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Cations and Ribosome Structure. I. Effects on the 30S Subunit of Substituting Polyamines for Magnesium Ion†

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ABSTRACT: The polyamines putrescine ($NH_3^+(CH_2)_4NH_2^+$) and spermidine ($NH_3^+(CH_2)_3NH_2^+(CH_2)_4NH_3^+$) have been used to replace the Mg^{2+} normally associated with the 30S ribosomal subunit of *Escherichia coli*. By means of equilibrium dialysis, 30S particles, purified by sucrose gradient centrifugation, have been prepared with various levels of bound Mg^{2+}. Stoichiometric replacement of Mg^{2+} by either polyamine had no effect on the ability of the particles to synthesize polyphenylalanine as long as the level of bound Mg^{2+} was above a critical level (Mg^{2+}/RNA-P = 0.115). Further replacement resulted in gradual loss of activity until none remained when only polyamines were bound to the particles. This inactivation was accompanied by a concomitant conformational alteration in the particles which resulted in an increase in their sensitivity to attack by ribonuclease, a decrease in their sedimentation coefficients, and an increase in their reduced viscosities. The inactive preparations sedimented as a single boundary in the ultracentrifuge. Putrescine appeared to be somewhat less effective than spermidine in maintaining ribosome structure and function. Neither native structure nor function could be restored by dialysis against buffers containing Mg^{2+}. However, inactive particles, after isolation from sucrose gradients, could be fully reactivated by a brief treatment at 40° under specific ionic conditions, implying that inactivation was reversible and did not involve covalent modification or loss of essential material from the particles. These results suggest that a critical level of bound Mg^{2+} is necessary for the maintenance of the structure and function of the 30S ribosomal subunit.

Magnesium ion (Mg^{2+}) is a required growth factor for all cells. This ion is important for the activity of many enzymes (Wacker, 1969) and also appears to be necessary for the stability of ribosomes both *in vitro* and *in vivo*. McCarthy (1962) demonstrated that cells of *Escherichia coli*, when starved for Mg^{2+}, fail to maintain intact ribosomes. Tempest and his coworkers, while studying bacteria whose growth was limited by magnesium ion, demonstrated a definite stoichiometry between cellular Mg^{2+} and RNA which was independent of growth rate (Tempest and Strange, 1966; Tempest and Meers, 1968). This observation supported the hypothesis of a required interaction between Mg^{2+} and RNA. The removal of ribosomally associated Mg^{2+} *in vitro*, either through the use of chelating agents (Weller and Horowitz, 1964; Cammack and Wade, 1965; Gesteland, 1966a; Weller *et al.*, 1968; Miall and Walker, 1969; Eilam and Elson, 1971) or by displacement with high concentrations of monovalent cations (Elson, 1961; Spirin *et al.*, 1963; Maruta *et al.*, 1969; Spitnik-Elson and Atsmon, 1969; Ghysen *et al.*, 1970), results in disruption of the native structure. These observations suggest that one of the primary roles of intracellular Mg^{2+} is in the maintenance of ribosome structure and function.

The changes in ribosome structure observed upon the removal of magnesium ion have been interpreted as the formation of more asymmetric and distended structures. This interpretation has been based primarily on studies of the hydrodynamic properties of the resulting particles. Although the exact nature of the derived particles appears to be a complex function of ionic strength and magnesium levels, it seems from the above-mentioned studies that a number of intermediate conformational changes can exist during the unfolding of these particles. These structural changes appear to take place with no loss of ribosomal protein. During the early stages of unfolding, there is very little disruption of the structure of the rRNA as detected by optical means (Miall and Walker, 1969; Eilam and Elson, 1971). However, in the more highly unfolded particles, it is clear that there is extensive disruption of base pairing (Eilam and Elson, 1971). These changes in ribosome structure on removal of Mg^{2+} have been interpreted as resulting from electrostatic repulsion,

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the implication being that bound multivalent cations are necessary for effective shielding of the RNA phosphates in the ribosome. In general, these changes in the tertiary structure of the ribosome cannot be reversed when Mg^{2+} is restored by dialysis. However, readdition of Mg^{2+} to EDTA-treated 30S subunits at high ionic strength and high temperature can return this particle to its original compact, active form (Traub and Nomura, 1969; Eilam and Elson, 1971).

The polyamines, putrescine ($NH_3^+(CH_2)_4NH_3^-$) and spermidine ($NH_3^+(CH_2)_3NH_2^+(CH_2)_4NH_3^+$), have been shown to bind electrostatically to nucleic acids in a manner similar to Mg^{2+} (Felsenfeld and Huang, 1961; Hirschman *et al.*, 1967; Choi and Carr, 1967). The ability of polyamines to partially replace Mg^{2+} in promoting the aggregation of ribosomal subunits (Cohen and Lichtenstein, 1960; Silman *et al.*, 1965; Moller and Kim, 1965; Norton *et al.*, 1968; Colbourn *et al.*, 1971) is probably a reflection of this binding. Polyamines will also replace Mg^{2+}, at least partially, in some of the processes in protein synthesis (Nathans and Lipmann, 1961; Martin and Ames, 1962; Takeda, 1969; Takeda and Igarshi, 1969). However, Moller and Kim (1965) and a previous report from this laboratory (Weiss and Morris, 1970) suggested that only part of the Mg^{2+} requirement for the maintenance of ribosome structure and function could be replaced by polyamines. The remainder of this requirement appeared to be specific for Mg^{2+} or possibly other closely related ions.

This paper will demonstrate that a distinct level of bound Mg^{2+} is required for the maintenance of functionally active 30S ribosomal subunits. Polyamines are unable to replace this critical Mg^{2+} without structural and functional alterations of the 30S ribonucleoprotein particle.

Methods

Isolation of Ribosomal Subunits and Supernatant Factors. *E. coli* A19, a strain deficient in ribonuclease I (Gesteland, 1966b), was used in these studies. Bacterial growth and isolation of 70S ribosomes and supernatant factors (S150) were performed as previously described (Weiss and Morris, 1970). For isolation of ribosomal subunits, a sample of 70S ribosomes (300–450 mg) was dialyzed for 16 hr against two changes of 4 l. each, of buffer B-3 [10 mM Tris-HCl (pH 7.5), 60 mM KCl, 1 mM magnesium acetate, and 6 mM 2-mercaptoethanol]. Following centrifugation at 30,000*g* for 30 min, the ribosome solution was diluted to 3 mg/ml by addition of buffer B-3 (assuming A_{260} of 15 to be equivalent to 1.0 mg/ml). This was layered on a 1500-ml radially linear 5–20% sucrose gradient (in buffer B-3) constructed over a 100-ml cushion of 35% sucrose (in buffer B-3) in a BIV zonal rotor (Spinco). Centrifugation was carried out for 7 hr at 40,000 rpm in a Spinco Model L4 ultracentrifuge. The gradient was then removed from the rotor by displacement with a 35% sucrose solution, and 25-ml fractions were collected. The fractions containing 30S and 50S subunits were detected by their absorbance at 260 nm and pooled, attempting to minimize cross-contamination.

Pooled subunit fractions were immediately supplemented with $MgCl_2$ to a final concentration of 10 mM, and concentrated by centrifugation at 80,000*g* for 20 hr. Subunit pellets were resuspended in buffer B (identical with buffer B-3 except containing 10 mM magnesium acetate) and clarified by centrifugation at 30,000*g* for 30 min. The subunit suspensions were then dialyzed overnight against buffer B and stored at −70°. In some cases this last dialysis was omitted with no discernible effect on the subunit preparations.

Analytical ultracentrifugation and phenylalanine polymerization activity detected less than 1% cross-contamination in the 30S preparation and less than 4% in the 50S preparation. Yields were consistently 50–70% for 30S subunits and 60–80% for 50S subunits. The phenylalanine polymerizing activity of the purified subunits was >80% of that of the original 70S ribosomes (33 pmol of phenylalanine/min per mg of 70S ribosomes).

Equilibrium Dialysis. Suspensions of 30S ribosomal subunits were diluted to 4–5 mg/ml with buffer B. Samples were then placed in EDTA-treated dialysis tubing (Weiss and Morris, 1970) attached to a Crowe–Englander Micro-Dialyzer (Hoefer Scientific Instruments). The dialyzer was then rotated rapidly at 4° in a 500-ml graduated cylinder containing 500 ml of the appropriate buffer [TS: 10 mM Tris-HCl (pH 7.5), 60 mM KCl, 1 mM spermidine–3HCl, and 6 mM 2-mercaptoethanol; TP: 10 mM Tris-HCl (pH 7.5), 4 mM putrescine–2HCl, and 6 mM 2-mercaptoethanol]. Desired levels of bound Mg^{2+} were obtained by supplementing these buffers with defined amounts of $MgCl_2$. After various times of dialysis, samples could easily be removed for analysis, but in most cases the dialysis time was 12 hr (see Results).

Cation Determinations. Magnesium ion was estimated with a Perkin-Elmer Model 303 atomic absorption spectrophotometer. Samples were prepared by dilution with distilled water and were burned directly without deproteinization. Control experiments demonstrated that polyamines did not interfere with the determination and that values observed by direct burning were identical with those obtained by first extracting the magnesium from the ribosomes with trichloroacetic acid. This latter procedure is known to extract all the bound Mg^{2+} (Edelman *et al.*, 1960). Polyamines were estimated with a modified Technicon AutoAnalyzer as previously described (Morris *et al.*, 1969; Morris, 1971). This procedure showed that the polyamine-containing solutions had less than 2 mol % cross-contamination by other polyamines.

The level of bound cations after equilibrium dialysis was estimated by subtraction of the free cation concentration, obtained by analysis of a sample of the dialyzing buffer, from the cation concentration in a sample of the subunit solution. Possible artifacts due to the Gibbs–Donnan effect (Donnan, 1911) were ruled out since the calculated value of Mg^{2+}/RNA-P was independent of ribosome concentration below 5 mg/ml.

Protein Synthesis. Phenylalanine polymerizing activity was measured using a 0.1-ml reaction mixture containing 0.1 M Tris-HCl (pH 7.8), 16 mM magnesium acetate, 0.1 M NH_4Cl, 1 mM ATP, 5 mM phosphoenolpyruvate, 0.05 mM GTP, 3.2 μg of pyruvate kinase (Calbiochem), 15 mM 2-mercaptoethanol, 0.065 A_{260} unit of S150, 4 μg of poly(U) (Miles Laboratories), 20 μg of *E. coli* K12 tRNA, 1.3 μM L-[^{14}C]phenylalanine (uniformly labeled) of specific activity 369 Ci/mol, 30 μg of 50S subunits, and 10–15 μg of 30S subunits. The reaction was initiated by addition of S150. After incubation for various lengths of time at 25°, trichloroacetic acid insoluble radioactivity was determined in aliquots of the reaction mixture using the method of Mans and Novelli (1960). The radioactive papers were placed in 5 ml of 2,5-diphenyloxazole–toluene scintillation fluid [5 g of 2,5-diphenyloxazole (New England Nuclear) in 1 l. of toluene] and counted using a Beckman CPM-100 liquid scintillation counter. Rates of protein synthesis were calculated from the linear portion of time courses of incorporation and expressed as counts per minute of [^{14}C]phenylalanine incorporated per minute per microgram of 30S subunits.

TABLE I: Stoichiometry of Magnesium Replacement.[a]

[Mg^{2+}][b]	Mg^{2+}/ RNA-P	Put^{2+}/ RNA-P[c]	Spd^{3+}/ RNA-P[c]	Total +/ RNA-P[c]
0.002	0.00	0.34		0.68
0.05	0.03	0.33		0.72
0.13	0.07	0.27		0.68
0.41	0.13	0.17		0.60
1.01	0.21	0.13		0.68
0.007	0.01		0.20	0.62
0.12	0.03		0.19	0.63
0.49	0.07		0.15	0.59
1.48	0.14		0.12	0.64
3.91	0.21		0.06	0.60

[a] See Methods for experimental details. [b] Concentration (mM) of $MgCl_2$ in polyamine buffer. [c] Abbreviations used are: Put^{2+}, putrescine; Spd^{3+}, spermidine; total +, total charge of bound polyvalent cations.

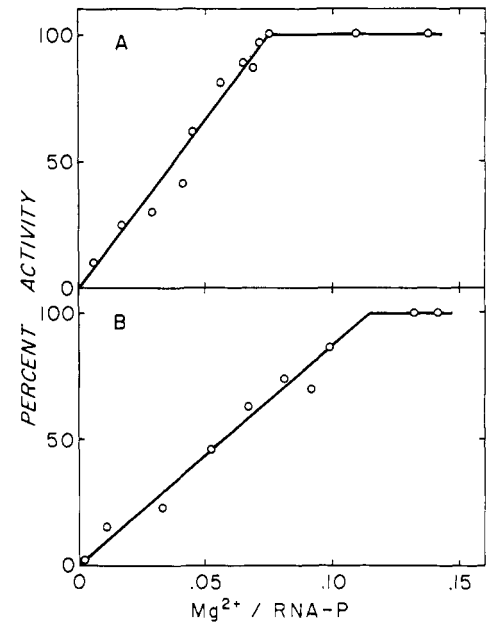

FIGURE 1: Inactivation of 30S subunits as a result of Mg^{2+} replacement by (A) spermidine and (B) putrescine. Preparation of particles, determination of bound Mg^{2+}, and measurement of phenylalanine polymerizing activity were performed as described in Methods. In each case, the activity was compared to that observed with particles which were kept at 4° in buffer B during the course of dialysis.

Physical Measurements. Sedimentation coefficients were determined using an An-D rotor in a Spinco Model E analytical ultracentrifuge equipped with Schlieren optics. The concentration of ribosomal subunits was 2.0 mg/ml. The bar angle was 40° and the temperature was maintained at 20 ± 0.1°. Pictures were taken at various intervals after reaching speed (42,040 rpm). Photographic plates were read using a microcomparator and sedimentation coefficients were calculated as described by Chervenka (1969). Maximum range of values on identical samples was ±0.5 S.

Viscosity was determined using an Ostwald capillary viscometer as previously described (Weiss and Morris, 1970). The concentration of ribosomal subunits was varied between 0.5 and 2.0 mg per ml to detect any large concentration dependence. The reduced viscosity was calculated as described by Schachman (1957). Values obtained within the above concentration range agreed to ±0.5 ml/g.

rRNA. RNA was prepared from 30S particles by the method of Stanley and Bock (1965), except that all extractions were done at room temperature. Solutions of isolated RNA were extensively dialyzed at 4° against 0.05 M Tris-HCl (pH 7.0) containing 0.1 M KCl. These RNA preparations were examined by analytical ultracentrifugation.

Ribonuclease Sensitivity. Preparations of ribosomal particles in the various buffers were diluted to 1.0 mg/ml, and 0.3-ml samples were then incubated at 37° for 2 min with 0.3 μg of pancreatic ribonuclease (Worthington). At this time a 0.2-ml aliquot was removed and immediately added to 2.0 ml of cold 0.25 N $HClO_4$. Following centrifugation at 10,000*g* for 30 min, the resulting supernatant solution was analyzed for material absorbing at 260 nm. Solubilization of 260-nm-absorbing material by this procedure was linear for all ribosome preparations for at least 2 min. Total A_{260} of the preparation was defined as that released by heating a 0.2-ml sample of the diluted ribosome solution at 70° for 30 min in 2.0 ml of 0.5 N $HClO_4$ and analyzing as above.

Reactivation of 30S Subunits. Aliquots (0.2 ml) of inactive particle preparations were layered on 10–30% sucrose gradients (4.8 ml) in the appropriate buffer and centrifuged for 3 hr at 48,000 rpm in a Spinco SW 50.1 rotor. Fractions were collected, and those containing the inactive particles were pooled and treated similarly to the procedure of Traub and Nomura (1969). Pooled fractions were dialyzed for 16 hr against two changes of standard buffer (5 mM H_3PO_4, brought to pH 7.4 with KOH, 20 mM $MgCl_2$, 0.286 M KCl, and 6 mM 2-mercaptoethanol). An aliquot was assayed for phenylalanine polymerizing activity and the remainder was heated to 40° for 25 min. A portion of the heated sample was then assayed for polymerizing activity while the remaining solution was concentrated by ultracentrifugation, suspended in buffer B, and then assayed. In all cases, the observed activity was compared to control particles which had received identical treatment except for dialysis against buffers containing polyamines.

Results

Stoichiometry of Mg^{2+} Replacement by Polyamines. Dialysis of 30S ribosomal subunits against either buffer TS (spermidine) or TP (putrescine) resulted in replacement of the ribosomally bound Mg^{2+} by the respective polyamine. Various degrees of replacement were achieved by supplementing the dialysis buffer with small amounts of $MgCl_2$. With both putrescine and spermidine, as the level of bound Mg^{2+} decreased, the level of bound polyamine increased (Table I). The ultimate result was the maintenance of a constant amount of bound charge equivalents of polyvalent cations.

Phenylalanine Polymerizing Activity. The ability to polymerize phenylalanine, using poly(U) as message, has been used to assay the functional integrity of 30S subunits during the replacement of Mg^{2+} with polyamines (Figure 1). A significant proportion of the bound Mg^{2+} on the 30S particle could be replaced by polyamines without affecting activity. However, when the bound Mg^{2+} was reduced below a critical level (Mg^{2+}/RNA-P = 0.075 for spermidine and 0.115 for putrescine), there was a gradual loss of activity which extrapolated to complete inactivation at Mg^{2+}/RNA-P = 0.

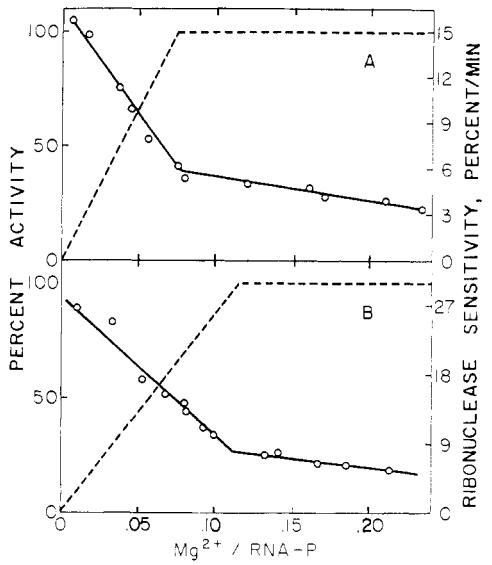

FIGURE 2: Ribonuclease sensitivity of 30S subunits at various stages of Mg^{2+} replacement by (A) spermidine and (B) putrescine. (---) Activity of the particles (Figure 1). Equilibrium dialysis, ribonuclease sensitivities, and calculation of Mg^{2+}/RNA-P were carried out as described in Methods.

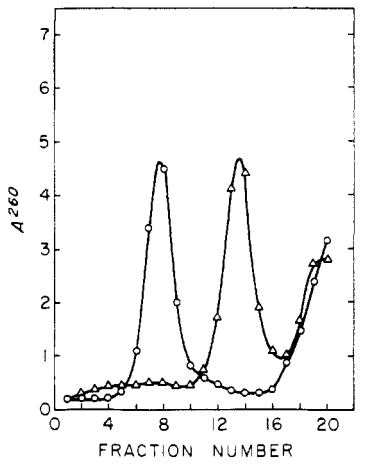

FIGURE 3: Sedimentation behavior of 30S particles in sucrose gradients following treatment with pancreatic ribonuclease. (○) Fully active particles in buffer TS plus 0.6 mM Mg^{2+} (see Methods) following a 30-min incubation at 0° in the presence of pancreatic ribonuclease. (Δ) Particles obtained after 12 hr of dialysis against buffer TS (see Methods) following incubation for 10 min at 0° in the presence of pancreatic ribonuclease. Particles at a concentration of 4 mg/ml were exposed to 1 μg of pancreatic ribonuclease/ml. A 0.1-ml sample was then immediately layered on a 4.9-ml sucrose gradient (10–30% in buffer B-3) and centrifuged for 2.5 hr at 48,000 rpm in a SW 50.1 rotor. Fractions were collected dropwise after piercing the bottom of the tube, and the absorbance at 260 nm of each fraction was determined.

The inactivation profiles were not altered by the presence or absence of KCl or by the use of phosphate in place of Tris buffer. The small amount of polyamines added to the assays system with the treated particles (less than 1 μmol/ml) was never observed to have any effect on the magnesium optimum for polymerization (Martin and Ames, 1962; Takeda, 1969). Thus, the observed inactivation appears to be a direct consequence of displacing ribosomally bound Mg^{2+} with polyamines.

Attempts were made to reverse the inactivation by dialysis against buffers containing K^+ and Mg^{2+} (buffer B or B-3). This procedure resulted in restoration of the original level of bound Mg^{2+} and concomitant removal of more than 95% of the bound polyamines. No change in the activity of the particles was observed even following preincubation at elevated temperatures (25–40°). The insensitivity of the polyamine-induced inactivation to the absence of K^+, and the inability of Mg^{2+} restoration to effect reactivation would seem to distinguish this process from the reversible alterations described by Zamir *et al.* (1971). Alterations such as those observed by these workers would have been reversed during the polymerization assay. However, incorporation was always observed to be linear for at least 20 min and proceeded without any detectable lag.

TABLE II: Properties of Unfolded 30S Particles.

Property	Control[a]	Polyamine Replacing Mg^{2+}	
		Putrescine	Spermidine
s_{20} (S)	30.5	21.5 (25.5)[b]	27.5 (29.5)
RNase sensitivity (%/min)	8.3	28.2 (18.0)	17.2 (16.2)
η_{sp}/c (ml/g)	8.0	13.8 (11.2)	9.4 (9.9)

[a] Untreated particles in buffer B-3. [b] Numbers in parentheses are the values observed for particles returned to buffer B-3.

Structural Properties. A previous report from this laboratory suggested that inactivation of 70S ribosomes through replacement of Mg^{2+} with polyamines was accompanied by structural changes (Weiss and Morris, 1970). Isolated 30S subunits also undergo structural alterations concomitant with inactivation. Replacement of ribosomally bound Mg^{2+} by either putrescine or spermidine led to particles with a greatly increased susceptibility to attack by pancreatic ribonuclease (Figure 2). A significant increase in ribonuclease sensitivity occurred at a value of Mg^{2+}/RNA-P essentially identical with that at which inactivation began. With both putrescine and spermidine, the ribonuclease sensitivity of the particles was directly related to the degree of inactivation. The slight increase in sensitivity at values of Mg^{2+}/RNA-P greater than that at which inactivation commences may be due either to ionic effects on the activity of the enzyme or to a *reversible* loosening of the subunit structure. It should be noted that replacement of Mg^{2+} with putrescine resulted in particles which were nearly two times more sensitive than those obtained when spermidine was used.

As indicated above, inactivation of the particles could not be reversed by simply returning them to buffer containing Mg^{2+}. When ribonuclease sensitivity was measured before and after dialysis into buffer B-3, no significant differences were observed with any fully functional particles, regardless of the extent of their exposure to polyamines. The sensitivity of inactive particles, on the other hand, was affected by this procedure to differing degrees, depending on the polyamine causing inactivation (Table II). Thus, although return to buffer B-3 caused only a 6% decrease in the ribonuclease sensitivity of particles inactivated by spermidine, the corresponding value for putrescine was 36%.

Figure 3 compares the effect of exposure to low concentra-

tions of pancreatic ribonuclease on the sedimentation behavior of control and spermidine-inactivated particles. The conditions indicated in the figure legend were chosen so that equivalent amounts of 260-nm-absorbing material were solubilized from both types of particles and therefore found at the top of the sucrose gradient. Despite solubilization of approximately 40% of the ultraviolet-absorbing material, both preparations exhibited single, apparently homogeneous peaks. Similar experiments using 30S subunits labeled with [^{3}H]-lysine indicated that both of these ribonuclease-treated species retained more than 90% of their ribosomal proteins. Although Mg^{2+} particles continued to sediment at approximately 30 S after RNase treatment, the spermidine particles were converted to a species of sedimentation coefficient approximately 20S. Ribonuclease digestion of spermidine particles after return to buffer B-3 also resulted in formation of the 20S species. This result suggests that the enzyme was attacking different sites in the spermidine particles or that these particles were more easily disaggregated after ribonuclease treatment.

Sedimentation velocity patterns of the inactive particles (Figure 4) suggest that the structural alteration resulted in a discrete particle. Putrescine particles (Figure 4A) showed one major boundary with a sedimentation coefficient of 21.5 S. When these particles were returned to buffer B-3 by overnight dialysis, a single major peak was observed (Figure 4B) and the observed sedimentation coefficient was changed to 25.5 S. Spermidine particles (Figure 4C) also had a decreased sedimentation coefficient (27.5 S) and a small amount of a faster sedimenting material was also observed. At low ribonucleoprotein concentrations, this latter material was not produced, and determination of the weight-average sedimentation coefficient of the entire particle population (Chervenka, 1969) suggested that this species was a dimer of the inactive particle (Schachman, 1959). When these preparations were dialyzed overnight against buffer B-3 in order to replace ribosomally bound spermidine with Mg^{2+}, a single species was observed with a sedimentation coefficient of 29.5 S (Figure 4D). This value is only slightly different than that observed with untreated particles in buffer B-3 (30.5 S).

When partially active particles were examined in the analytical ultracentrifuge, only a single species was observed throughout the process of inactivation with both putrescine and spermidine. The sedimentation coefficients of partially active putrescine particles were intermediate between those observed for totally inactive particles (21.5 S) and active subunits (30.5 S). For example, particles which exhibited only 65% of the control activity appeared homogeneous in the analytical ultracentrifuge and had a sedimentation coefficient of 27.5 S (Figure 4E). The apparent homogeneity of this peak was unaffected by changing the particle concentration from 0.5 to 4 mg per ml. Because of the smaller change observed when spermidine replaced Mg^{2+}, no reliable correlation could be made between sedimentation coefficient and activity in this case.

When partially active preparations were dialyzed against buffer B-3 the sedimentation behavior of spermidine and putrescine particles was different. As could be anticipated from the behavior of active and inactive spermidine particles (30.5 and 29.5 S), only a single boundary was observed when partially active preparations in buffer B-3 were examined in the analytical ultracentrifuge. With putrescine-inactivated preparations, however, two boundaries were observed which appeared to correspond to the 25.5S and 30.5S species observed with inactive and active particles in buffer B-3 (Figure 4F). The amount of material sedimenting at approximately 30 S appeared to be related to the remaining activity, but accurate determinations were not possible because the Schlieren peaks failed to resolve completely. Unfortunately, sucrose gradient centrifugation under these conditions also failed to resolve these species.

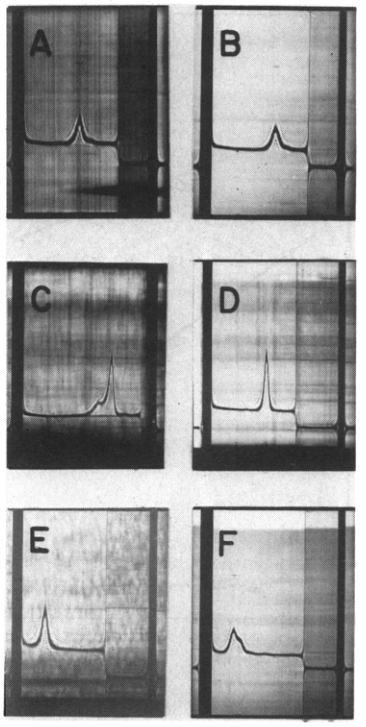

FIGURE 4: Sedimentation patterns of 30S particles following Mg^{2+} replacement by polyamines. (A) Particles in which Mg^{2+} had been totally replaced by putrescine; s_{20} = 21.5 S. (B) Particles from A dialyzed overnight against buffer B-3 (see Methods); s_{20} = 25.5 S. (C) Particles in which Mg^{2+} had been totally replaced by spermidine; s_{20} = 27.5 S. (D) Particles from part C following overnight dialysis against buffer B-3 (see Methods); s_{20} = 29.5 S. (E) Particles in which the Mg^{2+} had been partially replaced by putrescine, such that they exhibit 65% of the control activity; s_{20} = 27.5 S. (F) Particles from E following overnight dialysis against buffer B-3 (see Methods); s_{20} = 30.0 S (major species). Preparations of particles and analytical ultracentrifugation were carried out as described under Methods. Sedimentation is from right to left and pictures were taken 10 min after reaching speed.

The reduced viscosities of inactive particles were measured both in the absence of Mg^{2+} and following return to buffer B-3. The results of these determinations are compared in Table II with other structural characteristics of the particles. Spermidine particles showed approximately a 20% increase in reduced viscosity which was essentially unchanged when the particles were returned to buffer B-3. Putrescine particles showed almost a 75% increase in reduced viscosity. In this case, return to buffer B-3 led to a partial reversal of the viscosity increase. As noted above, this partial reversal with the putrescine particles was also observed in ribonuclease sensitivity and sedimentation coefficient (Table II).

Reactivation of Inactivated Particles. Preparations of inactive particles were obtained by replacement of Mg^{2+} by polyamines. Phenylalanine polymerization showed less than 2% residual activity, and Mg^{2+} analysis indicated that more than 99% of the ribosomally associated Mg^{2+} had been removed. The inactive particles were then isolated by sucrose gradient centrifugation in the appropriate polyamine buffer and treated by a modification of the procedure of Traub

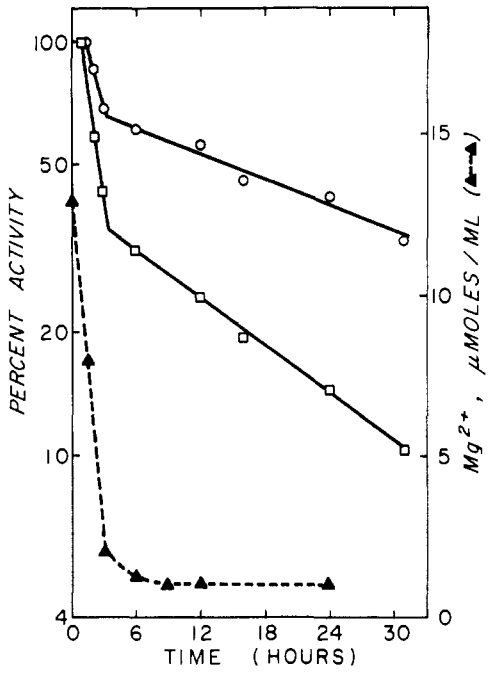

FIGURE 5: Inactivation profile of 30S subunits during and following partial replacement of Mg^{2+} by spermidine. Particles were dialyzed against buffer TS (see Methods) supplemented with 0.4 mM $MgCl_2$ (○; Mg^{2+}/RNA-P at 12 hr = 0.04) and with 0.2 mM $MgCl_2$ (□; Mg^{2+}/RNA-P at 12 hr = 0.02). (▲) Concentration of Mg^{2+} in the ribosome solution during dialysis against buffer TS. Activity determinations and Mg^{2+} analyses were performed as described under Methods and the legend to Figure 1.

and Nomura (1969) as described in Methods. Neither putrescine nor spermidine particles regained activity simply by dialysis into standard reactivation buffer. However, 90–100% activity was regained following heat treatment at 40° for 25 min.

Since the reactivation procedure did not involve separation of the rRNA from the ribosomal protein, nicks in the RNA could have been masked because the RNA fragments were maintained in the ribonucleoprotein matrix. However, if such nicks did occur, the reactivation experiment showed that they could not be the cause of the observed functional and structural changes. In addition, analytical ultracentrifugation of the 16S RNA isolated from polyamine particles and untreated 30S subunits failed to distinguish any differences between these RNA molecules.

Instability of the 30S Subunit as a Result of Mg^{2+} Replacement. Figure 5 shows the phenylalanine polymerizing activity of 30S subunits assayed both during the course of Mg^{2+} replacement by spermidine, and at various times after the establishment of a new value of Mg^{2+}/RNA-P at 8–12 hr. The upper curve illustrates the behavior of particles with a value of Mg^{2+}/RNA-P of 0.04 after 12 hr of dialysis, while the equivalent value for the particles shown in the lower curve was 0.02. Three distinct phases can be seen to occur. During the first hour of dialysis no loss of activity was observed; this probably being a reflection of the time required for equilibration of the polyamine and Mg^{2+} across the dialysis membrane. After this initial equilibration, a period of rapid inactivation ensued, the rate and extent of which apparently depending on the final level of bound Mg^{2+} to which the particles equilibrate. Replacement of Mg^{2+} by spermidine was proceeding rapidly and was more than 90% complete in the period over which rapid inactivation was observed. A slow decay in activity then commenced, the rate of which was also dependent on the value of Mg^{2+}/RNA-P (note half-lives of 16 and 30 hr in Figure 5). Since there was no further change in the value of Mg^{2+}/RNA-P over this period of slow inactivation, it appeared to represent an instability of the partially active preparations. When the ribonuclease sensitivity of putrescine and spermidine particles was followed during this slow decay, it related to the activity of the preparation precisely as described earlier for particles after 12 hr of dialysis (Figure 2). This was also true for the sedimentation coefficient of putrescine particles. Thus, at least by these gross criteria, the structural changes taking place during the two phases of inactivation were similar.

Discussion

These studies support our previous hypothesis that a functionally distinct class of magnesium ions exist on 70S ribosomes from *E. coli* (Weiss and Morris, 1970). These sites must be occupied by Mg^{2+}, or closely related divalent cations (Weiss *et al.*, 1973), for the activity and the native structure of the ribosome to be maintained. Displacement of this critical level of Mg^{2+} by polyamines leads to loss of structural and functional integrity of the particles. This specific class of bound Mg^{2+} interacts with approximately 15–23% of the total RNA phosphates of the 30S subunit. The physical interpretation of this critical class of bound Mg^{2+} is not clear at present. This specifically required level of Mg^{2+} may indicate the existence of a physically distinct group of cation binding sites on the ribosome. Alternatively, it may indicate a requirement for Mg^{2+} binding to approximately 20% of the total, but not specific, cation binding sites. The former model necessitates the existence of "structural magnesium" bound to sites unique from the bulk of the cation binding sites on the ribosome. The heterogeneity of divalent cation binding sites on ribosomes has not been firmly established (Edelman *et al.*, 1960; Rodgers, 1964; Sheard *et al.*, 1967; Walters and Van Os, 1971). However, Cohn *et al.* (1969) have presented convincing evidence for the existence of a class of strong, cooperative Mn^{2+} binding sites in isolated rRNA, which represent 19% of the total sites. The possible relationship between these sites in isolated RNA and the critical class of Mg^{2+} sites described here will require further investigation.

When this critical level of bound Mg^{2+} is replaced by polyamines, distinct structural alterations parallel the changes in activity. These alterations in the structure of the 30S subunit are reflected in the observed changes in sedimentation, reduced viscosity, and ribonuclease sensitivity. However, it is important for later discussion to point out that putrescine and spermidine produce decidedly different effects on the 30S subunit. The changes in the structural parameters measured are far more extensive in the case of putrescine. However, with both cations, the observed inactivation is not the result of compositional changes in the particles or covalent modification of critical components. This is illustrated by the fact that the polyamine-inactivated particles, isolated from sucrose gradients, can be reactivated by heating under the ionic conditions described by Traub and Nomura (1969). These observations lead to the conclusion that replacement of a critical level of bound Mg^{2+} with polyamines leads to conformational alterations in the 30S ribosomal subunit, producing more asymmetric particles with a higher degree of RNA exposure to the environment.

The continued presence of polyamines is not necessary for maintenance of the denatured state of the particles. Although replacement of polyamines by Mg^{2+} leads to some tightening of the structure, native active particles are not obtained. Only after heating particles to which Mg^{2+} has been returned is there a restoration of the native structure. This argues that there is a step with a high energy of activation involved in the renaturation process. This same conclusion was first reached by Traub and Nomura (1969) from studies on the reconstitution of the 30S particles from isolated proteins and RNA. These same workers also found the refolding of EDTA-unfolded particles required similar conditions.

Similar unfolding of the 30S subunit has been observed under conditions which result in lowering the level of Mg^{2+} bound to the ribosome (Spirin *et al.*, 1963; Gesteland, 1966a; Eilam and Elson, 1971). These changes have been attributed to less efficient neutralization of the negatively charged RNA phosphate groups and therefore increased electrostatic repulsion within the particle. However, this explanation cannot account for the unfolding observed in this work, since a constant level of bound multivalent cations is maintained. Two possibilities might be advanced as explanations for the induction of inactivation by polyamines. First, the act of removal of Mg^{2+} from a critical site or sites might cause a rearrangement of the ribosome structure regardless of the presence of a substituting polyvalent cation. The alternative explanation is that substitution of polyamines for Mg^{2+} disrupts local molecular interactions, because of the different structural properties of these cations. The second model is the most likely, since distinctly different particles are produced in the presence of putrescine and spermidine; a result not predicted by the first possibility. The property of the polyamines which is responsible for producing these structural changes is not certain from these studies. Perhaps the larger size of the organic cations produces distortion in critical regions of the particles. Alternatively, the separation of the charges in the polyamines, resulting in a lower charge density, may be the deciding factor. Regardless of the structural properties which lead to the observed functional differences between Mg^{2+} and polyamines, it is clear that a certain proportion of the cation binding sites of the 30S ribosomal subunit must be occupied by Mg^{2+} for structure and function to be maintained.

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