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The Sedimentation Behaviour of Ribonuclease-Active and -Inactive Ribosomes from Bacteria

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1. The '30s' and '50s' ribosomes from ribonuclease-active (*Escherichia coli* B) and -inactive (*Pseudomonas fluorescens* and *Escherichia coli* MRE600) bacteria have been studied in the ultracentrifuge. Charge anomalies were largely overcome by using sodium chloride–magnesium chloride solution, I 0·16, made 0–50mM with respect to Mg^{2+}. 2. Differentiation of enzymic and physical breakdown at Mg^{2+} concentrations less than 5mM was made by comparing the properties of *E. coli* B and *P. fluorescens* ribosomes. 3. Ribonuclease-active ribosomes alone showed a transformation of '50s' into 40–43s components. This was combined with the release of a small amount of '5s' material which may be covalently bound soluble RNA. Other transformations of the '50s' into 34–37s components were observed in both ribonuclease-active and -inactive ribosomes at 1·0–2·5mM-Mg^{2+}, and also with *E. coli* MRE600 when EDTA (0·2mM) was added to a solution in 0·16M-sodium chloride. 4. Degradation of ribonuclease-active *E. coli* B ribosomes at Mg^{2+} concentration 0·25mM or less was coincident with the formation of 16s and 21s ribonucleoprotein in *P. fluorescens*, and this suggested that complete dissociation of RNA from protein was not an essential prelude to breakdown of the RNA by the enzyme. 5. As high Cs^+/Mg^{2+} ratios cause ribosomal degradation great care is necessary in the interpretation of equilibrium-density-gradient experiments in which high concentrations of caesium chloride or similar salts are used. 6. The importance of the RNA moiety in understanding the response of ribosomes to their ionic environment is discussed.

Ribosomes isolated from bacteria, yeast, pea seedlings, rat liver and rabbit reticulocytes show remarkably similar properties in the ultracentrifuge (Ts'o, 1962; Arnstein, 1963), which might be expected from their function as sites of protein synthesis. To preserve their integrity in solution, it is essential to include millimolar concentrations of a bivalent cation such as Mg^{2+} or Ca^{2+} (Chao & Schachman, 1956; Chao, 1957). These cations interact with the negatively charged RNA phosphate groups (Hamilton & Petermann, 1959; Petermann, 1960; Rodgers, 1964) and probably play an essential role in the structure of the ribosome (Ts'o, Bonner & Vinograd, 1958). Ox-pancreas ribosomes do not show the usual susceptibility to dissociation as the Mg^{2+} concentration falls but this could be due to the significant amounts of extraneous protein attached to them (Madison & Dickman, 1963).

Several molecular species of ribosome exist and the distribution of these, as observed from their sedimentation-velocity boundaries, is mainly a function of the Mg^{2+} concentration. Tissières, Watson, Schlessinger & Hollingworth (1959) demonstrated the following series of reversible interactions with ribosomes from *Escherichia coli* in tris buffer of low ionic strength, where the Mg^{2+} concentration in the equation below is increased from 0·1 to 10mM proceeding from left to right:

$$\underset{0.76}{2('30s')} + \underset{2.0}{2('50s')} \rightleftharpoons \underset{3.4}{2('70s')} \rightleftharpoons \underset{6.8}{('100s')}$$

$$(10^{-6} \times \text{mol.wt.})$$

These were later confirmed by electron microscopy (Hall & Slayter, 1959; Huxley & Zubay, 1960). Each sedimentation coefficient in parentheses is the result of extrapolating the measurements to infinite dilution, which is essential with polyelectrolytes like ribosomes, particularly when solvents of low ionic strength are used. Under these conditions, the primary charge effect in sedimentation (Svedberg & Pederson, 1940) is very pronounced (cf. also Ts'o & Vinograd, 1961); not only does the sedimentation coefficient become very dependent on concentration but the resulting sharpening of the velocity boundaries produces a very misleading impression of physical homogeneity. When ribosomes are

provided with a counter-ion screen to suppress these charge effects by using 0·1–0·2 M-sodium chloride, univalent Na^+ displaces the Mg^{2+} from the RNA phosphate groups but is unable to fulfil the same structural function (Hamilton & Petermann, 1959), and a latent ribonuclease attached to '30s' ribosomes in *E. coli* (Elson, 1958, 1959; Elson & Tal, 1959; Spahr & Hollingworth, 1961; Wade, 1961; Tal & Elson, 1963) becomes activated, resulting in a rapid breakdown of the RNA.

The stability of bacterial ribosomes in sodium chloride solution, I 0·16, has been studied over a wide range of Mg^{2+} concentration in the analytical ultracentrifuge, and the results have been compared with those obtained at low ionic strength in tris buffer. To distinguish changes in molecular structure dependent on the ionic environment from those resulting from the ribonuclease activity, advantage has been taken of the fact that ribosomes from some bacterial species are free from detectable amounts of this enzyme (Wade & Robinson, 1963, 1965).

MATERIALS AND METHODS

Cultural conditions. Three strains of bacteria were used: *Escherichia coli* B, *E. coli* MRE600 and *Pseudomonas fluorescens* NCIB8248. *E. coli* MRE600 was selected from 13 strains that were examined for ribonuclease content; like *P. fluorescens* (Wade & Robinson, 1963), this strain displayed negligible ribonuclease activity.

The bacteria were grown (division time about 60 min.) by continuous culture (Wade, 1961) in a defined medium containing glucose (2%, w/v), $(NH_4)_2SO_4$ (0·05 M) and essential salts either alone (*E. coli* B) or with 15 mM-glutamate (*E. coli* MRE600 and *P. fluorescens*) at 37° (*E. coli*) or 30° (*P. fluorescens*). The culture (about 1% wet wt.) was chilled, treated with 10 mM-$MgCl_2$ and centrifuged in a continuous De-Laval centrifuge at 2–4°. The packed cells were stored at −20°.

Preparation of ribosomes. The bacteria were disrupted in a press (Hughes, 1951), and a suspension (10% wet wt.) of the product in a solution of NaCl (0·145 M) and $MgCl_2$ (5·0 mM) at pH 6–6·5 (solution A) at 2–4° was centrifuged at 25000g for 1 hr. in the no. 30 rotor of a Spinco model L centrifuge. The supernatant was centrifuged at 85000g for 7·5 hr. The tubes were drained at an angle of 45° for about 1 hr. to allow a thin layer of turbid gelatinous material to fall away, which contained about 15% of the total DNA in the cell. The underlying ribosomes were resuspended in solution A and washed twice (20 vol.) by centrifuging. The preparations contained 52–54% of RNA and less than 0·2% of DNA.

Separation of '30s' and '50s' ribosomes. The separation was achieved by differential centrifugation in the Spinco model L centrifuge at 2–4°. The time taken to obtain complete sedimentation of '50s' material and leave part of the '30s' component as a pure fraction in the supernatant was calculated from the measured S values. At the end of each sedimentation run both pellet and supernatant were examined in the analytical ultracentrifuge. At times the '30s' fraction in the supernatant was slightly contaminated with '50s' material, in which case the time for the preparative run on the redissolved pellet, enriched with '50s' material, was increased by 10%.

The ribosomes were suspended in solution A (about 1%, w/v), dialysed for 2 days against solution A and then centrifuged at 25000 rev./min. for 11 hr. in the SW25 rotor. The pellet enriched with '50s' material was extracted twice with solution A by centrifuging and the extracts were combined with the first supernatant to provide a suspension of '30s' ribosomes from which the ribosomes were recovered by centrifuging at 30000 rev./min. for 7·5 hr.

The pellet containing the '50s' material was washed three times by resuspending in solution A and centrifuging at 27000 rev./min. in the no. 30 rotor for two periods of 4·5 hr. and one of 3 hr. A suspension of the final deposit was cleared by centrifuging at this speed for 10 min. The ribosomes in the supernatant, comprising about 90% of the '50s' material (Fig. 3), were recovered by centrifuging at 27000 rev./min. for 5 hr. in the no. 30 rotor.

Ribosomal RNA. Ribosomal RNA was isolated from *P. fluorescens* ribosomes by precipitation with guanidinium chloride (Cox & Arnstein, 1962). After dialysing a sample against 0·01 M-sodium acetate, pH 4·6, containing NaCl (0·1 M), it was examined in the ultracentrifuge and showed two peaks at 15·2 and 19·6 s ($S_{20,w}$ values for a 0·2% solution; cf. Kurland, 1960); in addition to these components there was a third (15%) sedimenting at 28 s.

Ribonuclease. Pancreatic ribonuclease (42 units/mg.; Kunitz, 1946) was obtained from Boehringer und Soehne G.m.b.H., Mannheim, Germany).

Preparation of solutions for sedimentation analysis. The pellets of mixed '30s' and '50s' ribosomes, stored at 4° under toluene, were stable for several weeks. On redissolving in solution A they produced slightly turbid solutions that cleared rapidly with low-speed centrifugation to give naturally opalescent solutions. Solvents used for dialysis were replenished each day over 3–5 days, with a volume ratio of solvent to sample of about 100:1. The containers were agitated gently at 2–4° during this period on a reciprocating shaker. Diffusible degradation products, formed by the action of ribonuclease, were estimated from the extinction of the diffusate at 260 mμ.

Estimation of ribonucleoprotein by ultraviolet absorption. The routine determination of ribonucleoprotein concentration was based on the extinction of 260 mμ measured with a Unicam SP.500 spectrophotometer or an Optica CF4 recording spectrophotometer; matched silica cells (The Thermal Syndicate Ltd., Wallsend, Northumberland) were used for these measurements. An extinction ($E^{1\%}_{1\,cm.}$) of 133 was used and applied only to natural mixtures of the '30s' and '50s' sub-units in solution A.

Sedimentation analyses. These were performed in the Spinco model E ultracentrifuge (Beckman Instruments Inc., Belmont, Calif., U.S.A.) with the standard rotor and single- or double-sector 12 mm. cells. The double-sector cell was used to record an accurate base-line for the solvent when measuring the area of individual peaks. For concentrations greater than 0·2% the schlieren phase-plate optical system was used to record the sedimenting boundaries; at much lower concentrations, i.e. 0·005–0·01%, ultraviolet-absorption photographs were taken and after development these were scanned in the Spinco Analytrol to give a trace of the extinction against distance down the cell. In the calculation of S the statistical slope of $\log x$ against t was determined. To convert S into $S_{20,w}$ the buoyancy correction applied

was based on $\bar{v}=0{\cdot}64$ (Tissières *et al.* 1959). The correction for viscosity amounted to about $-12{\cdot}5\%$ since the RTIC (rotor-temperature-indicating and control) unit was set permanently at $25{\cdot}00\pm0{\cdot}05°$ for the convenience of studies on proteins. In many cases the boundary position was defined by the maxima of asymmetric or partly resolved boundaries, and therefore S can only be regarded as approximate.

Measurement of the refractive increment. The refractive increment between solution and solvent was measured either directly in a differential refractometer (Brice & Halwer, 1951) or indirectly in the analytical ultracentrifuge. In the differential refractometer the solution and solvent were accommodated in a specially divided optical chamber and measurements made by using a 5461 Å Hg-green illuminated slit. The specific refractive increment of a solution of freeze-dried ribosomes was found to be $0{\cdot}172\,g.^{-1}ml.$ In the ultracentrifuge, the refractive increment was obtained by measuring the area beneath the dn/dx against x diagram and converting this to Δn by using the appropriate optical factors. The sedimentation diagrams were traced under a photographic enlarger and the area of each peak was found by planimetry. Correction for radial dilution was made for each boundary with the factor $(\bar{x}/x_0)^2$, where $\bar{x}$ and x_0 are the distance of the boundary and meniscus from the axis of rotation.

Moving-boundary electrophoresis. Dialysed solutions were analysed in the 11 ml. long-limbed cell of the Spinco model H electrophoresis–diffusion instrument. Schlieren and Rayleigh interference optical systems were used. The Ag–AgCl electrode assembly was sealed off from the atmosphere on the cathode (descending-boundary) side of the apparatus; the anode side was open. Conductivity measurements were made at 0° by using a Wheatstone bridge (Doran Instrument Co. Ltd., Stroud, Glos.) and a conductivity cell similar to that described by Jones & Bollinger (1931). The cell constant was determined with standard 0·1 M-KCl solution of specific conductivity $0{\cdot}00715\Omega^{-1}$ (0°).

Zonal electrophoresis. Instructions given by Smith (1960) were followed. Strips (18·0 cm. × 2·5 cm.) of paper (Whatman no. 1) and cellulose acetate (Oxoid) were both used as supporting material for 0·16 M-sodium propionate buffer at pH 6·0. A current of 1·7–2·0 mA for each strip was applied over a period of 4 hr. Protein was detected on paper with lissamine green and on cellulose acetate with Ponceau S. Before the staining, the strips were examined under ultraviolet light and the absorbing area (RNA) was marked off in pencil.

RESULTS

Sedimentation patterns of ribosomes from E. coli *B*

Sedimentation in 0·145 M-*sodium chloride containing* Mg^{2+} (5·0 mM) (*solution A*). Suspensions of disrupted cells in solution A showed the usual '30 s' and '50 s' sub-units of ribosomes (Fig. 1*a*); traces of '70 s' material were sometimes observed but this boundary was usually unstable, showing spikiness typical of convection. The remainder of the soluble material in the suspension, consisting largely of protein, RNA and DNA, gave a polydisperse 4–6 s boundary only partially resolved from the meniscus. The S values of the ribosome sub-units were depressed to 28·1 and 42·9 s, partly through sedimenting in a fairly high concentration of 4–6 s material. Purified ribosomes (Figs. 1*b* and 1*c*) contained small amounts of 4–6, 15 and 21 s components, which increased with the period the ribosome pellet was stored at 4°.

The shape of the '50 s' boundary in Figs. 1(*b*) and 1(*c*) showed the presence of more than one component and depended on the period of storage of the ribosome pellet. After 14 days it was resolved into two maxima, 39·7 and 40·6 s (Fig. 1*b*), whereas its heterogeneity could only be inferred from the asymmetry of the major 44·0 s boundary (Fig. 1*c*) after a shorter period of 5 days. Failure to find any evidence of heterogeneity in the unfractionated suspension (Fig. 1*a*) indicated a change in the '50 s' material during its isolation.

The analysis of solutions of about 0·005% in solution A by using the ultraviolet-absorption optics did not provide such a clear indication of heterogeneous boundary shape. The S values determined in these very dilute solutions were in fair agreement with $S^0_{20,w}$ values given by Tissières *et al.* (1959); the range for '30 s' material was 31·9–33·2 s and that for '50 s' material was 50·8–51·6 s. The variation of S with the total concentration of '30 s' and '50 s' components (Fig. 2) showed that concentration-dependence is much less pronounced

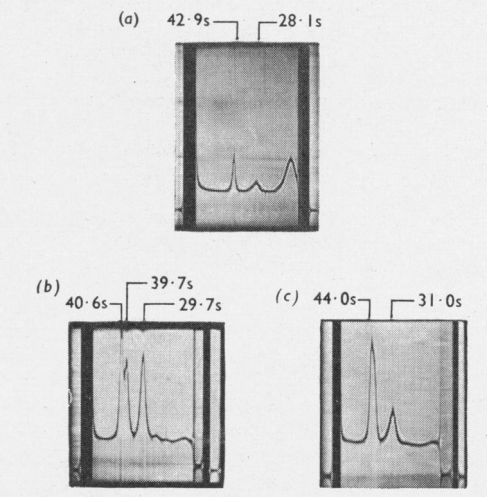

Fig. 1. Sedimentation diagrams of ribosomes from *E. coli* B in 0·145 M-NaCl containing Mg^{2+} (5 mM) (solution A), adjusted to pH 6·5. (*a*) Suspension of disrupted cells: approx. 20 mg. dry wt./ml.; 16 min. at 47 660 rev./min.; phase-plate angle 60°. (*b*) Purified ribosome pellet stored for 14 days at 4° (dialysed): 20 mg./ml.; 20 min. at 47 660 rev./min.; phase-plate angle 45°. (*c*) Purified ribosome pellet stored for 5 days at 4° (dialysed): 10 mg./ml.; 20 min. at 47 660 rev./min.; phase-plate angle 45°.

under these conditions compared with the conditions of low ionic strength used by Tissières *et al.* (1959).

Sedimentation in sodium chloride–magnesium chloride solutions, I 0·16, *made* 0·05–50mM *with respect to* Mg^{2+}. Some idea of the stability of ribosomes at high salt concentration was obtained from their dialysis against sodium chloride solutions containing increasing amounts of Mg^{2+}. The ionic strength of the solution was kept constant (*I* 0·16) and the Na^+/Mg^{2+} ratio adjusted to between 0·2 and 3000 (Table 1). The actual identity of the '70s' and '100s' aggregates was not of prime importance.

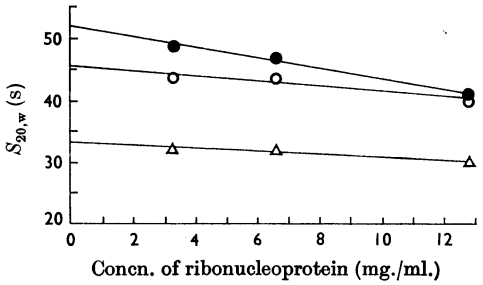

Fig. 2. Plots of corrected sedimentation coefficient against combined concentration of a natural mixture of '30s' and '50s' *E. coli* B ribosomes in 0·145M-NaCl containing $MgCl_2$ (5mM) (solution A). ●, Normal '50s' component; ○, derived '50s' fraction produced on aging the pellet or solution for 14 days or longer at 4°; △, normal '30s' component.

In Table 1, however, ribosomes that sedimented in the range 65·9–67·0s were assumed to be '70s', whereas those that sedimented in the range 73·9–75·7s were either the '80s' dimer of the '50s' component (Huxley & Zubay, 1960), or a very concentration-dependent '100s' dimer of the '70s' component (Tissières *et al.* 1959). Increasing association of the '30s' and '50s' sub-units at Mg^{2+} concentrations above 5mM led to a progressive dilution of these species, and therefore their *S* values showed a tendency to increase (Table 1).

Instability in the presence of sodium chloride was first evident at Mg^{2+} concentrations less than 5mM or Na^+/Mg^{2+} ratios greater than 30 (cf. Hamilton & Petermann, 1959). In 2·5mM-Mg^{2+}, there was a significant and irreversible decrease in *S* for both sub-units to 22·4s ('30s' component) and 35·3s ('50s' component), and the amount of '5s' material increased (cf. Elson, 1961, 1964). Degradation of ribosomes became progressively more extensive as the Mg^{2+} concentration was further decreased. At Mg^{2+} concentrations below 1mM and Na^+/Mg^{2+} ratios greater than 160, the '5s' material represented about 70% of the total; residual ribonucleoprotein sedimented as a rather broad 30s boundary. Finally, at the highest Na^+/Mg^{2+} ratios, nucleotides from degraded ribosomal RNA diffused through the dialysis sac and insoluble protein appeared inside.

Substitution of caesium chloride for sodium chloride in a similar series of experiments showed rather less degradation in the presence of Cs^+ at Cs^+/Mg^{2+} ratios greater than 30. Identical results were obtained at Cs^+/Mg^{2+} ratios less than 30.

Table 1. *Effect of* Mg^{2+} *concentration on the sedimentation properties of ribosomes from* E. coli *B in sodium chloride–magnesium chloride solutions, I* 0·16, *adjusted to* pH 6·0–6·5

The percentage of each component is based on the area below the peak corrected for radial dilution but not the Johnston–Ogston effect (Schachman, 1959); after correction for the latter, the ratio of slow to fast component will decrease according to the magnitude of the concentration-dependence of *S*.

Concn. of $MgCl_2$ (mM)	Concn. of NaCl (M)	Na^+/Mg^{2+} ratio	$S_{20,w}$ (s) '5s' component	'30s' component	'50s' component	'70–100s' component
0·05	0·16	3197	←Extensive degradation→			
0·25	0·159	637	←Extensive degradation→			
1·00	0·157	157	4·9 (70%)	←Partial degradation (30·2s, 30%)→		
2·5	0·153	61	5·0 (5%)	22·4 (20%)	35·3 (75%)	— —
5·0	0·145	29	5·0 (trace)	31·2 (27%)	37·8←44·8 (73%)	70 (trace)
10·0	0·130	13	—	32·4 (26%)	46·2 (74%)	70 (trace)
25·0	0·085	3·4	—	33·3 (8%)	54·1 (30%)	65·9, 73·9 (62%)
50·0	0·010	0·2	—	33·3 (3%)	53·0 (10%)	67·0, 75·7 (87%)

Sedimentation at low ionic strength. To obtain patterns similar to those in Figs. 1(*b*) and 1(*c*) in the more conventional 0·01 M-tris buffer, pH 7·4, 1 mM-Mg^{2+} was required. In the tris buffer, concentration-dependence was more pronounced and the boundaries were therefore sharper.

A preparation of '50 S' ribosomes (containing about 90% of '50 S' material) was analysed both in solution A (Fig. 3*a*) and in the tris buffer (Fig. 3*b*). In both, two peaks were resolved: in solution A these were at 40·4 and 47·2 S, and in tris buffer at 39·3 and 44·0 S. Both showed traces of an unstable '70 S' component. Decrease of the Mg^{2+} concentration in tris from 1·0 to 0·1 mM (Fig. 3*c*) produced a pronounced transformation of the heterogeneous '50 S' boundary to give a very sharp and symmetrical 27·5 S peak with a trace of 38·9 S material. Under these conditions of low ionic strength with insufficient Mg^{2+} to neutralize the RNA phosphate groups, boundary sharpening was very marked and the appearance of the peak gave an impression of homogeneity. A pattern very similar to the one in Fig. 3(*c*) was obtained in sodium chloride solution, *I* 0·16, containing Mg^{2+} (2·5 mM) (Fig. 3*d*; cf. also Table 1), but in this case the major 35·1 S boundary (formerly 27·5 S) was more diffuse and the minor component was at 46·8 S instead of 38·9 S.

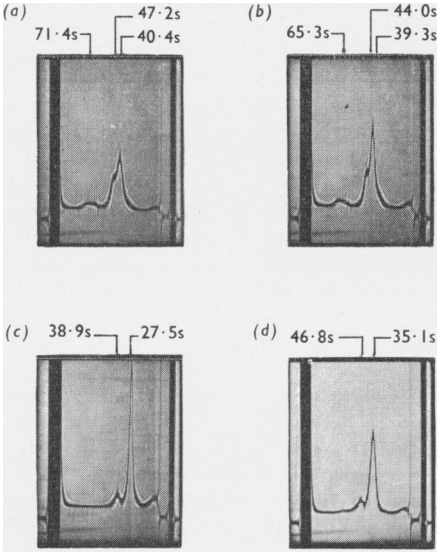

Fig. 3. Sedimentation diagrams of a partially purified '50 S' fraction from *E. coli* B ribosomes dialysed against: (*a*) 0·145 M-NaCl containing Mg^{2+} (5 mM) (solution A); (*b*) 0·01 M-tris buffer, pH 7·4, containing Mg^{2+} (1 mM); (*c*) 0·01 M-tris buffer, pH 7·4, containing Mg^{2+} (0·1 mM); (*d*) 0·153 M-NaCl containing Mg^{2+} (2·5 mM). The sedimentation conditions in each case were: approx. 9 mg./ml.; 37 min. at 29500 rev./min.; phase-plate angle 45°.

As further evidence of the diversity of the sedimentation properties of ribosomes, a natural mixture of the '30 S' and '50 S' sub-units in a 100-fold dilution of solution A (*I* 0·0016) was found to give very complicated sedimentation patterns (e.g. Fig. 4*a*) that could, nevertheless, be restored almost completely to the original pattern in undiluted solution A (Fig. 4*b*). The solution in the solvent of low ionic strength was stable and the same pattern was observed 1 month later after storage at 0°. A pure fraction of '30 S' material treated similarly was resolved into two components, 25·8 and 22·7 S, demonstrating its independent contribution to the complexity of the pattern.

Sedimentation patterns of ribosomes from ribonuclease-deficient species

Comparison of the sedimentation behaviour of ribonuclease-active and -inactive ribosomes. Wade & Robinson (1963) found that the ribosomes from *P. fluorescens* were deficient in ribonuclease. This provided an opportunity to differentiate molecular changes involving only weak, ionic, types of interaction from those resulting from the enzymic breakage of covalent links.

The sedimentation diagrams of ribosomes from *E. coli* B (ribonuclease-active) and *P. fluorescens* (ribonuclease-inactive) ribosomes were compared (Fig. 5). The much sharper '50 S' boundary in *P. fluorescens* [(i) in Fig. 5(*b*)] with only a trace of a second component on the trailing side is in direct contrast with the normal 'two-component' system for *E. coli* B [(i) in Fig. 5(*a*)]. These were retained as controls covering the 13-day period of an experiment that compared the effects of lowering the concentration of Mg^{2+} at *I* 0·16 (Table 2 and Fig. 5). During this period the '50 S' component of *E. coli* B changed further until there was a complete resolu-

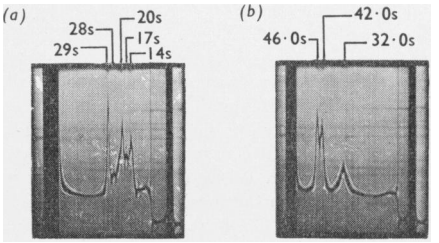

Fig. 4. Sedimentation diagrams of ribosomes from *E. coli* B dialysed: (*a*) first against a solvent of low ionic strength, namely 1·45 mM-NaCl containing Mg^{2+} (0·05 mM) (solution A diluted 100-fold); (*b*) and then 0·145 M-NaCl containing Mg^{2+} (5 mM) (solvent A). The sedimentation conditions in each case were: approx. 10 mg./ml.; 20 min. at 47660 rev./min.; phase-plate angle 45°.

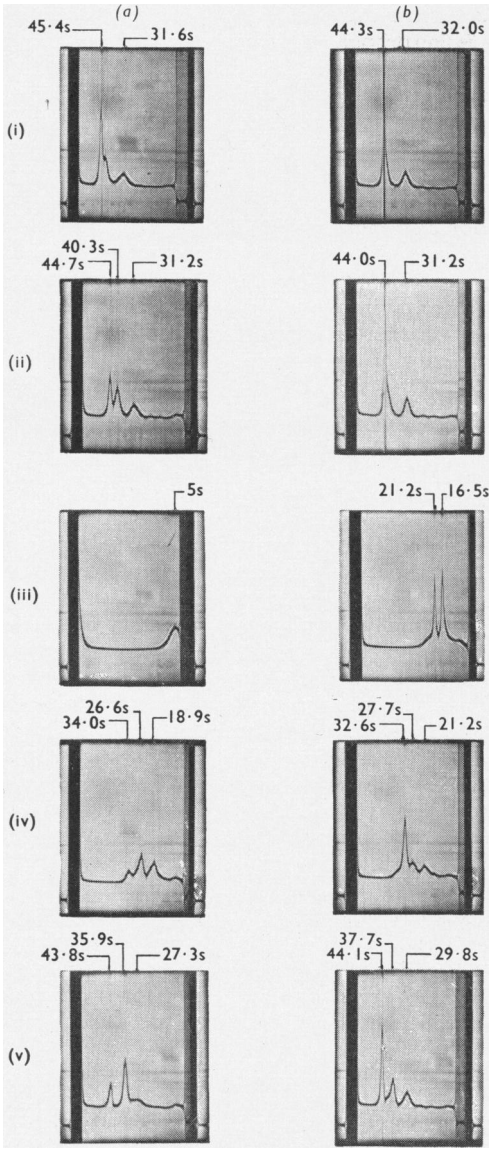

Fig. 5. Sedimentation diagrams of (*a*) ribonuclease-active ribosomes (*E. coli* B) and (*b*) ribonuclease-inactive ribosomes (*P. fluorescens*) in NaCl–$MgCl_2$ solutions, *I* 0·16, adjusted to pH 6·0–6·5: (i) Control in 0·145 M-NaCl containing Mg^{2+} (5 mM) (solution A); (ii) control in solution A stored for 13 days at 4°; (iii) in 0·16 M-NaCl containing no Mg^{2+}; (iv) in 0·157 M-NaCl containing Mg^{2+} (1 mM); (v) in 0·153 M-NaCl containing Mg^{2+} (2·5 mM). The sedimentation conditions in each case were: 10–15 mg./ml. (before dialysis); 18 min. at 47 660 rev./min.; phase-plate angle 60°.

tion into 40·3 and 44·7 s components [(ii) in Fig. 5(*a*)], as shown above in Fig. 1(*b*); however, there was no comparable change in the '50 s' component of *P. fluorescens* [(i) and (ii) in Fig. 5(*b*)], although there was evidence of a second component.

As a result of ribonuclease activity, ribosomes from *E. coli* B were degraded completely at Mg^{2+} concentration 0·25 mM or less [Table 2 and (iii) in Fig. 5(*a*)], whereas the ribosomes from *P. fluorescens* dissociated into two sharp peaks of 16·5–16·8 and 21·2–21·7 s [Table 2 and (iii) in Fig. 5(*b*)]. In *P. fluorescens* partial reversibility was obtained by dialysing the solution to bring the Mg^{2+} concentration back to 5 mM (solution A), when 23 s and 32 s components were re-formed in a ratio comparable with that of the original sub-units. The bulk of the macromolecular material was still present [see (iii) in Fig. 5(*b*)] since 90% of the differential refractometer measurement, Δn, was accounted for in the ultracentrifuge. The sharp peaks in (iii) in Fig. 5(*b*) were superimposed on what appeared to be more polydisperse material.

At higher Mg^{2+} concentrations (greater than 0·25 mM) the influence of ribonuclease became less pronounced [Table 2 and (iv) and (v) in Figs. 5(*a*) and 5(*b*)], and a distinct parallelism existed for ribonuclease-active and -inactive ribosomes between 1·0 mM- and 5·0 mM-Mg^{2+}. Complete reversibility to the normal pattern in solution A was possible only with the ribonuclease-inactive ribosomes at 2·5 mM-Mg^{2+}.

The addition of pancreatic ribonuclease to the ribonuclease-inactive *P. fluorescens* ribosomes, followed by incubation under solvent conditions matching those in Table 2 and Fig. 5, showed that the ribosomes were fairly resistant to enzyme in solution A (Fig. 6) at 5 mM-Mg^{2+} with only a marginal breakdown of the ribosomal RNA, whereas at 2·5 mM-Mg^{2+} and lower concentrations there was a very rapid increase both in the amount and rate of breakdown. At much higher concentrations of pancreatic ribonuclease (greater than 10 μg./ml.) 5 mM-Mg^{2+} offered no protection against the enzyme after incubating for 1 hr. Ribosomal RNA, dissociated and isolated from protein, was susceptible to enzyme under any of the conditions specified in Table 2.

Electrophoresis of dissociated ribonuclease-inactive ribosomes. There was an interesting similarity between the 16 s and 21 s components obtained at Mg^{2+} concentration 0·25 mM or less (*I* 0·16) and the two species of ribosomal RNA described by Kurland (1960), which had $S^0_{20,w}$ values '16 s' and '23 s' in 0·01 M-sodium acetate, pH 4·6, containing sodium chloride (0·1 M). The nature of the new components was therefore examined by electrophoresis to explore their possible identity with free RNA.

The dialysis of *P. fluorescens* ribosomes in 0·16 M-sodium propionate buffer, pH 6·0, gave the same characteristic dissociation products described in Table 2 and (iii) in Fig. 5(*b*), with *S* values 16·9 and

Table 2. *Comparison of the sedimentation properties of ribonuclease-active and -inactive bacterial ribosomes in sodium chloride–magnesium chloride solutions*, I 0·16, *adjusted to* pH 6·0

The percentage loss during dialysis is calculated from the combined measurements of extinction at 260mμ on the diffusates without any correction for hypochromicity. $S_{20,w}$ values were determined on ribosome solutions containing 10–12mg. of ribonucleoprotein/ml.

			E. coli B (ribonuclease-active)			*P. fluorescens* (ribonuclease-inactive)		
				$S_{20,w}$(s)			$S_{20,w}$(s)	
	Concn. of Mg^{2+} (mM)	Na^+/Mg^{2+} ratio	Loss during dialysis (%)	'30s' fraction	'50s' fraction	Loss during dialysis (%)	'30s' fraction	'50s' fraction
(Control: 0 days)	5·0	29·0	—	31·6	45·4	—	32·0	44·3
(Control: 13 days)	5·0	29·0	—	31·2	40·3←44·7	—	31·2	44·0
	0	∞	48·3	5		0·99	16·5 21·2	
	0·05	3197	26·2	5		2·67	16·5 21·6	
	0·25	637	1·9	5–10		0	16·8 21·7	
	1·0	157	2·95	18·9		4·52	21·2	
				26·6			27·7	
				34·0			32·6	
	2·5	61·0	10·2	27·3	35·9←43·8	2·47	29·8	37·7←44·1
	5·0	29·0	3·89	31·7	40·8←45·2	1·07	31·4	44·4
	10·0	13·0	3·86	32·4	43·5←46·6	3·55	32·3	45·6

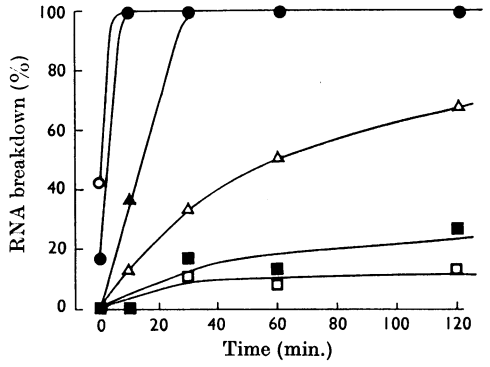

Fig. 6. Susceptibility of ribonuclease-free ribosomes to ribonuclease. Ribosomes from *P. fluorescens* (0·05mg./ml.) were incubated (at 37°) at pH 6–6·5 with 1μg. of pancreatic ribonuclease/ml. with different concentrations of $MgCl_2$ (0·05–10mM) and NaCl, *I* 0·16. The decrease in hypochromicity observed (from $E^{1\,cm.}_{260m\mu}$ 0·70 to 0·97) was used as a direct assessment of the degree of breakdown. Concentrations of Mg^{2+}: □, 10mM; ■, 5mM; △, 2·5mM; ▲, 1·0mM; ●, 0·25mM; ○, 0·05mM.

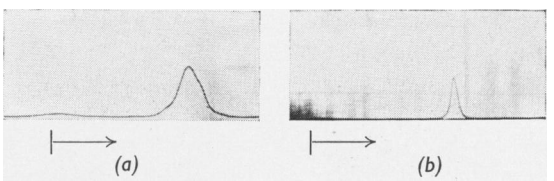

Fig. 7. (*a*) Moving-boundary electrophoresis diagram of dissociated ribonuclease-inactive ribosomes from *P. fluorescens* after dialysis against 0·16M-sodium propionate–propionic acid buffer, pH 6·0. The conditions were: 10mg./ml.; field strength 2·94v cm.$^{-1}$; migration 267min. towards anode; phase-plate angle 75°. (*b*) Moving-boundary electrophoresis diagram of ribosomal RNA from *P. fluorescens* ribosomes in 0·16M-sodium propionate–propionic acid buffer, pH 6·0. The conditions were: 3mg./ml.; field strength 3·01v cm.$^{-1}$; migration 203min. towards anode; phase-plate angle 70°. The diagrams show the cathode limb (descending) only and the arrows underneath indicate the position of the initial boundary and direction of migration.

21·5s, in excellent agreement with those in Table 2 obtained with 0·16M-sodium chloride. On both paper and cellulose acetate the zones migrated towards the anode. The area taking up the protein stain in both instances coincided with that of ultraviolet absorption, suggesting that both components were still ribonucleoprotein in character. This was confirmed by moving-boundary experiments with the dissociated ribosomes and ribosomal RNA (Figs. 7*a* and 7*b*). The former showed a partial resolution into two or more components of mobility $7{\cdot}0 \times 10^{-5}$–$9{\cdot}0 \times 10^{-5}$cm.^{2}sec.$^{-1}$v^{-1}. All the material under test was accounted for in this boundary, since the count of the Rayleigh interference fringes agreed well with the theoretical figure calculated from a Δn measurement; there was no evidence therefore of a very heterogeneous protein fraction (cf. Waller & Harris, 1961; Spitnik-Elson, 1962*a*,*b*, 1963, 1964), which might otherwise have escaped detection. Ribosomal RNA migrated as a single boundary of mobility $10{\cdot}5 \times 10^{-5}$cm.^{2}sec.$^{-1}$v^{-1} in 0·16M-sodium propionate buffer, pH 6·0 (Fig. 7*b*).

Table 3. *Sedimentation properties of* E. coli *MRE* 600 *(ribonuclease-inactive) ribosomes in sodium chloride–magnesium chloride solutions,* I 0·16, *adjusted to* pH 6·0

Concn. of ribonucleoprotein (mg./ml.)	Concn. of Mg^{2+} (mM)	Na^+/Mg^{2+} ratio	$S_{20,w}$(s)			'30s' fraction	'50s' fraction
12·3	5·0	29·0				30·5	43·4
—	0	∞	19·8	26·0			
10·1	0·05	3197	19·9	26·5			
10·5	0·25	637	19·8	27·0			
10·7	1·0	157			20·8		
					28·0		
					34·9		
10·2	2·5	61·0				28·5	36·7*←44·4

* At 2·5 mM-Mg^{2+} the normal '50s' component is partially transformed into a new component (cf. Fig. 8).

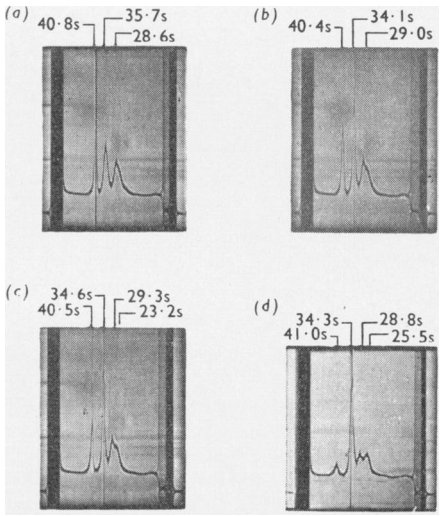

Fig. 8. Sedimentation diagrams of ribosomes from *E. coli* MRE 600 in 0·16 M-NaCl to which was added EDTA (0·2 mM), the solutions then being aged for: (*a*) 1 hr. at room temperature; (*b*) 24 hr. at 0°; (*c*) 48 hr. at 0°; (*d*) 7 days at 0°. The sedimentation conditions in each case were: 18 mg./ml.; 18 min. at 47 660 rev./min.; phase-plate angle 60°.

Ribonuclease-inactive ribosomes from E. coli *MRE* 600. The ribosomes from *E. coli* MRE 600 permitted an even closer comparison with *E. coli* B. As expected, the sedimentation properties of these ribosomes were very similar to those of *P. fluorescens* (cf. Tables 2 and 3), except that the fully dissociated components appearing at low Mg^{2+} concentrations (0·25 mM or less) had higher S values (19·8–19·9 and 26·0–27·0 s).

The rate of dissociation in low concentrations of Mg^{2+} was investigated by adding EDTA. The ribosome pellet was dissolved in 0·16 M-sodium chloride at 18–22° and EDTA (0·2 mM) was added; the latter was thought to be sufficient to chelate Mg^{2+} still firmly bound to RNA phosphate. Complete dissociation to the condition in (iii) in Fig. 5(*b*) was not observed. After the solution had been aged for 1 hr. at 18–22° (Fig. 8*a*) transformation of the nomal '50s' component from 40·8 s to 35·7 s had taken place. After a further period of 23 hr. at 4° the 34·1 s peak was the larger of the two and evidence for a similar transformation of the 28·9 s ('30s') component could be seen (Fig. 8*b*). After 48 hr. a new component, 23·2 s, derived from the '30s' component had been resolved, whereas the transformation of the '50s' component continued (Fig. 8*c*). At the end of 7 days the transformation of the '50s' component was almost complete and about 50% of the original '30s' component remained (Fig. 8*d*). Partial reversibility was obtained when Mg^{2+} (5 mM) was added to the 7-day solution and the product aged for 24 hr.

DISCUSSION

A precise definition of the ionic environment of ribosomes seems essential in attempting to relate physical properties to function in protein biosynthesis. The bivalent cation concentration is generally accepted as being the predominant factor, but the present results emphasize that the ionic strength and Na^+/Mg^{2+} ratio must be taken into account. The interpretation of the sedimentation diagrams is simplified by suppressing excessive concentration-dependence of S by using solvents of adequate, high, ionic strength and preserving an approximate relationship between S and the molecular size and shape of the particle. The choice of solvent, however, is severely restricted by the presence of ribonuclease which becomes active once the Na^+/Mg^{2+} ratio exceeds about 60 (Tables 1 and 2). By using ribonuclease-inactive strains such as *P. fluorescens* (Wade & Robinson, 1963) and *E. coli* MRE 600, this problem can be avoided and very useful information on the nature of the physical breakdown as distinct from enzymic breakdown of ribosomes obtained. With

both ribonuclease-active and -inactive ribosomes, physical instability of the '30s' and '50s' sub-units occurs at Na^+/Mg^{2+} ratios greater than 30.

The fact that, in a similar series of experiments, Cs^+ could substitute for Na^+ to give essentially the same result suggests that more attention needs to be given to the interpretation of sedimentation analyses of ribosomes in caesium chloride equilibrium-density gradients. Ratios of Cs^+/Mg^{2+} as high as 60–320 have been used (Brenner, Jacob & Meselson, 1961), and recent work (Meselson, Nomura, Brenner, Davern & Schlessinger, 1964) has shown a conversion of normal '30s' and '50s' sub-units into more dense 23s and 43s particles (cf. the changes at 1·0–2·5mM in Tables 1 and 2), deficient in protein, at Cs^+/Mg^{2+} ratios 80–1600. Since there are no indications that the strain of *E. coli* they used is free of ribonuclease, it is possible that the conversions they observe may involve partial attack by this enzyme.

A comparison of the sedimentation properties of ribonuclease-active and -inactive ribosomes suggests that there may be two distinct types of transformation of '50s' components, one of which depends on marginal ribonuclease activity in solution A [Fig. 1 and (i) and (ii) in Fig. 5(*a*)]. This particular transformation into a 40–43s species is accompanied by a release of 5s material and corresponds to the transformation described by Elson (1961, 1964); in that case the 5s material was identified as transfer RNA linked non-covalently to '50s' sub-units (cf. also Elson, 1962). The absence of a similar transformation in *P. fluorescens* [Table 2 and (i) and (ii) in Fig. 5(*b*)] encourages the view that ribonuclease is involved, and there exists the possibility that at least part of the '5s' material released may have been bound covalently to '50s' sub-units, although here there is no evidence to show whether this portion split off by enzyme is transfer RNA, the 5s material described by Rosset, Monier & Julien (1964), or a fragment derived from ribosomal RNA. Evidence against a covalent linkage of transfer RNA to ribosomes is based mainly on studies on the association and dissociation of initially separated ribosomes and transfer RNA (Cannon, Krug & Gilbert, 1963). Because of the relatively small amount of 5s material released from '50s' components the decrease in S for the latter is unlikely to be due to a decrease in mass alone (cf. Morgan, 1962); a significant change in the molecular shape must also occur and this could be induced by the rupture of only a few nucleotide bonds.

The process of activating the normally latent ribonuclease attached to '30s' components could be linked to the molecular changes that occur at Na^+/Mg^{2+} ratios greater than 30 [Table 2 and (iii), (iv) and (v) in Figs. 5(*a*) and 5(*b*)]. Ribosomes are comparatively resistant to attack by exogenous pancreatic ribonuclease in sodium chloride solution at Mg^{2+} concentration 5mM or less (Fig. 6), suggesting that degradation does not necessarily follow immediately the enzyme is released. But hypochromicity, as opposed to viscosity, is a rather insensitive measure of the breakdown of ribosomal RNA (cf. Littauer & Eisenberg, 1959), and could not be expected to reveal the marginal effects on the '50s' components where possibly only a few covalent links were broken. The appearance of 16s and 21s material in the ribonuclease-inactive ribosomes at Mg^{2+} concentration 0·25mM or less [(iii) in Fig. 5(*b*) and Table 2] is coincident with the rapid breakdown of the ribonuclease-active ribosomes [(iii) in Fig. 5(*a*) and Table 2]; since these components are also very susceptible to exogenous enzyme (Fig. 6), both release of enzyme and the molecular state of the substrate are important in determining breakdown. Free RNA is very rapidly digested under any of the conditions defined in Table 2 and Fig. 5. However, the electrophoresis results indicate that 16s and 21s material are ribonucleoprotein complexes and not RNA. Their susceptibility to ribonuclease could be due to some unfolding of the polynucleotide.

In addition to the ribonuclease-induced transformation of '50s' components in solution A, a transformation into 34·2–37·7s material occurs at I 0·16 with 2·5mM-Mg^{2+} [Fig. 3(*d*) and (v) in Figs. 5(*a*) and 5(*b*)] or EDTA (Fig. 8). The same component but with S depressed to 27·5s appears in 0·01M-tris buffer, pH 7·4, containing Mg^{2+} (0·1mM) (Fig. 3*c*). Similar transformations of '50s' material from *E. coli* into a 30s (extrapolated) component, brought about by lowering the Mg^{2+} concentration, have been reported (Rodgers, 1964; Weller & Horowitz, 1964). Weller & Horowitz (1964) inhibited ribonuclease activity with bentonite and dialysed a purified '50s' fraction against a solution of EDTA, obtaining 30s particles that were exceptionally concentration-dependent in sedimentation compared with those examined by Rodgers (1964), suggesting a possible structural difference between them. Rodgers (1964) concluded that the derived 30s material is a '30s' ribosome sub-unit of '50s' material, although the EDTA experiment in Fig. 8 shows complete resolution of the boundaries for native '30s' components and the derived particles.

In comparing these results with those of Rodgers (1964) and Weller & Horowitz (1964) some caution is necessary since the ionic conditions are not comparable. It is known that ribonucleoprotein particles show greater asymmetry in media of low ionic strength (Ts'o & Vinograd, 1961; Kisselev & Spirin, 1964) and therefore $S^0_{20,w}$ is expected to be a function of ionic strength.

RNA is a polyelectrolyte. Its molecular conformation depends largely on the degree of intra-

molecular hydrogen-bonding and the cross-linking of negatively charged RNA phosphate groups by Mg^{2+} or some other bivalent cation (Spirin, 1963). Variables such as pH, ionic strength, bivalent cation concentration and temperature are all known to influence the viscosity, sedimentation, hypochromicity and optical rotatory dispersion of RNA (Cox & Littauer, 1959, 1962; Littauer & Eisenberg, 1959; Boedtker, 1960; Spirin, 1963). Within the ribosome, both RNA and protein exhibit some degree of structural independence, as shown by X-ray-diffraction (Zubay & Wilkins, 1960; Klug, Holmes & Finch, 1961; Langridge & Holmes, 1962) and hypochromicity studies (Schlessinger, 1960) on ribosome gels and ribosomal RNA. It seems likely therefore that, over a limited range of ionic conditions, RNA in the ribosome may respond to the ionic environment almost independently of the protein attached to it in the ribonucleoprotein complex, and could determine the stable conformation of '30s' or '50s' components under any particular set of conditions (cf. also Bogdanova, Gavrilova, Dvorkin, Kisselev & Spirin, 1962).

To produce anything from a minor physicochemical change to extensive degradation through ribonuclease activity requires only a small adjustment in the ionic environment, and none of the ionic conditions specified in the present work is improbable in relating the results to a biological system. Uncontrolled ribonuclease activity in experiments on ribosomes can obviously lead to erroneous conclusions, as already emphasized by Spirin (1963) in the study of the physical properties of RNA.

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