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Dissociation of Ribosomes from Oocytes of *Xenopus laevis* into Active Subparticles

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Ribosomes from oocytes of *Xenopus laevis* possess low endogenous activity *in vivo* and *in vitro*, yet are readily stimulated by poly(U). The ease with which these ribosomes dissociate into active subparticles under conditions where polyribosomes and active monoribosomes are stable supports the view that the majority are unprogrammed.

In common with other Amphibia, the eggs of the South African clawed toad, *Xenopus laevis*, contain about 10^{12} ribosomes compared with about 3×10^6 ribosomes in the cells of other tissues. The majority (over 90%) of these ribosomes are monomers and are inactive in protein synthesis at any one time (Brown & Littna, 1964); the small proportion of polyribosomes present are as active as polyribosomes isolated from the liver (Ford, 1966). After fertilization and until gastrulation the ribosomes utilized in protein synthesis are maternal in origin (Brown & Littna, 1964). Thus the egg may contain a store of ribosomes sufficient for perhaps 30000 cells (Brown & Gurdon, 1964, 1966) and it is likely that a high proportion of these ribosomes are devoid of mRNA (Davidson, 1968).

The aim of this paper is to demonstrate that ribosomes from large oocytes of *X. laevis*, before ovulation, readily dissociate into biologically active subparticles under conditions where polyribosomes or active monoribosomes are stable. The majority of the ribosomes are recovered as active subparticles when zone-centrifuged under prescribed conditions. No preincubation is required. The ease with which ribosomes of oocytes of *X. laevis* dissociate is interpreted as further evidence for the view that the majority are unprogrammed with mRNA.

MATERIALS AND METHODS

Chemicals. Glutathione was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; ATP (dipotassium salt) from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; GTP (sodium salt) and poly(U) from Sigma Chemical Co., St Louis, Mo., U.S.A.; ^{14}C-labelled amino acids from The Radiochemical Centre, Amersham, Bucks., U.K.; heparin (trade mark, Pularin) from Evans Medical Co. Ltd., Speke, Liverpool, U.K.

Determination of protein and RNA. The amount of protein in the pH 5-enzyme fraction and the concentration of ribosomes in 0.25 M-sucrose–25 mM-KCl–1 mM-$MgCl_2$–50 mM-tris–HCl buffer, pH 7.6, were measured by means of a nomograph based on the extinction coefficients of enolase and nucleic acid (Calbiochem, Los Angeles, Calif., U.S.A.).

Standard cell-free system. The procedure of Arnstein, Cox & Hunt (1964) was followed. The incubation mixture (0.5 ml) used for testing the incorporation of labelled amino acids into protein had the following composition: 50 mM-KCl–12 mM-$MgCl_2$–10 mM-glutathione–25 mM-tris–HCl buffer, pH 7.6, at 20°C; 0.05 ml of an amino acid mixture containing [^{14}C]phenylalanine (specific radioactivity 100 mCi/mmol) and 19 unlabelled amino acids (0.1 μmol of each); ribosomes (0.25 mg); pH 5 enzymes (1 mg) and an ATP-generating system consisting of 2.5 μmol of sodium phosphoenolpyruvate, 0.125 μmol of ATP, 0.03 μmol of GTP and 25 μg of pyruvate kinase. The incubation was carried out in air at 37°C for 1 h.

Isolation of labelled protein from the incubation mixture. After incubation, 0.2 ml of 1 M-NaOH containing 25 μmol of unlabelled phenylalanine/ml was added to the reaction mixture (0.5 ml), which was then incubated at 37°C for 5 min and then at room temperature for 15 min. After the addition of 10% (w/v) trichloroacetic acid (2.5 ml) the mixture was kept at 4°C overnight; the protein was then filtered on to an Oxoid membrane (3 cm diam.) and washed with cold 5% (w/v) trichloroacetic acid (2 × 5 ml) and cold water (2.5 ml).

Radioactivity measurements. An automatic ultra-thin end-window counter (model C-110B; Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.) was used. The background was low (about 1.5 c.p.m.). The efficiency for ^{14}C was 15% and the radioactivity of the samples was counted for at least 4000 counts.

Isolation of ribosomes and pH 5*-enzyme fraction from the unfertilized eggs of* X. laevis. The ovaries of mature South African toads were used; the animals had not ovulated during the previous 3–4 months and hence the majority of oocytes were fully grown. Ribosomes and cell sap were prepared as described by Cox, Ford & Pratt (1970). Ribosome pellets were overlaid with 0.25 M-sucrose–25 mM-KCl–1 mM-$MgCl_2$–50 mM-tris–HCl buffer, pH 7.6, quickly frozen in an ethanol–solid CO_2 freezing mixture

and stored at −20°C. They retained their activity for over a month. Samples of the pH 5-enzyme fraction were stored under liquid N_2 and retained their activity for several weeks although it did diminish.

Isolation of liver polyribosomes and the pH 5*-enzyme fraction.* The liver was removed into ice-cold homogenizing medium (0.25 M-sucrose–100 mM-KCl–5 mM-$MgCl_2$–50 mM-tris–HCl buffer, pH 7.6) and weighed. The tissue was homogenized in 2.5 vol. of medium with four passes of a loose-fitting (radial clearance approx. 100 μm) Teflon plunger rotating at 1500 rev./min. The homogenate was filtered through glass wool to remove the connective tissue and then centrifuged at 20000 *g* for 15 min ($r_{av.}$ = 5.4 cm). The supernatant was removed with a syringe, made 1% with Triton X-100 and then layered over an equal volume of homogenizing medium made 1 M with respect to sucrose. The polyribosomes were centrifuged through this medium at 100000 *g* for 3.5 h ($r_{av.}$ = 6.4 cm) and the pellets were overlaid with 0.25 M-sucrose–25 mM-KCl–1 mM-$MgCl_2$–50 mM-tris–HCl buffer, pH 7.6, and stored at −20°C.

Dissociation of egg ribosomes into active subparticles. Subparticles were separated in the MSE 65 BXIV zonal rotor; capacity 675 ml.

The zonal gradient was made up as follows: 5% of the gradient was 15% sucrose in the appropriate salts, 50% of the gradient a 15–40% sucrose linear gradient and the remainder of the gradient a cushion of buffer containing 50% sucrose; 140 ml of buffer was used as overlay. Fractions (15 ml) were collected after centrifugation and the extinction of each fraction at 260 nm was determined by using a Unicam SP. 500 spectrophotometer.

Analysis of sucrose density gradients. Sucrose density gradients were analysed at 260 nm in the Perkin–Elmer 137 UV continuous-recording spectrophotometer by displacing the contents of each tube through a variable path flow cell. All E_{260} profiles were converted into a 1 cm cell path length. The monomeric ribosomes and large and small subparticles will be referred to in this paper as 80 S, 60 S and 40 S particles respectively.

RESULTS

Dissociation of oocyte ribosomes by high salt concentration. We found that oocyte ribosomes dissociated into active subparticles when treated with 0.88 M-potassium chloride–15 mM-magnesium chloride and zone centrifuged at room temperature (Martin, Rolleston, Low & Wool, 1969). Subparticles recombined in the ratio 1 E_{260} unit of 40 S to 2.5 E_{260} units of 60 S had activities comparable with the original undissociated ribosomes. However, the method had two disadvantages: the 60 S peak was always contaminated by 15–20% of the 40 S subparticle and an anomalous shoulder of active material sedimenting more slowly than the small subparticle consistently appeared in these gradients (see Fig. 1 for a typical gradient profile). When the potassium chloride concentration in the gradient was decreased to 0.5 M, the contamination of the 60 S peak was decreased to under 5% and the anomalous 40 S shoulder disappeared.

Dissociation by low salt concentration. It was decided, however, that such high concentrations of potassium chloride were probably inappropriate in our system. After it had been shown (Lawford, 1969; von der Decken, Ashby, McIlreavy & Campbell, 1970) that rat liver and skeletal-muscle ribosomes could be dissociated in low salt concentrations (0.15 M-potassium chloride) once the stabilizing nascent polypeptide chain and peptidyl-tRNA had been removed by incubation with puromycin, we applied oocyte ribosomes without any prior incubation to a zonal gradient containing 0.15 M-potassium chloride–1 mM-magnesium chloride and run at room temperature. The activity profile of the gradient is shown in Fig. 2; the 60 S peak appears to be contaminated with 40 S subparticles to the extent of approx. 10%, because there is a small portion of active material sedimenting in the monomer region of the gradient which is not stimulated to the same degree by addition of the small subparticle. The area under each peak in Fig. 2 represents the total extinction of that subparticle; in this case the ratio of E_{260} units of 60 S/E_{260} units of 40 S was 2.8:1. The value of this ratio should be 2.4:1 for equimolar proportions of subparticles of *X. laevis* ribosomes because it reflects the ratio of the molecular weights of the two rRNA species (Loening, Jones & Birnstiel, 1969). The value 2.8:1 obtained indicates that only 86% of the ribosomes have dissociated in the low-salt gradient.

The activity of recombined subparticles was comparable with the original undissociated ribosomes and the isolated subparticles recombined to sediment as monomers in the absence of supernatant factors as reported for liver ribosomal subparticles (Falvey & Staehelin, 1970).

When oocyte ribosomes were exposed to concentrations of potassium chloride lower than 0.15 M and analysed on gradients containing an identical concentration of potassium chloride, in 1 mM-magnesium chloride–50 mM-tris–HCl buffer, pH 7.6, substantial dissociation (40–50%) was still observed when the concentration of potassium chloride was decreased to 50 mM irrespective of whether the ribosomes were treated at 4°C or 14°C.

Complete removal of magnesium by chelation with EDTA led to the production of inactive subparticles (cf. Gould, Arnstein & Cox, 1966).

Dissociation by heparin. Low concentrations of heparin (500 μg/ml) have been shown to dissociate those HeLa cell ribosomes not bound into active polyribosomes by mRNA (Miller, 1968). Preliminary experiments showed that a tenfold increase in the concentration of heparin to 5 mg/ml still did not completely dissociate rabbit reticulocyte polyribosomes. When *X. laevis* oocyte ribosomes were centrifuged through a zonal gradient containing 500 μg of heparin/ml active subparticles

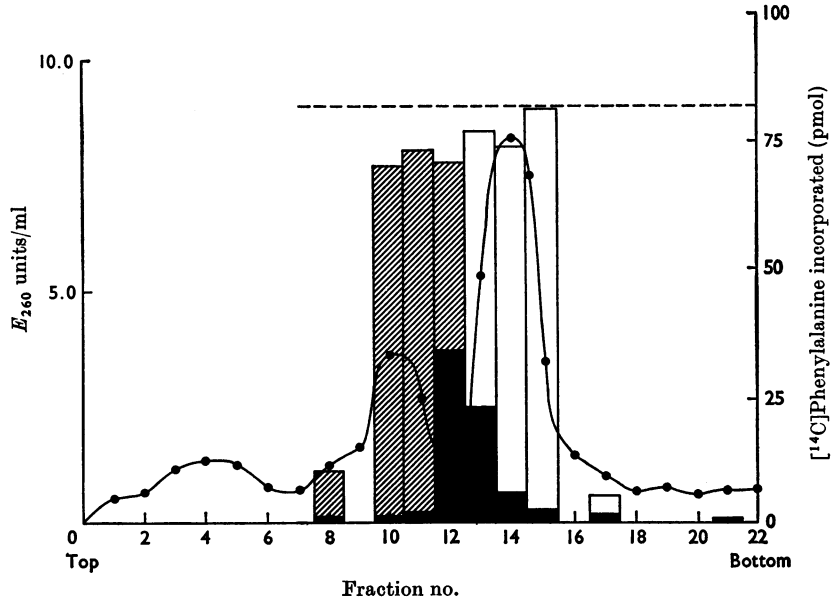

Fig. 1. Dissociation of *X. laevis* egg ribosomes with high salt (880 mM-KCl). Approx. 50 mg of ribosomes was suspended in 5 ml of 0.25 M-sucrose–25 mM-KCl–10 mM-$MgCl_2$–50 mM-tris–HCl buffer, pH 7.6, containing 20 mM-mercaptoethanol and made 1 M with respect to KCl and kept at 20°C for 10 min. The suspension was then layered on to a zonal gradient (for composition, see the Materials and Methods section) containing 15 mM-$MgCl_2$–880 mM-KCl–20 mM-mercaptoethanol–50 mM-tris–HCl buffer, pH 7.6, and centrifuged at 90000*g* ($r_{av.}$ = 4.3 cm) at 26°C for 2 h. Individual 15 ml fractions were dialysed against 0.25 M-sucrose–25 mM-KCl–1 mM-$MgCl_2$–50 mM-tris–HCl buffer, pH 7.6, to remove KCl, precipitated with 2 vol. of AnalaR methanol, dissolved in the dialysis medium and stored at −20°C. A 40S subparticle pool was formed from portions of fractions 9, 10 and 11 and a 60S subparticle pool from portions of fractions 13, 14 and 15. A 25 μg sample of each gradient fraction was assayed alone in the cell-free system and then together with either 25 μg of 40S subparticle pool or 50 μg of the 60S subparticle pool. A 25 μg sample of the 40S subparticle pool assayed alone in the cell-free system gave no incorporation. A 50 μg sample of 60S subparticle pool assayed alone incorporated 25 pmol of [^{14}C]phenylalanine. ■, [^{14}C]Phenylalanine incorporated (pmol) by each fraction assayed alone; ■, [^{14}C]phenylalanine(pmol) incorporated by each fraction due to stimulation by the 60S subparticle pool; □, [^{14}C]phenylalanine (pmol) incorporated by each fraction due to stimulation by the 40S subparticle pool; ----, [^{14}C]phenylalanine (pmol) incorporated by undissociated ribosomes assayed alone; ●——●, E_{260}; 100 pmol of [^{14}C]phenylalanine incorporated represents 1700 c.p.m. detected.

were formed without the need for prior incubation (Fig. 3). The subparticles were as active as those obtained previously though the resolution appeared to be improved with less contamination of the 60S subparticle peak. As in the previous gradient a small portion of active material that was not stimulated to the same extent by the addition of 40S subparticles, sedimented in the monomer region of the gradient. Dissociation was estimated to be about 85–90%. Subsequent experiments have shown that oocyte ribosomes can be dissociated to this extent by concentrations of heparin as low as 10 μg/ml.

The behaviour of *X. laevis* liver polyribosomes was then examined under similar conditions, by sedimenting polyribosomes through heparin (500 μg/ml) after various incubation treatments (Fig. 4). The hydrolysis of mRNA by mild ribonuclease treatment led to the production of monomers that did not completely dissociate into subparticles on zone centrifugation in the presence of heparin. 'Run off' ribosomes dissociated to a large extent in the presence of heparin (Fig. 4*a*) though the most extensive dissociation was obtained when liver polyribosomes were first incubated with puromycin and GTP (Fig. 4*b*).

Untreated liver polyribosomes did not dissociate in the presence of heparin.

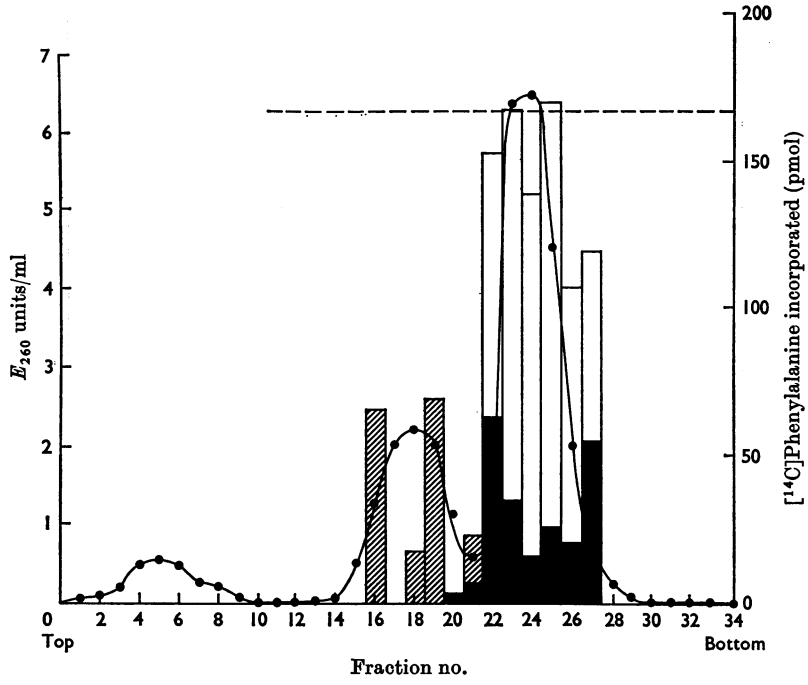

Fig. 2. Dissociation of *X. laevis* egg ribosomes with low salt (0.15 M-KCl). A 50 mg sample of ribosomes was suspended in 5 ml of 0.25 M-sucrose–25 mM-KCl–1 mM-$MgCl_2$–50 mM-tris–HCl buffer, pH 7.6, made 150 mM with respect to KCl and 10 mM with respect to mercaptoethanol, kept at 20°C for 10 min and then layered on a zonal gradient containing 150 mM-KCl–1 mM-$MgCl_2$–10 mM-mercaptoethanol–50 mM-tris–HCl buffer, pH 7.6, which was centrifuged at 90000*g* ($r_{av.}$ = 4.3 cm) at 26°C for 2.25 h. Fractions (15 ml) were collected and each fraction was centrifuged individually at 100000*g* for 16 h, dissolved in 0.25 M-sucrose–25 mM-KCl–1 mM-$MgCl_2$–50 mM-tris–HCl buffer, pH 7.6, and stored at −20°C. Fraction 19 was used as the 40S subparticle pool and fraction 24 as the 60S subparticle pool. A 25 μg sample of each gradient fraction was assayed alone in the cell-free system and then together with either 10 μg of the 40S subparticle or 30 μg of the 60S subparticle. A 10 μg sample of the 40S subparticle assayed alone in the cell-free system gave no incorporation. A 30 μg portion of 60S subparticle assayed alone incorporated 12 pmol of [^{14}C]phenylalanine. ■, [^{14}C]Phenylalanine (pmol) incorporated by each fraction assayed alone; ■, [^{14}C]phenylalanine (pmol) incorporated by each fraction due to stimulation by the 60S subparticle pool; □, [^{14}C]phenylalanine (pmol) incorporated by each fraction due to stimulation by the 40S subparticle pool; ----, [^{14}C]phenylalanine (pmol) incorporated by undissociated ribosomes assayed alone; ●——●, E_{260}; 100 pmol of [^{14}C]phenylalanine incorporated represents 1700 c.p.m. detected.

DISCUSSION

As the oocytes of *X. laevis* mature within the ovary they gradually become metabolically inert, and can remain in this state for many months, exhibiting low endogenous protein synthesizing activity both *in vivo* and *in vitro* (Ford, 1966). However, they contain all the ribosomes and some of the informational RNA molecules ultimately inherited by the embryo (Davidson, Crippa, Kramer & Mirsky, 1966; Davidson & Hough, 1971), the remainder of the informational RNA being synthesized during ovulation (Brown & Littna, 1966).

Since soluble factors and ribosomes from these oocytes can catalyse the formation of a peptide bound under conditions comparable with those reported for mammalian ribosomes (Cox *et al.* 1970), the lack of endogenous activity in these cells can be attributed to a deficiency or masking of the template RNA (Monroy, Maggio & Rinaldi, 1965; Kedes & Stavy, 1969) or else to an inefficient initiation step in polypeptide synthesis (Rinaldi & Monroy, 1969).

An important factor in maintaining the structural and functional integrity of ribosomes and also in controlling their dissociation into functional subparticles is the magnesium ion concentration in the medium. It appears likely that univalent cations cause dissociation of ribosomes by competing for

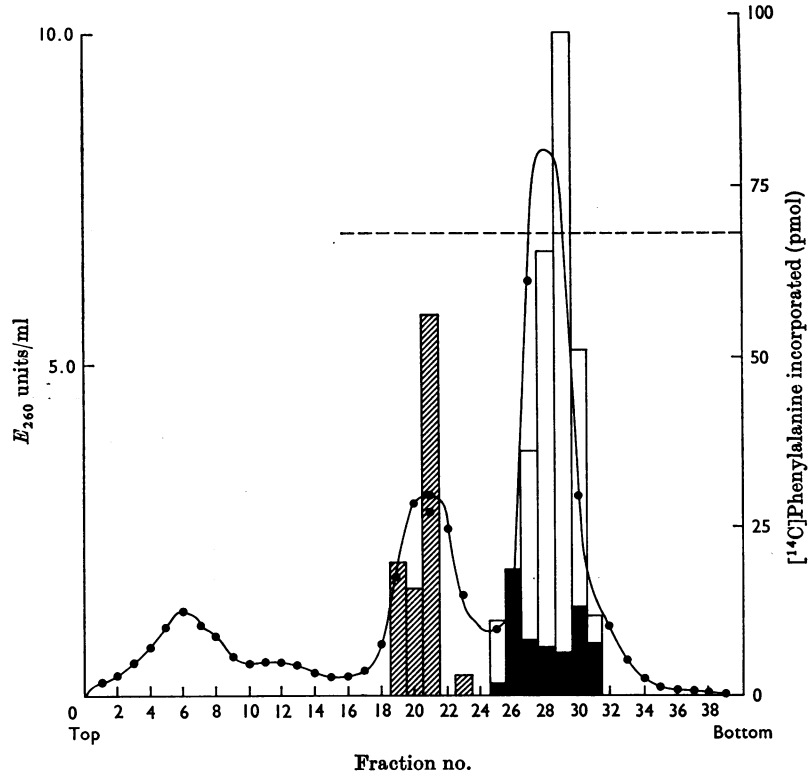

Fig. 3. Dissociation of *X. laevis* egg ribosomes with 500 μg of heparin/ml. Ribosomes were suspended in cold 0.25 M-sucrose–25 mM-KCl–1 mM-$MgCl_2$–50 mM-tris–HCl buffer, pH 7.6, containing 500 μg of heparin/ml, and layered on to a zonal gradient containing 50 mM-KCl–1.5 mM-$MgCl_2$–0.05% heparin–35 mM-tris–HCl buffer, pH 7.6. The gradient was centrifuged at 90000*g* ($r_{av.}$ = 4.3 cm) at 4°C for 3.5 h and fractions were recovered as described for the low-salt particles. Fraction 20 was used as the 40 S subparticle pool and fraction 28 as the 60 S subparticle pool. A 25 μg sample of each gradient fraction was assayed alone in a cell-free system and then together with either 15 μg of the 40 S subparticle or 30 μg of the 60 S subparticle. A 15 μg sample of the 40 S subparticle assayed alone in the cell-free system gave no incorporation; 30 μg of the 60 S subparticle assayed alone incorporated 10 pmol of [^{14}C]phenylalanine. ■, [^{14}C]Phenylalanine incorporated (pmol) by each fraction assayed alone; ■, [^{14}C]phenylalanine incorporated (pmol) by each fraction due to stimulation by the 60 S subparticle; □, [^{14}C]phenylalanine incorporated (pmol) by each fraction due to stimulation by the 40 S subparticle; ----, [^{14}C]phenylalanine incorporated by undissociated ribosomes assayed alone; ●——●, E_{260}; 100 pmol of [^{14}C]phenylalanine incorporated represents 1700 c.p.m. detected.

magnesium-binding sites and that the important parameter for obtaining functional dissociated subparticles is the univalent/bivalent cation ratio. This ratio depends on the precise value of the univalent cation concentration. A nearly sixfold decrease in this ratio from 58.5:1 for the high salt to 10:1 for the lowest salt treatment did not impair the activity of the subparticles obtained, although the extent of dissociation ranged from approx. 85% to approx. 30%.

Heparin appears to be acting in an analogous manner to the 0.15 M-potassium chloride by dissociating only those ribosomes not stabilized by a nascent polypeptide chain, tRNA or mRNA. In view of its anti-ribonuclease activity (Palmiter, Christensen & Schimke, 1970) heparin could prove useful for isolating eukaryotic mRNA species.

The ease with which 85–90% of *X. laevis* oocyte ribosomes dissociate into subparticles, under the conditions described, supports the view that the majority are unprogrammed, i.e. devoid of mRNA.

Other evidence to support this view comes from studies of the soluble RNA content of eggs of *X. laevis* by Brown & Littna (1966), who estimated that only one ribosome in ten has 4 S RNA associated

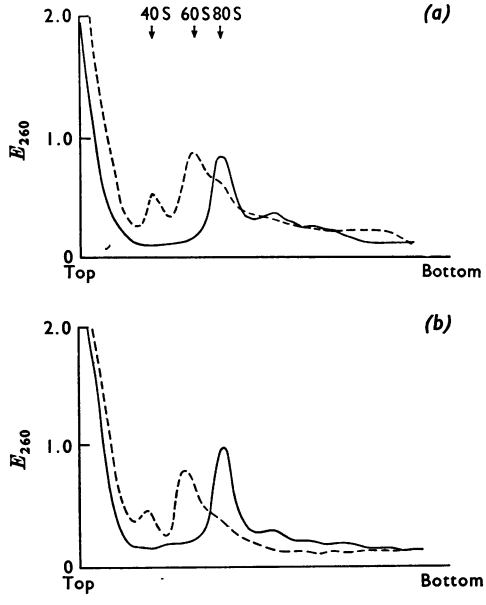

Fig. 4. (*a*) Dissociation of 'run off' liver ribosomes with heparin (500 μg/ml). A 2 mg sample of liver polyribosomes was incubated in a cell-free system at 37°C for 1 h. One sample was taken and layered on a 28 ml linear 15–45% sucrose density gradient in 50 mM-KCl–1.5 mM-$MgCl_2$–35 mM-tris–HCl buffer, pH 7.6; another sample was made 500 μg/ml in heparin and layered on an identical gradient containing 500 μg of heparin/ml. The gradients were run at 4°C at 40 000*g* ($r_{av.}$ = 9.1 cm) for 15 h in a no. SW25 head of a Spinco model L centrifuge. The gradients were analysed at 260 nm by displacing the contents of the tubes upwards through a variable path flow cell of the Perkin–Elmer 137UV recording spectrophotometer. The extinction profiles were adjusted to a 1 cm light-path. ——, Liver ribosomes run under control conditions; – – – –, liver ribosomes treated with heparin and run in heparin gradients. (*b*) Dissociation of puromycin-treated liver ribosomes in heparin. A 2 mg sample of liver polyribosomes was incubated in the cell-free system containing 1 mM-puromycin but devoid of pH 5 enzymes at 37°C for 1 h. Two samples were treated as indicated in (*a*). (85% displacement of the nascent polypeptide chains as a result of this puromycin treatment was estimated by incubating liver polyribosomes in the complete cell-free system containing labelled amino acids and measuring the proportion of radioactivity displaced by an identical puromycin treatment.)

with it. This lack of tRNA is to be expected if the ribosomes are unprogrammed. Ribosomes from the encysted gastrulae of *Artemia salina* evidently exist in a similar state to those from amphibian oocytes. The majority of these ribosomes are isolated as monomers with low endogenous activity, are readily stimulated by poly(U) and show a similar ease of dissociation into subparticles, probably also due to the absence of any mRNA (Hultin, Näslund & Nilsson, 1969). Pools of these inactive, unprogrammed monomers can also be identified in cells that synthesize large quantities of protein intermittently (Hogan & Korner, 1968; Fleck, Shepherd & Munro, 1965), suggesting that this may be a typical 'storage' form of the ribosome that can be activated reversibly for protein synthesis. Treatment of the derived liver ribosomes with heparin suggests that the oocyte ribosome of *X. laevis* is analogous to the particle that can be induced *in vitro* by intensive incubation of polyribosomes with puromycin, and that exists *in vivo* as a ribosome which has 'run off' a mRNA molecule after a cycle of protein synthesis.

These particles will readily dissociate to a substantial degree under mild conditions and will reassociate to form 80S couples in the absence of supernatant factors as reported for derived liver ribosomal subparticles (Falvey & Staehelin, 1970). This ease of interconversion between ribosomes and subparticles suggests that the distinction made between subparticles and monomers may well be trivial if rapid interconversion is possible *in vivo*. In fact the isolation of 80S couples from these oocytes could be an artifact due to an aggregation of subparticles in media of lower ionic strength than is physiological (Zylber & Penman, 1970).

In this study the use of poly(U)-directed protein synthesis as a criterion for biological activity has the disadvantage that it provides no information as to the capacity of the system for initiating the translation of natural messengers. Further experiments with a natural messenger or a synthetic polynucleotide containing an initiator codon are required to assess whether the factors within the oocyte are capable of forming an initiation complex with the ribosome and mRNA.

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