while at 10 mM Mg^{+2} large amounts of ribosomes were bound to the clay (0.7-1.8 mg/mg bentonite).

After this work was completed, a study by Dunn and Hitchborn (1965) concerned with the isolation and purification of virus particles from cellular extracts appeared. In this study the binding of cabbage ribosomes to bentonite was

examined and reported to be dependent on the Mg^{+2} concentration in agreement with our observations with E. coli ribosomes.

The binding of ribosomes to bentonite at 10 mM Mg^{+2} was partially reversed by decreasing the Mg^{+2} concentration in the suspension to 10^{-1} mM. Typical 30S, 50S and 70S ribosomal boundaries could be seen in the ultracentrifuge pattern of a solution containing the particles released from the bentonite. However, as will be discussed later, the interaction of ribosomes with bentonite apparently damages the 30S and 50S subunits.

Ribosomal RNA did not bind to the bentonite at 10 mM Mg^{+2} so that the binding of ribosomes to bentonite appeared to be due to the protein moiety of the ribosome. A plausible mechanism for the binding of ribosomes to bentonite would involve the exchange of the ribosomes for Mg^{+2} or Na^{+} on the surface of the clay. The role of Mg^{+2} in the binding would then be to reduce the negative charge on the ribosomal RNA allowing the cationic (basic) protein of the ribosomes to bind to the negatively charged surface of the bentonite.

The Mg^{+2} dependency and apparently reversible nature of ribosomal binding to bentonite were interesting because of the possibility of using bentonite as an ion exchanger or adsorbent for column chromatography of ribosomes. Bentonite, itself, could not be packed into a column because the flow of eluent through the column was too slow. Therefore, a

chromatographic material was prepared by trapping the clay in 5 percent agar gel (BAG). The BAG column exhibited properties similar to the bentonite alone since ribosomes were bound at 10 mM Mg^{+2} and eluted by buffered solutions containing lower levels of Mg^{+2}. Two main fractions of nucleoprotein particles were obtained if the ribosomes were eluted by decreasing the Mg^{+2} concentration in the buffered solution from 10 mM to 10^{-1} mM in steps of 5, 1 and 5 x 10^{-1} mM Mg^{+2}. One fraction was eluted either at 5 mM or 1 mM Mg^{+2} while the other was eluted at 10^{-1} mM Mg^{+2}. Both fractions contained 30S and 50S particles although the ratio of the components were different in the two fractions. A mixture of the material from the two fractions was dialyzed 24 hours against 10 mM Mg^{+2}, but increasing the Mg^{+2} level did not result in formation of 70S particles. The increased level of Mg^{+2}, however, appeared to cause aggregation of the 30S particles forming a particle sedimenting about 50-55S because the ratio of 30S:50S decreased in the sample on increasing the level of Mg^{+2} and no mass was lost during the dialysis. Therefore, the interaction of ribosomes with bentonite or BAG had apparently damaged the ribosomal subunits since the subunits did not associate to form 70S particles on increasing the Mg^{+2} concentration to 10 mM. Chemical estimates of the relative amounts of RNA and protein in the 30S and 50S particles eluted from the BAG column indicated the particles were richer in RNA than normal ribosomal subunits. The particles contained 75 percent RNA

compared with 65 percent RNA for normal ribosomes. However, the estimate of RNA may be high because of contamination of the nucleoprotein solutions by five particles of agar despite precautions taken to reduce the possibility. Carbohydrates interfere with the determination of ribose (RNA) by the orcinol method (Mejbaum, 1939). Therefore, any agar contaminating the nucleoprotein solutions would cause the estimate of RNA to be high.

An estimate of the ribosomal bound RNase activity showed the RNP-particles after elution from the BAG column exhibited about 35-45 percent of the original RNase activity. Thus, some of the RNase bound to the 30S ribosomal subunit (Tal and Elson, 1963) apparently was removed from the ribosomes on interaction with the BAG.

Does the fact that two peaks of 30S and 50S particles were obtained by chromatography on BAG mean that ribosomal subunits are structurally heterogeneous? The answer to this question might be obtained by examining the chemical composition of the RNA and protein from the particles in the two peaks. Disc-gel electrophoresis of the protein isolated from the particles may give valuable information concerning the kind and function of any protein lost from the ribosomes by interaction with the BAG column.

The major use of BAG may well be as a trap or filter for ribosomes. Applications could include: (1) isolation of ribosomes from cellular extracts, and (2) separation of some

virus particles from ribosomes and any other cellular components that bind to bentonite since some virus particles do not bind to bentonite at 10 mM Mg^{+2} (Dunn and Hitchborn, 1965). Kaiser (1965) has used BAG columns to trap ribosomes coded with synthetic messenger RNA's in an attempt to isolate specific transfer RNA molecules.

SUMMARY

Removal of Mg^{+2} from ribosomes by dialysis against 5 mM EDTA in the presence of bentonite (0.5 mg/ml) and in the cold (4-6°C) led to two transformations of the 50S ribosomal subunits; (1) a decrease in sedimentation constant (sedimentation coefficient corrected for concentration) from 50S to 33-43S that was reversed to about 50S on restoration of Mg^{+2} (0.1-10 mM) or on the addition of KCl (0.2 M), and (2) a larger decrease in sedimentation constant to 20S that was increased to about 30S (30*S) instead of 50S on restoration of Mg^{+2} or on the addition of KCl or NaCl. As more Mg^{+2} was removed from the ribosomes, a greater proportion of the 50S subunits were converted to 30*S particles until only the 30*S boundary could be seen in the ultracentrifuge pattern. Treatment of the 30S ribosomal subunit with EDTA resulted in a decrease in the sedimentation constant from 30S to 20S that was reversed to approximately 30S (30*S) by Mg^{+2} (0.1-10 mM) or KCl (0.2 M).

The 30*S particles derived from 50S ribosomes contained 23S RNA, like the 50S subunits. After treatment with EDTA the 30S ribosomal subunit appeared to still contain 16S RNA. Therefore, the decreased sedimentation rates of the ribosomal subunits on removal of Mg^{+2} could not have been due to dissociation of the ribosomes into lower molecular weight nucleoprotein subunits because dissociation would have split the r-RNA (one continuous polynucleotide chain) into slower

sedimenting fragments. The 30*S particles prepared from purified 50S ribosomes contained the usual relative ratio of RNA to protein for ribosomal particles, 65 percent RNA and 35 percent protein.

Other properties of the 30*S particles showed the decrease in sedimentation rates of the ribosomes on removal of Mg^{+2} resulted from an increase in the frictional coefficient of the nucleoprotein particles. The sedimentation constant of the 30*S particles decreased steadily from 26S at 10^{-4} mM Mg^{+2} to 20S at 10^{-5} mM Mg^{+2}. Restoration of the Mg^{+2} to 10^{-1} mM increased the sedimentation constant of the particles to 33S. The intrinsic viscosity of 30*S particles prepared from purified 50S ribosomes varied from 0.35 dl/g at low Mg^{+2} (i.e., in EDTA) to 0.23 dl/g at 10^{-1} mM Mg^{+2}. These values were several times higher than the 0.04 dl/g determined for purified 50S ribosomes at 10^{-1} mM Mg^{+2}. Calculation of molecular weights from $S^{0}_{20,w}$ and [η] using the Scheraga-Mandelkern equation showed the 30*S particles derived from 50S ribosomes had approximately the same molecular weight as the 50S ribosomes, 1.8×10^{6}. The 30*S particles prepared from purified 50S ribosomes were several times more sensitive to RNase degradation than 50S ribosomes which were relatively resistant to attack by the nuclease.

The optical properties of the 30*S particles indicated the changes in structure of ribosomes on the removal of Mg^{+2} caused little, if any, change in the secondary structure of

RNA in the particles. An ultraviolet absorption spectrum typical of ribosomes was determined for 30*S particles and the extinction coefficient of the 30*S particles and ribosomes at 260 mμ were essentially the same, 7500 liter/mole P x cm or $E_{260}^{0.1\%} = 16$. The ultraviolet ORD curves for the 30*S particles and ribosomes were almost identical.

A concentration of bentonite of 3 mg/ml at pH 7.4 was sufficient to protect high molecular weight E. coli ribosomal RNA at low Mg^{+2} (i.e., 5 mM EDTA) and at high Mg^{+2} (0.1-10 mM) from degradation by RNase at 3 μg/ml.

The interaction of ribosomes with bentonite was examined and found to be a function of Mg^{+2} concentration. Large amounts of ribosomes were bound to the bentonite at 10 mM Mg^{+2} (0.7-1.8 mg/mg bentonite) while at 10^{-1} mM Mg^{+2} only trace amounts were bound (0.03-0.06 mg/mg bentonite). Ribosomal RNA did not bind to bentonite at 10 mM Mg^{+2} so that the binding of ribosomes to bentonite appeared to be due to the protein of the ribosomes. The binding of ribosomes to bentonite at 10 mM Mg^{+2} was partially reversed (i.e., up to 80 percent) by decreasing the Mg^{+2} in the suspension to 10^{-1} mM Mg^{+2}. Therefore, the possibility of employing bentonite as an ion exchanger or adsorbent for chromatography of ribosomes was examined. The clay was trapped in 5 percent agar gel (BAG) to improve the flow rate of the eluent. When 30S and 50S ribosomal subunits in the usual ratio (i.e., 1:2, 30S:50S) were bound to the BAG column at 10 mM Mg^{+2} and then eluted with

decreasing levels of Mg^{+2}, two main fractions of 30S and 50S particles were obtained. One peak was eluted at 5 mM or 1 mM Mg^{+2} and the other at 10^{-1} mM Mg^{+2}. Characterization of the particles in the two fractions indicated the particles may be slightly richer in RNA than the usual ribosomal subunits. Estimates of RNase activity showed that the RNP-particles had lost 55-65 percent of the original RNase activity to the BAG column. The sedimentation constants of the particles were about 30S (31-33S) and 50S at 10^{-1} mM Mg^{+2}, like the usual ribosomal subunits. However, the 30S and 50S particles from the BAG column did not associate to form 70S ribosomes at 10 mM Mg^{+2}. Therefore, it appeared the interaction of ribosomes with the BAG had damaged the ribosomal subunits.

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