Reassembly of the Peptidyltransferase Centre of Larger Subparticles of Rabbit Reticulocyte Ribosomes from a Core-Particle and Split-Protein Fraction

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We report the reconstruction, from a core-particle and split-protein fraction, of the larger subribosomal particle of rabbit reticulocytes. The reassembled particle was active in poly-phenylalanine synthesis and in the puromycin reaction. The core-particles and split-protein fractions were obtained by treatment of the larger subparticle with salt solutions containing NH_4^+ and Mg^{2+} in the molar ratio 40:1 over the range 2.25–2.75 M- NH_4Cl /56–69 mM- MgCl_2 at 0°C. This treatment led to the loss of about eight proteins (approx. 17% of the protein moiety), which were found wholly or largely in the split-protein fraction as shown by two-dimensional gel electrophoresis. The core particle retained 5S rRNA and had much decreased (no more than 10% of control) ability to function in the puromycin reaction or in poly(U)-directed polyphenylalanine synthesis. Activity was recovered when the recombined core-particle and split-protein fractions were dialysed overnight at 4°C against 0.3 M- NH_4Cl /15 mM- MgCl_2 /1 mM-dithiothreitol/15% (v/v) glycerol/20 mM-Tris/HCl, pH 7.6, and then heated for 1 h at 37°C. The recovery was 40–80% of the original activity. Raising the concentration of MgCl_2 to 300 mM in 2.5 M- NH_4Cl led to the removal of seven rather than eight proteins, and the core particle remained active in the puromycin reaction. We infer that the protein retained by raising the concentration of Mg^{2+} is an essential component of the peptidyltransferase centre of the ribosome.

The ability to take ribosomes apart and to reassemble them from their RNA and protein components has advanced our understanding of the functional role of the components of bacterial ribosomes (for reviews, see Nomura, 1973; Nomura & Held, 1974).

Cytoplasmic ribosomes of eukaryotes are structurally more complicated than those of bacteria, and comparable progress has not been made in establishing structure–function relationships for the individual components. A bar to progress has been the absence of suitable core particles and their corresponding split-protein fractions. Attempts to produce core particles have been based on manipulations of the ionic conditions (see, e.g., Lerman, 1968; Reboud *et al.*, 1969; Clegg & Arnstein, 1970; Grummt & Bielka, 1970; Ford, 1971; Terao & Ogata, 1971; Reboud *et al.*, 1972).

The present paper reports the production of a core-particle and split-protein fraction of the larger subribosomal particle of rabbit reticulocytes by means of treatment with 2.25–2.75 M- NH_4Cl /56–69 mM- MgCl_2 , and the reassembly of functional subribosomal particles from their component fractions.

The basis for the method was provided by a study of the effect of MgCl_2 on the conformation of isolated rRNA [see the first of the present group of papers, Cox & Hirst (1976)], which suggested that rRNA

could be maintained in its 'native' conformation, provided that the molar ratio $\text{Mg}^{2+}/\text{NH}_4^+$ (or K^+) was maintained at (or greater than) 1:40. The study also provided support for the notion that the native conformation is essential for biological activity and that the Mg^{2+} concentration influences ribosome function through its effect on the conformation of the rRNA moiety. It was further shown, in the preceding paper (Cox *et al.*, 1976b) that when 0.5 M- NH_4Cl was the solvent the intact larger subparticle ('L-subparticle') was functional at a $\text{Mg}^{2+}/\text{NH}_4^+$ ratio of 1:40, but it was inactivated (and its conformation was altered) when the Mg^{2+} concentration was lowered to 2 mM. The inactive form was metastable, because activity was not recovered by restoring the concentration of Mg^{2+} ; it was also necessary to heat at 37°C for about 1 h. These earlier studies on the effects of Mg^{2+} enabled us to find both the conditions suitable for the production of core-particle and split-protein fractions amenable to reconstruction, and the conditions necessary to reconstruct a functional subparticle.

The capacity to produce suitable core-particles allows structure–function relationships to be explored and could lead to the identification of the functional role of individual ribosomal proteins. Further, the technique could help us to define those features of eukaryotic ribosomes that have been con-

served and those that have diverged during evolution, and to relate them to the evolution of the rRNA moiety (Cox *et al.*, 1973a, 1976a). Also ribosomes may become modified in certain pathological conditions (e.g. diabetes; Wettenhall *et al.*, 1974) and such alterations may be open to further study by the technique of partial reconstruction.

Methods

General methods

U.v. spectrophotometry. Unicam SP.500 and SP.1800 spectrophotometers were used for routine measurements with 1 cm cells (3.0 or 0.5 ml capacity). Concentrations of L-subparticle solutions were measured by using the value $E_{260}^{1\%} = 140$. This is based on ϵ_p (the molar extinction coefficient per gram of phosphorus in an RNA species) of 7750 litre·mol⁻¹·cm⁻¹ at 260 nm (Cox, 1970) and a molecular mass of 1 720 000 daltons (Loening, 1968) for rabbit L-rRNA, together with a chemically measured RNA/protein mass ratio of 3:2 (R. A. Cox & E. Godwin, unpublished work) for rabbit L-subparticles. Wool & Stöffler (1974) give very similar values for rat liver L-subparticles.

Ultracentrifugation. Polyribosomes and ribosomal subunits were pelleted by using an 8×25 ml rotor ($g_{av.} = 105\,000$ at 40 000 rev./min) in MSE Superspeed 40 or Superspeed 50 centrifuges. Ribosomal subunits were separated by zonal centrifugation in a B XIV titanium rotor in a Superspeed 65 centrifuge. Core particles were pelleted in an MSE 8×10 ml or 10×10 ml rotor ($g_{av.} = 105\,000$, $g_{max.} = 145\,000$ at 40 000 rev./min). Analytical ultracentrifugation was done with a Beckman model E centrifuge fitted with u.v. optics and a photoelectric slit scanner, as described previously (Cox *et al.*, 1976b).

Preparation of rabbit reticulocyte polyribosomes and supernatant enzyme fractions

These were prepared from phenylhydrazine-treated rabbits as described previously (Cox & Hirst, 1976; Arnstein *et al.*, 1964).

Separation of ribosomal subparticles

The polyribosome fraction was dissociated by incubation at 37°C for 30 min in 0.5 M-KCl/3 mM-MgCl₂/1 mM-dithiothreitol/20 mM-Tris/HCl, pH 7.6, together with 0.2 mM-GTP and 0.2 mM-puromycin. The subunits were then separated by zonal centrifugation in a 15–45% (w/v) sucrose gradient containing 0.3 M-KCl/3 mM-MgCl₂/1 mM-dithiothreitol/20 mM-Tris/HCl, pH 7.6, as described by Cox *et al.* (1976b). The pooled fractions of L-subparticles were pelleted by centrifuging at 105 000g for 17 h, resuspended in storage buffer at 150 or 250 E_{260} units/ml, rapidly frozen in a solid CO₂/ethanol bath and stored at -80°C. Analytical ultracentrifugation of the sub-

units after treatment with 1% sodium dodecyl sulphate revealed single rRNA components of sedimentation coefficient 28S and 18S for the L-subparticle and the smaller subribosomal particle ('S-subparticle') respectively. On this basis cross-contamination of the subunits is estimated to be less than 5%.

Buffers

'Storage buffer' for stock solutions of L-subparticles was 0.1 M-NH₄Cl/2 mM-MgCl₂/1 mM-dithiothreitol/15% (v/v) glycerol/20 mM-Tris/HCl, pH 7.6 (at 20°C), derived from Falvey & Staehelin (1970) and modified by the inclusion of glycerol as a cryoprotectant. 'Sample buffer' for dilution of L-subparticles and core particles before assay for polyphenylalanine synthesis was 25 mM-KCl/5 mM-MgCl₂/1 mM-dithiothreitol/0.25 M-sucrose/50 mM-Tris/HCl, pH 7.6.

'Activation buffer' for re-activation at 37°C of non-functional L-subparticles or reconstituted cores as described in the preceding paper (Cox *et al.*, 1976b) was 0.3 M-NH₄Cl/15 mM-MgCl₂/1 mM-dithiothreitol/15% (v/v) glycerol/20 mM-Tris/HCl, pH 7.6.

Poly(U)-directed polyphenylalanine synthesis

The incorporation of [¹⁴C]phenylalanine into acid-insoluble material by L-subparticles in the presence of S-subparticles, supernatant factors etc. at 37°C was measured as described by Cox & Hirst (1976). Samples were generally 50 µl of 2.0 E_{260} units/ml in an assay mixture of 0.5 ml.

Assay of peptidyltransferase by the puromycin reaction

The transfer of acetyl[³H]leucine groups from acetyl[³H]leucyl-tRNA to puromycin, catalysed by L-subparticles at 20°C in 30% (v/v) methanol, was described by Cox *et al.* (1976b). Samples were generally 5 or 10 µl of 50 or 30 E_{260} units/ml in an assay mixture of 50 µl.

Preparation of core-particle and split-protein fractions

Typically, L-subparticles at 100 or 250 E_{260} units/ml in storage buffer at 0°C was diluted to 30 or 50 E_{260} units/ml by rapid mixing with the appropriate volume of salt solution at 0°C. The salt solution contained 1 mM-dithiothreitol/20 mM-Tris/HCl, pH 7.6, and sufficient NH₄Cl (up to 4.5 M) and MgCl₂ (up to 0.625 M) to give the final concentration indicated in the Figures. In some cases the solution also contained 15% (v/v) glycerol added as a cryoprotectant, but this did not apparently alter the effect of the salt-shock. After the appropriate time (up to 2 h), samples were diluted directly into sample buffer and assayed for polyphenylalanine synthesis. The high salt content prevented direct assay of the salt-shock

reaction mixtures in the puromycin reaction. In some cases, the mixture was dialysed against activation buffer at 0°C for direct reconstruction experiments.

Core-particle and split-protein fractions were separated by differential centrifugation at approx. 0°C, generally for 30 min or 1 h at 40000 rev./min in the MSE 8×10 ml rotor ($g_{\max.} = 145000$), usually after a 1 h period of salt shock (but see individual Figure legends). Spectrophotometric measurements with ribosomes and cytochrome *c* added as a marker showed that the separation of the core particles into the pellet, and the split proteins into the supernatant, was substantially complete with the volumes used in these experiments. Reaction mixtures of 0.5–2.0 ml were centrifuged in MSE 10 ml polycarbonate tubes. Smaller volumes (250–500 μ l) were centrifuged in 2.0 ml polypropylene tubes [Precision Machinery Engineers (Harrow) Ltd., Harrow, Middx. HA3 7DX, U.K.] designed for use in microcentrifuges (e.g. Jobling model 320). They were placed within the 10 ml tubes and surrounded by ice-cold water up to the level of the sample.

After removal of the supernatants, the pellets were resuspended in a volume of storage buffer or activation buffer equal to the volume of the salt-shock mixture. Often difficulty was experienced in resuspending core particles formed in the highest salt concentrations, particularly of Mg^{2+} . This problem was alleviated on heating at 37°C in activation buffer, or on dilution into sample buffer. However, for direct assay of the cores in the puromycin reaction, the particulate material (up to 40% of the total E_{260}) was allowed to sediment by standing for 10 min, and samples of the cloudy suspension were taken for the assay, and for measurement of E_{260} after 50- or 100-fold dilution into water.

Determination of protein by filter assay of Amido Black complex

The concentration of protein in salt extracts of ribosomes, or dissolved in urea sample buffer for gel electrophoresis, was measured by a modification of the method of Nakao *et al.* (1973), as described previously (Cox *et al.*, 1976b). The protein content of L-subparticle or core-particle solutions was not measured by this method, because the presence of rRNA substantially decreases binding of the dye to the protein (P. Greenwell, unpublished work). For L-subparticle solutions, the protein content was calculated on the basis that $E_{260}^{1\%} = 140$ and that the subunits are 40% (w/w) protein (see under 'U.v. spectrophotometry' in the General Methods section).

Isolation of ribosomal proteins for gel electrophoresis

Ribosomal proteins were extracted from L-subparticles and core particles (usually 30–50 E_{260} units/ml) by adjusting the solution to 0.2 M- Mg^{2+} with 1 M- $MgCl_2$, and adding 2 vol. of acetic acid. The

mixture was stirred at 0°C for 40 min, after which the precipitate of rRNA was removed by centrifuging at 12000 g for 40 min at 4°C in MSE model HS18 or Sorvall RC2-B centrifuges. The supernatant was mixed with 5 vol. of ice-cold acetone in a Correx glass centrifuge tube (to see the pellet more easily) and the precipitate of the protein allowed to form for at least 1 h at –20°C, before centrifugation at 12000 g for 20 min. The pellet and tube were carefully rinsed with ice-cold acetone, to extract residual acid. The pellet was then dissolved in 8 M-urea/10 mM-2-mercaptoethanol (1.5–2.5 μ l/ E_{260} unit of L-subparticles extracted, giving 20–10 μ g of protein/ μ l) and stored at –80°C.

The split-protein fractions from the salt-shock supernatants were also dissolved in the same volume of urea solution as were the proteins extracted from core particles, on the basis of the initial E_{260} units of L-subparticles used to prepare the sample. However, these lesser quantities of protein were not precipitated by acetone, because the large amounts of salt were also precipitated. Instead 2 vol. of ice-cold 15% (w/v) trichloroacetic acid was added, and the mixture stored overnight at 4°C, before centrifuging, acetone rinsing and dissolving of the precipitate as above.

Analysis of ribosomal proteins by two-dimensional polyacrylamide-gel electrophoresis

Initially in this work protein samples were analysed by two-dimensional gel electrophoresis in 6 M-urea, essentially by the modification by Howard & Traut (1973) of the original method of Kaltschmidt & Wittmann (1970a). The tube gels for the first dimension were 4% (w/v) acrylamide, pH 8.7, made 11.5 cm long in 12 cm×0.3 cm Perspex tubes. Two portions (10–30 μ l) of each protein sample were electrophoresed on separate gels with opposite polarity because a small number of the rabbit L-subparticle proteins were anionic in the running buffer (pH 8.2). The anionic proteins were run for only half the period of 7 h at 200 V used with the cationic proteins. Each tube gel was then run separately in an 18% (w/v) acrylamide slab, pH 4.5, 0.2 cm thick and 14.0 cm square, cast in a horizontal flat-bed apparatus (Yeda Scientific Instruments, Rehovot, Israel). Running time was 1 h at 50 V, then approx. 18 h at 130 V until the tracking dye, Pyronine Y, had migrated across the slab.

The gel was stained for 4 h with shaking in 1 litre of 0.1% Coomassie Brilliant Blue R (R. A. Lamb Ltd., London N.W.10, U.K.) in methanol/acetic acid/water (20:3:17, by vol.). It was then destained electrophoretically in the same solvent at 12 V for 45 min. The voltage was applied across vertical stainless-steel mesh electrodes, 15 cm square and 2 cm apart, with the gel supported between by nylon mesh. After final clarification by shaking for about 1 h in 0.5 litre of the same solvent, the gel was

mounted on Whatman 3MM paper, and covered with plastic film (of the 'cling-wrap' type) on the porous polyethylene top of a drying box. Drying was accomplished at decreased pressure (water pump) after 24–36 h at room temperature.

Subsequently Knopf *et al.* (1975) developed a new acidic-buffer system for the first dimension of the protein analysis, which leads to a broadly similar pattern of spots, but which has the advantage that all the ribosomal proteins move cationically in the first dimension, and can be analysed in a single gel slab. Therefore the first-dimension gels were changed to 4% (w/v) acrylamide, 0.066% methylenebisacrylamide, 6M-urea and 38mM-Bis-tris [bis-(2-hydroxyethyl)imino-tris(hydroxymethyl)methane] adjusted to pH 5.5 with acetic acid. The upper-chamber buffer contained 10mM-Bis-tris adjusted with acetic acid to pH 3.7 and the lower-chamber buffer was 10mM-Bis-tris, pH 7. Samples were electrophoresed at 100 V for 1 h, then at 200 V until a cytochrome-*c* marker on a parallel gel had migrated 7.5 cm. Subsequent procedures for running, staining and drying the two-dimensional gel slab were as described above.

Isolation and gel electrophoresis of rRNA

RNA was isolated from solutions of ribosomal subparticles or core particles by phenol/sodium dodecyl sulphate extraction at room temperature (approx. 20°C). An equal volume of 0.6% (w/v) sodium dodecyl sulphate/0.4M-sodium acetate/4mM-disodium EDTA was added, and this aqueous layer was vigorously mixed with an equal layer of water-saturated redistilled phenol. The separated aqueous phase was washed twice with an equal volume of diethyl ether to remove phenol, and the RNA precipitated by the addition of 3 vol. of ethanol and storage overnight at –20°C. The RNA was collected by centrifugation at 1500g for 5 min and dissolved at 50–100 E_{260} units/ml in the sample buffer for gel electrophoresis, 0.1M-NaCl/1mM-disodium EDTA/10% (v/v) glycerol/20mM-Tris/acetate, pH 8.5.

Gel electrophoresis was carried out essentially as described by Loening (1967), by using 3% (w/v) acrylamide and 0.15% bisacrylamide buffered with 20mM-sodium acetate/2mM-disodium EDTA/40mM-Tris/acetate, pH 7.4. The gel was cast as a vertical slab 14 cm wide, 11 cm high and 0.15 cm thick in an apparatus of the type described by Reid & Bielecki (1968). After pre-electrophoresis of the slab at 27 mA for 1 h with the same buffer in the chambers, the 1 cm sample slots were loaded with 0.3–0.7 E_{260} unit of RNA in 10–20 μ l of sample buffer, and electrophoresis was conducted for 2.5–3.0 h at 42 mA until the Bromophenol Blue tracking dye was 3 cm from the bottom of the gel. The slab was stained in 0.05% (w/v) Toluidine Blue in 50mM-sodium acetate/0.1mM-disodium EDTA adjusted to pH 5.5 with acetic acid, and destained in several changes of water before

being dried as described above for two-dimensional protein gels.

Materials

L-[³H]Leucine (specific radioactivity 17 Ci/mmol) and L-[U-¹⁴C]phenylalanine (specific radioactivity 495 mCi/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. Puromycin dihydrochloride, poly(U), phosphoenolpyruvate, GTP, horse heart cytochrome *c* (type III), Tris and Bis-tris were supplied by Sigma (London) Chemical Co. Ltd., Surrey KT2 7BH, U.K. Pyruvate kinase and ATP were obtained from Boehringer Corporation (London) Ltd., London W5 2TZ, U.K. Tracking dyes, urea and acrylamide for gels were from BDH Chemicals Ltd., Poole, Dorset, U.K., as were all other reagents (AnalaR grade).

Results

Effect of salt-shock treatment on the activity of L-subparticles in polyphenylalanine synthesis

The first experiments were of simple design to find the most suitable range of NH_4Cl concentrations for the production of cores. A stock solution of 4M- NH_4Cl /0.1M-MgCl₂/1mM-dithiothreitol/20mM-Tris/HCl, pH 7.6, was diluted with suitable volumes of 1mM-dithiothreitol/20mM-Tris/HCl, pH 7.6, such that salt-shock mixtures could be prepared at 0°C as described in the Methods section, with 30 E_{260} units of L-subparticles/ml and up to 3M- NH_4Cl at a constant cation molar ratio for $\text{Mg}^{2+}/\text{NH}_4^+$ of 1:40. After 2 h, samples of the mixtures were diluted 15-fold into sample buffer and assayed for polyphenylalanine synthesis in the standard cell-free system. This procedure detects only those changes in the ribosome that are not reversed during dilution and subsequent storage at 0°C or during the assay incubation at 37°C. Initially the extent of reassembly at 0°C was underestimated, and the variability in the results of Fig. 1(a) is attributable, at least in part, to variations in the period of storage at 0°C.

The results of six independent experiments using different batches of ribosomes are given in Fig. 1(a), which reveals that full activity was recovered after exposure to 1.8M- NH_4Cl /45mM-MgCl₂. The loss of activity was abrupt as the salt concentration increased, since 50% of activity was lost after exposure to 2.1M- NH_4Cl /53mM-MgCl₂ and only approx. 10% activity remained after treatment by 2.75M- NH_4Cl /69mM-MgCl₂.

The profile of percentage activity against NH_4Cl concentration was not dependent on the concentration of ribosomes over the range tested (30–100 E_{260} units/ml). The temperature of the salt-shock treatment had a more dramatic effect on the extent of inactivation. At 25°C a profile comparable with that in Fig. 1(a) was found, except that the mid-point for

Fig. 1. Capacity of reaction mixtures of salt-shocked L-subparticles to function in poly(U)-directed polyphenylalanine synthesis

(a) In six independent experiments, L-subparticles at 30–100 E_{260} units/ml were salt-shocked for 2 h in 0.1–3.0 M NH_4Cl /2–75 mM $MgCl_2$ /15% (v/v) glycerol/1 mM dithiothreitol/20 mM Tris/HCl, pH 7.6. The molar ratio Mg^{2+}/NH_4^+ was maintained at 1:40. Samples were diluted to 2.0 E_{260} units/ml in sample buffer and assayed for polyphenylalanine synthesis as described in the Methods section. The assay measures inactivation of L-subparticles that is not reversed on diluting out the high salt concentrations at 0°C, or during the 30 min assay incubation at 37°C. The activities are expressed as percentages of the activity of control L-subparticles which were maintained at 0°C in sample buffer (controls incorporated 15–50 mol of phenylalanine/mol of L-subparticles). The average value is given with bars to denote where necessary the spread of results from different experiments. The broken line indicates the percentage of total protein in the split-fraction protein (see Fig. 2). (b) An example of the kinetics of the salt-shock treatment at 0°C. Samples were taken from a salt-shocked reaction mixture with 2.25 M NH_4Cl /56 mM $MgCl_2$ at the times indicated, and assayed for polyphenylalanine synthesis as described for Fig. 1(a).

50% loss of activity was shifted down to 1.8 M NH_4Cl /45 mM $MgCl_2$, and very little activity remained after shock treatment at 2 M NH_4Cl /50 mM $MgCl_2$. At 37°C, 50% inactivation was achieved after only 10 min at 1 M NH_4Cl /25 mM $MgCl_2$. The effect of tempera-

ture was not pursued further, and all subsequent experiments were done at 0°C.

The time-course for the inactivation by 2.25 M NH_4Cl /57 mM $MgCl_2$ at 0°C is given in Fig. 1(b). This shows that the effect of the salt shock was substantially complete after 1 h, although a period of 2 h (including 1 h for centrifugation of the core particles into a pellet) was used during most of the present work. However, such a long exposure to high salt concentrations was later found to decrease the potential of the cores for reassembly (see below and Fig. 9).

Salt-shock treatment and the elution of proteins from L-subparticles

Salt-shock mixtures were prepared at 0°C and separated into core-particle and split-protein fractions by differential centrifugation as described in the Methods section, and the amount of protein in the supernatants was measured. As shown in Fig. 2, the amount of protein split from the L-subparticles increased slowly above about 1.5 M NH_4Cl /38 mM $MgCl_2$, and did not reflect the abrupt loss of activity shown in Fig. 1(a). Despite this, the split-protein fraction formed at 2.5 M NH_4Cl /63 mM $MgCl_2$ (when the core particle is almost completely inactivated) was shown by two-dimensional electrophoresis (see below and Fig. 3) to comprise a small group of eight major protein components rather than a non-specific sample of all the L-subparticle ribosomal proteins.

Fig. 2. Elution of ribosomal protein from L-subparticles as a function of salt concentration during shock treatment

Samples of L-subparticles at 50 E_{260} units/ml were salt-shocked in 0.1–3.0 M NH_4Cl /2–75 mM $MgCl_2$ /15% (v/v) glycerol/1 mM dithiothreitol/20 mM Tris/HCl, pH 7.6 (cation ratio $Mg^{2+}/NH_4^+ = 1:40$) for 1 h at 0°C, and the core particles isolated by centrifuging over 1 h at 0–4°C as described in the Methods section. Samples of the supernatants (30–150 μl , containing 0–20 μg of protein) were assayed for protein content by the Amido Black-binding assay, and the eluted percentage of total protein was calculated, as described in the Methods section (see also preceding paper, Cox *et al.*, 1976b).

The elution of protein was also examined at constant 2.5M-NH₄Cl as a function of MgCl₂ concentration (see Fig. 8 below). Decreasing the Mg²⁺ concentration below 50mM, the point of substantially complete inactivation, increased the amount of protein in the split fraction from about 17% up to about 45% at 5mM-MgCl₂ (cf. Godwin *et al.*, 1974). On the other hand, an increase in the concentration of Mg²⁺ in the shock mixture resulted in only a small decrease in the elution of protein, to about 14% at 2.5M-NH₄Cl/0.3M-MgCl₂, even though the activity of L-subparticles (in the puromycin reaction) was largely preserved. This difference is apparently due

to one protein that is retained on the core particle at the higher concentrations of Mg²⁺ (see Plate 1).

Analysis of the split-protein fraction by two-dimensional gel electrophoresis

The two-dimensional gel system of Knopf *et al.* (1975), in which the first-dimension gel is buffered at pH 5.5, gives a pattern of stained spots very similar to that obtained with the first dimension gel at pH 8.7 (in the system of Howard & Traut, 1973) with both *Escherichia coli* S- and L-subparticle proteins (Knopf *et al.*, 1975; Kenny *et al.*, 1975). This is because the order of migration of the proteins at pH 5.5 or 8.7 in

Fig. 3. Analysis of total ribosomal proteins and 2.5M-NH₄Cl split-protein fractions from rabbit L-subparticles by two-dimensional gel electrophoresis

Proteins were isolated as described in the Methods section, and analysed in the two-dimensional urea gel system of Knopf *et al.* (1975), with separation in 4% (w/v) acrylamide at pH 5.5 followed by 18% (w/v) acrylamide at pH 4.5. Gels were stained with Coomassie Blue, dried and photographed (see also Plate 1). A tracing of a well-resolved protein pattern was the basis for this schematic drawing, which shows all spots that were seen consistently, although for clarity some of them were very faintly stained. The spots are tentatively numbered L1-L46 along horizontal lines starting at the top left, although the letter L, signifying the origin of the spots in the larger ribosomal subunit, is omitted from the diagram. The 'solid' spot L13, and the seven other solid spots, were seen consistently and prominently in the split-protein fraction isolated after salt-shock with 2.5M-NH₄Cl/63mM-MgCl₂ for 2h at 0°C. Spot L13 was largely retained on the core particle when salt-shock was conducted in 2.5M-NH₄Cl/300mM-MgCl₂ (see Plate 1).

EXPLANATION OF PLATE 1

Two-dimensional gel electrophoresis of the proteins derived from the split-protein and core-particle fractions of rabbit L-subparticles prepared with 2.5M-NH₄Cl/50mM-MgCl₂ and 2.5M-NH₄Cl/300mM-MgCl₂

Samples of L-subparticles at 50E₂₆₀ units/ml were salt-shocked in 2.5M-NH₄Cl/1mM-dithiothreitol/20mM-Tris/HCl, pH 7.6, with 50mM- or 300mM-MgCl₂ for 1 h at 0°C, and the core particles were isolated by centrifugation for 1 h as described in the Methods section. The core particles were resuspended in storage buffer and assayed for activity in the puromycin reaction relative to untreated L-subparticles (12% at 50mM-MgCl₂, 71% at 300mM-MgCl₂). Proteins were isolated from the core particle and split-protein fractions, and analysed by two-dimensional gel electrophoresis as described in the Methods section (see also Fig. 3). (a) and (b), split-protein fractions prepared at 50mM- and 300mM-MgCl₂ respectively. (c) and (d), proteins from core particles prepared at 50mM- and 300mM-MgCl₂ respectively. On each gel the position of spot L13 is indicated by an arrow.

the first dimension appears to be similar, and the conditions of the second dimension are the same in the two systems. When our preparations of rabbit L-subparticle proteins were run in the two systems as described in the Methods section, broadly similar but not identical patterns were obtained. With the first dimension at pH 8.7, four spots were found to have migrated anionically (results not shown). At pH 5.5 all the proteins migrated cationically, and could therefore be analysed in a single slab, so this was the system of choice for the present work.

The results are shown schematically in Fig. 3, in which are drawn all the spots that appeared reproducibly when the total L-subparticle protein samples were run as described by Knopf *et al.* (1975) at various loadings. The spots, which had a wide range of staining intensities, have been given tentative numbers, L1–L46 (L signifies the larger ribosomal subparticle), along horizontal lines commencing at the top left (cf. Kaltschmidt & Wittmann, 1970b). The numbering is tentative because the pattern has not been examined systematically for satellite spots or contamination by S-subparticle proteins [cf. the studies by Sherton & Wool (1974) on rat ribosomal proteins]. However, our samples of L-subparticles were substantially free of S-subparticles, since they did not contain appreciable amounts of S-rRNA (see Fig. 4), and they had very little activity (<3%) in polyphenylalanine synthesis in the absence of added S-subparticles (routine control assays). (S-rRNA and L-rRNA are the major RNA species of the S- and L-subparticles respectively.) Because of its outstanding position as the most acidic spot in the pattern, spot L7 (which sometimes appeared to be two spots incompletely resolved) can probably be identified with the rat liver proteins L40/L41 (nomenclature of Sherton & Wool, 1974). These in turn have been identified both immunologically and functionally (Stöffler *et al.*, 1974) with the *E. coli* proteins L7/L12 (nomenclature of Kaltschmidt & Wittmann, 1970b), which are closely involved in those ribosomal functions that use GTP.

The proteins of the split fraction obtained by salt shock of L-subparticles at 2.5M-NH₄Cl/63mM-MgCl₂ were analysed on two-dimensional gels, and located in the overall pattern by their relative positions (the absolute migration distances were rather variable). The most noticeable component was spot L13 (see Plate 1a), represented as the solid spot indicated by an arrow in Fig. 3. Seven other spots which were prominent in the split fraction (but also appeared to some extent in the core fraction) are also shown as solid spots in Fig. 3. They include spot L7 and two other spots which moved anionically when the first dimension was run at pH 8.7 (results not shown).

Spot L13 was largely retained in the core fraction when the salt shock was conducted at 2.5M-NH₄Cl/

0.3M-MgCl₂ (see Plates 1b and 1d). As the elevated Mg²⁺ concentration also preserved the activity of the core in the puromycin reaction, it appears that protein L13 is important to the peptidyltransferase function of the L-subparticle. However, the function of the split proteins will most probably be established with greater certainty by experiments with the purified proteins.

Retention of 5S rRNA in the core particles

The core particles obtained after salt-shock treatment (2 h at 0°C) were resuspended in storage buffer. The RNA moiety was extracted by the phenol method and then analysed by gel electrophoresis as described in the Methods section. The results (see Fig. 4) showed that 5S rRNA was retained with the core particle at all salt-shock conditions tested (up to 3M-NH₄Cl/75mM-MgCl₂). The conditions of the salt shock were designed to retain the rRNA (including 5S rRNA) in the native conformation, but the possibility remains that the 5S rRNA was detached, and then merely co-precipitated with the core particle because of the high salt concentration (cf. the production of 4M-LiCl cores from *E. coli* L-subparticles; Nierhaus & Dohme, 1974). The absence of S-rRNA from the RNA samples shows that the L-subparticles were substantially freed of S-subparticles by the sucrose-gradient zonal centrifugation.

Activity of core particles in poly(U)-directed polyphenylalanine synthesis

Suitable conditions for re-activation of L-subparticles inactivated, without loss of protein, by exposure to low Mg²⁺ concentrations at 0.5M-NH₄Cl were found to be 0.3M-NH₄Cl/15mM-MgCl₂ by Cox *et al.* (1976b). The core particles obtained by the high-salt shock treatment described in the Methods section were therefore dialysed overnight against activation buffer at 4°C with shaking, and then heated at 37°C for 1 h to optimize recovery of any latent activity. A sample was then assayed in the cell-free system. The results are given in Fig. 5 for activity in the cell-free system (after re-activation) over a wide range of salt concentrations in the shock treatment. More than 90% of activity was retained after treatment with 1.75M-NH₄Cl/44mM-MgCl₂, when about 7% of the total protein was lost. Approx. 25% of activity was retained at 2.25M-NH₄Cl/56mM-MgCl₂, and 5% activity was left after shock-treatment with 2.5M-NH₄Cl/63mM-MgCl₂. Thus shock treatment at 2.5M-NH₄Cl/63mM-MgCl₂ is sufficient to lead to core particles that are inactive in polyphenylalanine synthesis despite preincubation under re-activation conditions.

Activity of core particles in the puromycin reaction

The inability of L-subparticles to synthesize polyphenylalanine after salt-shock treatment could arise

Fig. 4. Salt-shock treatment does not remove 5S rRNA from L-subparticles

Core-particles were prepared from L-subparticles over the range 0.1–3.0M-NH₄Cl with Mg²⁺/NH₄⁺ = 1:40 (see Fig. 2). As described in the Methods section, the core particles were treated with sodium dodecyl sulphate and phenol to isolate the rRNA components, which were analysed by electrophoresis in a 3% acrylamide-gel slab. Each sample slot received 0.7 E₂₆₀ unit of RNA in 10 µl of buffer, except for the 2.5M-NH₄Cl sample, which was in 30 µl of buffer, and the markers, which were approx. 0.3 E₂₆₀ unit of *E. coli* tRNA and rabbit S-rRNA in 10 µl. The gel was stained with Toluidine Blue, dried and photographed.

Fig. 5. Activity in polyphenylalanine synthesis after reconstruction of core particles alone, and of unfractionated salt-shock mixtures

Salt-shock mixtures with L-subparticles at 50 E₂₆₀ units/ml were prepared at 0°C over the range 0.10–2.75M-NH₄Cl with Mg²⁺/NH₄⁺ = 1:40. After 1 h, one half of each mixture was centrifuged for 1 h to pellet the core particles as described in the Methods section. The core particles were resuspended in activation buffer and dialysed at 0–4°C overnight against the same buffer. After 2 h, the remaining half of each mixture was dialysed directly against activation buffer. All the dialysed solutions were heated at 37°C for 1 h and then assayed for polyphenylalanine synthesis as described in the Methods section. Each result was normalized to account for differences in the E₂₆₀ of the dialysed samples, and expressed as a percentage of the activity of a control which was incubated at 0°C in storage buffer, then dialysed and heated in activation buffer. ○, Activity of the core-particles alone; Δ, activity of the reconstructed salt-shocked mixtures. The broken line indicates the percentage of total protein in the split-protein fraction (see Fig. 2).

Fig. 6. Activity in the puromycin reaction after reconstruction of core particles alone, and of unfractionated salt-shock mixtures

See the legend to Fig. 5 for the preparation and reconstruction of salt-shock mixtures and core particles over the range 0.10–2.75M-NH₄Cl. After dialysis and heating, the samples were assayed in the puromycin reaction as described in the Methods section. The results were normalized and expressed as a percentage of the control (cf. Fig. 5). ○, Activity of the core particles alone; Δ, activity of the reconstructed salt-shock mixtures. The broken line indicates the percentage of total protein in the split-protein fraction (see Fig. 2).

for several reasons, e.g. inability to bind S-subparticle, elongation factors or aminoacyl-tRNA, as well as the loss of the catalytic function of peptidyl-transferase. Therefore the effect of salt-shock treatment on the peptidyltransferase centre of the ribosome was studied more selectively by means of the

Fig. 7. Correlation between polyphenylalanine synthesis and the puromycin reaction with salt-shocked core particles and reconstructed salt-shock mixtures prepared over the range 0.1–3.0M-NH₄Cl, with Mg²⁺/NH₄⁺ = 1:40

Samples of core particles or reconstructed salt-shock mixtures of L-subparticles, prepared as described in the Methods section, in several experiments (including the experiment of Figs. 5 and 6), were assayed for polyphenylalanine synthesis, and in the puromycin reaction. For every sample, each activity was expressed as a percentage of control (intact L-subparticles) and plotted against the other. The theoretical line for complete correlation is also shown.

puromycin reaction. The results (see Fig. 6) revealed that the loss of function in the puromycin reaction was parallel with the loss of function in poly(U)-directed polyphenylalanine synthesis. The correlation between the two activities is apparent from Fig. 7. Thus when the standard salt-shock treatments (at an Mg²⁺/NH₄⁺ molar ratio of 1:40) are severe enough to cause inactivation, they affect the peptidyltransferase catalytic centre of L-subparticles. Other functions may be affected as well if they are closely integrated, as in *E. coli* L-subparticles (Möller, 1974), but we have not tested other partial reactions of the core particles, nor explored the range of Mg²⁺/NH₄⁺ molar ratios over which the activities in polyphenylalanine synthesis and the puromycin reaction remain correlated.

Activity in the puromycin reaction of core particles prepared at higher Mg²⁺ concentrations

The activity of the 2.5M-NH₄Cl core particles was found to vary with Mg²⁺ concentration. Raising the concentration of MgCl₂ to 300mM protected the peptidyltransferase centre, and 80% activity was

retained (see Fig. 8). Over the same range of Mg²⁺ (50–300mM) the amount of protein eluted in the split-protein fraction diminished by about 3% of the total. As shown in Plate 1(a) and 1(b), the most noticeable change in the split-protein fraction was that spot L13 (indicated by an arrow) was very much decreased in intensity at 0.3M-MgCl₂, but in addition other spots were also somewhat fainter. Conversely, although the gel of the active core fraction from 2.5M-NH₄Cl/0.3M-MgCl₂ (Plate 1d) was more extensively destained, it clearly contained spot L13, whereas the less extensively destained gel of the inactive core from 2.5M-NH₄Cl/0.05M-MgCl₂ (Plate 1c) contained very little of spot L13 material. The inference is that the protein of spot L13 is closely involved in the peptidyltransferase activity of the ribosome, but the rather variable staining patterns of the two-dimensional gels can only give preliminary evidence of such a correlation. Experiments with purified split proteins are needed to establish their functional significance.

Reassembly of functional L-subparticles without first separating the core-particle and split-protein fractions

We were not able to re-activate the core particles to any appreciable extent by heating at 37°C in activa-

Fig. 8. Activity in the puromycin reaction and protein loss of salt-shocked L-subparticle core-particles prepared in 2.5M-NH₄Cl with a range of MgCl₂ concentrations

Samples of L-subparticles at 50E₂₆₀ units/ml were salt-shocked in 2.5M-NH₄Cl/1mM-dithiothreitol/20mM-Tris/HCl, pH7.6, including 5–500mM-MgCl₂, for 1 h at 0°C, and the core particles isolated by centrifugation for 1 h as described in the Methods section. The percentage of total protein eluted into the supernatant was measured (cf. Fig. 2) and the core particles prepared with 50–500mM-MgCl₂ were resuspended in storage buffer and assayed in the puromycin reaction as described in the Methods section. The activities are expressed as percentage of a control processed throughout in storage buffer. The individual points show the average result of three to five separate experiments, with a bar to denote (where necessary) the spread of results. □, Activity of the core particles in the puromycin reaction; ○, percentage of the total ribosomal protein eluted into the split-protein fraction.

tion buffer. However, L-subparticles active both in polyphenylalanine synthesis and in the puromycin reaction could be reassembled when the split-protein fraction was present. The procedure followed was that after the shock treatment (2 h at 0°C) a sample of the solution was dialysed (with agitation) against activation buffer at about 4°C overnight. The solution was then heated for 1 h at 37°C. Samples of this solution were then assayed in polyphenylalanine synthesis and the puromycin reaction. The results, given in Figs. 5 and 6, show that this procedure yielded 45–55% of the activity of the control samples (which were also dialysed and heated) when the core particles alone were almost inactive. Substantial activity was recovered by the dialysis step alone (results not shown but cf. Fig. 10 below), but the heating step increased the yield of activity by about 50%. Studies in the analytical ultracentrifuge (results not shown) revealed that the isolated core particles were very heavily aggregated, but that the cores dialysed together with split proteins had sedimentation profiles with boundaries corresponding to monomers, dimers, trimers and higher aggregates. When the latter samples had been heated for 1 h at 37°C, the yield of monomer particles was increased. These results suggest that heating may increase the yield of activity by disaggregating the inactive core particles, thereby facilitating their reassembly with the split proteins.

Reassembly of functional L-subparticles from separated core-particle and split-protein fractions

Attempts to reconstitute L-subparticles from separated core-particle and split-protein fractions were not reproducible at first. This led us to re-examine the kinetics of the salt-shock treatment. The result of an experiment at 2.75M-NH₄Cl/69mM-MgCl₂ is given in Fig. 9. We found that the inactivation of the L-subparticle was 90% complete within 20 min (plus 30 min centrifugation), but that the ability of the cores to reassemble also declined in a slower process, with a half-life of 40–50 min. This result is explained if an essential split-protein fraction is removed very rapidly under the conditions of the salt shock, and the core particle then undergoes a further change which hinders the reconstruction step. It was shown (see Table 1) that the core particle obtained after 2 h shock treatment was unable to yield active subparticles irrespective of the formation period (5 min or 2 h, plus 30 min centrifugation) of the split-protein fraction offered. However, active L-subparticles were reconstituted from the core particle obtained after the brief shock treatment on dialysis with either split-protein fraction. Therefore the longer shock treatment did not adversely affect the split-protein fraction, but did have a detrimental effect on the core particles.

Fig. 9. Activity in polyphenylalanine synthesis of the 2.75M-NH₄Cl/69mM-MgCl₂ L-subparticle core particle and reconstructed salt-shock mixture as a function of time of salt shock

L-subparticles at 50E₂₆₀ units/ml were salt-shocked in 2.75M-NH₄Cl/69mM-MgCl₂/15% (v/v) glycerol/1mM-dithiothreitol/20mM-Tris/HCl, pH 7.6, at 0°C. Duplicate samples were taken at the times indicated. One sample was dialysed overnight against activation buffer at 4°C and heated for 1 h at 37°C to yield reconstructed salt-shock mixture. The other sample was centrifuged for 30 min to isolate core particles as described in the Methods section. After resuspension in activation buffer the core particles also were dialysed and heated. Portions of each sample were diluted to 2.0E₂₆₀ units/ml in sample buffer, and assayed for polyphenylalanine synthesis as described in the Methods section. Results are expressed relative to a control sample which was maintained at 0°C in storage buffer, then dialysed, heated and assayed as for the other samples. □, Activity of core particle; ○, activity of reconstructed salt-shocked mixture.

Stoichiometry of the reassembly process

The core-particle fraction and different volumes of the supernatant containing the split-protein fraction (0.5–2.5 equivalents, where one equivalent was calculated as that volume of supernatant made with E₂₆₀ units of L-subparticles equal to the E₂₆₀ units of core being reassembled) were mixed at 0°C and dialysed overnight against activation buffer. One sample was then assayed without further treatment for activity in

Table 1. *Response of core-particle fractions from 5 min and 2 h salt-shock treatment to reassembly with split proteins, as judged by poly(U)-directed polyphenylalanine synthesis*

The core-particles and split-protein fractions were isolated after 5 min and 2 h salt-shock treatment of L-subparticles at $50 E_{260}$ units/ml in 2.75 M-NH₄Cl/69 mM-MgCl₂/15% (v/v) glycerol/1 mM-dithiothreitol/20 mM-Tris/HCl, pH 7.6, by centrifugation for 30 min as described in the Methods section. Samples of the core-particle fractions were mixed with 1.5 equivalents (as defined in Fig. 10) of the supernatant protein solutions from 5 min or 2 h shock treatment, or the same volume of salt-shock buffer. All the mixtures were dialysed overnight at 4°C against activation buffer, then heated for 1 h at 37°C. Samples were diluted to $2.0 E_{260}$ units/ml for assay as described in the Methods section.

Core particle	Split proteins	Dialysed and heated	[¹⁴ C]Polyphenylalanine synthesis	
			(nCi/assay)	(% of untreated)
Untreated L-subparticles	None	No	3.60	100
Control L-subparticles	None	Yes	3.53	98
Buffer blank	None	Yes	0.10	3
5 min core	None	Yes	0.31	9
5 min core	5 min split	Yes	1.30	36
5 min core	2 h split	Yes	1.87	52
2 h core	None	Yes	0.03	1
2 h core	5 min split	Yes	0.21	6
2 h core	2 h split	Yes	0.03	1

polyphenylalanine synthesis, and another sample was heated for 1 h at 37°C before assay. As shown in the dose-response curves of Fig. 10, the recovery of activity reached maximum at about 1.5–2.0 equivalents of split protein. However, the heat treatment increased the yield of activity by about 50%.

Kinetics of the heat-activation step

The dialysis procedure at 4°C, or indeed simply diluting out the excess of salt with a suitable buffer, leads to substantial reassembly of L-subparticles. As reported above, heat treatment at 37°C increases the recovery of activity, possibly by decreasing the aggregation of the core particles. Fig. 11 demonstrates that this beneficial effect of heating was complete after 40 min at 37°C.

Discussion

The results are consistent with the formation of a well-defined inactive core-particle and split-protein fraction from the larger subparticle of rabbit reticulocyte ribosomes. This was achieved by NH₄Cl salt-shock treatment of the L-subparticles in the presence of a critical minimum molar ratio of Mg²⁺ ion. The split-protein fraction comprised mainly eight ribosomal proteins and could be used to re-activate the core particle. Although Lerman (1968) has reported the reconstruction of both smaller and larger subparticles of rat liver ribosomes after CsCl salt-shock treatment, this approach does not seem to have been exploited (see the review by Wool & Stöffler, 1974).

Preparation of core particles and the conformation of rRNA

The importance of maintaining a concentration of Mg²⁺ ion adequate to protect the native rRNA structure throughout the isolation and study of ribosomes was suggested by observations of the effect of Mg²⁺ on the conformation of isolated rRNA from both rabbit and *E. coli* ribosomes (Cox & Hirst, 1976). Regions of the secondary and tertiary structure of the K⁺ or NH₄⁺ salt of rRNA are changed by the addition of Mg²⁺ ion, e.g. from conformation N_I (Mg²⁺-depleted, 'denatured') to conformation N_{II} ('native') according to eqn. (1):

A quantitatively very similar but metastable equilibrium holds for the rRNA within the intact rabbit L-subparticles (see also Cox *et al.*, 1976b). The use of a Mg²⁺/NH₄⁺ molar ratio of 1:40 in salt-shock treatments was originally based on the relative proportions of Mg²⁺ and NH₄⁺ needed to yield 67% of conformation N_{II}, by using the approximation that $m = n = 1$ in eqn. (1). It was then shown (Cox & Hirst, 1976) that $n \approx 1$, but $m \approx 1.3$, so that the concentration of Mg²⁺ required to preserve the native rRNA conformation is slightly underestimated by the approximation. Nonetheless it has proved to be a useful guide in the present work up to 3 M-NH₄Cl, which represents an extrapolation beyond the 1.2 M-KCl and 2.1 M-NH₄Cl concentrations used by Cox & Hirst (1976) to establish the relationship. Thus we infer that the rRNA conformation within the core particle was kept largely in the native state at the concentra-

Fig. 10. Stoichiometry of the reconstruction of *L*-subparticles from core particles and the split-protein fraction, as judged by activity in polyphenylalanine synthesis

Core-particle and split-protein fractions were prepared from *L*-subparticles at $50E_{260}$ units/ml as described in the Methods section, after 5 min salt shock at 0°C with $2.75\text{M-NH}_4\text{Cl}/69\text{mM-MgCl}_2$. The pelleted core particles were resuspended at $10E_{260}$ units/ml in activation buffer, and samples (0.5 ml) were mixed with 0.25 ml of supernatant solution (diluted as necessary with salt-shock buffer) to include up to 2.5 equivalents of split proteins as shown. (One equivalent of the split-protein fraction is defined as the amount of protein that could be obtained from an equivalent number of E_{260} units of the original *L*-subparticles.) The samples, including untreated controls, were dialysed overnight at 4°C against activation buffer. Samples were then taken from each sample both before and after an incubation for 1 h at 37°C , and diluted to $2.0E_{260}$ units/ml in sample buffer before assay for polyphenylalanine synthesis. The activities are expressed relative to that of the dialysed heated control. □, Samples reconstructed by dialysis at 4°C ; ○, samples reconstructed by dialysis at 4°C followed by incubation at 37°C .

tions of Mg^{2+} ion used in our salt-shock experiments. Consistent with this view is the ease with which the core-particle and split-protein fractions recombined (in part) on dilution of the salt at 0°C , or dialysis at 4°C , to yield particles with activity in polyphenylalanine synthesis. This result contrasts with the need for a reactivation step at 37°C when ribosomes had been inactivated by 'denaturing' the rRNA at low concentrations of Mg^{2+} ion (Cox *et al.*, 1976b). The increased efficiency of reassembly in the present experiments after heating at 37°C could represent restoration of a fraction of the rRNA which had become denatured, or it could represent increased

solubility and disaggregation of the core particles, as suggested in the Results section.

Another aspect of the rRNA component of the core particles was the presence of 5S rRNA. To achieve a partial reconstruction of the peptidyl-transferase centre of rabbit *L*-subparticles, similar to those achieved with *E. coli* ribosomes (see Nomura & Held, 1974), it seemed important that the 5S rRNA should be retained in the core particle. For example, Yu & Wittmann (1973) have obtained evidence that the inability of core particles of *E. coli* *L*-subparticles (prepared in 2M-LiCl) to reassemble as active sub-

Fig. 11. Kinetics of the effect of incubation at 37°C on *L*-subparticle reassembly

Core particles and split-protein fractions were prepared from *L*-subparticles at $50E_{260}$ units/ml as described in the Methods section, by centrifugation for 30 min, after 5 min salt shock at 0°C with $2.75\text{M-NH}_4\text{Cl}/69\text{mM-MgCl}_2$. The pelleted core particles were resuspended at $10E_{260}$ units/ml in activation buffer. A large portion was mixed with 1.5 equivalents (as defined in Fig. 10) of split-protein fraction, and the remainder was diluted to the same extent with salt-shock buffer. Both portions, and untreated controls, were dialysed against activation buffer at 4°C overnight. The core particles alone and the controls were incubated for 1 h at 37°C , before dilution to $2.0E_{260}$ units/ml in sample buffer at 0°C and assay for polyphenylalanine synthesis. The reconstruction mixture was incubated at 37°C , and samples were taken at the times shown for dilution into sample buffer at 0°C and subsequent assay. The activities (○) are expressed relative to that of the dialysed heated control, as is the activity of the core particle alone (●).

units is due to structural disorientation of the 5S rRNA on reconstruction (on the other hand, the re-incorporation of 5S rRNA is apparently not a critical step in the total reconstruction of *Bacillus stearothermophilus* or *E. coli* L-subparticles; see Cohlberg & Nomura, 1976; Nierhaus & Dohme, 1974). Therefore, our procedure for making core particles has features in common with the use of CsCl equilibrium density gradients to produce a series of core particles containing 5S rRNA from *E. coli* L-subparticles. Thus the γ cores of Staehelin *et al.* (1969), prepared in an equilibrium density gradient generated from 5M-CsCl/10mM-MgCl₂, had no peptidyltransferase activity (cf. our cores made in 2.5M-NH₄Cl/50mM-MgCl₂) whereas the β cores (gradient generated from 5M-CsCl/50mM-MgCl₂) did have peptidyltransferase activity (cf. our cores made in 2.5M-NH₄Cl/300mM-MgCl₂).

Core particles obtained from rabbit L-subparticles in repeated experiments were found to vary in activity, as shown in Fig. 8. This is probably because, as noted by Nomura & Held (1974), the protein composition of core particles can vary considerably with slight variations in the critical conditions of preparation.

The present report is confined to studies of L-subparticles, but preliminary work (cf. Cox & Hirst, 1976) has revealed that the activity of rabbit S-subparticles in polyphenylalanine synthesis is also diminished over the range 2–3M-NH₄Cl/50–75mM-MgCl₂. The 50% activity of particles recovered from 3M-NH₄Cl/75mM-MgCl₂ may indicate that re-assembly is more readily achieved in this case.

Nature of the split-protein fraction

The split-protein fraction should be readily separated into its major components, in view of the broad range of molecular weights and electrostatic charges suggested by their widely scattered positions on the two-dimensional gels (see Fig. 3). Subsequent reassembly experiments should permit the study of the structure–function relationships of individual proteins. For example, the role of the protein of spot L13 (Fig. 3), which is not necessarily part of the enzyme site, but which is implicated in the expression of peptidyltransferase activity of rabbit ribosomes, should be open to direct examination. Further characterization of this protein should enable comparisons to be made with proteins that have been implicated in the peptidyltransferase activity of ribosomes from other sources.

Reassembly of core-particle and split-protein fractions

The most important factor influencing reassembly appears to be the period of the salt-shock treatment. The ability of the cores to recombine with the split-protein fraction was impaired on prolonged exposure to high concentrations of NH₄Cl (see Fig. 9). The

overnight dialysis step was necessary for most experiments to decrease the NH₄Cl to 0.3M, but may not be essential otherwise. The incubation step at 37°C helped reassembly and a 1 h period was ample (see Fig. 11). A slight excess of the split-protein fraction was beneficial (see Fig. 10).

The ionic conditions and temperature of incubation have not yet been systematically varied to optimize recovery of active L-subparticles during the re-assembly process. However, the conditions successfully used (0.3M-NH₄Cl/15mM-MgCl₂ at 37°C) were those used in the preceding paper to re-activate rabbit L-subparticles (Cox *et al.*, 1976b). Also they are quite similar to the optimum ionic conditions for reconstruction of *E. coli* S-subparticles (0.25M-KCl/20mM-MgCl₂; Traub & Nomura, 1969) and for the final stage of reconstruction of *E. coli* L-subparticles (0.4M-NH₄Cl/20mM-MgCl₂; Nierhaus & Dohme, 1974), but rabbit L-subparticles cannot withstand the higher incubation temperatures used with the *E. coli* subparticles (Cox *et al.*, 1973b). Therefore it seems probable that the presently used conditions for reassembly are quite close to the optimum.

Conclusions

The technique used to prepare core particles was based on the concept that L-rRNA needs a particular molar ratio of Mg²⁺ to NH₄⁺ (>1:40) to maintain the 'native' conformation. In solutions of up to 1.8M-NH₄Cl, L-subparticles may be protected against disassembly by raising the Mg²⁺ concentration to the appropriate value. In solutions more concentrated than 1.8M-NH₄Cl, the subparticle lost particular proteins even at very high (500mM) concentrations of Mg²⁺ and a well-defined core particle was obtained, e.g. at 2.5M-NH₄Cl, presumably with the rRNA moiety in the native conformation. Because different rRNA species respond to Mg²⁺ in much the same way (Cox & Hirst, 1976), we infer that this is a general method for producing core particles capable of reassembly with recovery of activity, when recombined with the split-protein fraction.

The value of the ability to produce core particles for use in reassembly experiments has been amply demonstrated for *E. coli* ribosomes. Reassembly experiments have established a range of structure–function relationships, including the roles of individual proteins in wild-type and mutant species. The application of this approach to eukaryotic ribosomes should not only clarify the roles of particular protein components (e.g. L13 appears to be associated with the peptidyltransferase centre of rabbit L-subparticles), but may also provide insight into pathological conditions where ribosomes are altered, as, for example, in diabetes (Wettenhall *et al.*, 1974). The reassembly technique may also be useful in studying more general problems such as the evolution of ribosomes. Compared with prokaryotes, there is a

quantum jump in the size of cytoplasmic eukaryotic ribosomes, and evolutionary divergence is marked, particularly in the L-rRNA moiety (for a discussion, see Cox *et al.*, 1973a, 1976a). Finally, the disassembly and reassembly of eukaryotic ribosomes could prove useful in studies of the control of protein synthesis at the translational level.

Note added in Proof (Received 7 October 1976)

Although K^+ and NH_4^+ ions were found to have very similar effects on L-rRNA conformation (Cox & Hirst, 1976), recent experiments have shown that K^+ is less effective than NH_4^+ in splitting proteins off rabbit ribosomal L-subparticles under the salt-shock conditions described in the present paper. The subparticles treated with 2.75 M-KCl/69 mM-MgCl₂ for 2 h at 0°C retained 86% of control activity in the puromycin reaction and lost 10% of the protein complement, whereas a parallel preparation in 2.75 M- NH_4 Cl/69 mM-MgCl₂ retained at most 20% of activity but lost 17% of proteins. Two-dimensional gel electrophoresis revealed that protein spot L13 was much less prominent in the K^+ -split protein fraction than in the NH_4^+ -split protein fraction.

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