Cations and Ribosome Structure. II. Effects on the 50S Subunit of Substituting Polyamines for Magnesium Ion†

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ABSTRACT: Purified 50S ribosomal subunits of *Escherichia coli* were dialyzed to equilibrium against Tris buffers containing different levels of spermidine ($NH_3^+(CH_2)_3NH_2^+(CH_2)_4NH_3^+$) or putrescine ($NH_3^+(CH_2)_4NH_3^+$) relative to $MgCl_2$. In this way particles with defined ratios of bound magnesium ion to RNA-phosphate (Mg^{2+}/RNA-P) could be isolated. Examination of the polyvalent cation content of these particles demonstrated that there was essentially a stoichiometric charge replacement of Mg^{2+} by polyamines. Neither spermidine nor putrescine could totally replace Mg^{2+} with maintenance of structural and functional integrity of the 50S subunit. In both cases, the ability of these particles to polymerize phenylalanine in the presence of poly(uridylic acid) was completely abolished as the Mg^{2+}/RNA-P was reduced from approximately 0.10–0.05. Loss of activity observed during the replacement of Mg^{2+} by polyamines was accompanied by conformational changes in the 50S subunit. Spermidine particles showed a decrease in sedimentation coefficient to 42 S, a 2-fold increase in sensitivity to pancreatic ribonuclease, and a 40% increase in reduced viscosity. Formation of putrescine particles resulted in species of 30 and 20 S concomitant with a sevenfold increase in reduced viscosity. Partially active particles were unstable despite the fact that the Mg^{2+}/RNA-P remained constant, the rate of decay increasing with lower Mg^{2+}/RNA-P. Loss of activity could not be attributed to components in the buffering system other than Mg^{2+} and polyamines. In addition, inactivation was not due to loss or major degradation of any of the ribosomal proteins or of the 5S or 23S RNA species. The original structural and functional properties of the 50S subunit could not be regained after restoring Mg^{2+} by dialysis. It was concluded that replacement of a critical level of Mg^{2+} by polyamines resulted in an irreversible unfolding and loss of polymerizing activity of the 50S subunit of *E. coli*.

A recent report from this laboratory suggested that the stoichiometric replacement of ionically bound Mg^{2+} by the polyamines putrescine ($NH_3^+(CH_2)_4NH_3^+$) and spermidine ($NH_3^+(CH_2)_3NH_2^+(CH_2)_4NH_3^+$) induced irreversible conformational alterations in the 70S ribosomes from *Escherichia coli* (Weiss and Morris, 1970). Since polyamines appear to bind to nucleic acids through electrostatic interactions as does Mg^{2+} (Felsenfeld and Huang, 1960, 1961; Hirschman *et al.*, 1967; Choi and Carr, 1967), this system offers an opportunity to investigate the effects of removing Mg^{2+}, while still maintaining the level of polyvalent cations bound to the rRNA. In the preceding paper, polyamines by themselves were shown to be ineffective in maintaining the integrity of the 30S subunit (Weiss and Morris, 1973). The purpose of the following work is to define the level of ribosomally bound Mg^{2+} specifically required to maintain the structural and functional properties characteristic of native 50S subunits from *E. coli*.

Methods

Unless otherwise noted, all preparations and methods were identical with those in the preceding paper (Weiss and Morris, 1973). Preparations of 50S subunits contained not more than 4% cross-contamination by 30S subunits as judged by analytical ultracentrifugation and activity assays. In the protein synthesis assays, approximately a 50% excess of 30S subunits was maintained at all times.

The buffers used in these expeiments are defined as follows: TK buffer: 10 mM Tris-HCl (pH 7.5), 60 mM KCl, and 6 mM 2-mercaptoethanol; buffer TK6: TK buffer plus 0.6 mM magnesium acetate; buffer TK1: TK buffer plus 1 mM magnesium acetate; buffer TK10: TK buffer plus 10 mM magnesium acetate; buffer S: TK buffer plus 2.0 mM spermidine-3HCl; buffer P: TK buffer plus 4.0 mM putrescine-2HCl.

Viscosity Measurements. These were made after further purification of the 50S subunits. Preparations of 50S subunits, isolated by sucrose gradient centrifugation, in buffer TK10 (see above) were dialyzed for 8 hr against a buffer containing 10 mM Tris-HCl (pH 7.4), 1 M NH_4Cl, 2 mM magnesium acetate, and 6 mM 2-mercaptoethanol. A 0.6- to 0.8-ml sample was layered over 4 ml of 50% sucrose (Mann, ribonuclease free) in the same buffer and centrifuged at 48,000 rpm for 20 hr in an SW 50.1 rotor. The supernatant solution was discarded and the ribosomal pellet was resuspended in buffer TK10 and frozen in appropriate aliquots at −60°. This procedure resulted in a 40% loss of the original material absorbing at 260 nm. Reduced viscosities were then determined as previously described (Weiss and Morris, 1970).

Preparation and Electrophoresis of 5S RNA. A 2-ml sample containing 8 mg of 50S subunits was dialyzed to equilibrium (12 hr) against the indicated buffer. In the following steps, sterile technique was used throughout. After dialysis, the sample was separated into pellet and supernatant fractions by centrifugation at 150,000*g* for 6 hr. The upper 80% of the

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supernatant fraction was stored frozen at −60° (fraction A). The pellet fraction was resuspended in 2 ml of 5 mM Tris (pH 7.5), 2 M LiCl, and 0.1 mM $MgCl_2$ and allowed to sit at 0° for 3 hr with occasional stirring. This procedure has been shown to remove the 5S RNA from the 50S subunit (Morell and Marmer, 1968). The suspension was then centrifuged at 150,000*g* for 6 hr and the upper 80% of the supernatant solution was saved (fraction B), the pellet being discarded. Fractions A and B were extracted two times with buffer-equilibrated phenol at 23°. The residual phenol in the remaining aqueous phase was removed by sparging with nitrogen. The final preparations were dialyzed against several changes of 10^{-4} M Tris-HCl (pH 7.0), to remove salts, and then concentrated by lyophilization. Dialysis tubing was prepared according to the procedure of Stanley and Bock (1965). The lyophilized samples were resuspended in 30 μl of running buffer for electrophoresis (see below) containing 10% sucrose.

Electrophoresis of the 5S RNA was conducted on polyacrylamide gels as described by Morell and Marmer (1968), except for the omission of sodium dodecyl sulfate, which interfered with the staining procedure. Acrylamide and *N,N'*-bisacrylamide were recrystallized by the procedures of Loening (1967). Buffers in the upper and lower chambers (running buffer) contained 0.05 M Tris-acetate (pH 7.5), 0.025 M sodium acetate, and 0.0025 M EDTA. A 30-μl sample (see above) was layered on a gel measuring 0.5 × 9.0 cm which contained 10% (w/v) acrylamide and 0.25% (w/v) bisacrylamide in running buffer. Buffer was layered over the sample and electrophoresis was carried out for 3–3.5 hr at 15° and at a constant current of 5 mA/gel. The gels were removed and stained according to Peacock and Dingman (1967). The tRNA used as marker in these gels was $tRNA_{fMet}$ originally prepared at Oak Ridge National Laboratory and kindly provided by Dr. M. P. Gordon.

Isolation of 23S RNA. With the exception of a few modifications, total RNA was extracted from preparations of 50S subunits by the phenol–cresol procedure of Pigot and Midgley (1968). In our hands, other procedures previously reported for the extraction of rRNA (Kurland, 1960; Stanley and Bock, 1965) were satisfactory for the 30S subunit, but resulted in very poor yields with the isolated 50S subunit (20–30%). The procedure outlined below gave better than 80% recovery. Samples were brought to 0.1% macaloid (National Lead Co.; Mandeles and Bruening, 1968) and 0.1% sodium dodecyl sulfate and extracted two times at 23° with the phenol–cresol mixture. Macaloid was also included in the second phenol extraction. RNA was precipitated from the aqueous phase with two volumes of absolute ethanol. The precipitate was lyophilized to dryness, resuspended in 0.05 M sodium acetate (pH 5.4), and stored at −60°.

Disc Gel Electrophoresis of Ribosomal Protein. Ribosomal proteins were extracted from preparations of 50S subunits by treatment with 67% acetic acid (Waller and Harris, 1961; Hardy *et al.*, 1969). The RNA precipitate was discarded and the supernatant fraction containing the protein was dialyzed against distilled water supplemented with 6 mM 2-mercaptoethanol. The protein was then concentrated by lyophilization. Acrylamide gels were prepared according to the specifications delineated by Traut (1966), except for the use of 6 M rather than 8 M urea. The urea was deionized with mixed-bed resin (Bio-Rad) and recrystallized before use. The buffer used in the upper and lower reservoirs contained 15.6 g of β-alanine (Calbiochem) and 4.0 ml of glacial acetic acid per l. (pH 4.5). Approximately 50 μg of ribosomal protein in 0.1 ml was applied to each gel and electrophoresed for 3.5–4 hr at 15° and a constant current of 4 mA/gel. For staining, gels were removed from their glass tubes and transferred to a solution containing 0.25% (w/v) Coomassie Brilliant Blue in methanol, deionized water, and glacial acetic acid in a ratio of 5:5:1. After 1 hr, gels were destained electrophoretically in a mixture of methanol, glacial acetic acid, and deionized water (2:3:35) using a Canalco horizontal destaining apparatus.

For the preparation of proteins released during magnesium depletion, approximately 3 mg of 50S subunits, at a concentration of 0.5 mg/ml or less, were dialyzed against buffer P or buffer S. After dialysis, the preparation was centrifuged at 100,000*g* for 6–8 hr. The pellet fraction was discarded and the upper 80% of the supernatant solution was saved. Salts were removed from the supernatant fraction by dialysis against distilled water and the proteins were concentrated by lyophilization. Disc gel electrophoresis of this fraction was carried out as outlined above. Generally, from one-half to three-fourths of the total sample was analyzed by electrophoresis for the presence of ribosomal proteins.

Preparation of Radioactive Ribosomal Subunits. E. coli K12 (strain A19) was grown in the Tris-salts medium of Watanabe (1957) using a procedure similar to that reported by Otaka, *et al.* (1968). The medium was supplemented with 5 μg/ml of [^{14}C]lysine (specific activity 0.04 Ci/g, Volk) or [^{3}H]-lysine (specific activity 0.3 Ci/g, New England Nuclear), 15 μg each of the other amino acids/ml, 0.3 mg of yeast extract/ml, and 0.4% (w/v) D-glucose. Cultures of 200 ml were grown for approximately nine doublings and then harvested in exponential phase at an absorbance of approximately 1.5 at 540 nm. Cooled cells were concentrated by low-speed centrifugation and washed two times with buffer TK10. The frozen pellets were ground with alumina and S30 extracts prepared as previously described (Weiss and Morris, 1970). Ribosomes were pelleted by centrifuging the S30 (final volume equal to 2 ml) at 150,000*g* for 12 hr in an angle-head 50 rotor using 2-ml tubes and adaptors. The pellets were resuspended in 0.4 ml of buffer TK1 and dialyzed against the same buffer for 12 hr at 3°. Samples of 0.15–0.20 ml were layered on 10–30% sucrose gradients and centrifuged at 25,000 rpm for 14 hr in an SW 25.3 rotor. Gradients were monitored for radioactivity and the peak 30S and 50S fractions were pooled separately and centrifuged for 24 hr at 50,000 rpm in an SW 50.1 rotor. The pellets were resuspended in buffer TK10 and frozen at −60°. The final yields for the 200-ml culture were approximately 4 mg of 50S subunits and 2 mg of 30S subunits. Cross-contamination was less than 5% in each case as determined by analysis of these preparations on sucrose gradients. The ^{3}H-labeled 50S subunits were found to have full polymerization activity with [^{14}C]phenylalanine when compared to nonradioactive subunits prepared in the conventional manner.

Disc Gel Electrophoresis of Radioactive Ribosomal Protein. Samples of 0.10–0.15 ml of radioactive 30S or 50S subunits (60–120 μg of ribosomal protein) were dialyzed against buffer S (plus $MgCl_2$, where mentioned) or buffer TK1 for 12 hr. These samples of ^{3}H- or ^{14}C-labeled subunits were centrifuged separately on 10–30% sucrose gradients (in the appropriate buffer) in an SW 50.1 rotor (50,000 rpm) for 2.5 hr in the case of 30S subunits and 1.5 hr in the case of 50S subunits. Fractions with maximal radioactivity were pooled. Fractions containing ^{3}H- and ^{14}C-labeled subunits were mixed and the ribosomal protein was prepared as described above. After electrophoresis, the gels were removed and divided into 1-mm slices. The slices were treated with NCS reagent (Nuclear-Chicago)

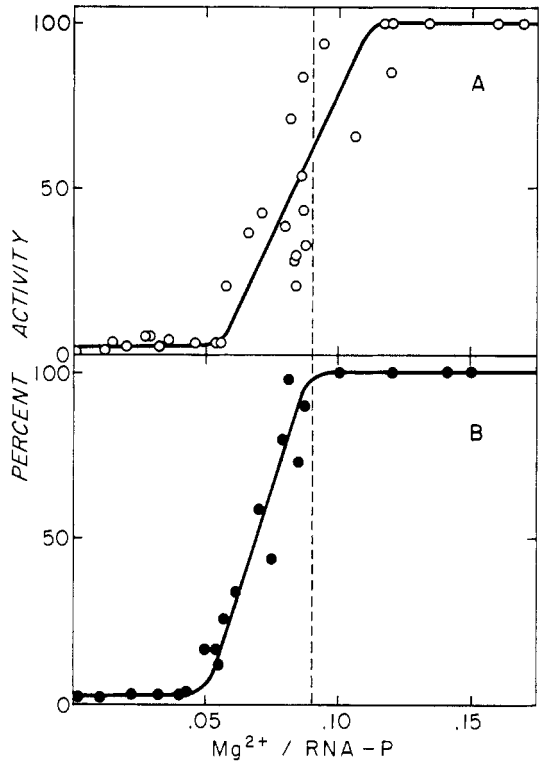


FIGURE 1: The change in polymerizing activity of the 50S subunit during the replacement of Mg^{2+} by putrescine (A) and spermidine (B). Solutions of 50S subunits (4.0 mg/ml) in buffer TK10 were dialyzed for 12 hr at 3° against buffer P or buffer S supplemented with 0 to 2.0 mM $MgCl_2$. Activity and Mg^{2+}/RNA-P were determined by methods previously described (Weiss and Morris, 1973).

and analyzed for radioactivity as described by others (Tishler and Epstein, 1968; Basch, 1968; Zaitlin and Hariharasubramanian, 1970). The ^3H and ^{14}C content of each slice was analyzed in a Beckman CPM-100 liquid scintillation counter.

Results

Stoichiometry of Replacement of Magnesium Ions by Polyamines. Suspensions of 50S ribosomal subunits were dialyzed against buffers containing spermidine (buffer S) or putrescine (buffer P) in the presence of varying concentrations of $MgCl_2$, to produce particles having different ratios of bound Mg^{2+} to RNA phosphate (Mg^{2+}/RNA-P). The time of dialysis required to reach a constant Mg^{2+}/RNA-P ranged from 4 to 8 hr and appeared to be partially dependent on the sample size, ribosome concentration and the particular preparation of dialysis tubing. The results in Table I show that as the $MgCl_2$ concentration in polyamine buffers was reduced, Mg^{2+}/RNA-P ratios were lowered. In the case of spermidine, it appears that bound Mg^{2+} is replaced by an equivalent level of bound polyamine, the ratio of total polycationic charge to RNA phosphate (+/RNA-P) remaining constant within the experimental error of the determinations. In similar experiments with putrescine, the same trend was observed, however, the stoichiometry was somewhat less than one to one (Table I). Nevertheless, under the conditions of these experiments, at least 80% of the vacant Mg^{2+} sites were filled with putrescine.

Activity as a Function of Mg^{2+}/RNA-P. 50S particles, with defined amounts of bound Mg^{2+} exchanged for putrescine or spermidine by equilibrium dialysis, were assayed for their capacity to synthesize poly(phenylalanine) in the presence of poly(U) and fully active 30S subunits. Full activity was defined as that displayed by 50S subunits dialyzed against buffer TK10 for 12 hr in the absence of added polyamines. Neither putrescine (Figure 1A) nor spermidine (Figure 1B) could totally replace Mg^{2+} without inducing a total loss of polymerizing activity. This loss of activity could not be attributed to components in the buffering system other than Mg^{2+} and polyamines. When KCl was removed from polyamine buffer or phosphate buffer used instead of Tris, identical inactivation profiles were observed. Furthermore, it could be demonstrated that the observed inactivation was not due to a displacement of the Mg^{2+} optimum for protein synthesis or to misreading of the message, since neither leucine nor isoleucine were incorporated in the presence or absence of phenylalanine.

It must be noted that while Mg^{2+}/RNA-P ratios were determined in polyamine buffers, the protein-synthesizing capacity of magnesium-deficient particles was measured in a buffer containing 16 mM magnesium acetate. The temperature of the assay (25 or 37°), together with the high level of Mg^{2+}, might be expected to promote a rapid replacement of bound polyamines with Mg^{2+}. Thus, the results in Figure 1 may more appropriately suggest the point at which inactivation on replacement of Mg^{2+} by polyamines became irreversible under the assay conditions. This point is particularly relevant in light of the monovalent cation-dependent changes in the activity of the 50S subunit observed by Miskin *et al.* (1970) which could only be detected in low-temperature assays.

Neither totally inactive nor partially active particles on which magnesium ion was replaced by putrescine or spermidine could be reactivated by dialysis into buffers containing Mg^{2+}. This dialysis procedure effectively removed over 98% of the bound polyamines and restored the Mg^{2+}/RNA-P of the 50S subunit to approximately 0.22. Other methods employing high temperature and ionic strength (Nomura and Erdmann, 1970) or sequential dialysis (Maruta *et al.*, 1969) have been equally unsuccessful in restoring activity.

Structural Properties of Magnesium-Deficient Particles. Ribonuclease sensitivity and sedimentation behavior were examined (Figure 2) during Mg^{2+} replacement by spermidine. At values of Mg^{2+}/RNA-P greater than 0.1, there was no de-

TABLE I: Stoichiometry of Mg^{2+} Replacement by Polyamines.[a]

Buffer	Free Mg^{2+}[b] (μmol/ml)	Mg^{2+}/RNA-P	Spd^{3+}[c] RNA-P	Put^{2+}/RNA-P	+/RNA-P
S	2.00	0.124	0.138		0.65
S	0.568	0.076	0.160		0.63
S	0.372	0.042	0.170		0.59
S	0.232	0.037	0.180		0.61
S	0.005	0.012	0.188		0.59
P	1.00	0.180		0.131	0.62
P	0.214	0.081		0.174	0.51
P	0.005	0.010		0.217	0.45

[a] All preparations dialyzed for 12 hr at 3° and a ribosome concentration of 4.0 mg/ml. [b] Concentration of Mg^{2+} measured by atomic absorption spectroscopy. [c] Abbreviations used are: Spd^{3+}, spermidine; Put^{2+}, putrescine; +/RNA-P total charge of bound polyvalent cations per rRNA-P residue.

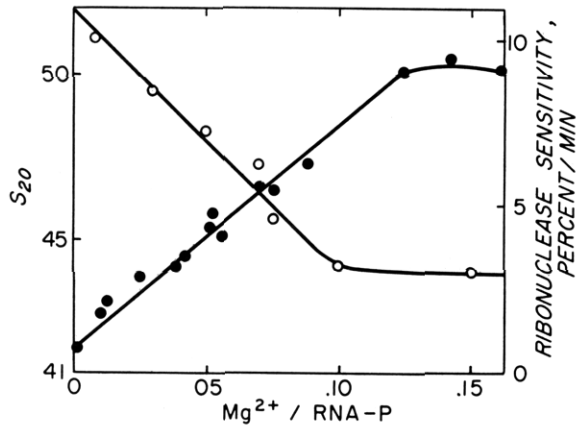


FIGURE 2: Changes in sedimentation coefficient (●) and ribonuclease sensitivity (○) of the 50S subunit during the replacement of Mg^{2+} by spermidine. Solutions of purified 50S subunits were dialyzed to equilibrium against buffer S containing 0–0.3 mM $MgCl_2$. After dialysis (12 hr at 3°), Mg^{2+}/RNA-P, sedimentation coefficient, at 20° and sensitivity to pancreatic ribonuclease at 37° were measured as previously described (Weiss and Morris, 1973).

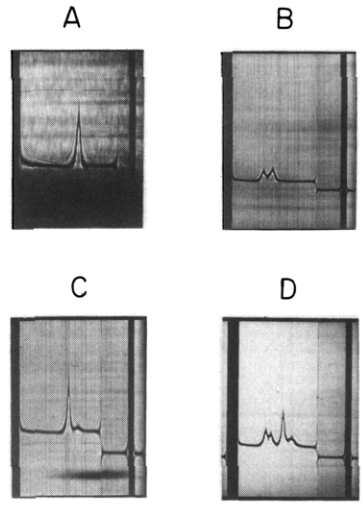


FIGURE 3: Sedimentation patterns of 50S subunits after replacement of Mg^{2+} by polyamines. Sedimentation is from right to left. Frame A represents a sample dialyzed against buffer S; Mg^{2+}/RNA-P <0.002, per cent activity <2%, s_{20} = 43.2 S. The sample in frame B was dialyzed against buffer S supplemented with $MgCl_2$ to produce a preparation with 50% activity and then the Mg^{2+}/RNA-P was restored to 0.22 by dialysis against buffer TK6 for 24 hr at 3°; s_{20} = 39.3 and 47.2 S. Frame C represents a sample dialyzed against buffer P; Mg^{2+}/RNA-P <0.005, per cent activity <2%, s_{20} = 20.5 and and 30.2 S. The sample in frame D was dialyzed to equilibrium against buffer P to produce a preparation with 40% activity; s_{20} = 20.1, 30.3, 42.6, 47.5 S. Sedimentation coefficients were determined as previously described (Weiss and Morris, 1973).

tectable change in either parameter. However, as the Mg^{2+}/RNA-P was reduced from approximately 0.1 to 0, the sedimentation coefficient of the 50S subunit continuously decreased from 50 to 42 S and the ribonuclease sensitivity of the particles continually increased. While neither of these changes in the 50S subunit paralleled the loss of activity seen in Figure 1, they were both initially manifested close to the point where activity was first seen to decay. Spermidine particles generally appeared as a single, sharp boundary in the analytical centrifuge (Figure 3A). Occasionally, a 74S species was observed which accounted for 5–10% of the material and could increase to levels as high as 30% on aging for 72 hr at 3°. Diluting buffer S in half showed no effect on the sedimentation coefficient of these particles. Extrapolation of the sedimentation coefficient of fully depleted particles to zero concentration yielded a value of 44.5 S.

If inactive spermidine particles were dialyzed against TK buffer containing 4, 1, and 0.6 mM magnesium acetate, their sedimentation behavior was distinctly different from control particles. In all three buffers, the control 50S subunit showed a sedimentation coefficient of 47–48 S. On the other hand, spermidine particles sedimented at 46 S in 4 mM Mg^{2+} and in 1 mM Mg^{2+} both 42S and 46S species were observed. In TK buffer containing 0.6 mM Mg^{2+} (buffer TK6) only a 40S species was observed. When Mg^{2+} was displaced by spermidine to give partially active particles and then further dialyzed against buffer TK6, two distinct species were produced, one sedimenting at 40 S and the other at 47–48 S (Figure 3B). Under these conditions, the decrease in the amount of 48S species paralleled the loss of activity (Figure 4). In addition, the increase in ribonuclease sensitivity measured in buffer TK6, is coincident with the decay of activity. These results suggest that a partially active preparation contains active and inactive species which sediment at 48 and 40 S, respectively, after dialysis into buffer TK6. This conclusion was confirmed by separation of the two species by sucrose gradient centrifugation. The 40S particle was totally inactive, whereas the 48S species showed the same specific activity as control particles.

When Mg^{2+} was completely replaced by putrescine, the native 50S species disappeared and a 30S particle plus a smaller amount of a 20S component were observed (Figure 3C). While totally inactive preparations contained only these two components, partially active preparations contained 47S, 42S, 30S, and 20S species (Figure 3D). These changes in sedimentation behavior were accompanied by an increase in sensitivity to pancreatic ribonuclease. These structural alterations of the particles in putrescine buffer were coincident with the loss of activity (Figure 5). When totally inactive or partially active particles were dialyzed back into buffer TK1, the sedimentation velocities of the various components and the ribonuclease sensitivity of the preparations were very close to those obtained in buffer P. Sucrose gradient

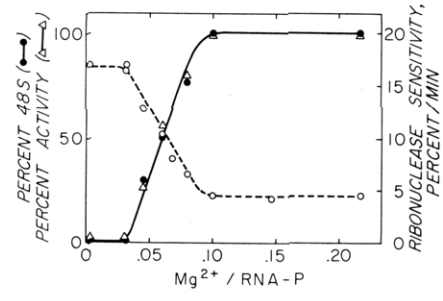


FIGURE 4: The structural and functional properties of spermidine 50S subunits after restoration of Mg^{2+}. Solutions of 50S subunits were dialyzed to equilibrium at 3° against buffer S supplemented with varying amounts of $MgCl_2$ and the Mg^{2+}/RNA-P determined. Each sample was then dialyzed into buffer TK6 for 24 hr at 3°. After this time, per cent activity, sedimentation behavior and sensitivity to pancreatic ribonuclease were determined as previously described (Weiss and Morris, 1973). The per cent 48S species was estimated by calculating the areas under the 40S and 48S schlieren peaks with the aid of a duPont curve analyzer. No corrections were made for radial dilution.

TABLE II: Summary of Physical Properties of the 50S Subunit after Mg^{2+} Replacement by Polyamines.

Buffer[a]	Mg^{2+} [b]/RNA-P	% Act.	s_{20} (S)	RNase Sensitivity (%/min)	Viscosity	
					mg/ml	η Red (cm^3/g)
S	0.15	100	49.2	3.0	0.88	6.8
S	0.05	<2	45.1	7.0	0.85	7.9
S	0.001	<2	42.1	10.7	2.05	9.8
S	0.001	<2			1.10	9.6
S → TK6	(0.15)	100	47.8	4.0	0.97	6.8
S → TK6	(0.001)	<2	39.4	17.0	1.95	15.0
S → TK6	(0.001)	<2			0.98	13.5
P	0.15	100	46.9	5.0	1.04	7.0
P	0.05	<2	30.5, 20.4	33.0	0.98	17.5
P	0.004	<2	31.2, 21.1	33.0	1.91	19.1
P	0.004	<2			1.02	16.7
P → TK1	(0.15)	100	47.2	4.0	1.00	6.5
P → TK1	(0.004)	<2	31.6, 21.7	26.0	1.92	16.7
P → TK1	(0.004)	<2			0.88	16.1

[a] Abbreviations used are: S → TK6, dialysis of particles from buffer S into buffer TK6; P → TK1, dialysis of particles from buffer P into buffer TK1. [b] Numbers in parentheses indicate Mg^{2+}/RNA-P of preparation before dialysis into buffer TK6 or buffer TK1.

analysis demonstrated that the 30S and 20S species were totally inactive. Unfortunately, the 47S and 42S components could not be resolved sufficiently to assess the functional state of the 42S particle.

To confirm that the observed structural changes of the 50S subunit in polyamine buffers were a true reflection of a change in shape of these particles, reduced viscosities were determined. The results of viscosity studies are provided in Table II along with a summary of other relevant data reflecting conformational alterations of the 50S subunit. It can be seen that replacement of Mg^{2+} by spermidine and purtrescine resulted in increases in the reduced viscosity of 40 and 120%, respectively. Values obtained for solutions of control 50S subunits (100% active) were essentially identical with those independently found by Hill *et al.* (1970). When Mg^{2+} was restored (S → TK6 or P → TK1 in Table II) by dialysis into Mg^{2+}-containing buffers, the reduced viscosities remained higher than comparable controls. These viscosity data were consistent with the results obtained for ribonuclease sensitivity and sedimentation behavior; when viscosity increased, ribonuclease sensitivity increased and sedimentation coefficient decreased.

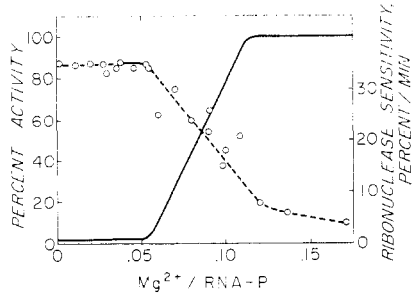


FIGURE 5: Sensitivity of the 50S subunit to pancreatic ribonuclease after replacement of Mg^{2+} by putrescine. Solutions of 50S subunits were dialyzed for 12 hr at 3° against buffer P containing 0–2.0 mM $MgCl_2$. At the end of this time, each sample was measured for Mg^{2+}/RNA-P and sensitivity to ribonuclease (O) as previously described (Weiss and Morris, 1973). The per cent activity (——) was taken from Figure 1.

Chemical Composition. By analogy to the work of others (Siddiqui and Hosokawa, 1968; Morell and Marmer, 1968) it was considered a strong possibility that replacement of Mg^{2+} by spermidine or putrescine might involve a loss of the 5S RNA. 50S subunits, dialyzed against buffer TK10, were compared to subunits which had their ionically bound magnesium totally removed by dialysis against buffers S and P. The particles were removed from solution by ultracentrifugation and the supernatant fractions saved for analysis. The ribonucleoprotein pellets were extracted with LiCl. After concentration, equivalent amounts of these two samples were analyzed for 5S RNA by electrophoresis in polyacrylamide gels (Figure 6). It can be seen that 100% of the 5S RNA in the case of spermidine and greater than 80% with putrescine remained in the pellet fraction and did not appear in the supernatant solution. Thus, replacement of Mg^{2+} by polyamines did not result in a significant loss of 5S RNA.

We have also investigated the possible degradation of the 23S RNA during removal of Mg^{2+}. RNA was prepared from untreated subunits and from magnesium-free particles which had been dialyzed for 24 hr against several changes of buffers S and P. These preparations were indistinguishable when analyzed for material absorbing at 260 nm after sucrose gradient centrifugation. Since it was possible that a break in one strand of a double-helical region in the rRNA might not be detected by the above analysis, rRNA preparations were also incubated for 15 min at 65° in 0.1 M sodium phosphate buffer (pH 7.7) and 1.1 M formaldehyde and then analyzed on sucrose gradients in the same buffer. This procedure effectively converts structured polynucleotides into random coils and the 23S and 16S RNAs sediment as 14S and 11S species (Boedtker, 1968). Analysis of the 23S RNA isolated from totally inactive putrescine and spermidine particles or totally active control particles showed identical distributions of ultraviolet-absorbing material.

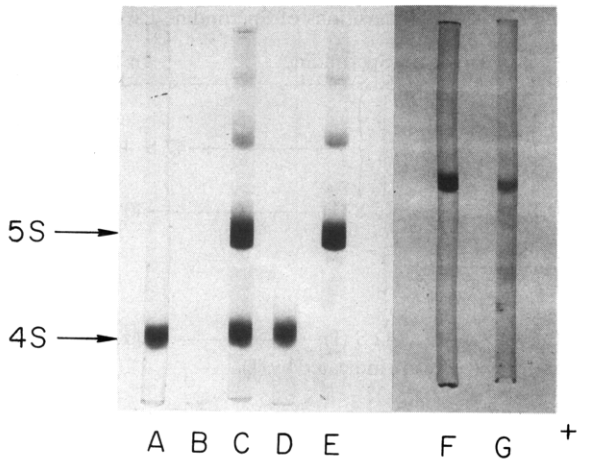


FIGURE 6: Location of 5S RNA after replacement of Mg^{2+} by polyamines. Solutions of 50S subunits were dialyzed for 20 hr at 3° against several changes of buffer TK10, buffer S or buffer P. After dialysis, each preparation was divided into supernatant and pellet fractions and each was analyzed for 5S RNA by acrylamide gel electrophoresis (see Methods). The patterns shown are as follows: (A) 15 μg of purified $tRNA_{fMet}$, 4S RNA; (B) TK10 supernatant fraction; (C) TK10 pellet fraction plus 4S RNA; (D) S supernatant fraction plus 4S RNA; (E) S pellet fraction; (F) P pellet fraction; (G) P supernatant fraction. The direction of electrophoresis is from − to + terminals.

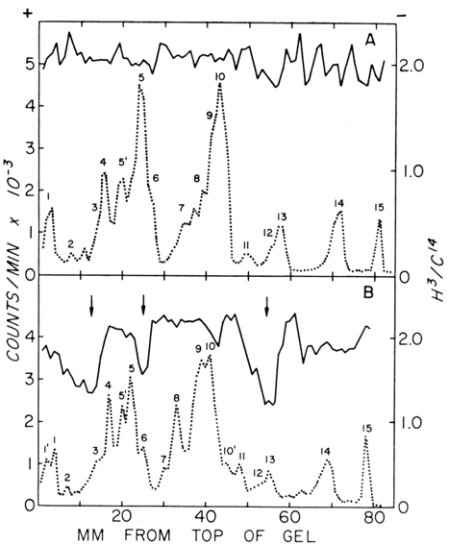


FIGURE 7: Comparison of total ribosomal proteins from active and inactive 50S subunits by polyacrylamide gel electrophoresis. 50S subunits labeled with [^{14}C]lysine and [^{3}H]lysine were dialyzed against buffer S supplemented with varying amounts of $MgCl_2$. In part A the proteins from ^{14}C-labeled active subunits (Mg^{2+}/RNA-P = 0.10) are compared to the proteins from ^{3}H-labeled inactive subunits (Mg^{2+}/RNA-P = 0.05). In part B the proteins from ^{14}C-labeled inactive subunits (Mg^{2+}/RNA-P = 0.05) are compared to ^{3}H-labeled inactive subunits (Mg^{2+}/RNA-P = <0.01). The dotted line represents the ^{3}H counts per minute and the solid line is the ^{3}H/^{14}C ratio. Direction of electrophoresis is from + to − terminals. For details, see Methods.

It was important to test for the possible loss of ribosomal proteins. Subunits labeled with either [^{14}C]- or [^{3}H]lysine were prepared at two different stages of magnesium depletion by dialysis against spermidine-containing buffers. All 50S ribosomal proteins contain significant amounts of this amino acid (Kaltschmidt *et al.*, 1970). The dialyzed particles were centrifuged to the middle of sucrose gradients to remove released proteins and particles from two separate gradients were mixed and protein extracted. The relative levels of the various protein components in these preparations were compared by monitoring the ^{3}H/^{14}C ratio after disc gel electrophoresis. It can be seen from Figure 7A that there was no apparent loss of protein during the inactivation phase of Mg^{2+} replacement (Mg^{2+}/RNA-P between 0.10 and 0.05). However, on further replacement of Mg^{2+} (Mg^{2+}/RNA-P less than 0.05) proteins were lost in regions 3, 6, and 12–13 of the electrophoretic gels (Figure 7B). Identical results were obtained if the ^{3}H and ^{14}C labels were reversed. Furthermore, when similar experiments were conducted with radioactive 30S subunits, no change in the ^{3}H/^{14}C ratio was observed, supporting the conclusions of the previous paper that 30S subunits do not lose any proteins after total exchange of polyamines for Mg^{2+} (Weiss and Morris, 1973). We confirmed the loss of proteins from both spermidine- and putrescine-dialyzed particles by electrophoresis of proteins in the supernatant fraction after removal of the ribonucleoprotein particles by centrifugation. These studies clearly showed the appearance in the supernatant of multiple bands near region 3 and 6 of the electrophoretic pattern. When Mg^{2+} was restored to these polyamine particles, the supernatant proteins disappeared. Thus, it appears that raising the Mg^{2+}/RNA-P to high values induces the reabsorption of these proteins to the ribonucleoprotein complex.

It was possible that those proteins which were lost on complete replacement of bound Mg^{2+} with polyamines were not truly of ribosomal origin. To test this possibility, 50S subunits were further purified by methods which are known to remove absorbed soluble proteins and membrane fractions which often copurify during conventional isolation procedures (Siekewitz, 1962; Kurland, 1966). When 50S subunits were washed with high concentrations of salt (1 M NH_4Cl), or treated with 0.2% deoxycholate, and then centrifuged through 50% sucrose, the pellet fractions were 60–100% active. After complete exchange of spermidine for Mg^{2+}, the supernatant fractions from these purified particles still showed the presence of stained bands near regions 3 and 6 and sometimes in region 12–13. Thus, there is reason to have confidence that at least some of the proteins lost from the 50S subunit upon complete Mg^{2+} replacement were of ribosomal origin. It should be emphasized that with both putrescine and spermidine, proteins were released only after inactivation had already occurred. Therefore, this protein loss was not a component of the inactivation process.

Instability of 50S Subunits in Polyamine Buffers. The experiments that have been described were designed to produce 50S subunits of defined Mg^{2+} content. While this was achieved, it was discovered that partially active preparations, at constant Mg^{2+}/RNA-P, were unstable when observed by both physical and functional measurements. Upon dialysis of 50S subunits against buffers supplemented with different amounts of $MgCl_2$, two phases of inactivation were observed (Figure 8). The first stage occurred very rapidly and appeared to correspond to the time during which Mg^{2+} was equilibrating across the dialysis membrane. However, once equilibrium was achieved, a slow, apparently first-order decay occurred which was characteristic of the Mg^{2+}/RNA-P of the preparation. The stability of the particles clearly decreased with lower Mg^{2+}/RNA-P (Figure 8).

The previous paper in this series showed that 30S par-

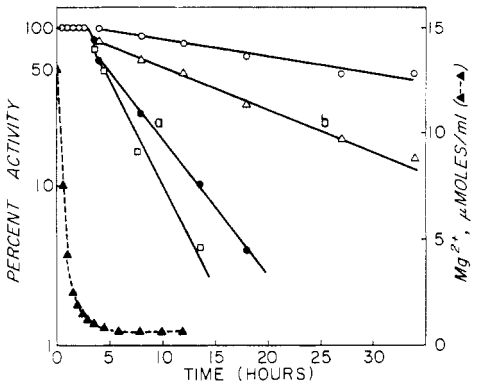


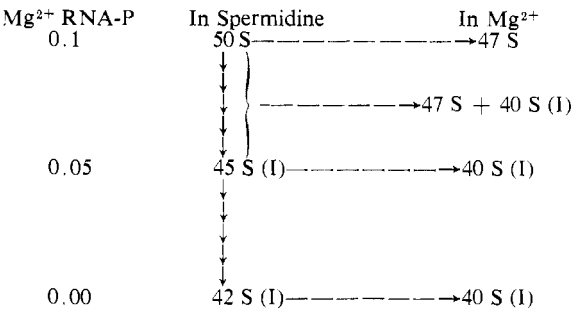
FIGURE 8: Decay of polymerizing activity during the dialysis of 50S subunits against buffer S. Solutions of 50S subunits (4.0 mg/ml) in buffer TK10 were dialyzed at 3° against buffer S supplemented with 0.6 mM $MgCl_2$ (○); 0.5 mM $MgCl_2$ (△); 0.4 mM $MgCl_2$ (●); 0.35 mM $MgCl_2$ (□). At various times during dialysis, small samples were removed from the dialysis bags and their polymerizing activity was measured (see Methods). Another solution of 50S subunits in buffer TK10 was dialyzed against buffer S and the concentration of Mg^{2+} inside the dialysis bag measured as a function of time (▲). The Mg^{2+}/RNA-P and half-life of polymerizing activity for each preparation were as follows: (○) Mg^{2+}/RNA-P = 0.080, $t_{1/2}$ = 28 hr; (△) Mg^{2+}/RNA-P = 0.067, $t_{1/2}$ = 9.5 hr; (●) Mg^{2+}RNA-P = 0.057, $t_{1/2}$ = 3.5 hr; (□) Mg^{2+}/RNA-P = 0.050, $t_{1/2}$ = 2.0 hr. Points a and b represent isoactivity points at different values of Mg^{2+}/RNA-P.

ticles exhibited a similar instability. It was suggested that particles which decayed to low activity at a relatively high Mg^{2+}/RNA-P were structurally similar to particles of equivalent activity but at low Mg^{2+}/RNA-P arrived at by short periods of equilibrium dialysis (Weiss and Morris, 1973). Identical results could be obtained for 50S subunits and it could be clearly shown by the criteria of ribonuclease sensitivity and sedimentation behavior that a particle at point a in Figure 8 was indistinguishable from a particle at point b. When dialysis was performed against spermidine-containing buffers, particles at points a and b in Figure 8 not only showed identical sensitivities to ribonuclease but also showed identical percentages of 48S and 40S species when Mg^{2+} was restored to each preparation by dialysis against buffer TK6. It would appear that the final particles produced by Mg^{2+} replacement of spermidine and by the instability process were indistinguishable by both structural and functional criteria, despite the fact that during decay the ionically bound Mg^{2+} remained constant.

Discussion

The studies reported here indicate that, at least qualitatively, the conclusions reached in the previous paper with regard to the interaction of polyamines and magnesium ion with the 30S ribosomal subunit (Weiss and Morris, 1973) also apply to the 50S particle. The basis of these conclusions has been discussed extensively elsewhere (Weiss and Morris, 1970, 1973) and the important facets, as they apply to the 50S subunit, will be listed only briefly here. Polyamines compete effectively for Mg^{2+} binding sites on the 50S subunit and replacement of Mg^{2+} by spermidine is stoichiometric and by putrescine nearly so. Therefore, the degree of binding of polyvalent cations to rRNA phosphate groups is essentially constant throughout the experiments described here. As was the case with the 30S subunit, there is a critical level of

SCHEME I: Structural Transitions of Spermidine Particles[a]



[a] Inactive particles are indicated by (I).

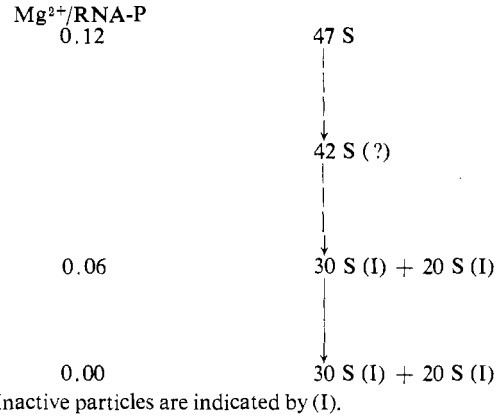
bound Mg^{2+} which cannot be replaced by polyamines without loss of activity and extensive structural changes in the particles. The critical level of Mg^{2+} amounts to 18–24% of the total available sites, and depends on whether spermidine or putrescine is used. This range of values is exceedingly close to that obtained for the 30S subunit (Weiss and Morris, 1973). The structural changes leading to inactivation are not due to loss or detectable modification of the protein and RNA components of the particle. Unfortunately this conclusion could not be confirmed by reconstitution, as was possible in the case of the 30S subunit. The conformational alterations in the 50S particles were much more extensive when Mg^{2+} was replaced by putrescine than with spermidine. This was also true with the 30S subunit and was used to argue that the changes observed were due to a structural property of the polyamines which rendered them unable to interact in an appropriate manner with the ribosome at approximately 20% of the total potential cation binding sites. Thus, the cation requirements for the maintenance of structurally and functionally intact particles are very similar for both the 30S and 50S ribosomal subunits. However, there is one distinct difference which resides in the quantitative dependence on Mg^{2+}/RNA-P of the structural change leading to inactivation. With the 50S subunit, this change occurs over approximately a twofold replacement of bound Mg^{2+} with polyamines. On the other hand, activity loss with the 30S subunit begins at a critical level of bound Mg^{2+} and continues gradually to the point where all of the Mg^{2+} has been replaced. This result could indicate that the interactions between the components of the 50S subunit are stronger and more cooperative than is found in the 30S particle.

The structural changes which the 50S subunit is observed to undergo during replacement of magnesium ion with spermidine are summarized in Scheme I. From the point where loss of activity begins (Mg^{2+}/RNA-P = 0.1) there is a continual decrease in sedimentation coefficient which proceeds through the point of full inactivation (Mg^{2+}/RNA-P = 0.05) to a sedimentation coefficient of 42S at full magnesium replacement. Paralleling this decrease in sedimentation coefficient is a continual three- to fourfold increase in sensitivity to ribonuclease. Additional information as to the mechanism of this structural transition is gained on returning the particles to a buffer containing magnesium ion. Partially active preparations are seen to consist of a mixture of fully active 47S particles and a totally inactive 40S component. Therefore, the continuum of structural states observed in the spermidine-containing buffer during inactivation must consist of either a mixture of inactive and active particles of very similar sedimentation behavior or, alternatively a homogeneous popu-

lation of particles which can follow two routes of refolding upon returning magnesium ion.

The structural changes observed on replacing magnesium ion with putrescine are in distinct contrast to the situation with spermidine. Instead of the apparent progression of conformational states observed with spermidine, the putrescine particles show a discrete structural change coincident with the loss of activity (Scheme II). The resultant 30S and

SCHEME II: Structural Transitions of Putrescine Particles[a]



[a] Inactive particles are indicated by (I).

20S particles are considerably more unfolded than the spermine particles, as indicated not only by sedimentation coefficient but also by ribonuclease sensitivity and by reduced viscosity. Upon returning magnesium to the putrescine particles, there is very little observable change in their structure. This is in clear contrast to the spermidine particles. The nature of the 42S species observed in partially active preparations is not clear. It may represent an intermediate in the unfolding process or it may be an alternate molecular form of the active 47S particle.

It is apparent from the studies reported here, as well as those of others, that the ribosomal particles can exist in a variety of stable or quasi-stable conformations, depending on the type of treatment they have received. Meaningful comparison of the various unfolded species with one another and with the native forms requires careful studies of the interconvertibility of the different particles and also awaits more sensitive probes of the molecular interactions within the ribosomal particle. However, it is interesting to note that in contrast to other types of unfolded 50S subunits, the spermidine particles and even the extensively unfolded putrescine particles still largely retain their 5S RNA. Removal of Mg^{2+} from the 50S particle through treatment with EDTA or high salt, results in the loss of 5S RNA concomitant with unfolding of the particle. For example, Morell and Marmer (1968) showed that on treatment of 50S particles with high concentrations of cesium chloride, the 5S RNA could only be retained in the presence of moderate concentrations of Mg^{2+}. The present studies demonstrate that Mg^{2+} is not unique in stabilizing the interaction between 5S RNA and the 50S particle. However, although the 5S RNA is clearly associated with the unfolded polyamine particles, it is known that this RNA species can exist in more than one molecular configuration (Aubert *et al.*, 1968). It is conceivable that at least one facet of polyamine inactivation of the 50S particle is a change in conformation of the 5S RNA. Furthermore, despite the fact that inactivation does not appear to involve the loss of 50S ribosomal proteins, it is possible that during the exchange of polyamines for Mg^{2+}, some proteins redistribute to new positions as suggested by Traub and Nomura (1969) for EDTA-treated 30S subunits. If these proteins formed stable, but incorrect, interactions in the unfolded ribonucleoprotein complexes, then restoration of Mg^{2+} by simple dialysis might not be sufficient for redistribution to the native structure.

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