A Comparison of the Unfolding and Dissociation of the Large Ribosome Subunits from *Rhodopseudomonas spheroides* N.C.I.B. 8253 and *Escherichia coli* M.R.E. 600

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1. The behaviour of the large ribosomal subunit from *Rhodopseudomonas spheroides* (45S) has been compared with the 50S ribosome from *Escherichia coli* M.R.E. 600 (and *E. coli* M.R.E. 162) during unfolding by removal of Mg^{2+} and detachment of ribosomal proteins by high univalent cation concentrations. The extent to which these processes are reversible with these ribosomes has also been examined. 2. The *R. spheroides* 45S ribosome unfolds relatively slowly but then gives rise directly to two ribonucleoprotein particles (16.6S and 13.7S); the former contains the intact primary structure of the 16.25S rRNA species and the latter the 15.00S rRNA species of the original ribosome. No detectable protein loss occurs during unfolding. The *E. coli* ribosome unfolds via a series of discrete intermediates to a single, unfolded ribonucleoprotein unit (19.1S) containing the 23S rRNA and all the protein of the original ribosome. 3. The two unfolded *R. spheroides* ribonucleoproteins did not recombine when the original conditions were restored but each simply assumed a more compact configuration. Similar treatments reversed the unfolding of the *E. coli* 50S ribosomes; replacement of Mg^{2+} caused the refolding of the initial products of unfolding and in the presence of Ni^{2+} the completely unfolded species (19.1S) again sedimented at the same rate as the original ribosomes (44S). 4. Ribosomal proteins (25%) were dissociated from *R. spheroides* 45S ribosomes by dialysis against a solution with a Na^+/Mg^{2+} ratio of 250:1. During this process two core particles were formed (21.2S and 14.2S) and the primary structures of the two original rRNA species were conserved. This dissociation was not reversed. With *E. coli* 50S approximately 15% of the original ribosomal protein was dissociated, a single 37.6S core particle was formed, the 23S rRNA remained intact and the ribosomal proteins would reassociate with the core particle to give a 50S ribosome. 5. The ribonuclease activities in *R. spheroides* 45S and *E. coli* M.R.E. 600 and *E. coli* M.R.E. 162 50S ribosomes are compared. 6. The observations concerning unfolding and dissociation are consistent with previous reports showing the unusual rRNA complement of the mature *R. spheroides* 45S ribosome and show the dependence of these events upon the rRNA and the importance of protein-protein interactions in the structure of the *R. spheroides* ribosome.

One approach to the study of the structure of the bacterial ribosome involves a loosening and unfolding of the ribosome structure by the progressive removal of Mg^{2+} ions. This is usually achieved by chelation with EDTA. The typical *Escherichia coli* 50S ribosome unfolds in a co-operative process forming at first a 35–40S component which is further unfolded on the removal of more Mg^{2+} ions to form a 19–21S ribonucleoprotein species. The 30S ribosome similarly unfolds via a discrete 26S intermediate to a ribonucleoprotein species sedimenting at approximately 16S (Gesteland, 1966b; Gavrilova *et al.*, 1966; Weller *et al.*, 1968; Tal, 1969; Miall & Walker, 1969; Roberts & Walker, 1970). Throughout these transitions both the RNA and protein components of the

original ribosomes remain intact and associated (Weller *et al.*, 1968; Miall & Walker, 1969; Roberts & Walker, 1970; Eilam & Elson, 1971). Although differences have been reported in the numbers of intermediate species and the degree of reversibility of these transitions the basic structural implication is that the 50S ribosome of *E. coli* consists of a single ribonucleoprotein strand held in a specific, compact, tertiary structure. Mg^{2+} ions act as counterions to the phosphate groups in the RNA decreasing the electrostatic free energy of the ribosome and allow other forces, mediated by the protein components, to maintain the tertiary structure of the ribosome. It is assumed that the unfolding sequence is dependent upon the presence of an intact 23S rRNA species.

Another approach to ribosome structure, i.e. the progressive dissociation of proteins from *E. coli* 50S

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ribosomes by exposure to increasing concentrations of univalent cations, is also well documented (Meselson *et al.*, 1964; Lerman *et al.*, 1966; Atsmon *et al.*, 1967; Itoh *et al.*, 1968; Spitnik-Elson & Atsmon, 1969; Atsmon *et al.*, 1969; Staehelin *et al.*, 1969; Homan & Nierhaus, 1971). The high concentration of univalent cations weakens both the electrostatic repulsion between the phosphate groups in the RNA and the possible electrostatic attraction between these groups and basic groups in the ribosomal proteins. Mg^{2+} ions are also competitively displaced from the ribosome and a co-operative loss of distinct groups of proteins ensues in a series of discrete steps. The 50S ribosome gives rise first to a 43–46S core particle and approximately 20% of the original protein may be detached depending upon the conditions employed.

The ribosomes of *Rhodopseudomonas spheroides* resemble those of *E. coli* and other bacteria in their gross composition and in consisting of a large (45S) and a small (29S) subunit. Friedman *et al.* (1966) showed that these will combine under certain conditions to give a 66S ribosome (cf. 70S in *E. coli*). However, the large ribosome subunit (45S) is atypical in that its high-molecular-weight rRNA complement consists of two different RNA species, i.e. one sedimenting at 16.25S ($s_{20,w}^0$ value) and one at 15.0S ($s_{20,w}^0$ value) (Marrs & Kaplan, 1970; Robinson & Sykes, 1971). This ribosome therefore presents a useful experimental system, generated *in vivo* for the examination of ribosome structure via the approaches outlined above. Accordingly in the present investigation the behaviour of *R. spheroides* 45S ribosomes has been compared with that of *E. coli* 50S ribosomes during unfolding by dialysis against EDTA and in dissociation of the ribosomes by high concentrations of univalent cations. The nature of the products formed and the reversibility of these procedures with these ribosomes have also been examined.

Materials and Methods

Bacterial strains and growth

R. spheroides N.C.I.B. 8253 was used throughout this study. The organism was grown at 30°C heterotrophically in batch cultures on the glutamate–malate medium described by Lascelles (1956) and supplemented with trace metals as described by Robinson & Sykes (1971). The level of ribonuclease activities in cell homogenates, ribosomes and soluble fractions from this organism in 0.01M-Tris–HCl–0.1mM-magnesium acetate buffer (pH 7.5) has been shown to be low (Robinson & Sykes, 1971). In the present experiments the nuclease activity of the ribosome preparations in the buffers employed and the specific tests under optimal conditions for ribonucleases I

and II gave results comparable with *E. coli* M.R.E. 600 (see the Results section).

E. coli M.R.E. 600 was chosen for comparison since this organism is deficient in ribonuclease I activity (Cammack & Wade, 1965) and has a single, covalent 23S rRNA species in the 50S ribosome. This organism was batch grown at 30°C on a medium containing per litre: 10g of Bacto-peptone (Difco Laboratories Inc., Detroit, Mich., U.S.A.), 9g of KH_2PO_4 , 1g of $(NH_4)_2SO_4$, 0.8g of $MgSO_4$ and 5g of glucose at pH 7.5. Cultures were grown aerobically in 250ml batches in 2-litre conical flasks on a Gyrotary Shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.) or in a 10-litre fermentor with forced aeration (L. H. Engineering Co. Ltd., Stoke Poges, Bucks., U.K.). *E. coli* M.R.E. 162, a strain with high ribonuclease I activity (Wade & Robinson, 1966), was used for certain comparative experiments and was grown in an identical manner.

Exponential-phase cells were harvested by centrifugation at 5°C.

Isolation of the large ribosomal subunit

R. spheroides 45S and *E. coli* 50S ribosomes were prepared by differential centrifugation as follows. Harvested-cell pastes were immediately placed in the Hughes bacterial press at –20°C and then crushed. The crushing process was repeated once more with *R. spheroides* to ensure a high degree of cell breakage (Robinson & Sykes, 1971). If not used immediately the crushed bacterial cells were stored at –20°C. Crushed cells (6–10g) were evenly dispersed in 3.5vol. of ice-cold 0.01M-Tris–HCl–0.1mM-magnesium acetate buffer (pH 7.5) containing 10µg of ribonuclease-free deoxyribonuclease/ml (EC 3.1.4.5; Worthington Biochemicals Corp., Freehold, N.J., U.S.A.)/g. The mixture was dispensed into centrifuge tubes at 4°C and during this time the viscosity of the mixture was appreciably lowered. The preparation was centrifuged at 54333g_{av.} for 35min at 5°C (Beckman model L2-50 ultracentrifuge, angle 50 rotor). The supernatant was carefully decanted from the pellet of unbroken cells and cell-wall debris and re-centrifuged at 122250g_{av.} for 90min in the angle 50 rotor. The pellet was resuspended in 0.01M-Tris–HCl–0.1mM-magnesium acetate buffer (pH 7.5) by using a small hand homogenizer. Any insoluble material was removed by a low-speed centrifugation and the clear suspension was centrifuged at 122250g_{av.} at 5°C for a further 60min. The pellet was well drained and the hard-packed glassy material carefully resuspended in 0.01M-Tris–HCl–0.1mM-magnesium acetate buffer (pH 7.5) and again centrifuged at 122250g_{av.} for 1h. No braking force was applied to decelerate the rotor in the above centrifugations. The final hard-packed pellet was

suspended in 3–4 ml of 0.01 M-Tris-HCl–0.1 mM-magnesium acetate buffer (pH 7.5) and insoluble material removed by a low-speed centrifugation. This suspension was the purified *R. spheroides* 45S or *E. coli* 50S ribosome preparation (see Fig. 1a) and was diluted to give an absorbance of 42.5–50.0 at 260 nm, i.e. equivalent to approx. 5 mg of ribosomes/ml.

Unfolding of the ribosomes by dialysis against EDTA

The method employed was essentially that of Gesteland (1966b). All dialysis tubing was boiled in 0.01 M-EDTA for 10 min, well rinsed in glass-distilled water and finally in the buffer to be used. For the unfolding experiments a series of approximately 1.0 ml samples of the ribosome preparation (*E. coli* 50S or *R. spheroides* 45S) were placed in separate dialysis sacs and dialysed in 200 vol. of 0.01 M-Tris-HCl–1 mM-EDTA buffer (pH 7.5) at 4°C. At the times indicated in the figure legends and the Results section the contents of an individual dialysis sac were removed and analysed. Attempts to re-form 50S or 45S ribosomes were made by removing unfolded samples in their dialysis sacs from the 0.01 M-Tris-HCl–1 mM-EDTA buffer (pH 7.5) and immediately dialysing against two or three changes of 200 vol. of 0.01 M-Tris-HCl–1 mM-magnesium acetate buffer (or 1 mM-NiSO₄), pH 7.5.

Partial dissociation of protein from the large ribosomal subunits

To dissociate some of the ribosomal proteins from the ribosomes, the ribosomes were dialysed against 0.01 M-Tris-HCl–1 mM-magnesium acetate–0.25 M-NaCl buffer (pH 7.5). The details of the dialysis procedure are the same as described in the section above. The ribosomes for these experiments were isolated as described above except that the solvent used throughout the preparation was 0.01 M-Tris-HCl–1 mM-magnesium acetate buffer (pH 7.5).

Attempts were made to reassociate the protein-deficient particles and dissociated protein molecules obtained by dialysis by redialysing unopened dialysis sacs against 0.01 M-Tris-HCl–1 mM-magnesium acetate buffer (pH 7.5) at 4° and 30°C.

Analytical ultracentrifugation

The sedimentation properties of the ribosome preparations were examined in the Spinco model E analytical ultracentrifuge equipped with u.v. and schlieren optics. Sedimentation coefficients are reported in Svedberg units corrected to 20°C. The use of standard and 1° positive wedge dural cells enabled

the simultaneous analysis of comparable *R. spheroides* and *E. coli* preparations.

Electrophoretic analyses

The RNA components of the native ribosomes, the unfolded ribosomes and the protein-deficient core particles were examined by polyacrylamide-gel electrophoresis. The RNA was first dissociated from the protein in the ribosomes by adjusting the ribosome concentration to approximately 2 mg/ml and then adding sodium dodecyl sulphate (specially pure grade; BDH Chemicals Ltd., Poole, Dorset, U.K.) to a final concentration of 0.2% (w/v) (Noll & Stutz, 1968). Sucrose, to a final concentration of 20% (w/v), was also added to the mixture to facilitate the layering of the electrophoresis buffer over the samples placed on cylindrical 2.4% polyacrylamide gels. Polyacrylamide gels were prepared, used and scanned as described by Robinson & Sykes (1971). The electrophoresis buffer was 0.04 M-Tris-HCl–0.02 M-sodium acetate–1 mM-EDTA buffer (pH 7.8). The gels were pre-run for 1 h before layering a volume of sample equivalent to 60–70 µg of rRNA on to the gels. The gels were subject to electrophoresis for 2.5 or 3 h at 5 mA/gel and 4°C.

Isolation of ribosomes and ribosome derivatives on rate-zonal sucrose gradients

For certain experiments the native ribosomes and their derivatives were reisolated on 5–20% (w/v) linear sucrose gradients in 0.01 M-Tris-HCl–1 mM-magnesium acetate buffer, pH 7.5 (for ribosomes), 0.01 M-Tris-HCl–1 mM-EDTA buffer, pH 7.5 (for unfolded derivatives), and 0.01 M-Tris-HCl–1 mM-magnesium acetate–0.25 M-NaCl buffer, pH 7.5 (for protein-deficient core particles). The Spinco SW39 rotor was used at 118000g_{av} at 5°C and the rotor was allowed to coast to rest. A 2 h centrifugation was used for the isolation of native ribosomes and a 3 h centrifugation for the isolation of the unfolded *R. spheroides* ribosomes. The *R. spheroides* protein-deficient core particles were centrifuged for 4 h and the *E. coli* 37.8S core particles for 2.25 h. The gradients were analysed after centrifugation by puncturing the base of the tubes and measuring the absorption of the effluent at 254 nm (Coleman & Sykes, 1966). The appropriate fractions were collected from the gradients and thoroughly dialysed against 0.01 M-Tris-HCl–1 mM-magnesium acetate buffer (pH 7.5) to ensure removal of the sucrose. RNA was estimated by the orcinol reaction (Schneider, 1957) with *E. coli* highly polymerized RNA (BDH) or yeast RNA (Calbiochem Ltd., London W.1, U.K.) as standard. Protein was estimated by the Folin-Ciocalteu reaction (Lowry *et al.*, 1951) with crystalline bovine serum albumin as standard (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.).

Enzyme assays

Ribonuclease I activity in the preparations was estimated as described by Gesteland (1966a, assay C) (assay 1) and Spahr (1966) (assay 2). These assays also detect latent RNAase* I since in assay (1) urea causes activation of latent enzyme and in assay (2) EDTA causes activation in addition to inhibiting RNAase II. For assay (2) the reaction mixture volume was 5.0ml containing 10.0mg of ribosomes (or derivatives), 0.1M-Tris-HCl buffer, pH7.5, 0.2M-NaCl and 0.05M-EDTA. The reaction was carried out at 4° and 30°C, and 0.5ml samples were taken at timed intervals and added to 4.5ml of ice-cold 0.5M-HClO₄. The samples were centrifuged in the cold and the absorbances of the supernatants at 260nm recorded.

Ribonuclease II activity was determined by the method of Singer (1966). The reaction mixture totalled 5.0ml and contained 10.0mg of ribosomes (or derivatives), 0.1M-Tris-HCl buffer, pH7.5, 0.1M-KCl and 1.5mM-MgCl₂. The sampling, assay temperatures etc. were as described above for RNAase I.

Non-specific ribonuclease activity in the various ribosome preparations in the buffers used to unfold or dissociate was similarly estimated by using 5.0ml of reaction mixtures containing 10.0mg of ribosomes in the appropriate buffer. The release of u.v.-absorbing material during the dialyses to unfold/dissociate was also recorded.

Results

Sedimentation properties of the ribosomes

The sedimentation profiles of the *R. spheroides* and *E. coli* ribosome preparations prepared as described in the Materials and Methods section in 0.01M-Tris-HCl-0.1mM-magnesium acetate, pH7.5, are shown in the Schlieren diagrams in Fig. 1(a). It is evident that at the same ribosome concentration (E_{260} 42.5) that the *R. spheroides* subunit has a lower sedimentation coefficient than the equivalent *E. coli* subunit. The sedimentation coefficients of these ribosomes in this solvent, determined by using the u.v.-absorption optics of the model E analytical ultracentrifuge with ribosome preparations having an E_{260} of 1.13 are 45.7S ($s_{20,w}^0$ value) $\pm$ 0.49 (s.e., four determinations) for *R. spheroides* and 49.9S ($s_{20,w}^0$ value) $\pm$ 0.34 (s.e., four determinations) for *E. coli*. The preparations do not contain significant amounts of other cell components.

Unfolding of the ribosomes

Dialysis of the ribosomes against 0.01M-Tris-HCl-1mM-EDTA (pH7.5). *R. spheroides* 45S and *E. coli*

* Abbreviation: RNAase, ribonuclease.

50S ribosomes in 0.01M-Tris-HCl-0.1mM-magnesium acetate buffer (pH7.5) were dialysed against 0.01M-Tris-HCl-1mM-EDTA buffer (pH7.5) as described in the Materials and Methods section. Samples were removed for examination in the ultracentrifuge at the times stated in Fig. 1. After 2h of dialysis there was very little unfolding of either ribosome preparation. The sedimentation profile of *R. spheroides* ribosomes was only slightly altered after 4h of dialysis (Fig. 1b) with only a small amount of slow derivatives (17.4S and 14.9S) present. In contrast *E. coli* 50S ribosomes began to unfold at this time. A 36.9S derivative was formed as the main unfolded species together with slower-sedimenting species at 22.4S and 18.4S. After 6h of dialysis (Fig. 1c) there was a marked change in the sedimentation of approximately one-third of the *R. spheroides* 45S

Fig. 1. Schlieren diagrams of the effect of dialysis of *R. spheroides* 45S and *E. coli* 50S ribosomes in 0.01M-Tris-HCl-0.1mM-magnesium acetate buffer, pH7.5, against 0.01M-Tris-HCl-1mM-EDTA buffer, pH7.5

Samples in individual dialysis sacs were removed and examined at the times shown. Sedimentation is to the right and a speed of 50740rev./min was used. Photographs were taken after 14min at speed. Upper trace is of *E. coli* and lower trace is of *R. spheroides* in every frame. All ribosome solutions had an absorbance of 42.5 at 260nm. Sedimentation coefficients corrected to 20°C in water are given for the major species present. (a) Samples before dialysis. Bar angle of 60°. *E. coli* 44.5S; *R. spheroides* 41.9S. (b) 4h of dialysis. Bar angle of 50°. *E. coli* 42.5S, 36.9S, 22.4S and 18.4S; *R. spheroides* 40.4S, 17.4S and 14.9S. (c) 6h of dialysis. Bar angle of 55°. *E. coli* 42.0S, 35.9S and 21.4S; *R. spheroides* 39.2S, 17.0S and 14.4S. (d) 8½h of dialysis. Speed of 59780rev./min for 8min. Bar angle of 60°. *E. coli* 19.1S; *R. spheroides* 40.1S, 16.6S and 13.7S.

ribosomes. A slow derivative was also formed which tended to resolve into two components sedimenting at 17.0S and 14.4S. Virtually all the 50S ribosomes of *E. coli* had unfolded at this time, forming a 21.4S species with only a small amount of the 50S and 35.9S components remaining. After 8.25h of dialysis (Fig. 1d) there was still a small amount of *R. spheroides* 45S ribosomes remaining. A major *R. spheroides* unfolded derivative sedimenting at 13.7S and a smaller, faster product at 16.6S were now evident. In contrast all the *E. coli* ribosomes had unfolded at this time to produce a single 19.1S derivative.

The time-course of unfolding was found to vary slightly with different preparations. Nonetheless *E. coli* 50S ribosomes invariably unfolded via several discrete intermediates, principally a 36–37S component and finally to a single component (19.1S; see Fig. 1d). The 50S ribosomes from *E. coli* M.R.E. 162 also showed this unfolding pattern. However, at no time during the dialysis were any such discrete intermediate unfolded species seen in the *R. spheroides* preparations. Here two final unfolded species were produced abruptly from the 45S ribosome; one sedimenting at 13.7S and the other at 16.6S. The nature of the two unfolded species derived from the *R. spheroides* ribosomes is examined in the following experiments.

Characterization of the derivatives produced by EDTA treatment of the ribosomes. The changes in sedimentation properties of the ribosomes during dialysis against the Tris–EDTA buffer may be due to the removal of protein and/or RNA or to conformational alteration of the ribosomes while conserving the rRNA primary structure and total protein complement of the original ribosomes. The latter possibility has been shown to be the case for the formation of the single slowly sedimenting derivatives of *E. coli* ribosomes (e.g. Fig. 1d) (Weller *et al.*, 1968; Miall & Walker, 1969; Roberts & Walker, 1970; Eilam & Elson, 1971). In the case of the *R. spheroides* 45S ribosomes corrected area measurements obtained by planimetry of the peaks in enlarged (5×) schlieren diagrams of the native ribosomes and ribosomes dialysed for 9h against the Tris–EDTA buffer showed that there was no decrease in total area under the peaks after dialysis, i.e. there was conservation of ribosomal material during this process. There is also no evidence of slowly sedimenting material accumulating at the meniscus in the analytical-ultracentrifuge cell during dialysis (see Fig. 1).

R. spheroides native 45S ribosomes before and after dialysis against the Tris–EDTA buffer were re-isolated on sucrose gradients and their RNA and protein contents determined by the orcinol and Folin–Ciocalteu reactions. For this 7.33h of dialysis against EDTA was used which unfolded approximately 50% of the original 45S ribosomes. During the subsequent layering and centrifugation of these ribo-

somes in linear rate-zonal 5–20% (w/v) sucrose gradients in 0.01 M-Tris–HCl–1 mM-EDTA buffer (pH 7.5) the unfolding was completed. The slowly migrating boundary in the sucrose gradient which was collected for analysis showed a tendency to resolve into two components. The RNA content of this fraction was 58.9% compared with 58.6% for native ribosomes similarly isolated from sucrose gradients (averages for two separate experiments in each case). The gross RNA and protein composition of the original ribosomes is therefore conserved during the unfolding by dialysis against 0.01 M-Tris–HCl–1 mM-EDTA buffer, pH 7.5.

The primary structure of the RNA in the ribosomes was also found to be conserved. Indirect evidence for this was obtained by treating the products formed after 6h of dialysis of both *E. coli* and *R. spheroides* ribosomes (Fig. 1c) with pancreatic ribonuclease (polyribonucleotide 2-oligonucleotide transferase, EC 2.7.7.16). In both instances the slowly sedimenting species were all susceptible to rapid degradation by the enzyme, indicating that these products contained polymerized but exposed RNA molecules. On the other hand the remaining native ribosomes, i.e. those apparently unaltered at this stage of dialysis, appeared to be resistant to enzyme digestion. Direct evidence that the RNA components of the ribosomes remained undegraded throughout the unfolding was obtained from polyacrylamide-gel electrophoresis of the RNA. *R. spheroides* 45S and *E. coli* 50S ribosomes were dialysed against 0.01 M-Tris–HCl–1 mM-EDTA buffer (pH 7.5) for 8h. During this time approximately 70% of the *R. spheroides* ribosomes were converted into the 16.6S and 13.7S derivatives and the *E. coli* 50S ribosomes were totally converted into the 19.1S derivative. The protein was dissociated from the RNA in the ribosome preparations by the addition of sodium dodecyl sulphate as described in the Materials and Methods section. Electrophoretograms of the RNA from detergent-treated preparations from *R. spheroides* native 45S ribosomes and the ribosomes dialysed against 0.01 M-Tris–HCl–1 mM-EDTA buffer (pH 7.5) were identical, both containing the 15.0S and 16.25S rRNA components (Fig. 2a). *E. coli* 50S ribosomes and their unfolded 19.1S derivatives also produced identical electrophoretograms with the anticipated 23S rRNA component and a very small amount of 16S RNA from the contaminating 30S ribosomes (Fig. 2b). The primary structure of the two rRNA species in *R. spheroides* 45S ribosomes is therefore conserved during unfolding and the ribonuclease activity in both preparations before and during dialysis is evidently low (see below).

These observations, taken with the above observation that two unfolded species are produced without observable intermediates, suggests that the *R. spheroides* 45S ribosome may straightway dissociate

Fig. 2. Polyacrylamide-gel electrophoretograms of the RNA isolated from ribosomes dialysed against the Tris-EDTA buffer

(a) *R. spheroides* 45S and (b) *E. coli* 50S ribosomes. The ribosomes were dialysed against 0.01M-Tris-HCl-1mM-EDTA, pH7.5, for 8h. The protein was dissociated from the ribosomes and the resulting mixture subjected to electrophoresis and the gels were scanned at E_{260} as described in the Materials and Methods section.

into two ribonucleoprotein units each containing one of the original RNA species. The relative amounts of the two units seen in Fig. 1(d) are distorted by a pronounced Johnston & Ogston (1946) effect, and this could be exaggerated by the two species differing in their protein/RNA ratios. These possibilities were tested and the 16.25S RNA species found to be associated with the fastest-sedimenting ribonucleoprotein unit as follows.

R. spheroides ribosomes were unfolded and then centrifuged in rate-zonal sucrose gradients as described above. The fractions from the gradient corresponding to the front, middle and rear of the partially resolved slowly moving peak were collected and concentrated by dialysis against polyethylene glycol P6000. The ratio of absorbancies at 260/280nm was 2.14 across the entire peak compared with intact and unfolded ribosome preparations having an absorbance ratio of 2.03 and 2.14 respectively. The concentrated samples were made 0.2% (w/v) with sodium dodecyl sulphate, well mixed and applied to 2.4% (w/v) polyacrylamide gels. The electrophoretogram from the pooled fractions from the front of the unfolded ribosome preparation gave a major ribonuclease-sensitive u.v.-absorbing band co-electrophoresing with 16.25S rRNA from *R. spheroides*. Conversely if the unfolded ribosome preparations were centrifuged in the moving-partition separation cell of the analytical ultracentrifuge and the fraction corresponding to the slowest-sedimenting species

was taken, treated with detergent and subjected to electrophoresis, a major band corresponding to the 15.0S rRNA species was obtained. The two species arising during the unfolding of *R. spheroides* 45S ribosomes are therefore ribonucleoprotein particles with similar RNA/protein ratios and having the 16.25S rRNA species associated with the fastest-sedimenting (16.6S) and the 15.0S rRNA species with the slower-sedimenting unit (13.7S).

Reversibility of the unfolding of the ribosomes. With the partially unfolded *E. coli* ribosome (i.e. after 3h of dialysis (Fig. 1b) total refolding to 50S-like ribosomes was achieved by dialysis against 0.01M-Tris-HCl-1mM-magnesium acetate buffer, pH7.5, for 16h at 4°C. These refolded ribosomes, like native 50S ribosomes, were relatively resistant to pancreatic ribonuclease (EC 2.7.7.16) whereas the various unfolded species are all extremely sensitive and rapidly degraded. However, this procedure was not sufficient to refold the totally unfolded 19.1S derivative; a further 1h of incubation at 30°C (in view of the known temperature effect in refolding during ribosome assembly *in vitro*, Nomura & Erdman, 1970) also failed to bring about refolding of this derivative. The inclusion of 1mM-NiSO₄ in this dialysis buffer (Tal, 1969) brought about the complete conversion of the 19.1S derivative into material sedimenting at 44S i.e. the sedimentation coefficient of native 50S ribosomes under these conditions. All of these procedures failed to reassociate the two unfolded species arising from *R. spheroides* 45S ribosomes. Thus although the conditions to achieve unfolding of *E. coli* and *R. spheroides* ribosomes are similar the conditions required to refold clearly differ.

Effect of partial dissociation of the protein from the ribosomes

Sedimentation profiles. The sedimentation profiles of *R. spheroides* 45S and *E. coli* 50S ribosomes, prepared in 0.01M-Tris-HCl-1mM-magnesium acetate buffer, pH7.5, and dialysed against 0.01M-Tris-HCl-1mM-magnesium acetate-0.25M-NaCl buffer (pH7.5) are shown in Fig. 3(a-d). After 1h of dialysis there was no alteration in the *R. spheroides* preparation, but a small 39.6S component was observed in the *E. coli* preparation (compare Fig. 3a and Fig. 1a). The proportion of this component increased in the *E. coli* preparations after 2h of dialysis and small amounts of species sedimenting at 27.3S and 20.9S appeared in the *R. spheroides* samples. These effects were pronounced after 4h of dialysis (Fig. 3b) together with an obvious decrease in the relative amount of the original ribosomes. After 7h of dialysis (Fig. 3c) none of the original *E. coli* 50S or *R. spheroides* 45S ribosomes remained. At this time the *R. spheroides* preparation contained a major heterodisperse product sedimenting at approximately

Fig. 3. Effect of dialysing *R. spheroides* 45S (lower trace) and *E. coli* 50S ribosomes (upper trace) against 0.01M-Tris-HCl-1mM-magnesium acetate buffer, pH7.5, containing 0.25M-NaCl

Samples from individual dialysis sacs were removed and examined at the times shown. Sedimentation is to the right and a speed of 50740 rev./min was used. Sedimentation coefficients, corrected to 20°C in water, are given for the major species present. All ribosome solutions had an absorbance of 50 at 260nm. (a) After 1h of dialysis. Bar angle of 60°, 14min at speed. *E. coli* 44.1S and 39.6S. *R. spheroides* 41.8S. (b) After 4h of dialysis. Bar angle of 50°, 16min at speed. *E. coli* 44.4S and 37.8S. *R. spheroides* 42.4S, 21.0S and 19.1S. (c) After 7h of dialysis. Bar angle of 50°, 16min. at speed. *E. coli* 37.6S and 32.6S. *R. spheroides* 21.2S and 14.2S. (d) Sample from (b) treated with pancreatic ribonuclease. Bar angle of 50°, 6min at speed. *E. coli* 46.3S and 33.1S, *R. spheroides* 44.7S.

21.2S partially resolved into two components. The major peak in the *E. coli* preparations sedimented at 37.6S together with a minor 32.6S species. The 21.2S species in the *R. spheroides* preparation was rapidly and completely degraded by pancreatic ribonuclease whereas the *E. coli* 37.8S species was not so readily digested and the original ribosomes remaining in both preparations were superficially unaltered by this enzyme treatment (Fig. 3d).

The changes in the sedimentation properties of the ribosomes during the dialysis were not due to degradation of the primary structure of their RNA components. Identical polyacrylamide-gel electrophoretograms were obtained with the RNA from native *R. spheroides* 45S ribosomes and the 21.2S dialysis product, each preparation giving a 16.25S and 15.0S RNA. Similarly *E. coli* 50S ribosomes and their 37.8S products gave 23S RNA.

The change in sedimentation properties was accompanied by a dissociation of a fraction of the ribosomal proteins ('split proteins') from the ribosomes. Direct evidence for this was obtained by determinations of the RNA and protein contents of the ribosomes and their derivatives isolated by pooling appropriate fractions from rate-zonal sucrose-gradient separations as described under the Materials and Methods section. In *R. spheroides* the partially resolved 21.0S and 19.1S peaks were pooled and in the *E. coli* preparations the 37.8S peak (Fig. 3b) was collected for analysis. The RNA content of *R. spheroides* 45S ribosomes was found to be $58.7\% \pm 1.17$ (s.e., eight determinations) and for *E. coli* 50S $60.1\% \pm 1.41$ (s.e., three separate determinations). A Student's *t* test revealed no significant difference between these values. Comparison of these values with the determinations on the products of dialysis showed that in the formation of the *R. spheroides* derivatives 25% of the ribosomal protein had been dissociated (21.4–27.8%, four determinations). In the formation of the *E. coli* 37.8S particle 15% of the original 50S ribosomal protein had been dissociated. Dialysis against 0.01M-Tris-HCl-1mM-magnesium acetate-0.25M-NaCl buffer (pH7.5) therefore results in a pronounced alteration in the sedimentation properties of both bacterial ribosomes accompanied by a partial loss of ribosomal proteins but the gross properties of the rRNA species are unaffected.

Reassociation of protein-deficient ribosomes and dissociated proteins. Ribosomes were dialysed as described above for 7h (see Fig. 3c) and without opening the dialysis sac the preparations were dialysed for a further 16h at 4°C against 0.01M-Tris-HCl-1mM-magnesium acetate (pH7.5). In the case of *E. coli* considerable reformation of 50S-like material was achieved and almost total re-formation took place if dialysis was for 20h followed by 1h at 30°C. With the *R. spheroides* preparations a major 34.2S component was formed in identical dialyses. The 50S particles re-formed in the *E. coli* preparations and the residual *R. spheroides* 45S ribosomes were not as sensitive to pancreatic ribonuclease digestion as the *R. spheroides* 34.2S species. Further, unlike the 45S ribosome, the *R. spheroides* 34.2S species was destroyed by a brief heat treatment (60°C, 3min). To provide an additional check on the degree of reassociation achieved in these dialyses the reformed *E. coli* 50S material and the *R. spheroides* 34.2S species were isolated by 2h (*E. coli*) or 3h (*R. spheroides*) centrifugations in linear 5–20% sucrose rate-zonal gradients in 0.01M-Tris-HCl-1mM-magnesium acetate buffer, pH7.5, and analysed for RNA and protein as described in the Materials and Methods section. In *E. coli* the native and re-associated 50S ribosomes had identical compositions, confirming that complete reassociation had taken place. However, the *R. spheroides* 34.2S species was

approximately 8% deficient in protein (7.8–9.2%, three determinations) compared with the native 45S ribosome. The 34.2S species therefore appears to be an association of the two protein-deficient particles with an incomplete reassociation of the dissociated protein.

Ribonuclease activities of E. coli and R. sphaeroides ribosomes

At 4°C the non-specific ribonuclease activities of both *R. sphaeroides* and *E. coli* M.R.E. 600 ribosomes before and after the dialyses to unfold or dissociate were very low, i.e. all less than 0.06 µmol of cold-acid-soluble ribonucleotide material released/h per mg of ribosomes. The activities in the specific assays for ribonuclease I (0.03–0.40 µmol of nucleotide released/h per mg of ribosomes) and ribonuclease II (<0.060 µmol of nucleotide released/h per mg of ribosomes) were also low in all circumstances in both preparations. At 30°C the former activities were comparable with the values reported by Gesteland (1966a) for ribosomes from ribonuclease I-deficient strains of *E. coli* (i.e. 0.2 µmol of nucleotide/h per mg of ribosomes). On the other hand the ribosomes from the ribonuclease I-active strain *E. coli* M.R.E. 162 had an activity of 2.19 µmol of nucleotide released/h per mg of ribosomes. The high ribonuclease I activity of *R. sphaeroides* cell extracts at pH 9 (Wade & Robinson, 1966) is clearly not expressed at pH 7.5 in the ribosomes isolated by repeated cycles of centrifugation from low-ionic-strength buffers, whereas significant amounts of activity remain with the *E. coli* M.R.E. 162 ribosomes. The low ribonuclease I activities found in *R. sphaeroides* and *E. coli* M.R.E. 600 ribosomes are not due to inhibition in the assay system since the addition of a final concentration of 1.2 µg of ribonuclease (EC 2.7.7.16)/ml at the conclusion of a 60 min assay-period gave an 8–20-times increase in cold-acid-soluble nucleotide material within 15 min. The ribosomal ribonuclease assay (assay C; Gesteland, 1966a) confirmed the above observations since there was a negligible release of u.v.-absorbing material on dialysis of the ribosomes for 16 h at 4°C. The low levels of ribonuclease activity found in the ribosome preparations from *R. sphaeroides* and *E. coli* 600 would not result in more than 1% of the total input of rRNA becoming degraded at 4°C during the periods of dialysis used to unfold or dissociate the ribosomes in these experiments. This conclusion is substantiated by the recovery of the intact, original rRNA species from the ribosomes with little sign of degraded material at the conclusion of the dialyses (e.g., see Fig. 2). It is therefore unlikely that the ribonuclease activities of the two ribosome preparations would account for the observed differences in their unfolding and dissociation, and especially since the *E. coli* M.R.E. 162 50S ribosomes were found to give

an identical unfolding pattern to *E. coli* M.R.E. 600 ribosomes.

Discussion

The pattern of unfolding with EDTA treatment and dissociation of proteins by salt from the *R. sphaeroides* 45S ribosome is quite distinct from that observed for *E. coli* M.R.E. 600 50S ribosomes under identical conditions. The pattern observed with the *E. coli* ribosomes is in substantial agreement with earlier reports (Gavrilova *et al.*, 1966; Gesteland, 1966b; Weller *et al.*, 1968; Miall & Walker, 1969; Tal, 1969; Roberts & Walker, 1970; Eilam & Elson, 1971). Since the ribonuclease activities of both ribosome species are very low under all circumstances reported here and the majority of their gross properties are similar, e.g. composition, association with 30S to give a 70S-type species etc., the differences observed may be attributed to the major difference in their respective rRNA complements. Thus *E. coli* ribosomes with a single covalent 23S rRNA species unfold via a series of discrete intermediates to give a single completely unfolded 19.1S ribonucleoprotein containing intact 23S rRNA. In identical circumstances the *R. sphaeroides* 45S ribosome containing two large rRNA species (Marrs & Kaplan, 1970; Robinson & Sykes, 1971) unfolds without observable intermediates to directly yield two unfolded ribonucleoproteins; one contains the original 16.25S rRNA species and the other the 15.0S rRNA species. In all cases the unfolded species present a more exposed rRNA and are dependent upon its observed integrity since they are rapidly degraded by ribonuclease (cf. original ribosomes).

The extent to which the unfolding process may be reversed also emphasizes the difference in basic structure of the two ribosome species. The initial partially unfolded intermediates from *E. coli* were readily re-folded to the original 50S species whereas the completely unfolded 19.1S species was not. Difficulty in reversing the complete *E. coli* unfolding sequence has been observed previously (Gavrilova *et al.*, 1966; Atsmon *et al.*, 1967; Morell & Marmur, 1968; Roberts & Walker, 1970). The apparent reversal with Ni^{2+} present, although in agreement with the observation by Tal (1969), should be viewed with caution in view of the tendency of this ion to increase the sedimentation coefficient of all species present. However, in total contrast the *R. sphaeroides* 45S ribosome was not re-formed from its two unfolded ribonucleoprotein derivatives under any circumstances. This difference persists in the dissociation experiments where in *E. coli* a single core particle was produced which recombined with the split protein whereas in *R. sphaeroides* two particles were produced which did not completely recombine with the split proteins. The absence of any reversibility in these two processes with *R. sphaeroides*

may reflect a failure to provide the correct conditions and/or the loss of an essential component. However, the dissociation and unfolding behaviour is consistent with the earlier reports demonstrating two rRNA components in the mature ribosome. Further, since the transient formation of a 23S rRNA occurs during the biogenesis of the 45S ribosome (Marrs & Kaplan, 1970; Robinson, 1971), the failure to reverse dissociation and unfolding may indicate the need for a transient 23S rRNA matrix to facilitate the protein-protein interactions clearly involved in the stabilization of the mature *R. sphaeroides* 45S ribosome. In a similar system generated *in vitro* with *E. coli* ribosomes split into two ribonucleoprotein components by controlled ribonuclease action and salt treatment Allet & Spahr (1971) found that certain split proteins would only reassociate in the presence of both core particles. The proteins of the ribosome are not randomly distributed and some are clearly bound to multiple sites.

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