Biochem. J. (1968) **110**, 87
Printed in Great Britain

The Production of Biologically Active Subparticles from Rabbit Reticulocyte Ribosomes

By SOPHIA BONANOU, R. A. COX, BETTY HIGGINSON AND K. KANAGALINGAM
National Institute for Medical Research, Mill Hill, London, N.W.7

(*Received* 19 *June* 1968)

The effect of exposing rabbit reticulocyte ribosomes to concentrated solutions of potassium chloride was examined by: (*a*) dialysis against 0·5 M-potassium chloride; (*b*) zone centrifugation through a sucrose gradient in 0·5 M-potassium chloride; (*c*) differential centrifugation of a solution made 0·5 M with respect to potassium chloride. The products of each treatment and their ability to support protein synthesis in a reticulocyte cell-free system, in the presence and in the absence of polyuridylic acid, were examined. The following results were found. (1) Exposing the polysomes to 0·5 M-potassium chloride was not a sufficient condition for the complete dissociation of ribosomes into subparticles; the reaction showed a concentration-dependence, implying the existence of an equilibrium between the various ribosomal species. Disturbance of the equilibrium by removing certain products, as in zone centrifuging, can lead to complete dissociation. (2) The subparticles produced by dialysis or sucrose-gradient fractionation were biologically inactive and unstable. (3) The pellet obtained by differential centrifuging consisted of subparticles, if suspended in a Mg^{2+}-free buffer; addition of Mg^{2+} converted about 30% of the material into heavier sedimenting species, and the preparation had 20–40% of the activity of the untreated control polysomes in the cell-free system. Addition of the 0·5 M-potassium chloride supernatant fraction resulted in further apparent reconstitution of subparticles into ribosomes and polysomes and in a 50–100% restoration of biological activity. When both polyuridylic acid and supernatant factors were present incorporations similar to or higher than those of the control were attained.

Ribosomes from bacterial sources, e.g. *Escherichia coli*, may be caused to dissociate into subparticles simply by a decrease in the concentration of Mg^{2+} in the medium (Tissières, Watson, Schlessinger & Hollingworth, 1959). When the original Mg^{2+} concentration is restored the subparticles recombine to form ribosomes and regain their ability to support protein synthesis in cell-free systems (Schlessinger & Gros, 1963; Okamoto & Takanami, 1963; Gilbert, 1963). The reconstitution of yeast (Chao, 1957) and pea-seedling (Ts'o, Bonner & Vinograd, 1958) ribosomes from their subparticles has also been reported, though the products were not tested for activity.

In contrast, attempts to prepare biologically active subparticles from mammalian ribosomes have generally been abortive (Lamfrom & Glowacki, 1962; Gould, Arnstein & Cox, 1966). Arlinghaus & Schweet (1962) reported that treatment of rabbit reticulocyte ribosomes with 0·1 M-potassium chloride resulted in the dissociation of the particles performing endogenous and the polyU*-directed incorporation of [^{14}C]phenylalanine in the cell-free system. When the potassium chloride concentration was increased to 0·5 M, differential centrifuging of the solution into a pellet and a supernatant fraction showed that normal haemoglobin synthesis, with new chain formation, occurred only when both the pellet and the supernatant fractions were present (Miller, Hausmann, Erickson & Schweet, 1965). Later it was briefly reported that the pellet consisted of ribosomal subparticles (Miller, Hamada, Yang, Cohen & Schweet, 1967), which reassociated into monomeric ribosomes active in haemoglobin and polyphenylalanine synthesis.

The aim of the present work was to study more closely the products of this exposure of rabbit reticulocyte ribosomes to concentrated potassium chloride solutions, and to define the conditions for the production of subparticles that can recombine with restoration of activity.

* Abbreviation: polyU, polyuridylic acid.

EXPERIMENTAL

Materials. The following were obtained from commercial sources: ATP (dipotassium salt) and GSH from Sigma Chemical Co. (St Louis, Mo., U.S.A.); GTP (trilithium salt)

and pyruvate kinase from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany); polyU and ^{3}H-labelled polyU from Miles Chemical Corp. (Elkhart, Ind., U.S.A.); L-[U-^{14}C]phenylalanine of high specific radioactivity from The Radiochemical Centre (Amersham, Bucks.); acrylamide and *NN*-methylenebisacrylamide from the British Drug Houses Ltd. (Poole, Dorset); sodium dodecyl sulphate from Matheson, Coleman and Bell Inc. (East Rutherford, N.J., U.S.A.); *NNN'N'*-tetramethylethylenediamine from Koch–Light Laboratories Ltd. (Colnbrook, Bucks.).

A solution of potassium phosphoenolpyruvate was prepared by treating its tricyclohexylammonium salt with ethanolic barium acetate and acidifying the barium salt with N-H_2SO_4. For use in the cell-free system an aqueous solution of phosphoenolpyruvate, ATP and GTP (in the respective proportions 100, 5 and 1·25 μmoles/ml.) was prepared and the pH was adjusted to 7·6 with N-KOH. All reagents for the cell-free system, ribosomes and enzyme fractions were stored frozen at −18°.

Preparation of rabbit reticulocyte ribosomes and pH 5 *enzymes.* The reticulocytes were collected and lysed as described by Arnstein, Cox & Hunt (1964). The pellet obtained by differential centrifugation of the postmitochrondrial supernatant at 105000*g* for 60min. was the polysome fraction. Where indicated it was washed free of contaminating non-ribosomal proteins by suspending it in medium A (0·25 M-sucrose–25mM-KCl–1mM-$MgCl_2$–50mM-tris–HCl buffer, pH 7·6) and centrifuging it through two layers of 0·5 M- and 2·0 M-sucrose solutions in the salts of medium A at 105000*g* for 4hr. by the procedure of Wettstein, Staehelin & Noll (1963). This treatment did not affect the activity of the polysomes in the cell-free system. The pH 5 enzyme fraction was prepared after removal of the ribosomes from the postpolysomal supernatant at 105000*g* for 2hr. as described by Arnstein *et al.* (1964).

Centrifuging. MSE low-speed (Major, Mistral and 13) and high-speed (40 and Superspeed 50) centrifuges and a Spinco model L ultracentrifuge were used for all preparative work.

Sucrose gradients were prepared by layering 7ml. portions of 30%, 25%, 20% and 15% (w/v) sucrose solutions in the appropriate buffers and leaving them at 0° for 4–24hr. for the formation of linear gradients. The tubes, after the addition of the samples in 1–2ml., were centrifuged in the Spinco swing-out SW 25.1 rotor. Fractions were collected with a siphon and the extinctions measured with a Unicam SP. 500 spectrophotometer. Dr Birnie, of the Imperial Cancer Research Fund, Mill Hill, kindly performed the zonal ultracentrifuge run shown in Fig. 3, with a Beckman–Spinco model Z.U. ultracentrifuge (rotor B4).

A Spinco model E ultracentrifuge, fitted with u.v. absorption optics, was used for analytical work. In the u.v. optical path the camera was replaced by a photoelectric cell that scanned the slit, and the movement of the sedimenting species was followed by means of a plot of percentage transmission against distance on an XY recorder. A 12mm.-path-length cell was used in all cases. All u.v. analytical studies were made at temperatures in the range 5–10° with a ribosome concentration of about 0·05g./l. When the schlieren optical system was used the phase-plate angle was set at 60° in all cases except Fig. 5, where the angle was 50°.

Determination and isolation of protein and RNA. For routine determinations samples were sufficiently diluted to give E_{260} between 0·3 and 1·0. The E_{260} and E_{280} values were measured and the concentrations of RNA and protein were determined by means of a nomograph based on the extinction coefficients of enolase and nucleic acid (California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.; cf. Warburg & Christian, 1942).

For more accurate work the protein content of a sample was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine serum albumin as standard. From the E_{260} value of the same sample, and assuming that rabbit reticulocyte ribosomal RNA has $\epsilon_{(P)}$ 7600, i.e. $E^{1\%}_{260}$ 230 (R. A. Cox, unpublished work), the RNA/protein ratio in each sample could be determined.

Ribosomal RNA was extracted by 6 M-guanidinium chloride as described by Cox (1966).

Ribosomal proteins were extracted for analysis by polyacrylamide-gel electrophoresis essentially by the method of Summers, Maizel & Darnell (1965).

Polyacrylamide-gel electrophoresis of ribosomal proteins. Ribosome fractions were suspended in 0·5 M-urea–0·5% sodium dodecyl sulphate–10mM-sodium phosphate buffer, pH 7·2, incubated for 1hr. at 37° and dialysed against the same buffer overnight at room temperature. Samples [30–100 μg. of protein in approx. 0·1ml. to which 0·02ml. of 50% (w/v) sucrose was added] were applied on 7cm. × 0·5cm. gels containing 10% (w/v) acrylamide, 0·27% (w/v) bisacrylamide, 0·075% (w/v) ammonium persulphate, 0·5% (w/v) sodium dodecyl sulphate, 0·5 M-urea and 0·1 M-sodium phosphate buffer, pH 7·2. Electrophoresis was carried out at 7·5mA/tube for about 5hr. in 0·1 M-sodium phosphate buffer, pH 7·2, containing 0·1% (w/v) sodium dodecyl sulphate. The gels were extruded by gentle suction, stained in 1% saturated Amido Black in 5% (w/v) acetic acid–40% (w/v) methanol, and de-stained in the dye-free medium. Their absorption in the visible region was scanned with a double-beam recording microdensitometer (Joyce, Loebl and Co. Ltd., Gateshead, Co. Durham).

Amino acid incorporation by the cell-free system. The assay of the incorporation of L-[U-^{14}C]phenylalanine into protein by nucleoprotein fractions was that described by Arnstein *et al.* (1964). Each tube (0·5ml.) contained approx. 0·25mg. of ribosomes, 1mg. of pH 5 enzymes, 25–50 μg. of pyruvate kinase, 5·0 μmoles of phosphoenolpyruvate containing 0·125 and 0·0315 μmole of ATP and GTP respectively, 5 μmoles of neutralized GSH, 25 μmoles of KCl, 2·5 μmoles of $MgCl_2$, 12·5 μmoles of tris–HCl buffer, pH 7·6, an unlabelled mixture of the 20 protein amino acids minus phenylalanine (0·01 μmole of each), and [^{14}C]phenylalanine (specific radioactivity 495mc/m-mole). (The effective specific radioactivity was diluted by the endogenous amino acid pool to approx. 30mc/m-mole, as calculated from pilot experiments by the formula given by Nair & Arnstein, 1965.) PolyU (20–50 μg./tube) and 0·5 M-KCl supernatant (20–50 μl./tube) were present where indicated. After incubation for 60min. at 37°, 0·2ml. of N-NaOH containing 5 μmoles of unlabelled DL-phenylalanine was added, and the incubation was continued for 5min. at 37° and for 15min. at room temperature and was terminated by the addition of 2·5ml. of 10% (w/v) trichloroacetic acid. The protein was isolated by filtration on 3cm.-diameter Oxoid membrane filters (Oxo Ltd., London, E.C. 4), washed once with cold (2·5ml.) and twice with hot (90°) 5% (w/v) trichloroacetic acid (5·0ml.) and finally twice with water (5·0ml.). The samples were counted at finite thinness with a low-background (1·5counts/min.) Geiger tube counter (model 181B; Nuclear–Chicago Corp.,

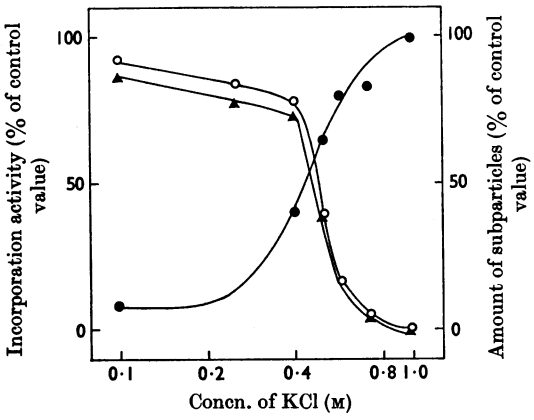

Fig. 1. Effect of dialysing samples (1ml.) of polysomes (6mg./ml.) against buffers (300ml.) containing various concentrations of KCl. After 2hr. at 4° the samples were analysed in the Spinco model E ultracentrifuge for the production of species sedimenting as less than 80s (●), and tested in the standard cell-free system for their ability to synthesize acid-insoluble proteins in the absence (○) and presence (▲) of polyU. The control polysomes incorporated 1·27 and 3·12mμc of acid-insoluble protein per tube respectively and contained approx. 8% of subparticles. All values are expressed as percentages of the control.

Des Plaines, Ill., U.S.A.), fitted with an ultra-thin Micromil window giving a counting efficiency of approx. 5%.

RESULTS

Attempts to produce ribosomal subparticles from the polysome fractions in concentrated chloride solutions included (*a*) dialysis against 0·5M-potassium chloride, (*b*) zonal centrifugation in sucrose gradients and (*c*) exposure to 0·5M-potassium chloride and differential centrifuging

Dialysis against 0·5M-potassium chloride

Samples (1ml.) of polysomes (6mg./ml.) in 10mM-potassium chloride–10mM-magnesium chloride–10mM-tris buffer, pH7·6, were dialysed against a 300-fold excess of a 10mM-tris buffer, pH7·6, containing an appropriate concentration of potassium chloride (0·1–1·0M). After 2hr. at 4° part of each sample was examined in the analytical ultracentrifuge and the remainder, after suitable dilution of the potassium chloride and addition of magnesium chloride, was tested for activity in the cell-free system. All assays were done within 5hr. of the preparation of extracts. The results are summarized in Fig. 1. It is apparent that exposure to 0·5M-potassium chloride was not the sole condition for the dissociation of polysomes into subparticles, though their yield rapidly increased between 0·4M- and 0·75M-potassium chloride. The diminished activity of the dialysed samples was closely paralleled by the loss of ribonucleoprotein sedimenting faster than 80s, e.g. at 0·5M-potassium chloride 40% of the original incorporation was observed when about 55% of the material had been converted into subparticles. The ratio of the radioactivity of protein synthesized in the presence of polyU to that of protein synthesized in the absence of polyU was constant for all samples, indicating that the progressive dissociation of polysomes into subparticles did not lead to an increased stimulation of the system by polyU. This is in contrast with the preferential response of native ribosomal subparticles to polyU reported for reticulocytes by Bishop (1965) and confirmed in this Laboratory (S. A. Bonanou & R. A. Cox, unpublished work). The omission of the pH5 enzyme fraction from the complete assay system similarly decreased the activity of all samples by the same factor.

It is concluded that dialysis against concentrated potassium chloride solutions appears to dissociate polysomes into inactive subparticles. The effect of dissociation was complicated by the instability of the products, which tended to degrade (particularly the smaller subparticle) and aggregate to give denatured precipitates.

Zonal centrifugation in sucrose gradients

Sucrose-gradient centrifugation through 0·5M-potassium chloride dissociated polysomes into subparticles; this is shown by the sedimentation properties of the RNA and the electrophoretic behaviour of the proteins isolated from the species produced.

In a typical experiment 6mg. of polysomes (previously washed through 2·0M-sucrose to remove non-ribosomal protein contaminants) in 25mM-potassium chloride–1mM-magnesium chloride–50mM-tris buffer, pH7·6, were layered on three 15–30% (w/v) sucrose gradients in the following buffers: 10mM-tris buffer, pH7·6, for tube (*a*), 0·50M-potassium chloride–5mM-magnesium chloride–10mM-tris buffer, pH7·6, for tube (*b*) and 0·50M-potassium chloride–10mM-tris buffer, pH7·6, for tube (*c*). The polysomes in tube (*a*) were made 0·75M with respect to potassium chloride and the gradients were centrifuged at 25000 rev./min. for 16hr. in the Spinco SW25.1 rotor. The results are shown in Fig. 2.

Gradients in 10mM-tris–hydrochloric acid buffer. The polysomes that were made 0·75M with respect to potassium chloride and centrifuged through a sucrose gradient in 10mM-tris buffer, pH7·6, produced a single peak (Fig. 2*a*), in contrast with EDTA-titrated polysomes, which, when centrifuged

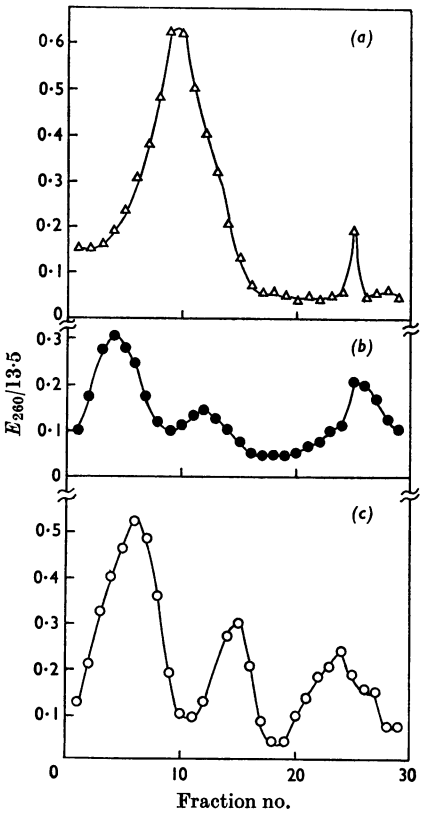

Fig. 2. Zone centrifugation of polysomes (approx. 6mg. portions) in 15–30% (w/v) sucrose gradients in the following buffers: (*a*) 10mM-tris, pH7·6; (*b*) 0·5M-KCl–5mM-$MgCl_2$–10mM-tris buffer, pH7·6; (*c*) 0·5M-KCl–tris buffer, pH7·6. The sample on gradient (*a*) was made 0·75M with respect to KCl before the centrifugation. The tubes were centrifuged at 25000rev./min. for 16hr. in the Spinco SW25.1 rotor. Fractions of volume 1ml. were collected and the E_{260} values measured. The direction of sedimentation is from right to left.

through the same buffer, give two distinct peaks (Gould *et al.* 1966). To check the composition of the peak a sample of tube 10 of the gradient given in Fig. 2(*a*) was diluted with a Mg^{2+}-containing buffer (25mM-potassium chloride-1mM-magnesium chloride–50mM-tris buffer, pH7·6) and examined in the analytical ultracentrifuge: although the buffer was expected to favour association, two components with $S_{20,w}$ values of 24·5s and 47·5s were present. The reason for the lack of resolution of these two species on the gradient is not understood.

Gradients in 0·5M-*potassium chloride.* When the gradients in 10mM-tris buffer, pH7·6, contained 0·5M-potassium chloride subparticles were formed; in the presence of 5mM-Mg^{2+} the two subparticles travelled 15% faster (Fig. 2*b*) than when no Mg^{2+} was in the gradient (Fig. 2*c*), though the extent of dissociation was smaller in the former case, with considerable amounts of aggregated material in the pellet. The $S_{20,w}$ values of the two subparticle peaks of the gradient in Fig. 2(*c*) were determined by analytical centrifuging in 0·5M-potassium chloride–10mM-tris buffer, pH7·6, to be 43s and 16s and in 25mM-potassium chloride–1mM-magnesium chloride–50mM-tris buffer, pH7·6, to be 47s and 28s for the larger and smaller subparticles respectively. Protein and a small RNA species were found to sediment after the smaller subparticle in the gradients in Figs. 2(*b*) and 2(*c*) and were designated the 'meniscus fraction'. When the concentration of potassium chloride was lowered (e.g. by dilution for u.v. extinction measurements) the protein was denatured and gave a coarse precipitate. When polysomes were incubated for 20min. in the cold with ^{3}H-labelled polyU and then centrifuged through a gradient similar to that used for Fig. 2(*b*), the radioactive peak travelled with or just ahead of the meniscus fraction. ^{3}H-labelled polyU centrifuged on its own travelled only 1–2 tubes from the top of the gradient.

Large-scale zonal centrifuging in 0·5M-*potassium chloride.* The existence of the meniscus fraction in gradients containing 0·5M-potassium chloride was confirmed by centrifuging 150mg. of washed polysomes in a zonal rotor (Beckman B4) for 4hr. The extinction profile is shown in Fig. 3. About 6% of the E_{260} travelled in advance of the larger subparticle into the region of intact ribosomes; this may be analogous to the persistence of 'stuck' 70s ribosomes reported for the *E. coli* system (Tissières, Schlessinger & Gros, 1960). The larger subparticle accounted for about 64% of the E_{260} compared with about 25% for the smaller subparticle. The remaining 4% of the E_{260} (varying in the range 3–6% in other experiments) made up of the slowest sedimenting species was located in a broad peak trailing behind the smaller subparticle.

The RNA/protein ratios were determined for the different species with an error of $\pm 0{\cdot}05$ to be approx. 0·98 for the washed polysomes, 1·07 and 1·04 for the larger and smaller subparticles respectively, and 0·35 for the meniscus fraction. Calculation shows that the meniscus fraction could account for 6–12% of the protein and not more than 1–3% of the RNA originally present in the polysomes.

Attempts to reconstitute active ribosomes. When the newly fractionated subparticles were mixed in equimolar amounts and dialysed against 25mM-potassium chloride–5mM-magnesium chloride–50mM-tris buffer, pH7·6, in the presence of the meniscus fraction, particles sedimenting as intact monomers and dimers were detected. They were, however, devoid of biological activity; the same was true for the

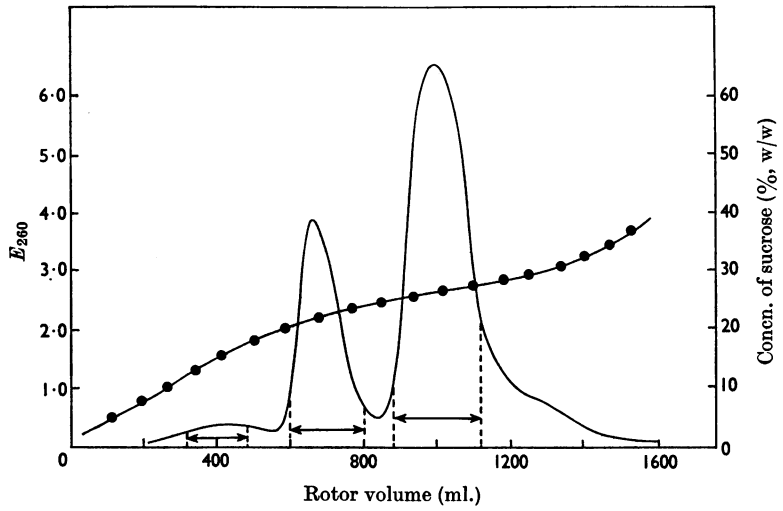

Fig. 3. Zone centrifuging (performed by Dr Birnie) of 150 mg. of washed polysomes in a Beckman Spinco model Z.U. ultracentrifuge (rotor B4). Centrifugation was for 4 hr. at 40 000 rev./min. The direction of sedimentation is from left to right. Fractions pooled for sedimentation are indicated by broken vertical lines. ●——●, Concn. of sucrose (%, w/w); ——, E_{260}.

dialysed 'stuck' ribosomes. Preincubation of the 'reconstituted' particles at 0° or 37°, in the presence or absence of pH 5 enzymes of polyU, did not alter their initial activity (1–2% of that of control polysomes). Inactive species sedimenting at 78 s and 94 s were also formed by simply freezing and thawing the subparticles, in the absence of Mg^{2+} and supernatant. In general, subparticles formed by zone centrifuging in 0·5 M-potassium chloride were difficult to recover because they tended to aggregate on standing or on dialysis against buffers of a wide range of ionic strengths, and to denature into an insoluble pellet after differential centrifugation.

Examination of the RNA and protein components. To check whether the cause of this instability was the degradation of ribosomal components, the RNA was isolated from the subparticles after the fractions had been pooled as indicated in Fig. 3 and sedimented at 78000*g* for 16 hr. RNA from the smaller subparticle sedimented as a single sharp boundary corresponding to 17 s, but degradation and aggregation gave polydisperse boundaries of about 20 s, 26 s and 35 s for the RNA from the larger subparticle. A single u.v.-absorbing material sedimenting at 5 s in 0·5 M-potassium chloride–10 mM-tris buffer, pH 7·6, was present in the meniscus fraction. After dialysis against 25 mM-potassium chloride–5 mM-magnesium chloride–50 mM-tris buffer, pH 7·6, most of the protein was precipitated, and analytical ultracentrifugation revealed two u.v.-absorbing species in the supernatant forming boundaries at 2 s and 4 s.

The ribosomal proteins were extracted by 0·5 M-urea–0·5% (w/v) sodium dodecyl sulphate–10 mM-sodium phosphate buffer, pH 7·2, and analysed by electrophoresis on polyacrylamide gels. Fig. 4

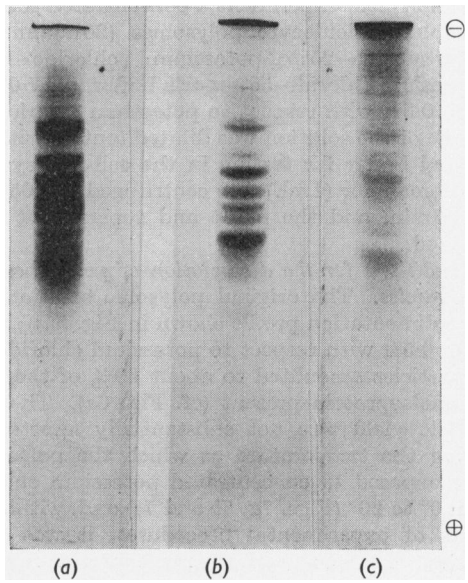

Fig. 4. Polyacrylamide-gel electrophoresis of ribosomal proteins (see the Experimental section) from the larger (*a*) and smaller (*b*) subparticles and the meniscus fraction (*c*). Proteins were extracted from the corresponding pooled and sedimented fractions as indicated in the zonal ultracentrifuge separation shown in Fig. 3. Electrophoresis was carried out (7·5 mA/tube) for 5 hr. at room temperature.

shows the marked heterogeneity of the bands obtained from the larger and smaller subparticles and the meniscus fraction, though the resolution of the system makes the quantitative measurement of the bands present in each fraction difficult.

The presence of certain ribosomal proteins in the meniscus was thus confirmed, but in the absence of similarly isolated proteins from the native rabbit reticulocyte subparticles it could not be decided from which subparticle they were derived. As, however, all the protein bands obtained were also present in unfractionated polysomes washed in 2·0 M-sucrose, we feel justified in calling them 'ribosomal'. It is realized that the term might cover both the strictly structural proteins of the ribosomes and the enzymes that are tightly bound to them.

The experiments for the fractionation of subparticles described so far were lengthy and so they might be expected to favour degradation; therefore quicker methods were sought.

Exposure to 0·5 M-potassium chloride and differential centrifuging

In a typical experiment a concentrated solution of rabbit reticulocyte polysomes (20 mg./ml.) in 0·25 M-sucrose – 25 mM-potassium chloride – 1 mM-magnesium chloride–50 mM-tris buffer, pH 7·6, was made 0·5 M with respect to potassium chloride. A sample of the solution was diluted tenfold with the original buffer for testing in the cell-free system. The remainder (1 ml.) was centrifuged at 105 000 *g* for 60 min. and the pellet and supernatant were retained.

Conditions for the dissociation of polysomes into subparticles. The original polysome solution had the sedimentation profile shown in Fig. 5(*a*); when made 0·5 M with respect to potassium chloride the subparticles amounted to about 60% of the total ribonucleoprotein present (cf. Fig. 6*a*). The subparticle yield was not substantially affected by raising the temperature at which the polysomes were exposed to concentrated potassium chloride from 0° to 20° (Figs. 7*a*, 7*b* and 7*c*) and, within the limits of experimental procedures, it was independent of time, though the nucleoprotein in 0·5 M-potassium chloride showed a marked tendency to aggregate (Figs. 6*a* and 6*b*). However, the proportion of subparticles formed depended on the concentration of the polysome solution made 0·5 M with respect to potassium chloride, increasing with decreasing concentration (Figs. 8*a* and 8*b*).

The composition of the pellet obtained by centrifugation of the polysome solution in 0·5 M-potassium chloride for 60 min. at 105 000 *g* depended on the buffer in which it was resuspended. To produce the maximum yield of subparticles it was essential to suspend the pellet in a Mg^{2+}-free medium (cf. Fig. 5*b*). If this step was omitted and the pellet was suspended in a buffer containing 1 mM-magnesium chloride, the proportion of subparticles was about the same as in 0·5 M-potassium chloride and poly-

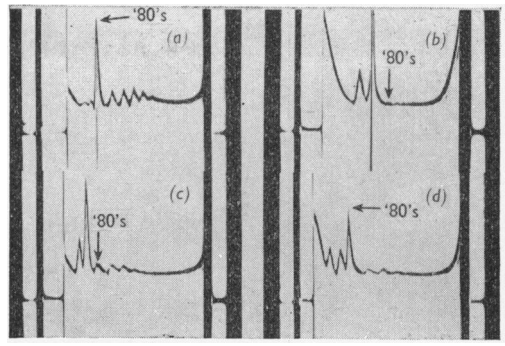

Fig. 5. Reconstitution experiments. All samples were run at 33 450 rev./min. The direction of sedimentation is from left to right. (*a*) Control polysomes (2·75 mg./ml.) in 25 mM-KCl–1 mM-$MgCl_2$–50 mM-tris buffer, pH 7·6. The photograph was taken 12 min. after full speed was attained. The temperature of the run was 2·8°. (*b*) 105 000 *g* × 60 min. pellet of polysomes exposed to 0·5 M-KCl, in 0·25 M-sucrose, The photograph was taken 54 min. after full speed was attained. The temperature of the run was 6·9°. (*c*) Portion of pellet (*b*) in 25 mM-KCl–1 mM-$MgCl_2$–50 mM-tris buffer, pH 7·6. The photograph was taken 12 min. after full speed was attained. The temperature of the run was 7·1°. (*d*) Portion of (*c*) after the addition of 0·5 M-KCl supernatant. The photograph was taken 13 min. after full speed was attained. The temperature of the run was 7·4°.

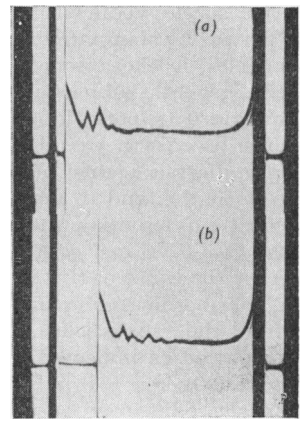

Fig. 6. Influence of time of exposure to 0·5 M-KCl on the polysome fraction. The photographs were taken 16 min. after full speed (33 450 rev./min.) was attained. The direction of sedimentation is from left to right. (*a*) Polysomes (2·5 mg./ml.) exposed to 0·5 M-KCl for 30 min. The temperature of the run was 7·7°. (*b*) Polysomes (2·5 mg./ml.) exposed to 0·5 M-KCl for 240 min. The temperature of the run was 10·1°.

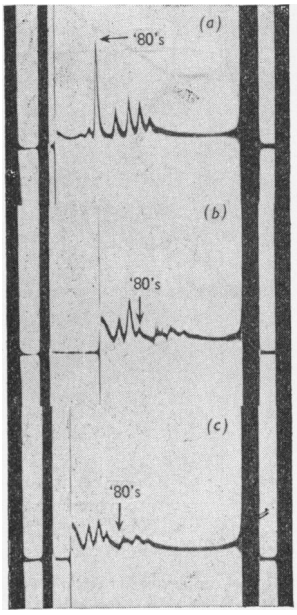

Fig. 7. Effect of temperature on the polysome fraction in concentrated KCl solutions. All the photographs were taken 10 min. after full speed (33 450 rev./min.) was attained. The direction of sedimentation is from left to right. (*a*) Stock polysomes (10 mg./ml.) in 50 mM-tris buffer, pH 7·6. The temperature of the run was 3·5°. (*b*) Stock polysomes (10 mg./ml.) exposed to 0·6 M-KCl–50 mM-tris buffer, pH 7·6 at 0°. The temperature of the run was 4·8°. (*c*) Stock polysomes (10 mg./ml.) exposed to 0·6 M-KCl–50 mM-tris buffer, pH 7·6, at 20°. The temperature of the run was 4·1°.

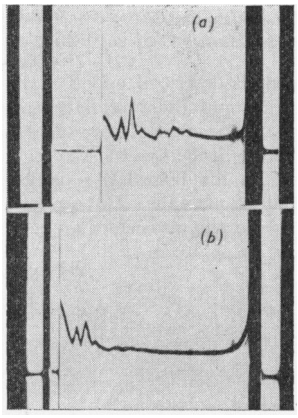

Fig. 8. Effect of polysome concentration on the production of subparticles. (*a*) Polysomes (10 mg./ml.) in 0·6 M-KCl–50 mM-tris buffer, pH 7·6. The photograph was taken 10 min. after full speed (33 450 rev./min.) was attained. The temperature of the run was 4·8°. (*b*) Polysomes (2·5 mg./ml.) in 0·5 M-KCl. The photograph was taken 12 min. after reaching full speed (33 450 rev./min.) was attained. The temperature of the run was 7·7°. Direction of sedimentation is from left to right.

somes might amount to 40–60% of the total, depending on the initial concentration of ribosomes in 0·5 M-potassium chloride (cf. Fig. 8).

Reconstitution of subparticles into ribosomes. (*a*) Sedimentation profiles. In the experiment described at the beginning of this section the original polysome solution (Fig. 5*a*) contained 8% of subparticles (44·6 s and 59·8 s), 52% of monoribosomes (76·2 s) and 40% of polysomes (112·3 s, 140·5 s, 162·6 s and 186·0 s). When the 105 000 g × 60 min. pellet obtained from the polysomes made 0·5 M with respect to potassium chloride was suspended in 0·25 M-sucrose, over 95% of the ribosomal material was in the form of subparticles with $S_{20,w}$ values of 31·2 s and 40·5 s (Fig. 5*b*). About 3–4% of the total ribonucleoprotein appeared as undissociated monoribosomes during the first 20 min. at full speed; after about 1 hr. at this speed, the slower limb of the heavier peak showed a slightly skewed appearance towards the base, representing 4% of material sedimenting at about 38 s. Essentially the same sedimentation profile as in 0·25 M-sucrose was obtained when the 105 000 g × 60 min. pellet was re-exposed to 0·5 M-potassium chloride, with a species moving just slower than the larger subparticle again discernible. In contrast, when the pellet originally resuspended in 0·25 M-sucrose was subsequently diluted with a Mg^{2+}-containing buffer to a final salt concentration of 25 mM-potassium chloride–1 mM-magnesium chloride–50 mM-tris buffer, pH 7·6, the sedimentation profile (Fig. 5*c*) showed that about 8% of the subparticles, now sedimenting at 35·1 s and 52·9 s, had been converted into ribosomes (75·2 s) and 20% into polysomes (109·8 s, 125·6 s and 152·3 s), suggesting reconstitution. Once more a species (about 10–12%), similar to that seen in 0·25 M-sucrose, sedimented at 47·5 s and was just distinguishable from the larger subparticle, after centrifuging for 1 hr. It is not clear whether the persistence of this 'inter-subparticle' peak is an artifact arising from interaction between the species in the cell or whether it represents some modified form of the larger subparticle. Whatever the case it does not alter the principal observation that these subparticles can recombine. The addition of an equivalent volume of the 0·5 M-potassium chloride supernatant increased the proportion of the reconstituted ribosomes (70·1 s) to about 50% of total ribonucleoprotein (Fig. 5*d*), whereas the amount of polyribosomes (104·7 s, 129·6 s and 162·4 s) remained about the same as in Fig. 5(*c*), and the subparticles (32·3 s and 52·3 s) amounted to 35% of the total. The significance of supernatant factors in the reconstitution of ribosomes was thus indicated.

Table 1. *Incorporation in the cell-free system of subparticles resuspended in* 0·25 M-*sucrose*

The experiment is described under 'Exposure to 0·5 M-potassium chloride and differential centrifuging' in the Results section. The amount of polysomes or derived subparticles was 0·25 mg./tube (see the Experimental section). Where indicated, 50 μl. of 0·5 M-KCl supernatant and 25 μg. of polyU were added per tube. The results were corrected for incorporations in the absence of an energy source and ribosomes.

Sample	Radioactivity of acid-insoluble protein (mμc/assay tube)
(1) Stock polysomes	8·8
(2) As (1)+0·5 M-KCl supernatant	9·2
(3) As (1)+0·5 M-KCl supernatant +polyU	17·0
(4) Stock polysomes in 0·5 M-KCl diluted tenfold	4·43
(5) Stock polysomes in 0·5 M-KCl sedimented at 105000g × 60 min. and resuspended in 0·25 M-sucrose	1·74
(6) As (5)+0·5 M-KCl supernatant	4·26
(7) As (5)+0·5 M-KCl supernatant +polyU	19·9

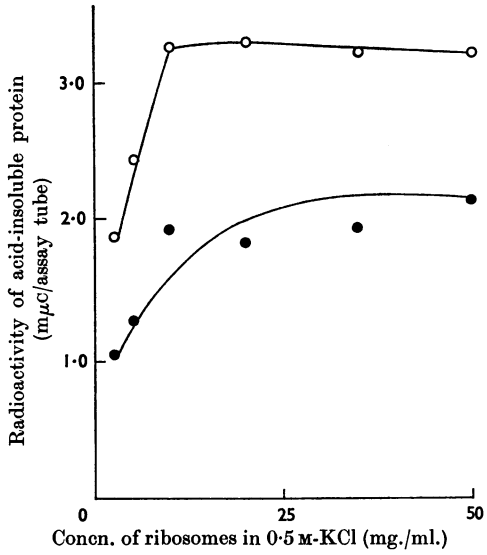

Fig. 9. Dependence on concentration of stock polysome solution, made 0·5 M with respect to KCl, of activity in the cell-free system of the 105000g × 60 min. pellet, which was redissolved in 0·25 M-sucrose–25 mM-KCl–1 mM-$MgCl_2$–50 mM-tris buffer, pH 7·6, and assayed both in the absence (●) and in the presence (○) of 0·5 M-KCl supernatant. The control activities were 2·74 and 2·96 mμc/tube respectively.

(*b*) Endogenous activity. The various samples shown in Fig. 5 were also tested for their ability to incorporate [^{14}C]phenylalanine into acid-insoluble protein in the standard cell-free system; the results are shown in Table 1. The polysome solution that was briefly exposed to 0·5 M-potassium chloride and diluted to the original salt concentration (0·25 M-sucrose–25 mM-potassium chloride–1 mM-magnesium chloride–50 mM-tris buffer, pH 7·6) had about half the activity of the control sample. The pellet obtained by centrifuging the polysomes in 0·5 M-potassium chloride for 60 min. at 105000g, which was resuspended in 0·25 M-sucrose and subsequently diluted in a Mg^{2+}-containing buffer, had about a fifth of the activity of the control. Addition of 0·5 M-potassium chloride supernatant almost trebled the incorporation into protein.

The 0·5 M-potassium chloride supernatant could substitute for part of the activity of the pH 5 enzyme fraction, as expected if some soluble enzymes adhere to the unwashed ribosomes. On its own no incorporation was detected, i.e. it could not substitute for any ribosomal fraction tested. It was not affected by preincubation at 37° for up to 1 hr., whereas the sedimented subparticles were completely inactivated after 10 min. at 37°. Addition of the supernatant to the control polysomes increased the cell-free system incorporation by up to 25%. Its effect on the 105000g × 60 min. pellet was less easily reproducible: the observed stimulations ranged from 10% to 60% of the initial incorporation by the pellet alone.

In general the activity of the pellet depended on the activity and the concentration of the initial ribosomal preparation and, provided that the assay was made shortly after exposure to 0·5 M-potassium chloride, the incorporation in the cell-free system corresponded to the proportion of undissociated or reassociated ribosomes calculated from the sedimentation profile. Thus the activity of the pellets initially suspended in a Mg^{2+}-containing buffer was usually 40–80% that of the control; when the pellets were initially suspended in a Mg^{2+}-free buffer their activity in the cell-free system was usually 20–40% of that of the control. Fig. 9 shows that, as the concentration of polysomes in solutions made 0·5 M with respect to potassium chloride decreased, so the activity of the 105000g × 60 min. pellets, resuspended in a Mg^{2+}-containing buffer to prevent further dissociation, decreased. Addition of the potassium chloride supernatant (pooled from the 10 mg./ml. and 20 mg./ml. solutions) increased the activity of all pellets by a constant factor, restoring the incorporation activity of pellets obtained from the more concentrated solutions to values similar to or higher than those of the control. Under the conditions used the effect of concentration on activity, both in the presence and in the absence of

Table 2. *Incorporation in the cell-free system of subparticles resuspended in* 0·5 M-*potassium chloride*

The stock polysome solution contained 15 mg./ml. in 0·25 M-sucrose–25 mM-KCl–1 mM-$MgCl_2$–50 mM-tris buffer, pH 7·6. After being made 0·5 M with respect to KCl the solution (1·3 ml.) was centrifuged at 105000*g* for 90 min. The pellet was resuspended in 0·5 M-KCl–10 mM-tris buffer, pH 7·6, and then diluted with a Mg^{2+}-containing buffer to the initial salt concentration. There was 0·25 mg. of polysomes or derived subparticles per tube. Where indicated, 25 μl. of supernatant and 30 μg. of polyU were added per tube. The results were corrected for incorporations in the absence of an energy source and ribosomes.

Sample	Radioactivity of acid-insoluble protein (mμc/assay tube)
(1) Stock polysomes	1·04
(2) As (1)+0·5 M-KCl supernatant	1·02
(3) As (1)+polyU	11·10
(4) As (3)+0·5 M-KCl supernatant	26·20
(5) Stock polysomes in 0·5 M-KCl sedimented at 105000*g* × 90 min. and resuspended in 0·5 M-KCl	0·20
(6) As (5)+0·5 M-KCl supernatant	0·35
(7) As (5)+polyU	11·60
(8) As (7)+0·5 M-KCl supernatant	26·90

supernatant, was abolished if the original ribosomal solutions contained more than 10 mg./ml.

(*c*) Polyphenylalanine synthesis. As shown by the results of two different experiments, summarized in Tables 1 and 2, in the presence of 0·5 M-potassium chloride supernatant the polymerization obtained by the dissociated and reconstituted subparticles was equal to or higher than that of the control. Other experiments showed that the addition of supernatant enhanced both the endogenous and the polyU-directed synthesis of all the fractions, though only the dissociated pellet showed a marked rise in the amount of acid-insoluble protein synthesized in the presence as compared with that in the absence of polyU. This behaviour was reminiscent of that of the native ribosomal subparticles (Bishop, 1966) that were implicated in the initiation of new polypeptide chains. Analysis of the complete incubation mixtures in a sucrose gradient showed that when the supernatant factors were added to the pellet the radioactive protein sedimented in advance of the subparticles, as would be expected if ribosomes and polysomes were formed.

DISCUSSION

Preparation of subparticles. The terms larger and smaller subparticles refer to two families of nucleoproteins, formed respectively from the 30 s and 17 s RNA components of ribosomes. The nucleoproteins in each family might differ slightly in the RNA/protein ratio. The action of concentrated potassium chloride solutions on the ribosomes is attributed to a substitution of K^+ ions for Mg^{2+} ions in the nucleoprotein, resulting in the formation of subparticles; these may subsequently unfold or lose protein, by analogy with the action of concentrated solutions containing univalent cations, e.g. lithium chloride (Itoh, Otaka & Osawa, 1968), caesium chloride (Lerman, Spirin, Gavrilova & Golov, 1966) or ammonium chloride (Gavrilova, Ivanov & Spirin, 1966), on *E. coli* ribosomes.

Our results are consistent with the notion that the dissociation of ribosomes and polysomes is governed by equilibria that may be represented as:

$$\text{Ribosomes} \rightleftharpoons \text{smaller subparticle} + \text{larger subparticle} + f_1 + f_2 + f_3 \text{ etc.} \quad (1)$$

$$\text{Polysomes} \rightleftharpoons \text{smaller subparticle} + \text{larger subparticle} + f_1 + f_2 + f_3 \text{ etc.} \quad (2)$$

where f_1, f_2, f_3 etc. are protein or RNA species.

Both eqns. (1) and (2) would be consistent with the recent evidence from the *E. coli* system that during protein biosynthesis ribosomal subparticles are stable and 'recycle' (Kaempfer, Meselson & Raskas, 1967), that 'native' and 'derived' subparticles are intrinsically equivalent, associating into ribosomes or polyribosomes and dissociating concomitantly with peptide chain initiation and termination, in the presence of the relevant factors (Schlessinger, Mangiarotti & Apirion, 1967), and that formation of a messenger RNA–30 s ribosome–formylmethionyl-transfer RNA complex is required (Nomura & Lowry, 1967) before formation of the 70 s ribosome and peptide synthesis can occur. There is no definitive evidence that ribosomes are formed as intermediates in the dissociation of polysomes.

By analogy with Ostwald's dilution law, the fraction of ribosomes or polysomes that are dissociated will depend on the total nucleoprotein concentration (cf. Fig. 9), provided that none of the products is removed. The different products obtained by exposing the ribosome fraction to 0·5 M-potassium chloride could be explained by the existence of equilibria of the type given in eqns. (1) and (2). Thus the limited dissociation into subparticles that was observed in the dialysis experiments (Fig. 1) would be expected if all the products remained within the dialysis sac. Separation of the products, as in zone centrifugation through 0·5 M-potassium chloride, would be expected to shift the equilibrium in favour of complete dissociation. This was observed (see Fig. 3). Any buffer that is free from Mg^{2+} or other bivalent cations is likely to favour

dissociation into subparticles, so that zone centrifugation of the ribonucleoprotein fraction first made 0·75 M with respect to potassium chloride through 10 mM-tris buffer, pH 7·6, would also be expected to lead to extensive dissociation. This was found to be the case (Fig. 2*a*), though the subparticles were not well separated. As a preparative method differential centrifuging may affect the equilibrium between the products formed by exposing the nucleoprotein fraction to 0·5 M-potassium chloride owing to the appreciable difference in the sedimentation ratio of the larger and smaller subparticles and the factors f_1, f_2, f_3 etc. (see Fig. 3). Provided that not all the slowest-sedimenting species has reached the bottom of the tube the composition of the pellet obtained on differential centrifuging will differ from that of the supernatant (for discussion see Arnstein, Cox, Gould & Potter, 1965), and a new equilibrium will be attained when the pellet is suspended in buffer. Thus at least four situations are possible. (*a*) A new equilibrium will be attained even though the pellet is resuspended in the same volume of the same buffer as the original supernatant; (*b*) resuspension in a buffer free from Mg^{2+} and other bivalent cations will shift the equilibrium in favour of subparticles; (*c*) the subsequent addition of Mg^{2+} to (*b*) will lead to a new equilibrium and the extent of recombination of the subparticles into ribosomes will depend on the concentration of the various components; some recombination is likely because even the slowest-sedimenting species will be present in the pellet; (*d*) when the pellet from (*a*) is resuspended in a buffer, such as 0·25 M-sucrose–25 mM-potassium chloride–1 mM-magnesium chloride–50 mM-tris buffer, pH 7·6, that favours the stability of polysomes, no further dissociation will take place and some recombination might be expected. In practice the equilibria attained in (*c*) and (*d*) may differ because the composition and properties of the nucleoprotein fraction may depend not only on the nature but also on the order in which various operations are performed. It appears that association of subparticles leads to the formation of ribosomes rather than polysomes. The ability of the subparticles to recombine is affected by prolonged handling, and both the protein and the RNA moieties might thus become damaged, e.g. the tendency of the particles to form aggregates that eventually become insoluble suggests that the protein moiety slowly denatures at 0°, and that protein-depleted particles may form. Since the starting preparations were not treated to remove nucleases the possibility that exposure to potassium chloride activates latent ribonucleases comparable with the *E. coli* exonuclease III (Robertson, Webster & Zinder, 1968), which is activated by 0·2 M-ammonium chloride, cannot be excluded and might account for the observed degradation of the larger RNA component. The marked effect of ribonuclease on the activity of ribosomes is shown in Table 3.

Table 3. *Effect of ribonuclease treatment on incorporation in the cell-free system*

There was 0·25 mg. of ribosomes per tube. Where indicated, 50 μl. of 0·5 M-KCl supernatant and 25 μg. of polyU were added per tube. The specific radioactivity of phenylalanine was 50 mμc/mμmole. A small correction was applied for incorporation observed in the absence of ribosomes or in the absence of an energy source.

Sample	Radioactivity of acid-insoluble protein (mμc/assay tube)
(*a*) Controls	
(1) Stock polysomes	2·19
(2) As (1)+0·5 M-KCl supernatant	2·41
(3) As (1)+polyU	15·2
(4) As (1)+polyU+0·5 M-KCl supernatant	20·4
(5) Stock polysomes in 0·5 M-KCl sedimented at 105 000*g* × 90 min. and resuspended in 0·25 M-sucrose	0·50
(6) As (5)+0·5 M-KCl supernatant	0·52
(7) As (6)+polyU	20·4
(*b*) Ribonuclease-treated	
(8) Stock polysomes digested with pancreatic ribonuclease for 15 min. at about 20°*	0·18
(9) As (8)+0·5 M-KCl supernatant	0·19
(10) As (8)+polyU	0·61
(11) As (9)+polyU	3·78
(12) Stock polysomes in 0·5 M-KCl sedimented at 105 000*g* × 90 min., resuspended in 0·25 M-sucrose and digested with ribonuclease as in (8)	0·078
(13) As (12)+0·5 M-KCl supernatant	0·083
(14) As (12)+polyU	0·3
(15) As (13)+polyU	6·7
(16) Stock polysomes treated as in (8), but for 60 min.	0·06
(17) As (16)+0·5 M-KCl supernatant +polyU	0·3
(18) Stock polysomes in 0·5 M-KCl treated as in (12) but for 60 min.	None detected
(19) As (18)+0·5 M-KCl supernatant	None detected
(20) As (18)+polyU	None detected
(21) As (19)+polyU	None detected

* 0·03 μg. of ribonuclease/10 mg. of ribonucleoprotein was used. After incubation the ribosome fraction was stored at 0–4°. The rate of hydrolysis was decreased but not eliminated by this procedure.

Reconstitution of subparticles into active ribosomes. As described in the Results section (Fig. 5) the dissociation of ribosomes and polysomes of the 105 000*g* × 60 min. pellet, dissolved in 0·25 M-sucrose, into subparticles was 95% complete as judged

by the boundaries observed on analytical centrifuging (Fig. 5*b*). When the concentration of salts in 0·25 M-sucrose was increased to 25 mM-potassium chloride–1 mM-magnesium chloride–50 mM-tris buffer, pH 7·6, four boundaries, amounting to 20–30% of the total mass, were observed; they were attributed to the presence of the factors f_1, f_2, f_3 etc. in low concentrations. About 70% of the mass sedimented apparently as subparticles (Fig. 5*c*). This nucleoprotein fraction had 20% of the original activity in the cell-free system (Table 1). The addition of supernatant factors led to a marked increase in the proportion of material sedimenting as ribosomes so that the total material sedimenting faster than 70S was 65%; there was a concomitant decrease in the proportion of subparticles to 35% of the total (Fig. 5*d*) and a parallel increase in the activity of the nucleoprotein in the cell-free system (50% of the control). These observations are entirely consistent with the reconstitution of ribosomal subparticles into active ribosomes.

Other explanations are possible but are considered less likely. Thus the biological activity might be explained if the 4–6% of ribosomes that appear to remain undissociated in 0·25 M-sucrose (Fig. 5*b*), or even after zone centrifuging through 0·5 M-potassium chloride (Fig. 3), together with supernatant factors, were the only active species (cf. Lamfrom & Glowacki, 1962). If this were the case it would be necessary to postulate that the ribosomes that are reconstituted are inactive.

Another possibility is that the species sedimenting at about 50S includes protein-deficient or conformationally changed ribosomes that fortuitously sediment at the same rate as the larger subparticle and that are restored to their original shape, sedimentation rate and activity by the addition of supernatant factors. Although this eventually cannot be eliminated, the evidence presented above supports the view that we were observing dissociation of ribosomes and polysomes into subparticles that can recombine with restoration of biological activity. Such behaviour has long been known to be a property of bacterial ribosomes and is to be expected if the major features of ribosome structure and function are universal.

Properties of the supernatant fraction. The relationship, if any, of the potassium chloride supernatant fraction to the enzymes TF-1 and TF-2, isolated at 40–70% ammonium sulphate saturation from the pH 5 enzyme fraction of rabbit reticulocytes and required for the transfer of amino acids from aminoacyl-transfer RNA to ribosome-bound peptidyl-transfer RNA in a two-step reaction dependent on polyU, GTP, bivalent and univalent cations and thiol groups (Arlinghaus, Shaeffer & Schweet, 1964), is not clear. Nor is it known whether the activity of the 0·5 M-potassium chloride supernatant is comparable with that of the protein fractions T_s, T_u and G, promoting polyU-directed phenylalanine synthesis (Lucas-Lenard & Lipmann, 1966) or the initiation factors F_1 and F_2 (Stanley, Salas, Wahba & Ochoa, 1966), isolated from 0·5 M-ammonium chloride, and F_3 (Brown & Doty, 1968), isolated from 1·0 M-ammonium chloride washes of ribosomes, all essential for the translation of natural messengers in the *E. coli* system. However, it is pertinent to mention the reports that enzymes TF-1 and TF-2 can be washed off rabbit reticulocyte ribosomes by treatment with deoxycholate and 0·1 M-potassium chloride (Arlinghaus, Favelukes & Schweet, 1963), that the proteins T_u and G appear to be absent from *E. coli* ribosomes washed in 0·5 M-ammonium chloride (Allende, Seeds, Conway & Weissbach, 1967), and that a peptidyltransferase is integrated in the structure of the *E. coli* 50S ribosomal subparticle (Monro, Maden & Traut, 1967).

Only fractionation and purification of the potassium chloride supernatant factors obtained from both washed and unwashed polysomes could elucidate their role and determine their origin and stoicheiometry of addition.

Note added after the submission of this paper. A report of the effect of high salt on reticulocyte ribosomes (Yang, Hamada & Schweet, 1968) and the description of some properties of the derived subparticles (Hamada, Yang, Heintz & Schweet, 1968) appeared after this manuscript was completed. Their results and ours are basically in agreement. The relationship of the 0·5 M-potassium chloride supernatant fraction described in this paper to the protein factor purified from a similar extract of reticulocyte ribosomes (Miller & Schweet, 1968) is not clear.

S.B. was a recipient of the Keddey–Fletcher Warr Studentship and was aided by a Fellowship from the European Molecular Biology Organization.

REFERENCES

Allende, J. E., Seeds, N. W., Conway, T. W. & Weissbach, H. (1967). *Proc. nat. Acad. Sci., Wash.*, **58**, 1566.

Arlinghaus, R., Favelukes, G. & Schweet, R. (1963). *Biochem. biophys. Res. Commun.* **11**, 92.

Arlinghaus, R. & Schweet, R. (1962). *Biochem. biophys. Res. Commun.* **9**, 482.

Arlinghaus, R., Shaeffer, J. & Schweet, R. (1964). *Proc. nat. Acad. Sci., Wash.*, **51**, 1291.

Arnstein, H. R. V., Cox, R. A., Gould, H. & Potter, H. (1965). *Biochem. J.* **96**, 500.

Arnstein, H. R. V., Cox, R. A. & Hunt, J. A. (1964). *Biochem. J.* **92**, 648.

Bishop, J. O. (1965). *Nature, Lond.*, **208**, 361.

Bishop, J. O. (1966). *Biochim. biophys. Acta*, **119**, 130.

Brown J. C. & Doty, P. (1968). *Biochem. biophys. Res. Commun.* **30**, 284.

Chao, F. C. (1957). *Arch. Biochem. Biophys.* **70**, 426.

Cox, R. A. (1966). *Biochem. Prep.* **11**, 104.
Gavrilova, L. P., Ivanov, D. A. & Spirin, A. S. (1966). *J. molec. Biol.* **16**, 473.
Gilbert, W. (1963). *J. molec. Biol.* **6**, 374.
Gould, H., Arnstein, H. R. V. & Cox, R. A. (1966). *J. molec. Biol.* **15**, 600.
Hamada, K., Yang, P., Heintz, R. & Schweet, R. (1968). *Arch. Biochem. Biophys.* **125**, 598.
Itoh, T., Otaka, E. & Osawa, S. (1968). *J. molec. Biol.* **33**, 109.
Kaempfer, R. O. R., Meselson, M. & Raskas, H. J. (1967). *J. molec. Biol.* **31**, 277.
Lamfrom, H. & Glowacki, E. (1962). *J. molec. Biol.* **5**, 97.
Lerman, M. I., Spirin, A. S., Gavrilova, L. P. & Golov, V. F (1966). *J. molec. Biol.* **15**, 268.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Lucas-Lenard, J. & Lipmann, F. (1966). *Fed. Proc.* **25**, 215.
Miller, R., Hamada, K., Yang, P. C., Cohen, B. & Schweet, R. (1967). *Fed. Proc.* **26**, 458.
Miller, R., Hausmann, E., Erickson, R. & Schweet, R. (1965). *Fed. Proc.* **24**, 283.
Miller, R. L., & Schweet, R. (1968). *Arch. Biochem. Biophys.* **125**, 632.
Monro, R. E., Maden, B. E. H. & Traut, R. R. (1967). In *Genetic Elements*, p. 179. Ed. by Shugar, D. New York: Academic Press Inc.
Nair, K. G. & Arnstein, H. R. V. (1965). *Biochem. J.* **97**, 595.
Nomura, M. & Lowry, C. V. (1967). *Proc. nat. Acad. Sci., Wash.*, **58**, 946.
Okamoto, T. & Takanami, M. (1963). *Biochim. biophys. Acta*, **68**, 325.
Robertson, H. D., Webster, R. E. & Zinder, N. D. (1968). *J. biol. Chem.* **243**, 89.
Schlessinger, D. & Gros, F. (1963). *J. molec. Biol.* **7**, 350.
Schlessinger, D., Mangiarotti, G. & Apirion, D. (1967). *Proc. nat. Acad. Sci., Wash.*, **58**, 1782.
Stanley, W. M., Salas, M., Wahba, A. J. & Ochoa, S. (1966). *Proc. nat. Acad. Sci., Wash.*, **56**, 290.
Summers, F. D., Maizel, J. V., jun. & Darnell, J. E., jun. (1965). *Proc. nat. Acad. Sci., Wash.*, **54**, 505.
Tissières, A., Schlessinger, D. & Gros, F. (1960). *Proc. nat. Acad. Sci., Wash.*, **46**, 1450.
Tissières, A., Watson, J. D., Schlessinger, D. & Hollingworth B. R. (1959). *J. molec. Biol.* **1**, 221.
Ts'o, P. O. P., Bonner, J. & Vinograd, J. (1958). *Biochim. biophys. Acta*, **30**, 570.
Warburg, O. & Christian, W. (1942). *Biochem. Z.* **310**, 384.
Wettstein, F. O., Staehelin, T. & Noll, H. (1963). *Nature, Lond.*, **197**, 430.
Yang, P. C., Hamada, K. & Schweet, R. (1968). *Arch. Biochem. Biophys.* **125**, 506.