Magnesium Ions and the Structure of *Escherichia coli* Ribosomal Ribonucleic Acid

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1. The effect of removing Mg^{2+} from a purified high-molecular-weight (1.07×10^6) fraction of *Escherichia coli* ribosomal RNA was examined by ultracentrifugation, thermal denaturation and optical rotation. 2. At moderate I (0.1 M-sodium chloride), EDTA at 2–50 mM has little effect on RNA; at low I , 0.01–0.04 (with tris as counter-ion), two boundaries appear. 3. The leading boundary, $S_{20,w}$ about 20s, is identified with the original material with counter-ion Mg^{2+} ('ionic atmosphere') removed, leading to an expanded form. 4. The slow boundary, 15–16s, is associated with a further loss of Mg^{2+} and a further expansion, sensitive to EDTA concentration: it is proposed that this Mg^{2+} is localized on the polynucleotide chain, i.e. 'site-bound'. 5. I is important and the EDTA effect at low I is reversible if Na^+ is added immediately after the EDTA: this Na^+ reversibility is lost on standing at 0°. It is suggested that changes in the tertiary structure may be associated with this loss of reversibility. 6. Thermal-denaturation studies show that there is no loss of secondary structure associated with these changes: change in the optical-rotatory-dispersion spectrum in the region of the Cotton effect may be associated with this change in tertiary structure.

Present ideas of the structure of ribosomal RNA envisage a continuous unbranched polynucleotide chain (Doty, Boedtker, Fresco, Haselkorn & Hall, 1959; Spirin, 1963) with short hydrogen-bonded helical regions, with loops (Fresco, Alberts & Doty, 1960) and separated by unpaired segments of the chain. Hydrodynamic and electromagnetic-scattering data are consistent with a model of a zigzag, possibly partly branched, array of short rods (Timasheff, 1964). The secondary structure is stabilized by metal ions, and possibly hydrophobic regions and outer water-bridges (Lewin, 1965).

Mg^{2+} is a particularly striking stabilizing agent for tobacco-mosaic-virus RNA and is some 25 000 times as effective (based on thermal-denaturation data) as Na^+ (Boedtker, 1960). Metal ions other than Mg^{2+} are also found and may be very firmly bound to RNA, e.g. Ca^{2+} , Sr^{2+} , Ba^{2+} , Al^{3+} , Cr^{3+} , Mn^{2+} , Fe^{2+} , Ni^{2+} , Cu^{2+} and Zn^{2+} (Wacker & Vallee, 1959). Fuwa, Wacker, Druyan, Bartholomay & Vallee (1960) have suggested a role for some of these 'intrinsic' metal ions in terms of a tertiary structure for RNA. Many experiments suggest that Mg^{2+} plays an important, though in isolated systems not specific, role in RNA and ribosome structure and particularly function. Thus Mg^{2+} is involved in the binding of polynucleotides (including the hypothesized 'messenger' RNA) to ribosomes (Takanami & Zubay, 1964), the association reactions of ribosomes (Tissieres, Watson, Schlessinger & Hollingworth,

1959), the activity of virus-induced RNA polymerase (Wilson & Bader, 1965) and amino acid incorporation into microsomes (Singh, Raghupathy & Chaikoff, 1965). The details of the structural relationships between Mg^{2+} and ribosomes or RNA are not clear: a knowledge of such details would seem to be essential for an understanding of the functioning of these molecules.

The present experiments were intended to assess the importance of Mg^{2+} in the RNA structure, in a general way. The method was to remove Mg^{2+} from the molecule and examine the resulting changes in RNA structure.

The results support the idea of an important role for Mg^{2+} in the structure of the high-molecular-weight (1.07×10^6) fraction of ribosomal RNA. At least two binding sites for Mg^{2+} are proposed, and removal of Mg^{2+} from one set of sites (possibly cross-linking different parts of the chain) results, it is suggested, in changes in the tertiary structure of the molecule. Under the experimental conditions, no loss of secondary structure was observed.

METHODS

Organisms. Growth of the organisms and preparation of the 70s ribosomes were performed as described previously (Rodgers, 1964), except that the cells were disrupted in a Hughes bacterial press at -25° .

Preparation of RNA. To 70s ribosomes (about 5 mg./ml.)

in 2.5 mM-Mg²⁺-tris buffer was added 0.1 vol. of sodium dodecyl sulphate (in 2.5 mM-Mg²⁺-tris buffer) at room temperature. After standing for 5 min. an equal volume of water-saturated phenol was added and the mixture rotated at 3° for 30 min. It was then centrifuged at 2000g for 10 min. at 3° and the upper layer removed. Sometimes this layer was re-extracted with phenol, but this made no difference to the preparation. RNA was precipitated with redistilled ethanol at 3°, the precipitate separated, redissolved in 2.5 mM-Mg²⁺-tris buffer and the precipitation repeated twice more. It was finally treated with bentonite and dialysed overnight at 3° against 5 l. of 2.5 mM-Mg²⁺-tris buffer. The high-molecular-weight (1.07×10^6) fraction of RNA was isolated on a sucrose gradient in the SW 39 rotor of the Spinco model L ultracentrifuge. The sucrose was pretreated with bentonite to remove traces of ribonuclease (Barlow, Mathias, Williamson & Gammack, 1963). The high-molecular-weight fraction was dialysed, to get rid of sucrose, against 2.5 mM-Mg²⁺-tris buffer and stored at -15°.

Velocity ultracentrifugation. This was carried out in the Spinco model E analytical ultracentrifuge at about 3° by using schlieren or u.v.-absorption optics. The u.v.-lamp housing was water-cooled and had the large aperture and ground-quartz screen recommended by Markham (1963). Films were analysed in the Analytrol densitometer: a Joyce-Loebl instrument was used occasionally. Areas under schlieren peaks were measured by planimetry of enlarged tracings, and were corrected for the Johnston-Ogston effect by the method of Trautman, Schumaker, Harrington & Schachman (1954).

Viscometry and pycnometry. These were carried out in a thermostatically controlled bath at 25.00°.

Thermal-denaturation measurements. These were made at 259 mμ in a Beckman model DU spectrophotometer with thermospacers, and heated or cooled by circulating an external supply of liquid. Samples were heated continuously and not cooled between readings. Denaturation curves starting at 0° were obtained by mixing the solutions in the cooled cells and then placing them in the cooled cell compartment. The cell temperature was measured with a Thermistor thermometer in a blank cell.

Optical rotation. This was measured in a Bendix Polaromatic 62 instrument cooled by an external water supply to about 5°. Rotations are reported at 5°, $[\alpha]_D^{25}$, and for wave numbers from 22 000 to 40 000 or 45 000 cm.⁻¹.

Chemicals. All reagents except sodium dodecyl sulphate were of A.R. grade.

Tris and EDTA were the purest grades of the Sigma Chemical Co., St Louis, Mo., U.S.A.

Tris-succinate buffer ('tris buffer') contained tris (5 mM) and succinic acid (2 mM), final pH 7.3-7.4. Magnesium acetate was dissolved in this buffer to give a final concentration of 2.5 mM or 1 mM.

In solutions where a final concentration of 0.1 M-NaCl was required, this was added as a 5 M solution in water with an Agla micrometer syringe.

EDTA-tris was prepared by suspending the weighed amount of EDTA (free acid) in a Pyrex beaker and titrating with standard tris solution in water to the required pH 7.3 with a pH-meter (Radiometer Ltd., Copenhagen, Denmark). A solution 1 mM in EDTA is 2.9 mM in tris at pH 7.3.

Bentonite (Hopkin and Williams Ltd., Chadwell Heath, Essex) was suspended thoroughly (20 g./100 ml.) in 2.5 mM-Mg²⁺-tris or EDTA-tris, and a fraction that is centrifuged

Fig. 1. Effect of EDTA-tris on *E. coli* ribosomal RNA, showing the change in the relative proportion of the components. (a) Original material in 2.5 mM-Mg²⁺-tris buffer; (b) in the presence of EDTA-tris (final concn. 5 mM); (c) in the presence of EDTA-tris (final concn. 10 mM); (d) in the presence of EDTA-tris (final concn. 10 mM) with NaCl (final concn. 0.1 M) added immediately afterwards. Photographs were taken after 30 min. at 52 640 rev./min. at 2.2-3.6°, and tracings made of suitable exposures. The RNA concentration was about 6 mg./ml.

down at 7500g in 10 min. was isolated by differential centrifugation.

RESULTS

Effect of EDTA-tris on ribosomal RNA. Freshly isolated *E. coli* ribosomal RNA in 2.5 mM-Mg²⁺-tris buffer showed the usual ultracentrifuge pattern (Fig. 1a). This Mg²⁺ concentration is optimum for the stability of the 70s ribosomes. Area measurements (corrected) from the schlieren diagrams show the leading component was 76% of the total; the $S_{20,w}$ was 24.8s. The slower component had $S_{20,w}$ 19.8s (values at 40 μg./ml. in the same buffer were 28.8s and 22.0s). This approximately 3:1 ratio for the amount of fast to slow components was reproducible over several preparations and is greater than that of the ratio (2:1) of 50s and 30s ribosomes, from which the RNA was derived, suggesting an aggregation of about 10% of the slow to the fast component. If EDTA-tris (final concn. 5 mM) was added at 0° the proportion of fast material fell to 70% and its $S_{20,w}$ to 18.4s; the $S_{20,w}$ of the slow component fell to 15.0s (Fig. 1b). If EDTA-tris was added to 10 mM (final concn.) the proportion of fast material fell to 55% and $S_{20,w}$ to 15.1s and 13.0s (Fig. 1c). Increase in EDTA-tris to 12.5 mM (final concn.) had no further effect. No intermediate stages were observed. The addition of sodium chloride to 0.1 M (final concn.) partially reversed the effect and the proportion of fast material increased to 62% (Fig. 1d); the $S_{20,w}$ also increased to 21.5s and 16.3s.

It is clear from these observations that a proportion of the fast component ($S_{20,w}$ 24.8s) has $S_{20,w}$ 13.0s in 10 mM-EDTA-tris (a fall of 48% in $S_{20,w}$) and is, under these conditions, ultracentrifugally indistinguishable from the slow component (original $S_{20,w}$ 19.8s), which has decreased in $S_{20,w}$ by 34%.

Since the most striking effect seemed to be on the fast fraction (mol.wt. 1.07×10^6), this was isolated and investigated in more detail.

Effect of EDTA on the high-molecular-weight fraction. High-molecular-weight (1.07×10^6) fraction of ribosomal RNA was isolated by sucrose-gradient centrifugation: all preparations were 90–95% pure, and in 1mM- or 2.5mM- Mg^{2+} -tris buffer had $S_{20,w}$ 28–29s. Experiments were conducted at 0–3° to decrease residual ribonuclease activity and to avoid changes in the secondary structure of the molecule (see below). Sedimentation properties were examined with u.v.-absorption optics at about 40 μg . of RNA/ml., to avoid the concentration-dependence of $S_{20,w}$ and spurious boundary-sharpening effects and to decrease charge and interaction anomalies.

Fig. 2 shows the effect of adding EDTA-tris to a typical preparation. Fig. 2(a) shows the initial preparation; the high-molecular-weight fraction was 91% of the total and its $S_{20,w}$ 28.8s. Fig. 2(b) shows the effect of adding EDTA-tris (final concn. 2mM) at 3°. Two main boundaries appeared with $S_{20,w}$ 20.2s and 15.2s. The weight fractions of these components (extinction basis) were 0.5 and 0.5. If EDTA-tris was added to 6mM (final concn.) the pattern shown in Fig. 2(c) resulted. Two main boundaries were again visible of $S_{20,w}$ 20.0s and 15.0s, very similar to the previous example, but the weight fractions of the components were 0.36

and 0.64 respectively. Inspection of the slow component in Figs. 2(b) and 2(c) showed that it consisted of two components, poorly resolved. The resolution was improved by using a Joyce-Loebl densitometer to analyse the films (Fig. 2e): clearly three components are visible in the mixture. No particular significance was attached to the fact that two slow boundaries exist although they are recognized experimentally.

If sodium chloride (final concn. 0.1M) was added immediately after the EDTA-tris (final concn. 6mM) the fast component was re-formed and a single sharp boundary appeared (Fig. 2d).

All boundaries were quite sharp in the presence or absence of sodium chloride, and the effect seems to be 'all-or-none', i.e. a molecule is either in the 20–20.5s form or in the 15–15.5s form. The poor resolution of the two slow boundaries suggests some heterogeneity between them, and hence they were treated as a single boundary in this work.

The sedimentation coefficients of the fast (20–20.5s) and the slow (15–15.5s) forms did not change in the range 2–10M-EDTA-tris, although the weight fractions changed considerably. Fig. 3 shows the variation in the weight fraction of the fast component with the final concentration of EDTA-tris in the solution. The weight fraction fell to a minimum value of about 0.33 (Fig. 2c) at a final EDTA-tris concentration of about 6mM. This weight fraction fell slightly, to 0.26, over a further 70hr. at 3°. The $S_{20,w}$ of the fast material also fell slightly from 20.2s to 19.2s over the same time. An increase in the final EDTA-tris concentration to

Fig. 2. Effect of EDTA-tris on the high-molecular-weight fraction of *E. coli* ribosomal RNA, showing the formation of the fast and slow boundaries. (a) Purified high-molecular-weight fraction (91% pure) in 1mM- Mg^{2+} -tris buffer; (b) in the presence of EDTA-tris (final concn. 2mM); (c) in the presence of EDTA-tris (final concn. 6mM); (d) in the presence of EDTA-tris (final concn. 6mM) with NaCl (final concn. 0.1M) added immediately afterwards; (e) in the presence of EDTA-tris (final concn. 6mM) but with a Joyce-Loebl microdensitometer being used to improve the resolution of the two slow boundaries. The RNA concentration was about 40 μg /ml. The u.v. photographs were taken after 14 min. for (a) and after 24 min. for (b), (c), (d) and (e) at 59780 rev./min. The temperature was approx. 3°.

Fig. 3. Variation of the weight fraction of the fast component of the high-molecular-weight fraction of *E. coli* ribosomal RNA with EDTA-tris concentration. Weight fractions were obtained from ultracentrifuge runs with u.v. optics, at an RNA concentration of about 40 μg /ml. Each point is the mean of two (in some cases three) measurements: $\circ$, in the presence of EDTA-tris; $\bullet$, in the presence of EDTA-tris with NaCl (final concn. 0.1M) added.

greater than 7 mM (7–50 mM) caused some reversal of the change; thus the pattern with 50 mM-EDTA-tris was similar to the original (except that $S_{20,w}$ was 23.9 s). If Na⁺ was added immediately after the EDTA-tris, to give 0.1 M (final concn.), the weight fraction of fast material was independent of the EDTA-tris concentration and was about 0.82–0.85. Thus the importance of the ionic strength of the solution, whether from tris cation or Na⁺, is clear from these curves.

A variation in the RNA concentration (EDTA-tris final concn. 6 mM) from 28 to 95 µg./ml. had no effect on the weight fraction of fast material (0.33 ± 0.03), and clearly the binding of Mg²⁺ from the RNA does not affect the free EDTA concentration significantly. Partial hydrolysis by (presumed) ribonuclease also did not alter the proportion of fast to slow material and hence neither was hydrolysed preferentially by the enzyme.

These results are interpreted most easily on the basis that the fall in $S_{20,w}$ of the fast component from 28.8 s to about 20 s (and the constancy of this value over a fivefold range of EDTA-tris concentration, namely 2–10 mM), during which the proportion of the components is changing considerably, represents the polyelectrolyte expansion usual in buffers of low ionic strength (10 mM-EDTA-tris, $I 0.04$) or, as in this case, when a good counter-ion (Mg²⁺) is replaced by a poor one. Tris is not a good counter-ion for RNA (Cox & Littauer, 1962). In addition to this effect, and at low ionic strength only ($I < 0.04$), two slower components are formed (mean $S_{20,w}$ 15–16 s). The latter effect is reversed by Na⁺ if added immediately after the EDTA-tris, when a single boundary is formed again, but with $S_{20,w}$ 26–27 s. Thus the initial expansion of all the RNA molecules occurs at a relatively low EDTA-tris concentration (less than 2 mM) and is independent of it, but the second expansion is dependent on EDTA-tris concentration and a solution of effectively low ionic strength. The dependence of the second expansion on the EDTA concentration suggests some heterogeneity in the binding capacity of these latter sites on different RNA molecules.

Effect of time on the EDTA-RNA relation. As discussed above (Fig. 3) the addition of sodium chloride to 0.1 M (final concn.) immediately after the addition of EDTA-tris to high-molecular-weight (1.07×10^6) RNA causes a re-formation of the fast component, to a weight fraction of about 0.82. The effect of time on this reaction was studied by adding EDTA-tris (final concn. 6 mM) to a RNA solution, and then adding sodium chloride, to 0.1 M (final concn.), at various times afterwards. All additions were carried out at 3°. Samples were run in the ultracentrifuge immediately, at 3°. Two controls were examined: (a) addition of sodium chloride at zero time, and examination after 90 min.;

Fig. 4. Effect of time on the ability of Na⁺ to reverse the formation of the slow boundary, as shown by the weight fraction of the fast component of the high-molecular-weight fraction of *E. coli* ribosomal RNA. The proportion of fast material in the mixture was determined in the ultracentrifuge at 59 780 rev./min. at 2–4°. All samples contained about 40 µg. of the high-molecular-weight fraction of ribosomal RNA/ml. O, RNA in 1 mM-Mg²⁺-tris buffer, with EDTA-tris (final concn. 6 mM) added at 0 min.; ●, RNA in 6 mM-EDTA-tris with NaCl (final concn. 0.1 M) added immediately afterwards; ▲, RNA in 6 mM-EDTA-tris with NaCl (final concn. 0.1 M) added at various times.

(b) examination, without sodium chloride, at 0, 60 and 150 min. The result is shown in Fig. 4. As before (in the absence of Na⁺) there is an immediate fall in $S_{20,w}$ from a single component, 28–29 s, to two components, 20.4 s and 15.8 s. After 60 min. these were 19.2 s and 14.7 s, and after 150 min. 20.3 s and 14.8 s, a fall of about 6.6% for the slow boundary. There was a corresponding fall in $S_{20,w}$ of both of the components after the addition of Na⁺, from 26.4 s and 20.0 s (weight fraction of fast material 0.82) at 0 min. to 23.7 s and 17.3 s (weight fraction of fast material 0.25) at 60 min. The addition of Na⁺ at 0 min. resulted in the formation of fast material to a weight fraction of 0.82, which after 90 min. at 3° had fallen to 0.76. During the 60 min. period there was no change in the weight fraction (0.25) of the fast material in the absence of Na⁺; after 2.5 hr. it had fallen to 0.18. A concentration of EDTA-tris that produces a higher weight fraction of fast material (e.g. 0.5 approx.) produces a similar effect; Na⁺ reversibility in this case is not lost for 5 hr. Thus, after 60 min. in the example discussed, Na⁺ will not re-form the single fast component, and it then acts as a simple counter-ion for the two (three) independent species present, with $S_{20,w}$ 23.7 s and (mean) 17.3 s.

Dialysis of the high-molecular-weight RNA fraction for 18 hr. against mM-EDTA-tris (51.) at 3° gives a pattern similar to that with 6 mM-EDTA-tris (Fig. 2c) with a weight fraction in the fast form

Fig. 5. Thermal-denaturation curves of the high-molecular-weight fraction of *E. coli* ribosomal RNA in various buffers: $\circ$, in mm- Mg^{2+} -tris buffer; Δ , after dialysis against tris buffer without Mg^{2+} ; $\bullet$, after dialysis against mm-EDTA-tris buffer; $\blacktriangle$, after dialysis against mm-EDTA-tris buffer followed by 2.5 mm- Mg^{2+} -tris buffer. All denaturation curves were started at about 3°.

about 0.25–0.30. The Na^+ reversibility is lost. Dialysis against fresh EDTA solution for a further 18 hr. at 3° leads to a much smaller loss of fast component, to a weight fraction 0.18–0.20.

Thermal denaturation. That the changes in $S_{20,w}$ described above are probably due to changes in tertiary structure and not to any significant loss of secondary structure is shown in Fig. 5. Thus the total extinction change of 27–29% for all samples suggests that no significant loss of helical structure takes place in EDTA-tris, provided that the temperature is maintained below 5°. A value of 29% was found by Schlessinger (1960) on heating *E. coli* ribosomal RNA at 85°, and 24–27% in 8M-urea. T_m is strongly affected: thus T_m for 28–29S RNA in mm- Mg^{2+} -tris buffer was 69.5°. Overnight dialysis against tris buffer, without Mg^{2+} , decreased this to 49.5°. Overnight dialysis against mm-EDTA-tris at 3° resulted in a further fall to T_m 28.5°, similar to the value obtained (30.0°) on the addition of EDTA-tris to 6 mM (final concn.).

Fig. 5 shows that, in EDTA-tris solution at 40°, about 78% of the extinction change has been lost (in some cases 85%). This is accompanied by a fall in $S_{20,w}$ to about 4S (measured at 40° by u.v.-absorption optics). A similar fall has been observed by Boedtker (1960) with tobacco-mosaic-virus RNA, and by Spirin (1963).

The loss of reversibility to Na^+ (Fig. 4) is not associated with any change in E_{259} at 2°. This was shown by adding EDTA-tris (final concn. 7 mM) to an RNA solution in 2.5 mm- Mg^{2+} -tris buffer in a

Fig. 6. Optical-rotatory-dispersion spectrum of the high-molecular-weight fraction of *E. coli* ribosomal RNA. $\bullet$, RNA in mm- Mg^{2+} -tris buffer; Δ , RNA in 10 mm- Mg^{2+} -tris buffer; $\circ$, RNA in EDTA-tris (final concn. 3 mM).

spectrophotometer cell at 2°. The E_{259} was measured at 10 min. intervals, and remained constant over a 2 hr. period (0.428, s.d. $\pm$ 0.001). In a control with RNA in 2.5 mm- Mg^{2+} E_{259} was constant over the same period (0.417, s.d. $\pm$ 0.003).

Thus, if it is accepted that changes in the u.v. extinction of RNA are associated with changes in its helical content, then the experiments described above do not involve any loss of secondary structure in the molecule.

Optical rotatory dispersion. Measurements of optical rotatory dispersion were made at 5°, and showed the characteristic nucleic acid pattern (Fasman, Lindblow & Seaman, 1965; Cheng, 1965; Samejima & Yang, 1964). It was not possible to obtain the pattern at wave numbers greater than about 44 000 cm^{-1} in the presence of Mg^{2+} and greater than about 40 000–41 000 cm^{-1} in the presence of EDTA-tris owing to the low transmission of light (7 mM-EDTA-tris starts to absorb strongly at about 40 000 cm^{-1}). With EDTA-tris, then, wave numbers greater than about 39 000 cm^{-1} are suspect. The optical-rotatory-dispersion spectrum was recorded from 22 000 cm^{-1} upwards, and shows the usual positive Cotton effect, a positive peak at 36 000 cm^{-1} and a negative trough at 40 000 cm^{-1} .

The result of adding Mg^{2+} (final concn. 10 mM) or EDTA (final concn. 3 mM) to a solution of RNA in mm- Mg^{2+} -tris buffer is shown in Fig. 6. There is no change in the position of the positive peak or the negative trough of the Cotton effect, but it is noted that with decreasing Mg^{2+} concentration: (a) there is a decrease in the peak-to-trough height of the Cotton effect, from $[\alpha]_D^5$ (total) 8350 in 10 mM- Mg^{2+} to 7750 in EDTA-tris; (b) there is a shift of the

Cotton effect in this region to more positive rotations. The structural significance of these observations is not clear. It is not likely that the decrease in the peak-to-trough height observed between mM-Mg²⁺ and 3 mM-EDTA represents a loss of secondary helical structure since: (a) a similar change occurs on transfer from 10 mM-Mg²⁺, which does not involve a change in extinction (and therefore secondary structure) (Cox & Littauer, 1962); (b) no change in hypochromicity is associated with this change in solvent at 2° (Fig. 5); (c) a comparable change has been noted with DNA (Cheng, 1965), whose helical structure is more stable than RNA. It is possible that optical rotatory dispersion measures all changes in structure, secondary and tertiary. Another possibility is that the sensitivity of optical-rotation measurements in this region is much greater than extinction measurements to changes in secondary structure. No explanation is offered for the shift in rotation in a more positive direction on removal of Mg²⁺; a similar phenomenon can be seen in the optical-rotatory-dispersion spectra of DNA obtained by Cheng (1965). The changes observed, then, do not seem to be connected specifically with changes in RNA structure, since they take place in DNA also.

DISCUSSION

Theoretical and experimental studies of ion distribution near polyelectrolytes (Harris & Rice, 1954*a,b*; Osawa, Imai & Kagawa, 1954) suggest that Mg²⁺ may be envisaged as binding to RNA in several ways: (a) as a non-specific counter-ion, owing to the large electrostatic field of the RNA on the ions in its vicinity ('ionic-atmosphere'-binding); (b) by specific association between counter-ions and the fixed phosphate groups along the polynucleotide chain: this association leads to ion-pair formation (Gross & Strauss, 1964; see Strauss & Leung, 1965) and loss of the hydration shell of the ion ('site-binding') (Strauss & Leung, 1965); (c) binding to adenine or guanine, which supply groups suitable for chelation (Zubay, 1959): this binding is strongest in the unpaired parts of the polynucleotide chain, (cf. denatured DNA); this may be regarded as a type of 'site-binding'; (d) formation of 'sandwich' complexes of the ferrocene type (Woodward, Rosenblum & Whiting, 1952) with a pair of bases (Wacker & Vallee, 1959).

It is not possible at present to apportion quantitatively the Mg²⁺ bound to RNA between different sites in this system; however, some qualitative statements may be made. That the $S_{20,w}$ of the faster component is constant over the range 2–10 mM-EDTA-tris, when the ratio of the faster to the slower material is changing, suggests that the fall in $S_{20,w}$ of the high-molecular-weight RNA

from 28.8s to 20.2s (Fig. 2) represents a polyelectrolyte expansion on removal of loosely bound ('ionic-atmosphere') Mg²⁺ from the molecule. The formation of a more slowly moving boundary in a proportion dependent on the EDTA-tris concentration suggests that further Mg²⁺ ('site-bound') is removed to form the new species and a further expansion of the molecule takes place, $S_{20,w}$ falling to 15–16s (Figs. 2*b* and 2*c*). The sites at which this Mg²⁺ is bound may be either particular phosphate groups, or chelation to adenine or guanine or both. 'Site-bound' metal ions have been proposed in DNA (Ross & Scruggs, 1964*a,b*). Ferrocene-type complexes have been associated (Wacker & Vallee, 1959) with metal ions that are very firmly bound. This second expansion would be facilitated if some of the Mg²⁺ bound at these sites provided cross-linkages between different parts of the polynucleotide chain. The importance of the conformation of the RNA for the removal of this 'site-bound' Mg²⁺ is illustrated in Fig. 3, which shows that the slowly sedimenting 15–16s material is formed only when the molecule is in the extended state, at low effective ionic strength.

By schlieren optics (Figs. 1*b* and 1*c*) the new component is indistinguishable from the true 16s RNA (mol.wt. 0.55×10^6) in sedimentation-velocity experiments. A comparison of the $S_{20,w}$ changes for these species suggests that the true 16s RNA may contain only 'ionic-atmosphere'-bound Mg²⁺, and hence that Mg²⁺ may not play an important part in its internal structure. There is good evidence that the 30s and 50s ribosomes from which the low-molecular-weight and high-molecular-weight ribosomal RNA fractions originate differ in several properties (Tal & Elson, 1959; Aronson, 1962; Takanami & Zubay, 1964).

The addition of sodium chloride (final concn. 0.1M) immediately after the EDTA-tris leads to recovery of the faster component (Figs. 1*d* and 2*d*), although $S_{20,w}$ is lower (26–27s compared with 28–29s). A reciprocal relationship between Mg²⁺ and Na⁺ has been noted by Boedtker (1960) for tobacco-mosaic-virus RNA. The Na⁺ may also cross-link phosphate groups in different parts of polyphosphate chains (Strauss & Ross, 1959).

The slow irreversible (in 0.1M-sodium chloride) change that takes place if sodium chloride is added later, or if the RNA solution is dialysed, may be due to a further slow loss of Mg²⁺ from 'site-bound', possibly cross-linking, positions or an internal molecular rearrangement, or both. If a molecular rearrangement takes place it has only a small effect on the overall shape of the molecule since $S_{20,w}$ changes only slightly (from 15.8s to 14.8s in the absence of sodium chloride, and from 20.0s to 17.3s in 0.1M-sodium chloride). An internal rearrangement has been suggested by Boedtker (1960) to

account for a small change (from 26.5 s to 28.3 s) in sedimentation coefficient that occurred during the thermal denaturation of tobacco-mosaic-virus RNA. Instability in microsomal RNA has also been observed by Hall & Doty (1959).

The implication of metal ions other than Mg^{2+} in this irreversible change is unlikely since A.R. grade reagents were used, but trace metal ions, possibly from bentonite, cannot be excluded. Hydrolysis of cross-linkages in the form of covalent branches is also unlikely since no unambiguous evidence for such cross-linkages has yet appeared (Michelson, 1963). The special type of end-to-end bond proposed by Midgeley (1965) is also unlikely, since rupture of this bond leads to the formation of two 16s molecules (mol.wt. 0.55×10^6).

A further important possibility is that these reactions are due to nuclease activity, known to be stimulated by EDTA; several pieces of evidence make this unlikely, however.

(a) No non-sedimentable u.v.-absorbing material is detected at the meniscus in the ultracentrifuge after the main boundaries have sedimented, in the presence of EDTA.

(b) The initial reaction forming the slow material (15–16s) occurs rapidly and depends on a low ionic strength (Fig. 3). Adding sodium chloride (final concn. 0.1 M), potassium chloride (final concn. 0.1 M) or ammonium chloride (final concn. 0.1 M) or increasing the EDTA concentration (and hence the concentration of the tris cation) inhibits the reaction: thus 20 mM-EDTA causes partial inhibition and 50 mM-EDTA complete inhibition, and the slow material is not produced in the latter case. If formation of the new boundary is due to EDTA-activated nuclease action, then the enzyme is stimulated by 2–10 mM-EDTA, partially inhibited by 20 mM-EDTA and completely inhibited by 50 mM-EDTA or by 0.1 M-sodium chloride, 0.1 M-potassium chloride or 0.1 M-ammonium chloride. This effect has not been observed with purified nucleases.

(c) The reaction forming the slow material is reversible by 0.1 M- Na^+ , 0.1 M- NH_4^+ etc., i.e. the products of the reaction are 'reassociated' by these ions. The ability of these ions to reverse the reaction is lost with time (Fig. 4): the stabilizing influence is not lost after the reversal has taken place. A higher concentration of Na^+ (0.8 M) will cause a small (10%) partial reversal of the reaction, even when 0.1 M-sodium chloride has no effect (after 60 min.).

These effects of ions in this system may be compared with the known effects of ions in ribonuclease I (yielding nucleoside 3'-monophosphates) and ribonuclease II (yielding nucleoside 5'-monophosphates) systems. Effects in these systems have been established by, e.g., Spahr & Hollingworth (1961), Spahr (1964) and Singer & Tolbert (1965). Ribo-

nuclease I is strongly inhibited by Mg^{2+} (hence the EDTA activation), but strongly stimulated by Na^+ or K^+ . Ribonuclease II is strongly stimulated by Mg^{2+} and K^+ or NH_4^+ ; Na^+ and Li^+ have no effect. The present reactions are strongly inhibited by Na^+ , K^+ , NH_4^+ or tris cation at concentrations that give maximal stimulation of ribonuclease I or II. The present reactions are also initially reversible. These data show that the effect of metal ions in this system has no parallel with the known effects of the same ions in ribonuclease I or II systems, and that the data cannot be simply interpreted in terms of nuclease action.

(d) An analogous formation of a slow boundary from the fast form in the absence of Mg^{2+} may be seen in the diagrams of Cammack & Wade (1965), using 50s ribosomes from *Pseudomonas fluorescens*, which does not contain nucleases [Fig. 5(b) (iii) in that paper].

Data presented in Fig. 5 suggest that no changes in the secondary structure of the molecule take place on the addition of EDTA-tris when the temperature is maintained at less than 5°; thus the changes in $S_{20,w}$ refer to the tertiary structure only. Similarly, no detectable change in the extinction of the solution during the loss of Na^+ reversibility occurred. The changes in the optical-rotatory-dispersion spectrum in the region of the Cotton effect at 36 000 and 40 000 cm^{-1} is therefore difficult to explain; presumably the optical-rotatory-dispersion spectrum in this region reflects changes in the 'total conformation', in this case in the tertiary structure, and not simply the secondary structure. Lewin (1965) has proposed that all conformational changes are detected by optical rotatory dispersion, as distinct from changes in $E_{259-260}$, which are specific for base-pairing. An alternative explanation is that optical rotatory dispersion is much more sensitive to changes in secondary structure than are extinction measurements.

The effect of ions on this system suggests that simple removal of Mg^{2+} does not cause dissociation of the RNA. There is conflicting evidence whether the high-molecular-weight (1.07×10^6) fraction can be dissociated at all (Hall & Doty, 1959; Boedtker, Möller & Klemperer, 1962; Petermann & Pavlovic, 1963; Spirin, 1963; Midgeley, 1965; Stanley & Bock, 1965). However, Mg^{2+} may cause some reversible aggregation of the slow (mol.wt. 0.55×10^6) to the fast (mol.wt. 1.07×10^6) RNA since the proportion of slow to fast material was less than the expected 1:2 ratio; a 1:3 ratio was reproducibly obtained. The difference may be accounted for by assuming that about 10% of the slow material is converted into fast material. This value is consistent with the fact that the immediate Na^+ reversibility of the EDTA-tris-induced change in $S_{20,w}$ is to only 90% of the original proportion of fast material.

Apart from the heterogeneity between Mg²⁺-binding sites on the same RNA molecule proposed, some evidence has been presented for heterogeneity between different high-molecular-weight RNA molecules. Heterogeneity in this respect is noteworthy since it suggests that ribosomes (i.e. the 50s ribosomes) are not identical in all respects. This need not imply differences in nucleotide sequence between RNA molecules since binding specificity may arise as a result of steric factors. It would be difficult to reconcile heterogeneity in ribosomes with the current view of the 'messenger' hypothesis although the 'faceless' ribosome is not an absolute requirement of this hypothesis.

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