Phytochemistry, 1976 Vol. 15. pp. 447–453. Pergamon Press. Printed in England.

EFFECT OF CATIONS ON THE STRUCTURE OF MUNG BEAN CYTOPLASMIC RIBOSOMES

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(*Revised received* 14 *November* 1975)

Key Word Index—*Phaseolus aureus*, Leguminosae; mung bean; structure; ribosomes; ions.

Abstract—The importance of the absolute and relative concentrations of monovalent and divalent cations and centrifugal speed (pressure) in the dissociation of mung bean 80S ribosomes has been examined. In the absence of Mg^{2+} ions, ribosome monomers yield 47S and 34S particles. Fixation with glutaraldehyde, however, indicates that this dissociation pattern is largely dependent upon high pressures developed during centrifugation and that in the absence of such artifacts the immediate product of Mg-free conditions is a 74S particle. Since 74S particles rapidly revert to the 80S form when Mg is replaced, this would appear to be a conformational change. Ribosomes were also dissociated in the presence of Mg^{2+} ions if the K^+ ion concentration was raised. Three major particles were produced, 38S and 49S from the small ribosomal sub-unit and 60S from the large sub-unit. A proportion of the 80S monomer population is more resistant to dissociation. Experiments with puromycin indicate that the more resistant fraction probably represents ribosomes complexed with nascent polypeptide resulting from polysome breakdown.

INTRODUCTION

Following the original observations of Chao [1] on the importance of magnesium ions in the dissociation and reassociation of yeast ribosomes, much work has been published on the effects of cations on ribosome structure. Recent work on higher plant cytoplasmic ribosomes under various ionic conditions has shown that they can be dissociated and also that the sedimentation coefficients of the monomers and sub-units can be apparently altered as the result of protein losses and conformational changes [2–9]. In addition, a degree of heterogeneity in ribosome monomer populations *in vitro* has been reported [6, 10, 11], those monomers that bear nascent polypeptide chains being more resistant to ionic dissociation than uncomplexed ribosomes.

In the present work the dissociation and reassociation of mung bean cytoplasmic ribosomes under varying ionic conditions has been examined, placing particular emphasis on (1) the monovalent:divalent cationic ratio in the competition of cations for the available negatively-changed sites on the ribosome, (2) the possibility of artefacts produced by pressure-induced dissociation during centrifugation, a problem largely ignored in previous studies on plant ribosome dissociation. Results indicate that within certain limits, the monovalent:divalent cationic ratio is more important than the absolute concentrations of these ions in determining dissociation/reassociation equilibria within the ribosome population *in vitro*. By using glutaraldehyde as a ribosome fixative [12], it has further been shown that under certain ionic conditions, dissociation of ribosomes is not solely a function of the ionic environment but requires the dissociative effect of the high pressures developed during sucrose gradient centrifugation.

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RESULTS AND DISCUSSION

When mung bean cytoplasmic ribosomes and *E. coli* ribosomes and sub-units were fractionated on isokinetic gradients based on Medium A (50 mM KCl, 10 mM Mg acetate), a comparison of particle mobilities using 30S, 50S and 70S bacterial particles as markers [15], showed that the mung bean ribosomes had a sedimentation coefficient of 80S, as is normally the case for cytoplasmic ribosomes from higher plants [16], and that furthermore, there was little contamination with 70S chloroplast ribosomes (Fig. 1). Further investigation showed that the chloroplast ribosomes were dissociated into 46S and 34S sub-units *in vivo*, and therefore were not pelleted through the sucrose cushion used in the ribosome purification procedure. Polyribosomes were observed in very small quantities only, this probably being due to the high nuclease activity associated with this tissue (Newbury and Callow, in preparation). Estimation of the RNA and protein contents of the mung bean 80S ribosomes by the orcinol [47] and Folin procedures [48] showed that they contained 48% RNA and 52% protein. RNA extracted from the ribosomes gave the standard $M_S = 1.3 \times 10^6$ and $M_S = 0.7 \times 10^6$ components on electrophoresis in polyacrylamide gel (Newbury and Callow, in preparation).

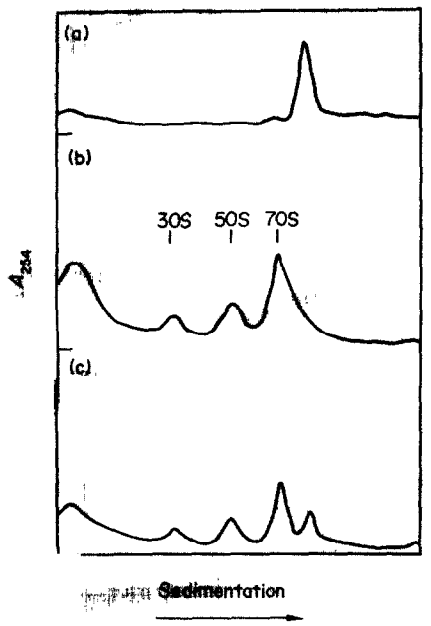

Fig. 1. Absorbance profiles of mung bean and *E. coli* ribosomes resuspended in Medium A (containing 0.05 M KCl, 10 mM Mg acetate), and centrifuged on 15–33% (w/v) sucrose gradients at 95000 g_{av} for 6 hr at 5°. (a) 90 μg mung bean cytoplasmic ribosomes; (b) 140 μg *E. coli* ribosomes and sub-units; (c) 35 μg mung bean ribosomes plus 85 μg *E. coli* ribosomes and sub-units.

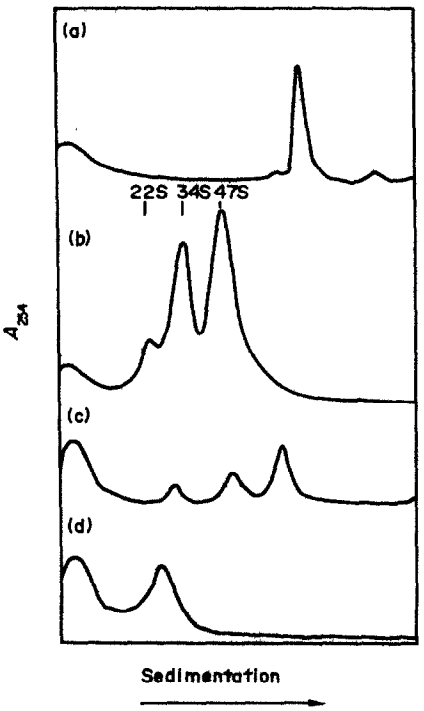

Fig. 2. Absorbance profiles of 110 μg mung bean ribosomes, resuspended in Medium A, fractionated on 15–33% (w/v) sucrose gradients based on Medium A (Fig. 2a); 215 μg mung bean ribosomes, resuspended in Medium B (minus magnesium), fractionated on gradients based on Medium B; 100 μg *E. coli* ribosomes and sub-units, fractionated on gradients based on Medium A; 80 μg *E. coli* ribosomes and sub-units fractionated on gradients based on Medium B. Gradients centrifuged at 95000 g_{av} for 6 hr at 5°. Using 30S, 50S, and 70S *E. coli* markers (2c), the peaks obtained in gradient 2(b) were estimated as 22S, 34S and 47S.

When cytoplasmic ribosomes were resuspended in medium B (no Mg^{2+}) and fractionated through a sucrose gradient [illegible] medium, the 80S particles were replaced by two major components of apparent sedimentation coefficients, 47S and 34S, with a minor component of 22S (Fig. 2). These values were calculated from parallel fractionations of *E. coli* markers in gradients based on medium A, since in medium B gradients, *E. coli* ribosomes were completely degraded to a 26S component (Fig. 2d), precluding the possibility of co-running plant and *E. coli* ribosomes. The same pattern of dissociation of mung bean 80S ribosomes was observed when plant ribosomes were resuspended in medium A and fractionated in gradients based on medium B. The 47S and 34S particles are derived from the large and small ribosomal sub-units respectively since the former contained $M_S = 1.3 \times 10^6$ RNA, and the latter $M_S = 0.7 \times 10^6$ RNA (Newbury and Callow, in preparation).

It has been shown that centrifugation speed may play an important part in the dissociation of animal and bacterial ribosomes [17–22]. Variation of sucrose gradient viscosity has shown that particle velocity is not an important factor in ribosome dissociation [18], and the conclusion of many workers is that the high pressures developed during centrifugation which may be as much as several hundred atmospheres [20], induce dissociation of ribosome monomers [18–22]. The theoretical basis on which these results may be explained is that the sum of the molecular volumes of the ribosomal sub-units is smaller than the molecular volume of the monomer, so that high pressures push the ribosome association:dissociation equilibrium to the right.

In order to ascertain the importance of pressure-induced dissociation of mung bean ribosomes in the above results, the following experiment was carried out. Cytoplasmic ribosomes were resuspended in Medium B, fixed with glutaraldehyde, and fractionated in a gradient based on medium B. The glutaraldehyde fixation technique cross-links the ribosomal protein, effectively preventing any change in the conformation or dissociation:association equilibrium of the ribosomes [12]. Thus, comparison of fixed and unfixed ribosomes on Medium B gradients should isolate effects due solely to the ionic environment, from the joint effects of ionic composition and centrifugation. Ribosomes resuspended in Medium B, and fixed with glutaraldehyde before fractionation in Medium B gradients, were observed as 74S particles (Fig. 3a) as determined by comparison with unfixed *E. coli* and mung bean ribosomes fractionated in parallel gradients based on Medium A (Fig. 3b, c). The apparent reduction in sedimentation coefficient from 80S to 74S was not caused by the glutaraldehyde *per se* since mung bean cytoplasmic ribosomes resuspended in Medium A, fixed and then fractionated on gradients based on Medium A, still sedimented as 80S particles, although it has been reported that glutaraldehyde fixation of bacterial ribosomes results in a slight increase in sedimentation coefficient [12].

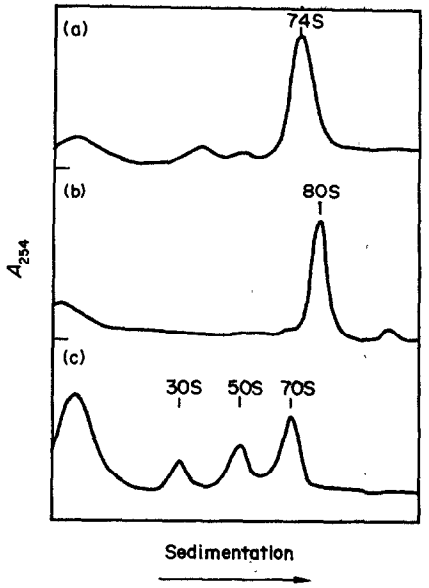

Fig. 3. Absorbance profiles of: (a) 150 μg mung bean ribosomes, resuspended in Medium B (minus Mg^{2+}), and fixed with glutaraldehyde. Centrifuged on gradients based on Medium B. Sedimentation coefficient of peak calculated to be 74S. (b) 110 μg mung bean ribosomes resuspended in Medium A, on Medium A-based gradients. (c) 150 μg *E. coli* ribosomes and sub-units on gradients based on Medium A. In all cases, 15–33% gradients were centrifuged at 95000 g for 6 hr at 5°.

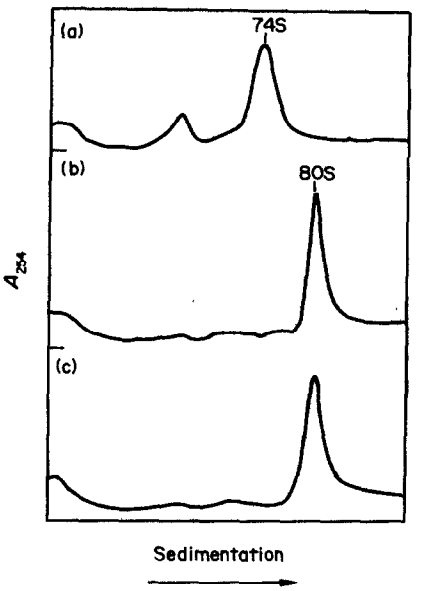

Fig. 4. Absorbance profiles of: (a) 120 μg mung bean ribosomes, resuspended in Medium B (minus Mg^{2+}), and fixed with glutaraldehyde; (b) 105 μg mung bean ribosomes resuspended in Medium A (50 mM KCl, 10 mM Mg acetate) and fractionated on Medium A-based gradients. (c) 110 μg mung bean ribosomes resuspended in Medium B and fractionated on Medium A-based gradients. In all cases, 15–33% sucrose gradients were centrifuged at 95000 g_{av} for 6 hr at 5°.

By preventing further changes in conformation and dissociation, the glutaraldehyde treatment shows that the dissociation pattern of unfixed ribosomes in gradients based on Medium B must have been dependent upon centrifugation. Thus under Mg^{2+}-free conditions, the 80S ribosome is modified to a 74S form which dissociates into two particles of apparent sedimentation coefficients 34S and 47S under the high pressures developed within the sucrose gradient.

Limited studies on the 74S particle have shown that it retains its stability in Medium B at 5° for at least 12 days, and that on fractionation through gradients based on Medium A, the particle reverts to its 80S form (Fig. 4). The 80S:74S transition would thus appear to be conformational in nature rather than due to a change in protein composition, since it is difficult to envisage the recombination of split protein and the 74S particle during the short period that they would exist together in dilute solution at the top of the gradient. Ionically-induced reduction of the sedimentation coefficients of plant ribosomes has also been noted [2, 3, 5, 9]. However, some workers state that the slower-sedimenting ribosomes previously reported are artefacts caused by lack of resolution of sub-units produced by dissociation during centrifugation, or populations of ribosomes and sub-units in a dynamic dissociation:reassociation equilibrium, sedimenting together at a rate which reflects the mean sedimentation coefficient of the particles involved[22, 23;46]. Whilst these may be fair criticisms of some previous reports of "unfolded monomers", they cannot apply here since the use of glutaraldehyde would preclude any such artefactual changes within the sucrose gradient.

Mung bean ribosomes may also be induced to dissociate in sucrose gradients under less extreme ionic conditions. When the monovalent ion molarity was maintained at 50 mM, and the divalent ion molarity varied between 40 mM and 4 mM (resulting in monovalent:divalent ionic ratios between 1.25 and 12.5), the ribosomes sedimented exclusively as 80S particles. When the Mg^{2+} concentration was reduced still further, resulting in ratios between 25 and 250, there was a progressive dissociation of the 80S ribosomes into sub-units (Fig. 5). Several workers have reported that ribosome dissociation occurs at a critical K^+:Mg^{2+} ratio [21, 24–27]. The relative importance of the monovalent:divalent ionic ratio in inducing dissociation, rather than the absolute concentrations of these ions *per se* was investigated by using higher absolute concentrations of K^+ and Mg^{2+} whilst maintaining molar ratios within the range previously employed. Cytoplasmic ribosomes fractionated in gradients containing 500 mM KCl and 10 mM Mg acetate (ionic ratio 50:1) produced an identical fractionation profile to ribosomes fractionated on gradients containing 500 mM KCl and 1 mM Mg acetate (ionic ratio also 50:1, Fig. 5c). A similar result was obtained when ribosomes were fractionated in gradients containing 1 M KCl and 10 mM Mg acetate, and 50 mM KCl and 0.5 mM Mg acetate (ionic ratios, 100:1).

The mobilities of the sub-units obtained in gradients based on 500 mM KCl and 10 mM Mg acetate (medium D, ionic ratio 50:1), or 50 mM KCl and 0.5 mM Mg acetate (medium C, ionic ratio 100:1) were compared with *E. coli* markers, and the following values were obtained: Medium C, 28S, 39S, 48S, 61S; Medium D, 28S, 37S, 49S, 59S (Fig. 6). The differences between the

two sets of results are well within the range of experimental error between duplicate determinations and the particles are thought to be direct equivalents. For convenience they have been termed 28S, 38S, 49S and 60S. The origins of these particles and their relationships to the 47S and 34S particles produced under Mg^{2+}-free conditions were determined in the following experiment. The 38S, 49S, 60S and 80S particles were collected separately from gradients based on Medium C, pelleted, resuspended in Mg^{2+}-free medium B, and fractionated in gradients based on Medium B. It is known that in Mg^{2+}-free gradients the large and small sub-units become 47S and 34S particles respectively (Fig. 2). Hence, in this experiment the derivatives of the 38S, 49S, 60S and 80S particles were compared in the same Mg^{2+}-free conditions with known derivatives of the large and small sub-units, indicating possible relationships. These results show that in Mg^{2+}-free conditions, the 38S particle is reduced to 34S (Fig. 7b), and the 60S particle is reduced to 47S (Fig. 7d), strongly suggesting that the 60S and 38S particles in Medium C-based gradients, represent the large and small ribosomal sub-units respectively. As expected, the derivatives of the 80S particle were 34S and 47S components (Fig. 7e). The derivative of the 49S particle in these conditions is more difficult to interpret (Fig. 7c), but seems to consist largely of 34S particles with slowly-sedimenting heterogeneous material which may have been lost by the 34S particle as it sedimented. The small amount of 47S material on this gradient is probably the result of contamination of the 49S particles with 60S particles on collection from the medium C-based gradient. The 28S particle produced in gradients based on Media C and D is only present in small quantities and could not be identified.

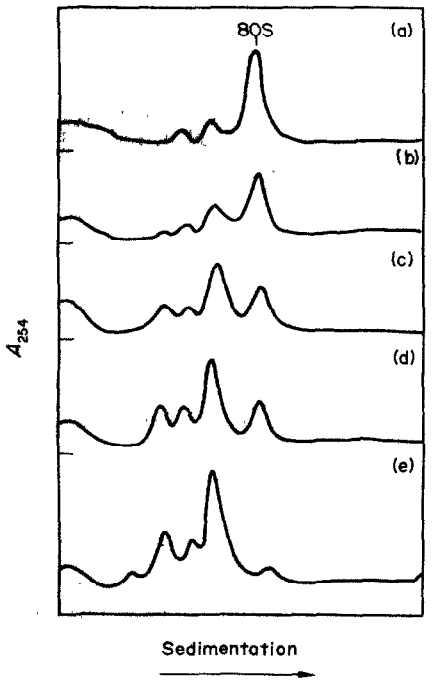

Fig. 5. Absorbance profiles of mung bean ribosomes resuspended in various media containing 0.025 M Tris–HCl, pH 7.4, 4 mM 2-mercaptoethanol and 50 mM KCl, but with different Mg acetate concentrations. (a) 125 μg ribosomes resuspended in medium containing 2 mM Mg acetate; (b) 125 μg: 1.5 mM Mg acetate; (c) 125 μg: 1 mM Mg acetate; (d) 140 μg: 0.5 mM Mg acetate; (e) 140 μg: 0.2 mM Mg acetate. In all cases, 15–33% sucrose gradients were centrifuged at 95000 g_{av} for 6 hr at 5°.

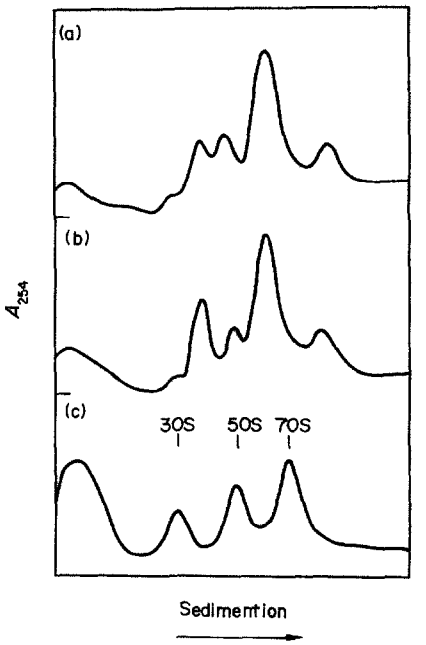

Fig. 6. Absorbance profiles of mung bean ribosomes (200 μg) resuspended in Medium C (containing 50 mM KCl, 0.5 mM Mg acetate, Fig. 6a) or Medium D (containing 500 mM KCl, 10 mM Mg acetate, Fig. 6b), and 150 μg of *E. coli* ribosomes in Medium A-based gradients (Fig. 6c). In all cases gradients were made up in the corresponding medium and centrifuged at 95000 g_{av} for 6 hr at 5°. The sedimentation coefficients of the various particles in (a) and (b) were estimated as 28S, 39S, 48S, 61S and 81S (Fig. 6a) and 28S, 37S, 49S, 59S and 80S (Fig. 6b).

The results from this experiment indicate that both 49S and 38S particles can be degraded to a 34S particle in Mg^{2+}-free conditions, and thus it seems that both are forms of the small ribosomal sub-unit. Similar results have been obtained with pea [5] and wheat [7, 8] ribosomes, and it has been shown that the two forms of small sub-unit contain the same RNA and protein components and are inter-convertible under suitable ionic conditions [7]. The reason for this duplicity is not understood.

The two groups of sub-units (i.e. the 22S, 34S and 47S particles obtained under Mg^{2+}-free conditions and the 28S, 38S, 49S and 60S particles obtained under high K^+/Mg^{2+} conditions) were next compared in their ability to reassociate under suitable ionic conditions. When the complete contents of Medium C-based gradients (i.e. 28S, 38S, 49S, 61S and 80S particles) were collected, pelleted, resuspended in a small volume of Medium A, stored 18 hr and fractionated in gradients based on Medium A, the fractionation profile showed that the proportion of 80S particles was considerably increased (Fig. 8c) indicating that these sub-units were capable of reassociation. When the same procedure was applied to the 22S, 34S and 47S contents of Medium B-based gradients, no 80S particles were formed (not shown), indicating that the sub-units were incapable of reassociation under these conditions.

Quantitative analysis of the progressive dissociations of the 80S ribosomes over a range of K^+/Mg^{2+} ratios (12.5–100), indicated that 70% of the 80S ribosomes dissociated between the ratios 12.5–50, but that a further increase in the ratio to 100 only resulted in a further 7% dissociation. The remaining 23% only dissociated at much higher ratios. Clearly some ribosomes were much more resistant to ionically induced dissociation than others. It is known from previous work with bacterial [28–33], fungal [34–36], animal [17, 25, 35, 37–40], and green plant [6, 10, 11, 41] ribosomes, that ribosomes active in protein synthesis, and therefore complexed with peptidyl *t*RNA and *m*RNA, are more resistant to ionically-induced dissociation than inactive ribosomes. To determine whether the ribosomes remaining after the ionic ratio was increased to 50:1 (medium D) were more resistant to dissociation because they were complexed with peptidyl *t*RNA and *m*RNA, an experiment was performed using puromycin. Since puromycin acts as an

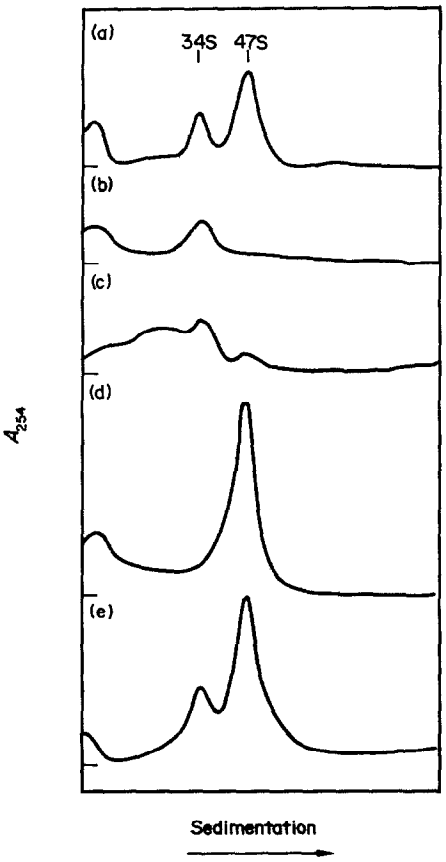

Fig. 7. Mung bean ribosomes were resuspended in Medium C (containing 50 mM KCl, 0.5 mM Mg acetate) and centrifuged at 95000 g_{av} for 6 hr at 5°. The four major particle types (i.e. 38S, 49S, 60S and 80S; as in Fig. 6a), were collected separately, pelleted at 200000 g_{av} for 90 min. The pellets were resuspended in Medium B (minus Mg^{2+}) and centrifuged on 15–33% gradients based on Medium B, for 6 hr at 95000 g_{av} at 5°. The gradient profiles shown represent: (a) 34S and 47S markers obtained by resuspending mung bean cytoplasmic ribosomes in Medium B and centrifuging 120 μg samples on a gradient based on Medium B; (b) 50 μg of "38S" particles; (c) 80 μg of "49S" particles; (d) 120 μg of "60S" particles; (e) 180 μg of "80S" particles.

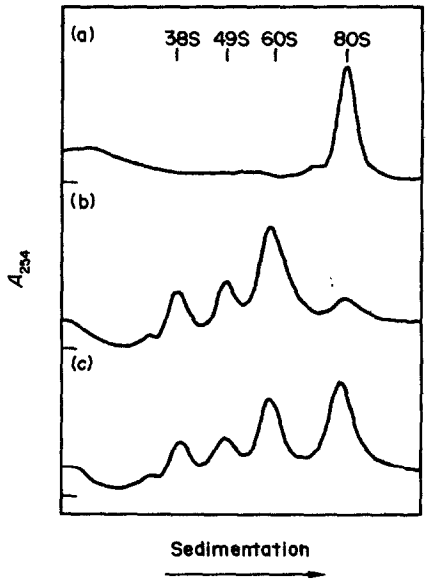

Fig. 8. Mung bean ribosomes were resuspended in Medium A (50 mM KCl, 10 mM Mg acetate) or Medium D (500 mM KCl, 10 mM Mg acetate) loaded onto 15–33% sucrose gradients and fractionated at 95000 g_{av} for 6 hr at 5°, (Figs. 8a and 8b respectively). The contents of three parallel gradients based on Medium D were fractionated, all particles were collected, pooled, pelleted at 200000 g_{av} for 90 min and the pellet resuspended in Medium A. 170 μg samples were then centrifuged on a gradient based on Medium A for 6 hr at 95000 g_{av}, 5°, (Fig. 8c).

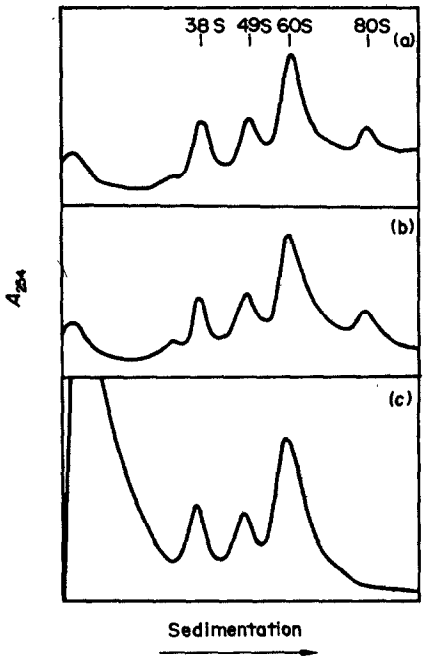

Fig. 9. Mung bean cytoplasmic ribosomes were resuspended in Medium D (containing 500 mM KCl, 10 mM Mg acetate) and loaded onto 15–33% gradients based on Medium D, after: (a) storage at 5° for 30 min; (b) incubation at 37° for 30 min; (c) incubation at 37° for 30 min with 2 mM puromycin and 1 mM GTP. Loadings were (a) 170 μg, (b) 190 μg, (c) 190 μg and centrifugation was for 6 hr at 95000 g_{av} and 5°. The high absorbance at the top of the gradient (c) is due to puromycin and GTP.

analogue of aminoacyl *t*RNA [42] and removes nascent peptidyl chains from active ribosomes [43, 44], active ribosomes so treated, should be dissociated as easily as inactive ribosomes. When ribosomes were incubated at 37° for 30 min with 2 mM puromycin (Calbiochem [24]), in Medium D, with 1 mM GTP (Sigma [45]), the 80S particles observed in control, medium D-based gradients (Fig. 9, a, b) were completely dissociated (Fig. 9c). This experiment would appear to indicate therefore that 80S ribosomes, undissociated at a K^+/Mg^{2+} ratio of 50:1, represent ribosomes active in protein synthesis and therefore are probably the products of polyribosome breakdown. However, the experiment does not yield information on the character of the more easily dissociated ribosomes. This problem is at present under investigation.

EXPERIMENTAL

Growth of plants and ribosome extraction. Seedlings of *Phaseolus aureus* (Roxb.) were grown in soil-less compost (Bower's, Lincoln, U.K.) in greenhouse conditions at a minimum temp. of 14°, and with supplementary lighting providing an 18 hr day. Primary leaves were harvested at full expansion, frozen with solid CO_2 and triturated in 8 g samples. Homogenisation medium was added (32 ml 0.1 M Tris–HCl, pH 7.4 containing 0.4 M sucrose, 10 mM 2-mercaptoethanol, 50 mM KCl and 10 mM Mg acetate. After thawing the brei was strained through two layers of muslin before successively centrifuging at 4000 g_{av} for 4 min and 30000 g_{av} for 30 min. The resulting supernatant was made to 0.4% (v/v) with Triton X-100 and layered onto 15 ml sucrose cushions (33%, w/v sucrose in 0.025 M Tris–HCl 7.4, containing 4 mM 2-mercaptoethanol, 50 mM KCl and 10 mM Mg acetate. Centrifugation was for 2.5 hr at 130000 g_{av}. The supernatant was then removed by aspiration, the cushion discarded, and the ribosome pellet rinsed and resuspended in medium appropriate to the experiment. Prior to layering onto a sucrose gradient, the ribosome preparation was centrifuged at 10000 g_{av} for 10 min.

E. coli *culture and ribosome extraction.* Exponential growth phase cells of *E. coli* (MRE 600), grown in a medium containing 1% peptone and 0.1% glucose, were harvested by centrifugation at 10000 g_{av} for 15 min, and were then either used immediately or stored at −20°. For ribosome extraction, 1 g of frozen cells was washed in homogenisation medium, pelleted by centrifugation and transferred to an ice-cold mortar. Alumina (2 g) was gradually ground in and the paste was ground for a further 15 min after which 5 ml of homogenisation medium (0.01 M Tris–HCl pH 7.4, containing 6 mM 2-mercaptoethanol, 50 mM KCl and 10 mM Mg acetate) was slowly added. After standing for 15 min the slurry was centrifuged at 20000 g_{av} for 15 min to remove alumina and large cell debris. The supernatant was collected, DNase (BDH, 2 μg ml^{-1}) was added and the mixture was allowed to stand for 5 min at 0° before centrifugation at 30000 g_{av} for 35 min. The upper two-thirds of the resulting supernatant was removed and used as a ribosome suspension.

Sucrose gradient fractionation. Convex, isokinetic 15–33% (w/v) sucrose gradients [13] were produced by pumping 19 ml 33% sucrose into a mixing chamber containing 18 ml of 15% sucrose, the contents of the mixing chamber being simultaneously pumped into a 23 ml centrifuge tube at the same rate. The stock sucrose solutions were based on the appropriate medium used to resuspend the ribosome pellets. After cooling, the gradients were loaded with 0.5 ml ribosome suspension and centrifuged at 95000 g_{av} in an MSE 3 × 23 ml SW rotor at 5°. The gradients were fractionated and simultaneously scanned at 254 nm. Discrete peaks were automatically collected using a peak analyser and an automatically-actuated fraction collector.

Glutaraldehyde fixation of ribosomes. Following the original method of ref. [12] as modified in ref. [14], 50% glutaraldehyde was mixed with an equal vol. of 1 M Tris and the pH adjusted to 7.4 with KOH. This mixture was diluted to 10% with the appropriate ribosomal resuspension medium and a suitable aliquot used to fix ribosomes at a final glutaraldehyde concn of 1%. The fixed ribosomes were maintained at 0° for 15 min before loading onto sucrose gradients.

Definition of media. Ribosome resuspension media were made up in 0.025 M Tris–HCl pH 7.4 containing 4 mM 2-mercaptoethanol, but in addition contained, 50 mM KCl, 10 mM Mg acetate (medium A); 50 mM KCl, (medium B); 50 mM KCl, 0.5 mM Mg acetate (medium C); 0.5 M KCl, 10 mM Mg acetate (medium D).

Acknowledgements—This work was carried out under the tenure of a Science Research Council postgraduate studentship to H.J.N.

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