Physical Properties of Ribosomal Ribonucleic Acid Isolated from Bacteria Deficient in Ribonuclease I

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1. Bacteria deficient in ribonuclease I were used as a source of stable ribosomal RNA. RNA was isolated from a ribosome fraction of *Pseudomonas fluorescens* N.C.I.B. 8248 and acetone-treated cells of *Escherichia coli* M.R.E. 600 by the method developed by Robinson & Wade (1968). 2. The $s_{20,w}$ of the 16S and 23S components can vary from 21S and 28S down to 4S depending on the RNA macro-ion concentration and the extent to which charge is suppressed by univalent Na^+ and tris^+ counter-ions or neutralized through the binding of bivalent Mg^{2+} to phosphate groups. 3. The primary charge effect in sedimentation and the frictional coefficient (which increases as the molecular conformation expands) both increase with charge and cause a decrease in s value. 4. RNA solutions heated to 80°C for 10 min show minor changes in s value and a detectable increase in polydispersity. Millimolar concentrations of Mg^{2+} promote heat-instability and so does treatment of RNA solutions with the nuclease adsorbent macaloid, which was found to contaminate the solutions with Mg^{2+} . 5. The stabilization of secondary structure by univalent and bivalent cations was investigated by optical methods. 6. The sedimentation properties of 30S and 50S ribosomal subunits and their constituent 16S and 23S RNA components were compared and discussed from the viewpoint of unfolding.

The physical stability and molecular conformation of the 30S and 50S subunits of ribosomes and their 16S and 23S RNA constituents are dependent on the ionic composition of the aqueous solvent. Bivalent cations such as Mg^{2+} and Ca^{2+} bind very strongly to RNA phosphate groups (Goldberg, 1966; Choi & Carr, 1967) and since the major part of RNA in the ribosome is also accessible to dyes it may be inferred that this RNA is located on the surface (Cotter, McPhie & Gratzer, 1967). Therefore the response of ribosomes and RNA to changes in the ionic environment prove to be very similar.

Partially reversible unfolding of the normally compact ribonucleoprotein strand constituting a 30S or 50S ribosomal subunit, when stabilizing Mg^{2+} cations are removed from solutions in 0.01–0.15M-salt, is a prime example of this dependence on ionic conditions (Spirin, Kisselev, Shakulov & Bogdanov, 1963; Cammack & Wade, 1965; Gesteland, 1966; Gavrilova, Ivanov & Spirin, 1966; Weller, Schechter, Musgrave, Rougvie & Horowitz, 1968). Another aspect is the tendency shown by subunits to undergo a stepwise dissociation in 3.0–5.0M-caesium chloride or -lithium chloride, losing part of their protein with conversion

of the 30S and 50S subunits into 23S and 42S ribonucleoprotein core particles of higher density and relatively higher RNA content (Meselson, Nomura, Brenner, Davern & Schlessinger, 1964; Lerman, Spirin, Gavrilova & Golov, 1966; Hosokawa, Fujimura & Nomura, 1966; Staehelin & Meselson, 1966). A complete dissociation of 30S subunits into 16S RNA and ribosomal protein fractions, followed by reassembly into particles which are again active in protein synthesis *in vitro*, is reported by Traub & Nomura (1968).

Although the characterization of the two RNA species has progressed well since the work of Kurland (1960), some of their physical properties are ill-defined and to some extent controversial (McIlreavy & Midgley, 1967). Partly responsible are the difficulties met in the non-ideal sedimentation of RNA and the early history of degradation through contamination by nucleases (Spirin, 1963; Stanley & Bock, 1965; Boedtker, 1968).

The extraction of the highly polymerized RNA from selected strains of bacteria that are deficient in ribonuclease I (Elson, 1961; Cammack & Wade, 1965; Gesteland, 1966) is a sensible precaution to take to minimize any possibility of degradation

but not absolutely essential. Much of the early success in isolating infective viral RNA is directly attributable to the use of bentonite as a nuclease adsorbent (Brownhill, Jones & Stacey, 1959); polyvinyl sulphate has also been used as an enzyme inhibitor (Boedtke, Möller & Klemperer, 1962); but the most successful work reported on undegraded and highly stable RNA is that by Stanley & Bock (1965), who used macaloid in place of bentonite. It is widely accepted that any spontaneous breakdown of rRNA into smaller subunits or fragments arises from nuclease contamination, and is not evidence of a hydrogen-bonded subunit structure for RNA (Bogdanova, Gavrilova, Dvorkin, Kisselev & Spirin, 1962; Spirin, 1963; Stanley & Bock, 1965; Boedtke, 1968).

The methods used here for isolating RNA from ribosomes of *Pseudomonas fluorescens* N.C.I.B. 8248 and *Escherichia coli* M.R.E. 600 are based on phenol (Kirby, 1965), but the large-scale purification method (Robinson & Wade, 1968) applied to *E. coli* M.R.E. 600 uses whole cells that are given an initial treatment with acetone, and the tedious procedure of purifying the ribosome fraction is eliminated. Both methods produce material that satisfies generally acceptable criteria of physical purity and stability. A fairly comprehensive programme of study in the analytical ultracentrifuge has produced a wide range of sedimentation coefficients in relation to the amount of Mg^{2+} bound to RNA, ionic-strength factors and the total concentration of RNA in solution. The stability of RNA solutions heated to 80°C was investigated in the ultracentrifuge to assess to what extent a sample may be degraded in plotting absorption-temperature profiles in the u.v. and in estimating 'melting' temperatures (T_m). In conclusion, the unfolding of 30S and 50S ribonucleoprotein strands is discussed from the viewpoint of secondary and tertiary structural changes in the molecular conformation of RNA.

MATERIALS AND METHODS

Material. A mixture of 30S and 50S ribosomal subunits was freshly prepared from a culture of *Ps. fluorescens* N.C.I.B. 8248 (Cammack & Wade, 1965) and stored overnight as pellets at 4°C. Resuspension in a solution containing 0.15M-NaCl and 5.0mM-MgCl₂ and examination in the ultracentrifuge showed the 70S ribosomes were completely dissociated into their subunits [e.g. Fig. 5b(i) in Cammack & Wade (1965)]. From 20g wet wt. of cells disintegrated in a Hughes press the yield of ribosomes was approx. 3g wet wt. or 1g dry wt. Sample RNA-9 was isolated from the ribosomes by method 2 described by Kirby (1965). The product was washed in 75% (v/v) ethanol before drying in a vacuum desiccator and storing at -20°C. The yield was approx. 480mg.

A larger sample, RNA-11, was extracted directly from

whole cells of *E. coli* M.R.E. 600 after prior treatment with chilled acetone. Fractionation of the crude bacterial RNA follows a procedure similar to the Kirby method and the yields are comparable (Robinson & Wade, 1968). From 1.0kg of acetone-treated cells a total of 23g of dried rRNA was obtained and stored at -20°C.

Precautions taken to preserve the stability of RNA solutions. As ribonuclease-deficient bacteria were used to prepare rRNA, the usual nuclease adsorbents, bentonite and macaloid, were omitted; nevertheless, the products were clearly susceptible to nuclease contamination from other sources. Wherever practical an aseptic technique was adopted. All the procedures outlined by Stanley & Bock (1965) are equally applicable here; these include protection of solutions from direct hand contact by the wearing of surgical gloves and the boiling of dialysis membranes in 0.01M-EDTA adjusted to pH 7.4. Diffusates after dialysis were checked for any u.v.-absorbing material derived from the breakdown of RNA.

Determination of 'free' Mg^{2+} and Mg^{2+} 'bound' to RNA. It is essential to state whether RNA solutions are dialysed to equilibrium or simply made up in the solvent since more Mg^{2+} is bound to the macro-ion after dialysis. The Mg^{2+} content of the diffusate and dialysed RNA solution was measured in an atomic-absorption spectrophotometer (Varian Techtron model AA-4, Melbourne, Vic., Australia). A practical detection limit (absorption 0.01) was found to be 0.02 μ g of Mg^{2+} /ml. Interference from RNA (4mg/ml) and 0.01M-sodium phosphate was checked and found to be negligible. Calculation of 'bound' Mg^{2+} was based on the difference in the results obtained for 'free plus bound' in the dialysis sac and 'free' in the diffusate (cf. Goldberg, 1966).

Sedimentation analysis. The methods used to measure the sedimentation coefficients with schlieren and u.v. optics in the Beckman Spinco model E ultracentrifuge are described in Cammack & Wade (1965). A partial specific volume of 0.53 (Kurland, 1960) was used for the buoyancy corrections. The RTIC unit was set to control the rotor temperature at 25.0 $\pm$ 0.05°C. To convert schlieren peak areas into RNA concentrations a specific refractive increment of 0.162g⁻¹ml was used. This was measured in a Brice-Phoenix differential refractometer for 546nm mercury green light.

Separation of 16S and 23S RNA components by zonal ultracentrifugation. A Beckman L4 zonal ultracentrifuge together with a standard B-IV rotor were used to obtain pure fractions of the 16S and 23S RNA (Hastings, Parish, Kirby & Klucis, 1966). Separations were carried out at room temperature (18-22°C) and the rotor was run at its maximum speed of 40000 rev./min for times ranging from 3.8 to 5.3h. The physical stability of samples of RNA-11 was checked in solutions containing 0.15M-NaCl, 5.0mM-MgCl₂ and 20% (w/v) sucrose (A.R.) and the samples were left for 3 days at room temperature before examination in the ultracentrifuge. Freshly prepared sucrose solutions were delivered from the gradient pump into the rotor spinning at 4000 rev./min to produce linear-with-radius gradients (5-20%, w/v). A 'cushion' of 250ml of 30% (w/v) sucrose was used at the periphery of the rotor. Sample solutions applied to the gradient contained 52-95mg of RNA in 50ml of solvent containing 2.5% (w/v) of sucrose and were followed by an overlay of 150ml of solvent.

After high-speed centrifugation the rotor contents were displaced at 4000 rev./min by pumping in 30% (w/v) sucrose and scanned in a Beckman DB spectrophotometer at 280–295 nm before collection in 10 ml fractions. Approx. 85–90% of the RNA was recovered from the rotor. A complete resolution of the 16S and 23S RNA was not obtained and approx. 15% was lost in selecting homogeneous fractions (see the Results and Discussion section, Fig. 5). They were recovered by precipitation with acetone (50%, v/v) at 4°C and kept for 6 h before centrifugation for 10 min at 6000g. The pellets of RNA were washed with 75% (v/v) ethanol before they were vacuum-dried and stored at –20°C.

Absorption measurements in the u.v. RNA concentrations were based on measurements of the extinction at 260 nm in a Unicam SP. 500 or Beckman DB spectrophotometer. The extinction coefficient (260 nm, 1 cm cell, 10 mg of RNA/ml) in unbuffered 0.15 M-NaCl or 0.02 M-tris-HCl buffer, pH 7.4, containing 0.1 M KCl was 207, a lower value than that (223) reported by Stanley & Bock (1965) in tris buffer. The molar extinction of RNA based on its phosphorus content is ϵ_p 7920.

Absorption as a function of temperature was measured in the Unicam SP.500 instrument fitted with a modified cell chamber that could be heated or cooled in the temperature range 10–95°C by circulating fluid from a temperature-controlled bath. Water-cooled thermospacers were added to protect the instrument. The silica cells (Thermal Syndicate Ltd., Wallsend, Northumberland, U.K.) were sealed by polytetrafluoroethylene stoppers and a self-curing rubber preparation (Silcoset, ICI). A therm-

istor probe was inserted into the reference cell and the temperature could be read off from a direct-reading telethermometer (model 42SC; Yellow Springs Instrument Co. Inc., Yellow Springs, Ohio, U.S.A.). Helium gas was bubbled through the experimental solution in ice for 20–30 min to displace dissolved air before heating (Felsenfeld & Sandeen, 1962).

Optical rotatory dispersion. Optical-rotatory-dispersion curves were plotted in a Bendix Polaromatic 62 spectropolarimeter in the wavelength range 220–320 nm by using 1 cm cells and RNA solutions of E 1.00–1.03 at 260 nm. Some were repeated in the FICA Spectropol 1b (Applied Research Laboratories, Luton, Beds., U.K.). The temperature was not accurately controlled near room temperature, although in heating experiments up to a maximum of 70°C the error was not greater than $\pm 1^\circ\text{C}$.

The specific rotation $[\alpha]_\lambda = \frac{\alpha_\lambda}{lc}$ is calculated, where α_λ is the measured rotation in degrees at wavelength λ nm, c is the concentration in g/ml and l the optical path in dm. Mean residue-weight molar rotations, $[m]_\lambda$ can be calculated from $[m]_\lambda = 3.17[\alpha]_\lambda$.

RESULTS AND DISCUSSION

Stability of RNA solutions and the effect of dialysis. RNA-9 (*Ps. fluorescens* N.C.I.B. 8248) and RNA-11 (*E. coli* M.R.E. 600) resolved into well defined 16S and 23S peaks in the ultracentrifuge (Figs. 1a and 2a). A continuous dialysis of RNA-9 for 2 weeks

Fig. 1. Stability of *Ps. fluorescens* rRNA (RNA-9) in 0.15 M-NaCl containing 5.0 mM-MgCl₂. Sedimentation diagrams were recorded after centrifugation for 26 min at 47 660 rev./min with the phase-plate set at 60°. The initial RNA concentration was approx. 4.2 mg/ml. $s_{20,w}$ values are indicated. (a) Undialysed solution after standing for 1 h at room temperature (18–22°C); (b) undialysed solution after storage for 14 days at 4°C; (c) solution dialysed for 14 days at 4°C.

Fig. 2. Stability of *E. coli* M.R.E. 600 rRNA (RNA-11) in 0.15 M-NaCl containing 5.0 mM-MgCl₂. Sedimentation diagrams were recorded after centrifugation for 26 min at 47 660 rev./min with the phase-plate set at 60°. The initial RNA concentration was approx. 4.4 mg/ml. $s_{20,w}$ values are indicated. (a) Undialysed solution after standing for 1 h at room temperature (18–22°C); (b) undialysed solution after storage for 15 days at 4°C; (c) solutions dialysed for 14 days at 4°C.

Fig. 3. Sedimentation coefficients of 16S(Δ) and 23S($\circ$) components of *Ps. fluorescens* rRNA (RNA-9) as a function of the total unfractionated RNA concentration in 0.15 M-NaCl containing 5.0 mM-MgCl₂.

Fig. 4. Sedimentation coefficients of 16S(Δ , $\blacktriangle$) and 23S($\circ$, $\bullet$) components of *E. coli* M.R.E. 600 rRNA (RNA-11) as a function of the total unfractionated RNA concentration in 0.15 M-NaCl containing 5.0 mM-MgCl₂ ($\circ$, Δ) and in 0.5 M-NaCl ($\bullet$, $\blacktriangle$).

in a solution containing 0.15 M-sodium chloride and 5.0 mM-magnesium chloride produced no visible effect on the peaks, but the slight increase in the amount of Mg²⁺ bound to RNA caused the s values to increase (Figs. 1b and 1c). RNA-11 tended to aggregate on dialysis and a new component, of $s_{20,w}$ 30.9S ($s_{20,w}^0$ 33.6S), was formed (Figs. 2b and 2c). Aggregation of RNA is frequently observed and is clearly linked to the neutralization of charge (Marcot-Queiroz & Monier, 1965; Taylor, Glasgow & Storek, 1967). Material that had been stored at -20°C for two years gave similar s values though the schlieren peaks were more diffuse. Polyacrylamide-gel electrophoresis of this same material produced very sharp 16S and 23S bands

and confirmed the absence of degradation products (Dr M. Spencer, Medical Research Council Biophysics Unit, King's College, London W.C.2, U.K.).

Dependence of s values on concentration. The sedimentation coefficient is very dependent on the RNA concentration, ionic strength and the extent to which charge is neutralized by bound Mg²⁺. In 0.15 M-sodium chloride containing 5.0 mM-magnesium chloride a good linear relationship of s value with the total concentration of unfractionated RNA was found with no evidence suggesting an inversion in the plots of s versus c at less than 2 mg/ml (Figs. 3 and 4; cf. Stanley & Bock, 1965). The statistical regression equations and the 95% fiducial limits, given in parentheses, for the intercept at $c = 0$ ($s_{20,w}^0$) and the slope are: RNA-9 (Fig. 3), $s_{20,w} = 19.18_3 (0.775) - 0.502 (0.089)c$ and $s_{20,w} = 26.20_1 (0.813) - 1.118 (0.094)c$; similarly, for RNA-11 (Fig. 4), $s_{20,w} = 19.38_3 (0.629) - 0.741 (0.062)c$ and $s_{20,w} = 25.72_4 (1.828) - 1.217 (0.194)c$.

In Fig. 4 it is evident that 0.5 M-sodium chloride in the complete absence of Mg²⁺ was more effective than 0.15 M-sodium chloride containing 5.0 mM-Mg²⁺ in suppressing the dependence of s on c and the regression equations are:

$$s_{20,w} = 18.36_0 (0.821) - 0.258 (0.076)c$$

and

$$s_{20,w} = 25.72_4 (1.828) - 1.217 (0.194)c$$

This solvent condition is very similar to that used by Spirin *et al.* (1963), who found that ribosomal subunits could be stabilized in the absence of Mg²⁺ at high ionic strength in 0.5 M-ammonium chloride. Measurements on very dilute RNA solutions (50–60 μ g/ml, of E 0.8–1.5 at 260 nm) gave s values that agreed well with the extrapolated $s_{20,w}^0$ values derived from the regression lines in Fig. 3 and Fig. 4.

Pure 16S and 23S RNA fractions from the zonal ultracentrifuge (Fig. 5) proved to be 90–95% homogeneous in the analytical ultracentrifuge. Results pooled from three preparative runs were used to measure s versus c at different concentrations (Fig. 6) for individual components. The regression equations are: $s_{20,w} = 18.40_7 (4.896) - 0.550 (1.044)c$ and $s_{20,w} = 25.10_2 (1.794) - 1.028 (0.218)c$.

Extent of the Johnston-Ogston error in measuring the 23S RNA/16S RNA ratio. Since the s value of RNA is so dependent on concentration it means that the ratio of the areas for the 'fast' and 'slow' peaks cannot provide an accurate estimate of the 23S RNA/16S RNA ratio unless the 'Johnston-Ogston error' is corrected (Trautman, Schumaker, Harrington & Schachman, 1954; Schachman, 1959; Midgley, 1965a; Petermann & Pavlovic, 1966). Apparent 23S RNA/16S RNA ratios based on the

Fig. 5. Separation of *E. coli* 16S and 23S RNA in the zonal ultracentrifuge in 0.15 M-NaCl with 0–20% (w/v) sucrose as density gradient. The B-IV rotor was spun at 40000 rev./min for 4.5 h at 18°C. —, E_{260} profile versus rotor volume; — — —, sucrose concentration versus rotor volume; ·····, resolution of profile into 16S and 23S RNA components.

Fig. 6. Sedimentation coefficients of *E. coli* M.R.E. 600 rRNA (16S and 23S RNA fractions isolated in the zonal ultracentrifuge in 0.15 M-NaCl containing 5.0 mM-MgCl₂) as a function of RNA concentration. Zonal runs: ○, ZU/4; △, ZU/5; □, ZU/6.

Fig. 7. Magnitude of the 'Johnston-Ogston error' at different RNA concentrations in attempting to measure the 23S RNA/16S RNA ratio from schlieren diagrams resolved in ultracentrifugation. ×, *Ps. fluorescens* RNA-9 stored dry for 3 months at -20°C and dissolved in 0.15 M-NaCl containing 5.0 mM-MgCl₂; ●, *E. coli* M.R.E. 600 RNA-11 stored dry for 5 months at -20°C and dissolved in 0.15 M-NaCl containing 5.0 mM-MgCl₂; ○, *E. coli* M.R.E. 600 RNA-11 in 0.15 M-NaCl.

measurement of the relative areas in schlieren diagrams after correction for sectorial dilution during the run are plotted as a function of the total RNA concentration (Fig. 7). Extrapolation of the apparent ratio to zero concentration provides a rather crude method of eliminating the 'Johnston-Ogston error' but a better procedure is to analyse

the extinction profile from a zonal-ultracentrifuge run (Fig. 5).

The value of 2:1 commonly quoted for the 23S RNA/16S RNA ratio is based on the following suppositions: (i) equimolecular amounts of 30S and 50S are produced by the cell; (ii) the 16S and 23S RNA species are derived quite independently from the respective 30S and 50S ribosomal subunits (Kurland, 1960; Green & Hall, 1961); (iii) the molecular-weight ratio for 23S RNA and 16S RNA is 2:1 (Kurland, 1960; Stanley & Bock, 1965). Each is questioned and discussed by Midgley (1965*a,b,c*) and McIlreavy & Midgley (1967), mainly on the basis of chemical evidence.

For ribosomes extracted from *Ps. fluorescens* the 50S subunit/30S subunit ratio 3.5:1 proved to be unusually high and might in fact indicate some loss of 30S material during washing of the mixed ribosome pellet (cf. Cammack & Wade, 1965). The 23S RNA/16S RNA ratio for RNA-9 derived from this ribosome fraction was 3:1. Ribosomes from *E. coli* M.R.E. 600 had a 'normal' 50S subunit/30S subunit ratio of 2:1 and the 23S RNA/16S RNA ratio was approx. 1.8:1. Thus, for both bacteria, there exists marginal evidence of slightly more 16S RNA being extracted from a mixture of ribosomal subunits than would be expected from the premises outlined in (ii) and (iii); however, there is no evidence of a major conversion of 23S RNA into 16S RNA (cf. McIlreavy & Midgley, 1967).

Polyelectrolyte character of RNA in media of low ionic strength. RNA is very sensitive to small amounts of bivalent cations (Lindahl, 1967) and chelating agents are used as a routine to remove these contaminants. Both RNA-9 and RNA-11 were recovered as sodium salts and contained less than a single Mg^{2+} cation bound to each polynucleotide strand (1500–3000 nucleotide residues).

Sedimentation analysis of RNA (4–8 mg/ml) dissolved in 0.01 M-tris-HCl buffer, pH 7.4, revealed a single sharp boundary of 4S; addition of 0.15 M-sodium chloride resulted in a marked decrease in

viscosity (cf. Cox, 1960) and in sedimentation the reappearance of the 16S and 23S boundaries. Very dilute solutions (50–60 μ g/ml) in 0.01 M-tris examined by u.v.-absorption optics also produced a 'two-component' boundary system, although the s values were somewhat depressed to 13S and 17S. At very low ionic strength, as in 0.1 mM-tris, the 'two-component' boundary system was transformed into a single boundary of $s_{20,w}^0$ 9.0S whereas in deionized water this value was decreased to $s_{20,w}^0$ 4.0S.

This type of reversible sedimentation behaviour is typical of a single-stranded RNA molecule and is due to the dependence of hydrodynamic shape (secondary and tertiary structure) and the magnitude of charge effects in sedimentation on the net effective charge (Littauer & Eisenberg, 1959; Cox & Littauer, 1959, 1962; Spirin, 1963).

Effect of Mg^{2+} binding on the sedimentation behaviour of RNA. At low ionic strength the Mg^{2+} cation binds very strongly to RNA and is therefore much more effective than higher concentrations of univalent counter-ions in suppressing charge. For example, Boedtker (1960) found 25000 times as much Na^+ as Mg^{2+} was needed to stabilize the molecular conformation of tobacco mosaic virus RNA and this difference cannot be explained simply on the basis of ionic strength (cf. Tanford, 1961; Felsenfeld & Miles, 1967).

RNA solutions (3–5 mg/ml) were dialysed to equilibrium in 0.01 M-tris-HCl buffer, pH 7.4, containing increasing amounts of Mg^{2+} up to a maximum concentration of 10 mM. Both the sac contents and the diffusate were analysed for 'free plus bound' and 'free' Mg^{2+} to obtain by difference a value for the 'bound' Mg^{2+} .

The sedimentation diagrams in Figs. 8(a), 8(b), 8(c) and 8(d) illustrate the dramatic nature of the changes observed in the ultracentrifuge as the Mg^{2+} bound to RNA increases. In each experiment the value for 'bound' Mg^{2+} is in excess of that given as

Fig. 8. Sedimentation properties of *E. coli* M.R.E. 600 RNA-11 in 0.01 M-tris-HCl buffer, pH 7.4, as a function of 'free' and 'bound' Mg^{2+} determined after equilibrium dialysis. Sedimentation diagrams were recorded after centrifugation at 47660 rev./min for (a) 140 min; (b) 76 min; (c) 44 min and (d) 29 min. The phase-plate angle was 60° and the RNA concentration was approx. 4 mg/ml. The concentration of RNA phosphate was approx. 13 mM; dialysis against 10–15 mM- $MgCl_2$ in 0.01 M-tris generally caused incipient or total precipitation.

Fig. 9. Sedimentation coefficient of *E. coli* M.R.E. 600 RNA-11 as a function of Mg^{2+} concentration. The solvent was 0.01 M-tris-HCl buffer, pH 7.4; Mg^{2+}/P gives mol of bound Mg^{2+} /mol of rRNA phosphate in the experiments where dialysis equilibrium was used to adjust the solvent condition. ▲, upper curve 23S RNA, lower curve 16S RNA, undialysed, or dialysed runs measured in the u.v. at approx. 50 μ g/ml; ●, upper curve 23S RNA, lower curve 16S RNA, dialysed solutions containing 4 mg/ml; abscissa gives 'free' Mg^{2+} concentration and right-hand ordinate indicates 'bound' as Mg^{2+}/P ratio; ■, upper curve 23S RNA, lower curve 16S RNA, undialysed solutions containing 4 mg/ml; ○, mol of Mg^{2+} bound/mol of rRNA phosphate ratio.

'free'; only the 'bound' Mg^{2+} is truly operative in neutralizing charge at these very low millimolar concentrations and the s value increases accordingly. When the charge on the macro-ion approaches zero, as in Fig. 8(d), 16S RNA and 23S RNA are completely resolved with s values 18.9S and 23.4S. With the gradual removal of 'bound' Mg^{2+} the peaks converge into a single peak sedimenting at about 4.7S (Fig. 8a); examination of the intermediate pictures in Figs. 8(b) and 8(c) shows increasing boundary sharpening and a complete reversal of the 23S RNA/16S RNA ratio as a result of the 'Johnston-Ogston error'.

The s values for RNA in Fig. 9 were determined with schlieren and u.v. optics as a function of the Mg^{2+} concentration in 0.01 M-tris-HCl buffer, pH 7.4. Dialysis is relatively unimportant for dilute solutions (50 μ g/ml) but a distinction between dialysed and undialysed solutions is essential at schlieren concentrations. As the Mg^{2+}/P molar ratio approaches 0.5 (i.e. zero charge) there is good agreement between the schlieren and u.v. results. Experiments beyond this stage were impractical because the RNA began to aggregate and precipitate. The highest s values were 20S (16S) and 29S (23S).

Fig. 10. *E. coli* M.R.E. 600 RNA-11 T_m values from extinction-temperature profiles in aqueous solvents of low and moderate ionic strength as a function of Mg^{2+} concentration. ○, 0.15 M-NaCl including Mg^{2+} concentration indicated; Δ, 0.01 M-tris-HCl buffer, pH 7.4, including Mg^{2+} concentration indicated.

Optical properties of RNA in a changing ionic environment. Measurements of absorption, optical rotation and circular dichroism in the u.v. are, with certain reservations (Gratzer & Cowburn, 1969), a function of the secondary or helical structure of RNA (Doty, Boedtker, Fresco, Haselkorn & Litt, 1959; Spirin, 1963; Samejima & Yang, 1964; Lambourg, Zamecnik, Li, Kagi & Vallee, 1965; Cox, 1965; Bush & Scheraga, 1967; Wolfe, Oikawa & Kay, 1968a,b). Thermal disorganization of helical structure, at neutral pH, occurs at a lower temperature if the ionic strength is decreased and bivalent cations are removed (Boedtker, 1960; Bogdanova *et al.* 1962; Rodgers, 1966). Thus in Fig. 10, the T_m value decreases from 71°C to 41°C, and it is clearly very sensitive to Mg^{2+} in the range 0.01 mM–10 mM (cf. Boedtker, 1960). Conditions that favour high values of s and T_m for rRNA are equally effective in stabilizing ribosomes and their subunits.

In 0.01 M-tris-HCl buffer, pH 7.4, the lowest ionic strength investigated, there was a 2–3% increase in extinction as the temperature was raised from 10°C to 20–25°C; at higher ionic strengths a change was not detectable below 40°C. Therefore any variation in $s_{20,w}^0$ measured with u.v. optics is likely to be the result of a tertiary structural change provided that $I \geq 0.01$ (cf. Rodgers, 1966). At the much higher RNA concentrations required for schlieren optics the essential T_m values are not obtainable. Here the s values are likely to be a more complicated function of both secondary and tertiary structure as well as charge effects (cf. Henley, Lindahl & Fresco, 1966).

Fig. 11. Optical-rotatory-dispersion curves for *E. coli* M.R.E. 600 RNA-11. Solvents: ■, water, 21°C; ○, 0.01 M-tris-HCl buffer, pH 7.4, 26–27°C; ●, 0.01 M-tris-HCl buffer, pH 7.4, containing 10 mM-MgCl₂, 26°C; △, 0.15 M-NaCl with and without 10 mM-MgCl₂, 23°C.

Optical-rotatory-dispersion curves in Fig. 11 were recorded over the range 225–370 nm at 20–25°C. Symmetrical Cotton effects are produced with a cross-over at about λ_0 265 nm, a peak $[\alpha]_{270 \text{ nm}} = +3550^\circ$ and a trough $[\alpha]_{250 \text{ nm}} = -3560^\circ$, for RNA in 0.15 M-sodium chloride, in 0.15 M-sodium chloride containing 10 mM-Mg²⁺, and in 0.01 M-tris-HCl buffer, pH 7.4, containing 10 mM-Mg²⁺. The amplitude of the Cotton effect was decreased by approx. 250° combined with a detectable red-shift in λ_0 to 267 nm when the experimental measurements were made in 0.01 M-tris with no Mg²⁺ added. RNA in water showed a very marked asymmetry in the Cotton effect with a peak $[\alpha]_{284 \text{ nm}} = +2420^\circ$ and a trough $[\alpha]_{257 \text{ nm}} = -4450^\circ$ and a larger red-shift in λ_0 to 273 nm; the amplitude of this displaced Cotton effect was 180° less than that in solvents containing 0.15 M-sodium chloride or 10 mM-Mg²⁺. As expected from the work of Lambourg *et al.* (1965) on *E. coli* tRNA, the heating of RNA in 0.15 M-sodium chloride produced a decrease in the amplitude of the Cotton effect analogous to the hyperchromic effect over the same temperature range.

The experimental optical-rotatory-dispersion curves in the range 187–320 nm produced by samples of RNA of different origin (Samejima & Yang, 1964; Lambourg *et al.* 1965; Bush & Scheraga, 1967; Wolfe *et al.* 1968a) show a marked sensitivity

to heat, ionic strength, pH, organic solvents and high concentrations of urea, which disrupt secondary structure. The results in Fig. 11 for *E. coli* M.R.E. 600 RNA are in substantial agreement with those of Wolfe *et al.* (1968a) and Bush & Scheraga (1967). For $I > 0.01$ the Cotton effect is symmetrical at λ_0 265 nm and its shape is independent of the Mg²⁺ concentration in 0.15 M-sodium chloride. In 0.01 M-tris the addition of 10 mM-Mg²⁺ produced an equivalent profile; in 0.01 M-tris without Mg²⁺, the small red-shift in λ_0 is consistent with the 2–3% increase in extinction at 260 nm at 25°C. Larger red-shifts coupled with a pronounced asymmetry of the Cotton effect may be an indication of the breakdown of helical structure (Wolfe *et al.* 1968a). A very intense circular-dichroism band at 187 nm is very sensitive to ionic strength (Wolfe, *et al.* 1968b), and if confirmed would be very useful in further studies on the molecular conformation of RNA.

Observations on thermal stability. Unfortunately the methods adopted for the recording of extinction-temperature profiles vary a great deal, and the extent to which the sample may be degraded is often uncertain. The time factor is important. Heating to 80°C and cooling slowly to room temperature may take as long as 90 min and in the case of RNA-11 caused severe breakdown of the 16S and 23S RNA species into a polydisperse material

Fig. 12. Thermal stability of *E. coli* M.R.E. 600 rRNA (3 mg/ml) in 0.01 M-tris-HCl buffer, pH 7.4, containing 0.15 M-NaCl with Mg^{2+} absent. Sedimentation diagrams were recorded after centrifugation for 26 min at 47660 rev./min with the phase-plate set at 60°. $s_{20,w}$ values are indicated. (a) Solution aged for 24 h at 4°C before examination at 25°C; (b) solution aged for 72 h at 4°C before being heated at 80°C for 10 min; (c) solution aged for 10 days at 4°C before being heated at 80°C for 10 min.

Fig. 13. Effect of Mg^{2+} on the thermal stability of *E. coli* M.R.E. 600 rRNA (4.5 mg/ml) in 0.01 M-tris-HCl buffer, pH 7.4, containing 0.15 M-NaCl. Sedimentation diagrams were recorded after centrifugation for 26 min at 47660 rev./min with the phase-plate set at 60°. $s_{20,w}$ values are indicated and expected to be higher when Mg^{2+} is present. 'Upper trace' shows the RNA solution containing 5.0 mM- $MgCl_2$ after being heated at 80°C for 10 min; 'lower trace' shows the control with no Mg^{2+} after being heated at 80°C for 10 min.

Fig. 14. Effect of treatment with macaloid (0.1%) on the heat-stability of *E. coli* M.R.E. 600 rRNA (3.0 mg/ml) in 0.01 M-tris-HCl buffer, pH 7.4, containing 0.15 M-NaCl. Sedimentation diagrams were recorded after centrifugation for 26 min at 47660 rev./min with the phase-plate set at 60°. $s_{20,w}$ values are indicated. 'Upper trace' shows the solution treated with macaloid after being heated at 80°C for 10 min; 'lower trace' shows the untreated solution after being heated at 80°C for 10 min.

sedimenting at 5.0–9.0S (mol.wt. 25000–150000). Nevertheless the hyperchromicity was reversible on cooling, thus demonstrating that the short helical segments in rRNA may re-form even though a number of covalent bonds have obviously been broken. Far less damage to RNA occurred when solutions were heated to 80°C within 2 min and cooled after 10 min at 80°C (Figs. 12a, 12b and 12c). The indicated s values are rather low since no Mg^{2+} was included in the solvent. Heating did not affect the s values very much but a significant increase in polydispersity is visible in the sedimentation diagrams. The results appeared to be unaffected by the age of the solution stored at 4°C for up to 10 days (Figs. 12b and 12c).

Covalent phosphodiester bonds are known to yield directly to thermal energy (Eigner, Boedtker & Michaels, 1961; Stanley & Bock, 1965; Lindahl, 1967) and traces of many bivalent cations (e.g. 0.1–10 μM - Ni^{2+} , $-Co^{2+}$, $-Zn^{2+}$, $-Fe^{2+}$, $-Cu^{2+}$) act as catalysts (cf. Fuwa, Wacker, Druyan, Bartholomay & Vallee, 1960; Huff, Sastry, Gordon & Wacker, 1964; Lindahl, 1967). Bivalent Mg^{2+} has a similar action at higher millimolar concentrations, which accounts satisfactorily for the more severe breakdown observed when RNA was heated rapidly to 80°C with 5.0 mM- Mg^{2+} present (Fig. 13).

Enzymic degradation of rRNA extracted from ribonuclease I-deficient strains of *E. coli* cannot be dismissed entirely since *E. coli* M.R.E. 600 and

Table 1. Comparison of sedimentation coefficients for *Ps. fluorescens* ribosomal subunits and RNA-9 in 0.15M-sodium chloride containing increasing concentrations of Mg^{2+}

Values for ribosomal subunits are taken from Cammack & Wade (1965).

Concn. of $MgCl_2$ (mM)	RNA				Ribosomal subunits	
	$s_{20,w}^0$ (schlieren optics, approx. 3.9 mg/ml) (S)		$s_{20,w}^0$ (u.v. optics, 50–60 μ g/ml) (S)		$s_{20,w}^0$ (schlieren optics, approx. 9 mg/ml) (S)	
	16S	23S	16S	23S	30S	50S
0	13.8	16.7	17.0	23.7	16.5	21.2
0.1	14.2	17.3	16.8	23.4	16.5	21.2
1.0	14.2	17.3	17.9	24.9	21.2, 27.7	32.6
5.0	17.5	21.5	18.7	26.4	31.4	44.4
10.0	17.5	22.4	21.2	28.9	32.3	45.6

E. coli Q13 are now known to contain Mg^{2+} -dependent endonuclease activity, i.e. ribonuclease IV (Spahr & Gesteland, 1968; Szer, 1969), which causes a splitting of 23S RNA into two similarly sized fragments sedimenting at about 16S (Kurland, 1960; McIlreavy & Midgley, 1967; Szer, 1969). However, the precaution of using macaloid in some of the heating experiments did not lead to any improvement in stability; in fact samples treated with macaloid were more degraded than the control solution (e.g. Fig. 14). This unexpected result appears to be due to Mg^{2+} being leached from the macaloid (cf. Fig. 13) and supernatants recovered after washing the material contained 1mM–10mM- Mg^{2+} . The unheated RNA solutions were quite stable at room temperature in the presence of macaloid.

Effect of Mg^{2+} on the sedimentation properties of rRNA and the 30S and the 50S ribosomal subunits. There exists an obvious parallel in the solvent conditions that are well known to stabilize ribosomal subunits and those producing maximum s values for the 16S and 23S RNA species. This is applicable at low ionic strength, in 0.01M-tris-HCl buffer, pH 7.4, and also at moderately high ionic strength in 0.15M-sodium chloride (Table 1).

By removal of Mg^{2+} and increase in the charge on the RNA moiety of the ribosomal subunits it might be expected that the unfolding of the ribonucleoprotein was simply promoted by the polyelectrolyte expansion of the RNA, but Gavrilova *et al.* (1966) have emphasized that the changes in s value denoting unfolding are discontinuous rather than continuous, and it is widely accepted that unfolding arises from a co-operative breakdown of weak protein-protein interactions.

In assessing the contribution rRNA makes to the unfolding of ribosomal subunits it is essential to specify the experimental conditions, especially the

ionic strength and the Mg^{2+} concentration. The removal of Mg^{2+} by chelation with EDTA and dialysis in low-ionic-strength tris buffer (Gesteland, 1966; Weller *et al.* 1968) will bring about a fivefold decrease in the s value of rRNA if the sedimentation diagrams are recorded by schlieren optics (Fig. 8); very dilute solutions analysed by u.v. optics may show only a 25–30% decrease in s value (Fig. 9) but on removal of all the Mg^{2+} the concentration of the tris⁺ counter-ion itself becomes critical. There are small changes in the optical properties of isolated rRNA at room temperature (20–25°C) in 0.01M-tris buffer that are indicative of a minor disruption of secondary structure. Both Gesteland (1966) and Weller *et al.* (1968) performed their sedimentation analyses at low temperatures (5–6°C).

Unfolding reported at a higher ionic strength, in 0.1–0.15M-salt (Spirin *et al.* 1963; Cammack & Wade, 1965; Gavrilova *et al.* 1966), is paralleled by comparatively minor changes in the sedimentation behaviour of the isolated rRNA (Table 1) and the RNA concentration is clearly far less important. Under these conditions the s values of the 16S and 23S RNA decrease sufficiently to indicate a change in tertiary structure, and the optical measurements confirm that the secondary structure remains intact even at 25°C.

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