Cations and Ribosome Structure. III. Effects on the 30S and 50S Subunits of Replacing Bound Mg^{2+} by Inorganic Cations†

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ABSTRACT: Using equilibrium dialysis, the physical and functional properties of purified ribosomal subunits from *Escherichia coli* were studied as ionically bound Mg^{2+} was replaced by divalent cations, Mn^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+}, and the monovalent cations, K^+ and NH_4^+. With the 30S subunit, Mn^{2+} and Ca^{2+} could totally replace Mg^{2+} without impairing phenylalanine polymerizing activity. Sr^{2+} was only partially effective in this capacity, and substitution by Ba^{2+} caused total inactivation as the Mg^{2+}/RNA-P was reduced from 0.10 to 0. Loss of function was accompanied by significant increases in sensitivity to pancreatic ribonuclease and decreases in sedimentation coefficient. All activity could be regained after treating these altered 30S particles in high ionic strength buffers at 40°. Only Mn^{2+} could totally replace Mg^{2+} bound to the 50S subunit without irreversible alterations in polymerizing activity. Total substitution of Ca^{2+} for Mg^{2+} resulted in particles with lower activity and small changes in ribonuclease sensitivity and sedimentation coefficient. Sr^{2+} and Ba^{2+} totally inactivated the 50S subunit as the Mg^{2+}/RNA-P was reduced from 0.075 to 0.025. With these latter two cations, there was a six- to eightfold increase in ribonuclease sensitivity and a transformation of the particle to multiple species with sedimentation coefficients of approximately 40 (major), 30, and 20 S. Neither the activity nor the structure of the native 50S subunit were regained after restoring Mg^{2+} by dialysis. There were no observable alterations of the rRNA species which could account for the irreversible nature of these changes. However, the possible loss of one or a few proteins was not eliminated. None of the monovalent cations tested could displace Mg^{2+} bound to either subunit without inducing loss of activity and extensive changes in sedimentation velocity and sensitivity to pancreatic ribonuclease. Inactivation by the monovalent cations K^+ and NH_4^+ was observed to begin as the Mg^{2+}/RNA-P was reduced below 0.20. These results, together with those reported in the other papers of this series, suggest that there are at least three distinct classes of cation binding sites on *E. coli* ribosomes which differ in their specificity. In addition, from estimations of the intracellular cation levels, it is proposed that the polyamines putrescine and spermidine may serve an essential role in preserving the structural and functional integrity of ribosomes *in vivo*.

A variety of studies clearly show that multivalent cations are an indispensible component of ribosomes (reviewed in Weiss and Morris, 1973). However, a systematic examination of the specificity of this requirement has not been made. Previous work in this laboratory has shown that at least one class of cation binding sites can be defined on the basis of its specificity (Weiss and Morris, 1970, 1973; Kimes and Morris, 1973). In replacing Mg^{2+} with the polyamines putrescine and spermidine, it was found that approximately 20% of the rRNA phosphate groups must continue to interact with Mg^{2+} for the functional integrity of the particles to be maintained. In light of these results, the present study was undertaken to further circumscribe the limits of the specificity of this cation requirement.

Methods

Unless otherwise stated, all preparations and procedures were as previously described (Weiss and Morris, 1973; Kimes and Morris, 1973). Magnesium and barium were determined with a Perkin-Elmer Model 303 atomic absorption spectrophotometer using ion-specific hollow cathode lamps. Sample preparation was as previously described (Weiss and Morris, 1973). Specific procedures were those described for the particular ion in the Perkin-Elmer Atomic Absorption Method Manual (1964). Standard solutions of $MgSO_4$ and $BaCl_2$ were prepared from reagent grade crystals following extensive drying *in vacuo* over P_2O_5.

Results

Inactivation of Ribosomal Subunits by Divalent Inorganic Cations. The ions examined here, Mn^{2+}, Ca^{2+}, Ba^{2+}, and Sr^{2+}, did not inhibit the phenylalanine polymerizing system when added at a concentration of 1 mM. Ni^{2+}, Zn^{2+}, and Co^{2+} were found to be inhibitory and therefore were not used. In addition, the cations which were used did not produce any effects on the 30S subunit which could not be reversed by the reactivation procedure of Traub and Nomura (1969) (see below).

Barium ion was used to examine the stoichiometry of replacement of Mg^{2+} by another divalent cation. At all levels of replacement, removal of Mg^{2+} bound to the ribosomal subunits was found to be accompanied by binding of an

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

Subunit	Mg^{2+} [b]	Mg^{2+}/ RNA-P	Ba^{2+}/ RNA-P	+[c]/ RNA-P
30 S	1.02	0.11	0.09	0.40
30 S	0.30	0.07	0.14	0.42
30 S	0.014	0.01	0.19	0.40
50 S	1.02	0.12	0.09	0.42
50 S	0.30	0.07	0.14	0.42
50 S	0.014	0.01	0.18	0.38

[a] Solutions of 50S and 30S subunits (5.0 mg/ml) were dialyzed for 12 hr at 3° against buffers containing 0.01 M Tris-HCl (pH 7.5), 0.06 M KCl, 1 mM $BaCl_2$, and the indicated concentrations of $MgCl_2$. [b] The concentration of free Mg^{2+} after dialysis (mM). [c] Abbreviations used are: +/RNA-P, total charge of bound Mg^{2+} and Ba^{2+} per RNA-P residue.

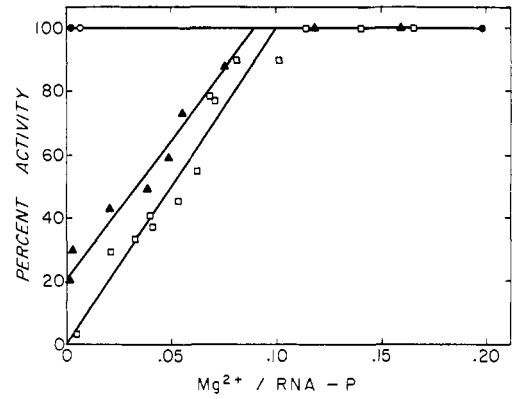

FIGURE 1: Activity of 30S subunit during the replacement of Mg^{2+} by divalent inorganic cations. 30S subunits (4.0 mg/ml) were dialyzed for 12 hr at 3° against buffers containing 0.01 M Tris-HCl (pH 7.5), 0.06 M KCl, 6 mM 2-mercaptoethanol, 1 mM of the indicated cation (choloride salt), plus varying amounts of $MgCl_2$ (0–2.0 mM). After dialysis, polymerizing activity was determined at 25° and Mg^{2+}/RNA-P estimated as previously described (Weiss and Morris, 1973). The curves represented are as follows: Mn^{2+} (●), Ca^{2+} (○), Sr^{2+} (▲), and Ba^{2+} (□).

equivalent amount of Ba^{2+} (Table I). Similar results have been obtained with Mn^{2+}, consistent with the results of Sheard *et al.* (1967). Ba^{2+} has the largest ionic radius of the cations chosen for use and therefore would be expected to interact the least effectively with the ribosome because of its more diffuse charge. Hence, since Mg^{2+} displacement by Ba^{2+} is stoichiometric, it is reasonable to assume that the same is true of the other divalent cations used.

Various degrees of Mg^{2+} replacement by the divalent cations Mn^{2+}, Ca^{2+}, Ba^{2+}, and Sr^{2+} were achieved by equilibrium dialysis. Equilibrium was attained after 6 to 8 hr of dialysis. The activities of the 30S and 50S subunits, using poly(U) as message, are compared with the amount of Mg^{2+} replacement in Figures 1 and 2. With neither subunit was any inactivation observed when Mn^{2+} was used to replace Mg^{2+}. Calcium ion, although having no deleterious effect on the activity of the 30S subunit, can be seen to cause some loss of 50S function when the value of Mg^{2+}/RNA-P is lowered below 0.06. In no case was complete inactivation observed with Ca^{2+}, even when more than 99% of the Mg^{2+} had been replaced.

Ba^{2+} and Sr^{2+} led to inactivation of both subunits. Inactivation of the 30S subunit was first observed at a value of Mg^{2+}/RNA-P of approximately 0.09 (Figure 1). On the other hand, the effect of Sr^{2+} on the 30S subunit was similar to that observed with Ca^{2+} on the 50S subunit. Thus, inactivation was incomplete when more than 98% of the Mg^{2+} had been replaced by Sr^{2+}. This residual activity was most prominent when the polymerizing assays were performed at 37°, and was almost nonexistent if the assays were carried out at 25°. This suggests that reactivation of the 30S subunit is taking place in the assay incubation. With the 50S subunit, both Ba^{2+} and Sr^{2+} show almost identical inactivation profiles (Figure 2). Activity began to be lost at a value of Mg^{2+}/RNA-P of approximately 0.075 and was complete at 0.025.

Attempts to regain activity by restoring the original level of ribosomally associated Mg^{2+} were only partially successful. Dialysis of any partially active or totally inactive 50S subunit preparation against buffers containing Mg^{2+} resulted in neither a gain nor a further loss of activity. Both Ba^{2+} and Sr^{2+} 30S subunits sometimes exhibited a small increase in activity when dialyzed against buffer B-3 [0.01 M Tris-HCl (pH 7.5), 0.06 M KCl, 1 mM magnesium acetate, and 6 mM 2-mercaptoethanol]. However, the amount of regained activity varied from experiment to experiment and in no case was complete reactivation observed.

Structural Alterations Accompanying Inactivation with Divalent Cations. The effects on the structure of the ribosomal subunits of replacing ribosomal Mg^{2+} with other divalent cations were examined by ribonuclease sensitivity and sedimentation velocity. No effects of the ions used on the activity of pancreatic ribonuclease itself could be demonstrated. Figure 3 indicates the ribonuclease sensitivity of both 30S and 50S subunits at various levels of Mg^{2+} replacement by Ba^{2+}. As can be seen, an increase in sensitivity occurred concomitantly with the loss of activity and continued with increasing inactivation. The decrease in ribonuclease sensitivity at values of Mg^{2+}/RNA-P higher than the point of inactivation was probably the result of the increase in the total amount of bound divalent cations which occurred above a value of 0.12. Similar results, with both subunits, were obtained with Sr^{2+}. The use of Ca^{2+} resulted in a very small change in the case of the 50S subunit and no changes in the 30S subunit. No increase in ribonuclease sensitivity for either sub-

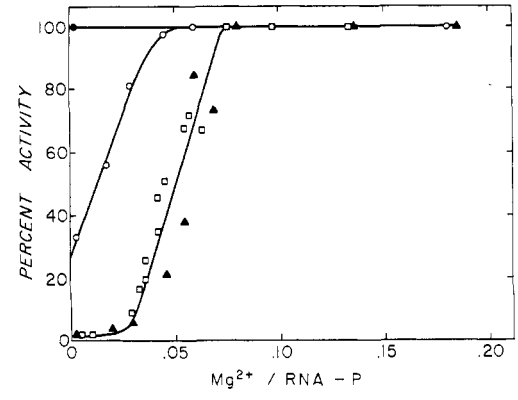

FIGURE 2: Activity of 50S subunits during the replacement of Mg^{2+} by divalent inorganic cations. The same procedure was used as described in Figure 1, except for the assay conditions which employed 37° instead of 25°. The curves represented are as follows: Mn^{2+} (●), Ca^{2+} (○), Sr^{2+} (▲), and Ba^{2+} (□).

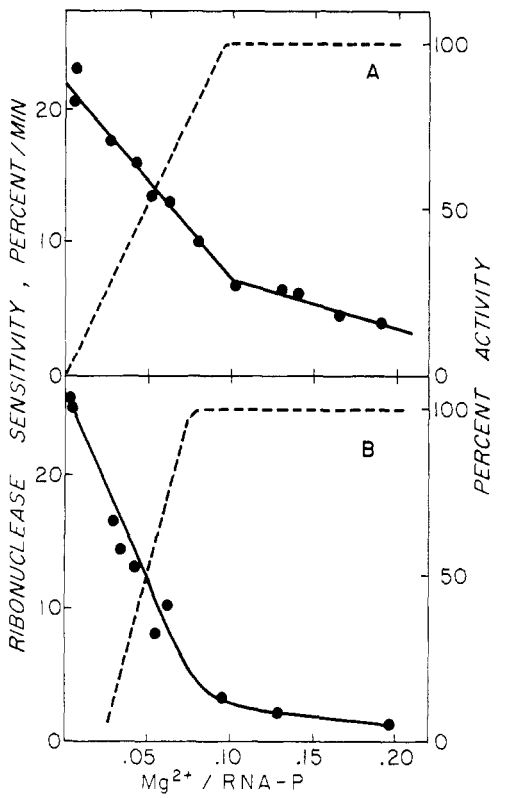

FIGURE 3: The change in ribonuclease sensitivity of 30S and 50S subunits during the replacement of Mg^{2+} by Ba^{2+}. Solutions of subunits were dialyzed as described in Figures 1 and 2. The sensitivity to pancreatic ribonuclease and the value of Mg^{2+}/RNA-P were determined as previously described (Weiss and Morris, 1973). Frame A shows results obtained with the 30S subunit and frame B with the 50S subunit. The curves are represented as follows: -----, activity; ●, ribonuclease sensitivity. The activity curves are taken from Figures 1 and 2.

TABLE II: Summary of Physical Properties of 30S and 50S Ribosomal Subunits after Replacement of Bound Mg^{2+} by Other Divalent Cations.[a]

		In Cation Buffer		In Buffer B-3	
Subunit	Cation	RNase (%/min)	s_{20} (S)	RNase (%/min)	s_{20} (S)
30 S	Mg^{2+}	8.0	30.5	8.0	30.5
30 S	Mn^{2+}	4.5	31.5	8.3	30.5
30 S	Ca^{2+}	8.1	30.5	8.2	30.5
30 S	Ba^{2+}	20.7	28.0	17.0	28.5
30 S	Sr^{2+}	20.5	27.5	15.5	29.0
50 S	Mg^{2+}	2.7	47.9	2.7	47.9
50 S	Mn^{2+}	2.0	49.6	3.0	48.4
50 S	Ca^{2+}	4.8	48.5	6.7	48.7 (40.1)
50 S	Ba^{2+}	26.1	42.0 (31.5, 22.6)	19.7	40.3 (28.9, 22.6)
50 S	Sr^{2+}	27.3	40.8 (32.5, 22.5)	25.0	40.0 (31.5, 22.3)

[a] Ribosomal subunits at a concentration of 4.0 mg/ml were dialyzed for 12 hr at 3° against buffers containing 0.01 M Tris-HCl (pH 7.5), 0.06 M KCl, and 1 mM of the chloride salt of the indicated cations. They were then dialyzed for 24 hr at 3° against buffer B-3 [0.01 M Tris (pH 7.5), 0.06 M KCl, 1 mM magnesium acetate, and 6 mM 2-mercaptoethanol]. At each stage of dialysis, the subunits were examined for their sensitivity to pancreatic ribonuclease and their sedimentation behavior by methods previously described (Weiss and Morris, 1973). Values in parentheses are for minor species.

unit was observed when Mn^{2+} replaced Mg^{2+}. The ribonuclease sensitivities for completely altered particles are summarized in Table II.

The sedimentation properties of Mg^{2+}-free 30S particles were also examined (Table II). As predicted from the activity studies, Ca^{2+} particles were indistinguishable from the control Mg^{2+} particles. Mn^{2+} led to a small increase in the observed sedimentation coefficient which was probably due to an increase in the total number of bound polyvalent cations, since Mn^{2+} appeared to have a greater affinity than Mg^{2+} for the binding sites on both 30S and 50S subunits. A similar increase in sedimentation coefficient could be observed when control particles were examined under conditions resulting in a higher level of bound Mg^{2+}. Both Ba^{2+} and Sr^{2+} 30S particles were found to sediment as single boundaries and to have reduced sedimentation coefficients (Table II). Although the decrease in sedimentation coefficients of the 30S subunits was only approximately 10%, this decrease was reproducible and was accompanied by a distinct broadening of the observed schlieren peaks.

The changes observed in the sedimentation behavior of the 50S subunit were also dependent upon the nature of the divalent cation (Table II). There was no appreciable alteration of the sedimentation coefficients when Mn^{2+} and Ca^{2+} were used as substitutes for Mg^{2+}. However, the use of either Sr^{2+} or Ba^{2+} led to the conversion of the 50S subunit to multiple species sedimenting at approximately 40, 30, and 20 S. The 30S and 20S components were minor species, comprising together no more than 20% of the total material.

When the original level of bound Mg^{2+} was restored (Table II), the slight changes observed on replacing Mg^{2+} with Mn^{2+} were readily reversed. In those cases where extensive structural changes did occur, simply restoring Mg^{2+} did not restore the native structure. This was consistent with the inability to restore functional activity under the same conditions.

Nature of Inactivation with Divalent Cations. In order to investigate whether the loss of 30S activity was due to compositional changes in the particles, attempts were made to reactivate the Mg^{2+}-free particles. Subunits inactivated by replacing Mg^{2+} by either Ba^{2+} or Sr^{2+} were isolated from sucrose gradients in the presence of the substituting cation. This procedure should separate the particles from any ribosomal proteins which might have been released during dialysis against Ba^{2+} or Sr^{2+}. The particles were then exposed to the reconstitution procedure of Traub and Nomura (1969) as previously described (Weiss and Morris, 1973). The particles were assayed for phenylalanine polymerizing activity both before and after heating at 40° for 25 min. The particles resulting from Mg^{2+} depletion by Sr^{2+} were totally reactivated without heating. The Ba^{2+} particles exhibited 80–100% activity prior to heating and were consistently fully active following the heat step. Thus, the transformations observed for the 30S subunit after replacement of Mg^{2+} by Ba^{2+} and Sr^{2+}

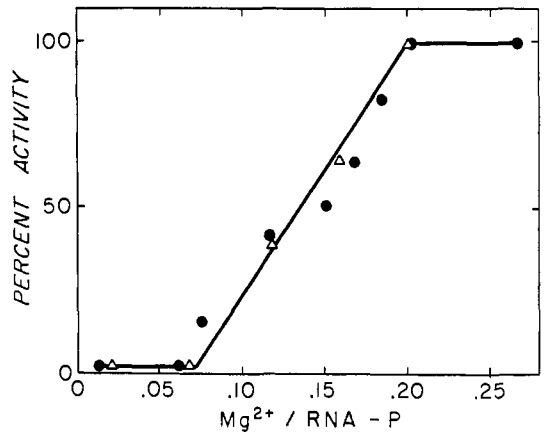

FIGURE 4: The change in activity of the 30S subunit during the displacement of Mg^{2+} by monovalent cations. Solutions of 30S subunits (4.0 mg/ml) were dialyzed against buffers containing 0.01 M Tris-HCl (pH 7.5), 6 mM 2-mercaptoethanol, and 0.06 M of each monovalent cation (chloride salt) plus 0–3.0 mM $MgCl_2$. Polymerizing activity was determined at 37° and Mg^{2+}/RNA-P determined as previously described (Weiss and Morris, 1973). The curves represented are K^+ (●) and NH_4^+ (△).

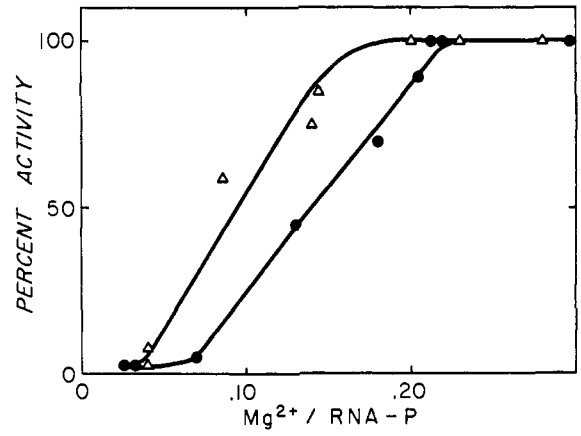

FIGURE 5: The change in activity of the 50S subunit during the displacement of Mg^{2+} by monovalent cations. Experiments were carried out under conditions identical with those described in Figure 4. The curves shown are K^+ (●) and NH_4^+ (△).

were not irreversible and therefore presumably did not involve either loss of ribosomal proteins or covalent alteration of any ribosomal component.

The altered 50S subunits could not be reactivated under the conditions tried (Nomura and Erdmann, 1970; Maruta *et al.*, 1969). However, in no case could loss of 5S RNA or major alteration of the 23S RNA be detected by methods previously reported (Kimes and Morris, 1973). Observations by disc gel electrophoresis (Kimes and Morris, 1973) suggested that replacement of Mg^{2+} by Ba^{2+} or Sr^{2+} may have resulted in the release of one protein, but rigorous studies have not been conducted to determine whether this protein was lost during or after inactivation. However, since the inactivation obtained with Ba^{2+} and Sr^{2+} was similar to that with spermidine (Kimes and Morris, 1973), it is likely that the protein release occurred after inactivation was complete.

Inactivation of Ribosomal Subunits by Monovalent Cations. Experiments similar to those described above were carried out using K^+ and NH_4^+ to displace ribosomally bound Mg^{2+}. The time of dialysis required to reach equilibrium was found to be 14 hr. Figures 4 and 5 show the polymerizing activity of 30S and 50S ribosomal subunits, respectively, when monovalent cations were used to lower the Mg^{2+}/RNA-P. While the 30S subunit showed identical responses to K^+ and NH_4^+, NH_4^+ appeared to be a better substitute for Mg^{2+} than K^+ in maintaining the function of the 50S subunit. It is clear from these results that monovalent cations began to induce loss of ribosome function at much higher values of Mg^{2+}/RNA-P (*ca.* 0.20) than any of the multivalent inorganic or organic cations previously tested. In addition, unlike those results obtained using multivalent cations, complete inactivation of the 30S subunit occurs before total displacement of the bound Mg^{2+}. Attempts to restore the function of either subunit by dialysis into Mg^{2+}-containing buffers were completely unsuccessful.

Structural Alterations Accompanying Inactivation with Monovalent Cations. As was the case when divalent cations were used, ribosomal particles showed a sharp increase in their ribonuclease sensitivity beginning at a value of Mg^{2+}/RNA-P similar to that at which inactivation was first observed. Results using K^+ are illustrated in Figure 6. NH_4^+ acted in a similar fashion. At approximately the point of complete inactivation, the increase in ribonuclease sensitivity ceased. Results with fully altered particles are summarized in Table III.

Inactivation resulted in structural alterations that were readily visualized by analytical ultracentrifugation. When more than 95% of the bound Mg^{2+} had been removed by dialysis against buffers containing K^+ or NH_4^+, the particle arising from the 30S subunit appeared homogeneous with a sedimentation coefficient of approximately 19 S. With the 50S subunit, particles sedimenting at approximately 25 and 18 S were observed. These species were in a ratio of roughly 2:1. If the level of ionically bound Mg^{2+} was restored to these altered 30S and 50S subunits by further dialysis into buffer B-3, sedimentation coefficients remained low and ribonuclease sensitivities high.

Nature of Inactivation by Monovalent Cations. The 19S species resulting from Mg^{2+} displacement from 30S particles

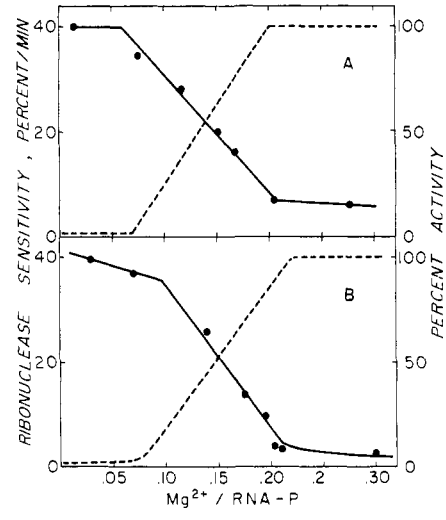

FIGURE 6: Ribonuclease sensitivity of 30S and 50S subunits during the displacement of Mg^{2+} by K^+. See Figure 4 for the conditions of dialysis. Sensitivities to pancreatic ribonuclease and Mg^{2+}/RNA-P were estimated as previously described (Weiss and Morris, 1973). Frame A shows results obtained for the 30S and B for the 50S subunit. The dotted lines represent activity taken from Figures 4 and 5.

TABLE III: Summary of Physical Properties of 30S and 50S Ribosomal Subunit after Displacement of Bound Mg^{2+} by Monovalent Cations.[a]

Subunit	Cation	RNase	s_{20} (S)
30S	K^+	40.2	19.2
30S	NH_4^+	38.0	18.8
50S	K^+	40.2	24.8, 18.1
50S	NH_4^+	38.0	26.2, 17.1

[a] Ribosomal subunits (4.0 mg/ml) were dialyzed for 18 hr at 3° against buffers containing 0.01 M Tris-HCl (pH 7.5), 0.06 M of the indicated cation (chloride salt), and 6 mM 2-mercaptoethanol. Each preparation was then dialyzed into buffer B-3. See Table II for further details, and values obtained with control particles.

TABLE IV: Relationship of the Crystalline Ionic Radius of a Divalent Metal Ion and Its Ability to Maintain Subunit Function.

Cation	Ionic Radius[a] (Å)	Activity[b] 30 S	50 S
Mg^{2+}	0.66	+	+
Mn^{2+}	0.80	−	+
Ca^{2+}	0.99	−	±
Sr^{2+}	1.12	±	−
Ba^{2+}	1.34	−	−

[a] Values for ionic radii were taken from the Handbook of Chemistry and Physics, The Chemical Rubber Co., 1968. [b] Abbreviations used are: +, full activity; ±, partial activity; −, no activity.

by K^+ and NH_4^+ were isolated from sucrose gradients in the absence of Mg^{2+}. They were then reactivated as described for the Ba^{2+} and Sr^{2+} particles and assayed for phenylalanine polymerizing activity. In contrast to the results obtained with Ba^{2+} and Sr^{2+}, essentially no reactivation was observed unless the particles were heated to 40° for 25 min. However, complete recovery of function was observed following this heat step. Using procedures previously described for the 50S subunit (Kimes and Morris, 1973), no detectable alteration of the 23S RNA or 5S RNA was found. However, one or two proteins were released after 95% displacement of Mg^{2+}, but it is not known whether this occurred during or after loss of 50S function.

Discussion

The results presented here suggest the existence of multiple classes of anionic sites on *E. coli* ribosomes which differ in their specificity for neutralization by cations. Occupation of approximately 80% of these sites by a combination of Mg^{2+}, Mn^{2+}, Ba^{2+}, Ca^{2+}, Sr^{2+} or the naturally occurring polyamines putrescine and spermidine (Weiss and Morris, 1970, 1973; Kimes and Morris, 1973) has no effect on the phenylalanine polymerizing activity of either the 30S or the 50S ribosomal subunit. The remaining 20% of the sites appear to be specific for a narrow range of inorganic divalent cations. Either Mg^{2+} or Mn^{2+} can satisfy this requirement for both subunits, and Ca^{2+} appears satisfactory for the function of the 30S subunit. The effects observed with Ca^{2+} on the activity of the 50S subunit suggest that it may be at least partially effective in maintaining 50S function. A similar hypothesis can be made for the effect of Sr^{2+} on the 30S subunit. These conclusions arise from the inability to completely inactivate the particular subunit following the displacement of more than 98% of the ribosomally associated Mg^{2+}.

These results with Mn^{2+} are in essential agreement with the observations of others. Tissieres *et al.* (1960) suggested that Mn^{2+} could replace Mg^{2+} in maintaining ribosome function. These authors failed, however, to determine the amount of residual Mg^{2+} which remained bound to their ribosome preparations. Sheard *et al.* (1967) indicated that *E. coli* ribosomes appeared normal by analytical ultracentrifugation following Mg^{2+} replacement by Mn^{2+}, but did not state whether the resulting ribosomes were in *in vitro* protein synthesis. Our results indicate that Mn^{2+} is fully capable of maintaining ribosomes which exhibit unimpaired phenylalanine polymerizing activity when assayed in the presence of Mg^{2+}. These results cannot rule out the possibility that the subunits are reactivated in the assay mixture when exposed to Mg^{2+}. Since Mn^{2+} cannot replace Mg^{2+} for some of the steps in protein synthesis (Gordon and Lipmann, 1967), this question will require further investigation. However, it should be mentioned that when ribosomal subunits, totally saturated with Mn^{2+}, are dialyzed against spermidine, the loss of activity plotted as a function of Mn^{2+}/RNA-P is nearly identical with those results obtained for the substitution of spermidine for Mg^{2+} (Kimes, 1971; Weiss, 1971). Thus, it seems likely that Mn^{2+} and Mg^{2+} interact at equivalent sites on the ribosome.

The inability of Ca^{2+} to maintain functionally active 50S subunits is in contrast to the results of Gordon and Lipmann (1967). However, Choi and Carr (1967) reported anomalous sedimentation behavior for Ca^{2+}-ribosomes. It is possible that the procedure used by Gordon and Lipmann may have failed to completely replace Mg^{2+} with Ca^{2+}. This would be consistent with the results described above which indicate that small amounts of residual Mg^{2+} maintain fully active 50S subunits. In addition, close examination of their data shows that the Ca^{2+} ribosomes had a lower specific activity than that observed with Mg^{2+} ribosomes. This may be a reflection of the residual activity observed here following extensive dialysis against buffer containing Ca^{2+}.

The inactivation profile of the 30S subunit in the presence of Sr^{2+} is similar to that of the 50S subunit when Ca^{2+} is used to replace Mg^{2+}. The similarity resides in the failure to observe complete inactivation following replacement of more than 98% of the bound Mg^{2+}. These results are indicative of a basic difference in the specificity of the cation requirements of the two subunits. However, with a given subunit, the results of the divalent cation studies correlate with the crystalline ionic radii of the cations, as shown in Table IV. Thus it appears that *ca.* 1.0 Å is the critical size; larger cations being unable to fulfill the structural requirements for stability of the particles. The correlation suggests that these sites, which were defined by their functional requirements, may be physically distinct from the remaining sites.

The natural extension of this argument is that there exists a special group of cation binding sites in the rRNA which

are important in maintaining the biologically active conformation of the molecule. The environment of these sites would be such that only divalent cations with an ionic radius of less than 1.0 Å could interact with this conformation of the RNA. It is clear from the studies of Sander and Ts'o (1971) that the conformation of an RNA molecule has an important influence on its binding of Mg^{2+}. In the case of tRNA, Mg^{2+} is required for the secondary and tertiary structure of the molecules (Adams *et al.*, 1967; Ishida and Sueoka, 1967; Reeves *et al.*, 1970) as well as for their biological activity (Adams *et al.*, 1967). This requirement seems to involve a small number of cooperative binding sites (Cohn *et al.*, 1969; Willick and Kay, 1971) and shows a degree of cation specificity (Robison and Zimmerman, 1971). With rRNA, the existence of a class of tightly bound Mg^{2+} was postulated by Rodgers (1964) on the basis of $^{28}Mg^{2+}$ exchange kinetics. This result has been challenged by Sheard *et al.* (1967) who failed to detect any differences in cation binding sites by proton relaxation studies of Mn^{2+} bound to *E. coli* ribosomes. These studies would not, however, have detected small differences. Recently, Cohn *et al.* (1969), using the same technique, described the existence of a special class of cooperative, high affinity Mn^{2+} binding sites in isolated rRNA which represented approximately 19% of the total sites. If our proposal is correct, one should be able to observe cation-specific conformational changes in isolated rRNA.

When K^+ and NH_4^+ were used to displace Mg^{2+} from the ribosomal subunits, inactivation occurred at values of Mg^{2+}/RNA-P of approximately 0.2 and lower. Thus, in the absence of other multivalent cations, 40% of the cation binding sites need to be occupied by Mg^{2+}. This result suggests the existence of at least three functionally distinct cation classes. One class (I) is that discussed above which requires specific divalent inorganic cations—Mg^{2+} or Mn^{2+} (with Ca^{2+} sufficing for the 30S subunit). A second class (II) must be occupied by multivalent cations, but appears to show no specificity. Class II sites can be occupied by any of the inorganic divalent cations tested or by polyamines (Weiss and Morris, 1970, 1973; Kimes and Morris, 1973) with no effect on the functional integrity of the ribosomal subunits. Class III sites appear to be even less specific and can be neutralized by K^+ or NH_4^+ and any of the multivalent cations tested. There are indications in the literature, however, that there may be a degree of specificity in the class III sites. Partial reactions of protein synthesis carried out by the 30S and 50S subunits are inactivated when the particles are dialyzed against buffers containing sufficient Mg^{2+}, but Na^+ or Li^+ as monovalent cations (Miskin *et al.*, 1970; Zamir *et al.*, 1971). However, it is difficult to compare these results with those presented here since no measurements of bound Mg^{2+} were made and the assays were carried out at 0°. Under our assay conditions, the inactivation observed by these authors would have been reversed. In this regard, it is interesting to note that we have observed that Na^+ is much less effective than K^+ or NH_4^+ in filling the class III sites on both subunits (Kimes, 1971; Weiss, 1971).

The distinction among these classes is entirely functional. Although some evidence suggests a physical distinction of class I sites, it seems unlikely that the distinction between classes II and III has any physical significance. The wide variety of cations (from inorganic divalent cations to the trivalent polyamine spermidine) which effectively occupy the class II sites share one common feature. They are multivalent, in contrast to K^+ and NH_4^+, and thus would be bound to the RNA. The distinction between classes II and III may therefore represent the necessity for tight binding of cations to a certain fraction of the RNA phosphate residues (*ca.* 40%) so that internal charge repulsion in the particles does not rise above a level consistent with stability. This conclusion is consistent with the relative effectiveness of monovalent and divalent cations in reducing the electrophoretic mobility of DNA (Ross and Scruggs, 1964).

In addition to providing structural information about the ribosome, these observations are potentially of physiological significance. Magnesium ion appears to be the most prevalent intracellular multivalent cation (Wacker, 1969). One of its primary functions appears to be the neutralization of RNA phosphate groups. Thus, when grown under a variety of growth-limiting conditions, *Aerobacter aerogenes* maintains a relatively constant ratio of Mg^{2+} to RNA phosphate (Tempest and Strange, 1966; Tempest and Meers, 1968). In addition, McCarthy (1962) demonstrated that *E. coli*, under conditions of Mg^{2+} starvation, fail to maintain intact ribosomes. The results presented here and in the accompanying papers indicate that only 20% of the rRNA phosphates require neutralization specifically by Mg^{2+}. This is consistent with the observation that RNA is not saturated with Mg^{2+} *in vivo* (Edelman *et al.*, 1960; Hurwitz and Rosano, 1967). In fact, the intracellular Mg^{2+} content of exponentially growing cells is insufficient to neutralize all the RNA-P groups (Watson, 1964; Lusk *et al.*, 1968). A variety of techniques designed to measure the level of ribosomally associated Mg^{2+} in intact cells have given rise to values of Mg^{2+}/RNA-P ranging from 0.08 to 0.17 (Hurwitz and Rosano, 1967; Lusk *et al.*, 1968). The results presented here indicate that such a level of ribosomally associated Mg^{2+} would either be insufficient or only marginally sufficient to maintain ribosome structure and function in the absence of other *multivalent* cations to fill the class II sites. It would appear that the only other multivalent cations which exist in exponentially growing *E. coli* at sufficient levels to occupy the class II sites are the polyamines putrescine and spermidine (Tabor and Tabor, 1964). Such a hypothesis is consistent with the observation of Hurwitz and Rosano (1967) that an increase in the level of spermidine associated with the ribosome occurs concomitantly with a decrease in bound Mg^{2+} during growth of *E. coli* under conditions of Mg^{2+} limitation. Similar results have been reported for putrescine in the case of a *Pseudomonas* species which lacks spermidine (Rosano and Hurwitz, 1969). In addition, a mutant of *E. coli*, which lacks >95% of the wild type level of intracellular putrescine, grows with a generation time which is essentially normal and contains increased levels of Mg^{2+} (Morris and Jorstad, 1970). This result is also consistent with the concept that the class II sites can be filled by either polyamines or Mg^{2+}.

In the case of the class III sites, there is clearly enough intracellular K^+ present to neutralize these sites (Solomon, 1962; Lubin and Ennis, 1964; Lusk *et al.*, 1968). Another facet of this high level of K^+ is that it would decrease the affinity of the ribosome for Mg^{2+}, thus helping to maintain a sufficient concentration of free Mg^{2+} for other metabolic processes that require this cation (Wacker, 1969). From studies with a mutant of *E. coli*, it has been suggested that K^+ limitation causes inhibition of protein synthesis which is known to require high concentrations of K^+ (Ennis and Lubin, 1961; Lubin and Ennis, 1964; Conway, 1964; Spyrides, 1964). An alternative interpretation is that during K^+ limitation, this cation no longer competes with Mg^{2+} for the class III RNA phosphates. Thus, an intolerably low level of unbound Mg^{2+} would be created.

The fact that polyamines, when substituted for Mg^{2+} in the class I sites, produce inactivation of the ribosomes, suggests that it is important that an intracellular balance between these cations be maintained. Thus Mg^{2+} starvation results in impairment of protein synthesis and eventual breakdown of ribosomal particles (McCarthy, 1962). We would interpret this result to be due to insufficient Mg^{2+} for occupation of the class I sites, leading to inactivation of both the 30S and 50S ribosomal subunits. The resulting ribosome degradation may be a consequence of the increased sensitivity of such particles to attack by ribonuclease described here. Conversely, Ezekiel and Brockman (1968) showed that high concentrations of spermidine in the growth medium inhibit protein synthesis in *E. coli*. Accordingly, we would suggest that this effect arises from displacement of Mg^{2+} from the critical class I sites by spermidine. One might expect that intracellular control mechanisms might exist to buffer these sorts of effects. In fact, the activity of biosynthetic arginine decarboxylase, the first enzyme in the conversion of arginine to putrescine (Morris and Pardee, 1966), is regulated *in vitro* by the balance between Mg^{2+} and polyamines in a fashion which might be predicted from these considerations (Wu and Morris, 1973).

The results presented in this series of papers suggest one, rather nonspecific function for polyamines in *E. coli*. However, the existence of mutants of this organism, which show a specific growth requirement for spermidine, even in the presence of high concentrations of Mg^{2+} in the culture medium, suggests that there are other biological roles for these compounds (Morris and Jorstad, 1973).

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