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Mouse egg ribosomes: evidence for storage in lattices

By R. BACHVAROVA,[1] V. DE LEON AND I. SPIEGELMAN
From the Department of Anatomy, Cornell University Medical College

SUMMARY

The ribosomes of mouse ova were labeled by exposure of growing ovarian oocytes to [^{3}H]uridine. The ribosomes of ova lysed and dispersed in hypotonic medium were contained in particles with an unusually low buoyant density in CsCl density gradients. These particles sedimented at 9000*g* or less, and electron microscopy of the pellet revealed ribosome-like particles embedded in a fibrillar network. These results indicate that the ribosomes are present in a proteinaceous superstructure, probably the lattices seen in situ by transmission electron microscopy.

INTRODUCTION

The eggs of most animals contain a large store of ribosomes not engaged in protein synthesis at the time of fertilization, but available to carry out translation during embryonic development. Since the mammalian egg is relatively small, and transcription of rRNA commences early in development (Knowland & Graham, 1972), it might be expected that a large stockpile of ribosomes would not be necessary. Nevertheless, 75–80 % of the ribosomes present in the mouse egg are not in polysomes at the time of ovulation and fertilization, but are apparently in reserve for later use (Bachvarova & De Leon, 1977).

When sectioned mouse eggs are examined in the electron microscope, clusters of ribosomes probably representing polysomes are quite sparse but readily observed, while single ribosomes are more rare (Calarco & Brown, 1969; Hillman & Tasca, 1969; Wassarman & Josefowicz, 1978), a finding inconsistent with our biochemical data. It has been proposed that the unusual fibrillar or lamellar structures prevalent in rodent eggs may consist of ribosomes embedded in a proteinaceous (nonmembranous) superstructure. These structures vary in different rodent species, from spiral lamellae to arrays of closely spaced parallel bilaminar sheets to the cross-linked fibrils or lattices of mouse eggs (Schlafke & Enders, 1967; Weakley, 1967, 1968; Calcarco & Brown, 1969; Zamboni, 1970; Burkholder, Comings & Okada, 1971; Kang & Anderson, 1975; King & Tibbitts, 1977; Wassarman & Josefowicz, 1978). In all cases the fibrils or

[1] *Author's address:* Department of Anatomy, Cornell University Medical College, 1300 York Avenue, New York, N.Y. 10021, U.S.A.

lamellae have a periodic structure suggesting that they are composed of particles on a string. The lamellar or fibrillar structures decrease in abundance during preimplantation development, probably dispersing completely around the time of implantation (Enders & Schlafke, 1967; Enders, 1971; McReynolds & Hadek, 1972; Dvorak, Travnik & Stankova, 1977). During their formation in growing oocytes, closely associated ribosomes are apparently incorporated into them (Zamboni, 1970; Kang & Anderson, 1975).

The most direct evidence for the presence of ribosomes in the lattices of mouse eggs comes from the electron microscopic work of Burkholder *et al.* (1971). They observed lattices in whole mounts prepared by spreading mouse eggs on an air-liquid interface. When partially disrupted by mechanical forces or protease digestion, the lattices were seen as chains of closely linked particles or beads. Lattices were not found in spreads prepared from eggs treated with RNase. Particles in lattices were smaller than released particles which had the dimensions of ribosomes. Ribosomes may undergo a reversible modification of structure during formation of the lattices.

In mammalian eggs other than rodent eggs, typical ribosomes are also quite sparse and usually found in clusters (polysomes) (Zamboni, Mishell, Bell & Baca, 1966; Zamboni, Thompson & Smith, 1972; van Blerkom, Manes & Daniel, 1973; Karp, Manes & Hahn, 1974). Whether an unrecognized storage form of ribosomes exists in these cases remains to be determined.

In our previous biochemical studies of ovulated mouse eggs, at least 90 % of the ribosomes in egg lysates moved as typical ribosomal monomers, subunits, or polysomes on sucrose gradients (Bachvarova & De Leon, 1977). Thus, if ribosomes are stored in lattices, these structures are very fragile and easily dispersed. However, the evidence suggested that stored ribosomes were unable to accept poly (U), consistent with the possibility that they have a modified structure. Using egg lysates prepared by gentle procedures, we report here that the bulk of mouse egg ribosomes are present in a proteinaceous superstructure, presumably the lattices seen in the electron microscope.

METHODS

Labeled superovulated ova of ICR mice were collected 8–19 days after injection of 40 μCi of [5,6-^{3}H]uridine (40–50 Ci/mM) into the ovarian bursa of females about 6 weeks old as described previously (Bachavarova, 1974). When fresh labeled ova were required, cumulus cells were dispersed by brief stirring in trypsin (2 mg/ml in Eagle's Minimal Essential Medium (MEM) plus 0·5 mM-pyruvate) at room temperature, and the zona pellucida removed by incubating in trypsin at 37 °C for 18 min. Ova were passed through three rinses of mammalian Ringer's solution containing 5–10 % fetal calf serum. To obtain frozen ova with zona pellucida, cumulus cells were removed by stirring in pronase (1 mg/ml in Ringer's solution) and rinsed as above.

For sedimentation analysis, samples of 150–200 labeled ova were placed in 0·2–0·4 ml of the appropriate buffer (buffer = salt of the indicated molarity, 20 mM-triethanolamine, pH 7·5, 2 mM-$MgCl_2$) containing 1 mM-2′3′CMP, 0·5 mM-phenylmethylsulfonyl fluoride, 0·1 mM-L-1-tosylamide-2-phenylethyl chloromethyl ketone and 5 μg/ml soybean trypsin inhibitor. The samples were incubated for 30 min at 37 °C except that samples exposed to Triton X-100 or 0·01 M-KCl or 0·01 M-NaCl buffer were incubated at room temperature. The eggs were lysed and the cytoplasm dispersed by gentle pipetting until no fragments could be observed under the dissection microscope. A few samples were lysed in Eagle's Minimal Essential Medium (MEM) by addition of 10 % guinea-pig serum containing complement and antibodies crossreacting with mouse egg antigens. (Addition of rabbit anti-mouse immunoglobulin did not significantly accelerate egg lysis.) The samples were transferred to 0·75 ml plastic microfuge tubes and centrifuged at 650*g* for 5 min in the JS 7·5 rotor of the Beckman JB4 centrifuge. The supernatant was recentrifuged at 9000*g* for 12 min. The second supernatant and the dissolved pellets (0·5 N PCA, 80 °C) were transferred to vials for counting.

For sucrose gradient analysis, 9000*g* supernatants plus carrier liver polysomes were centrifuged on 15–40 % sucrose gradients in the appropriate buffer for 100 min at 36 000 rev./min in the SW 41 rotor of a Beckman ultracentrifuge.

For electron microscopy, samples of 250–1500 unlabeled superovulated zona-free ova were incubated at 37 °C in 0·05 M-KCl buffer, dispersed as described above, and centrifuged at 9000*g* for 12 min. The pellet was fixed in 2 % glutaraldehyde in 0·05 M Sorenson phosphate buffer, and held in place by 1 % agarose or gelatin plugs. The samples were rinsed in buffer, postfixed in 1 % OsO_4, dehydrated in acetone, embedded in Araldite, sectioned, and examined in a Philips 200 electron microscope. Some samples were embedded in hydroxypropyl methacrylate (HPMA), which permits specific extraction with RNase (Leduc & Holt, 1965).

CsCl density gradient analysis was carried out essentially according to Henshaw (1968). Carrier polysomes were prepared from livers of rats starved for 40 h, suspended in the appropriate buffer, and clarified. Polysomes (2–3 O.D. units) were added to 0·45 ml samples of fresh lysed ova, formaldehyde (pH 7–7·5) added to a final concentration of 4 %, and the sample fixed for 2 h at 0–4 °C. Samples of frozen ova were suspended in the appropriate buffer containing carrier polysomes, incubated for 30 min at 0–4 °C, and fixed in 4 % formaldehyde for 2 h at 0–4 °C. The fixed sample in 0·2–0·5 ml was brought to a final volume of 2·5 ml and a density of 1·33 g/cm^3 by addition of 0·01 M-NaCl buffer containing 4 % formaldehyde and 0·8 % Brij, and 0·9 ml of saturated CsCl in the same solution. The mixture was layered over 2·5 ml of CsCl in the same solution, ρ = 1·7 g/cm^3. The tube was filled with mineral oil and centrifuged in the SW 41 rotor of the Beckman ultracentrifuge at

33 000 rev./min for 22 h at 20 °C. The optical density of the gradient was monitored at 257 mμ on an ISCO u.v. analyser, and fractions of 0·15–0·25 ml collected. Total counts in each fraction were determined after addition of 0·8 ml of water and 15 ml of aquasol. The density gradient formed was monitored by weighing 100 μl of alternate fractions or by measuring the refractive index in an Abbe refractometer. A gradient similar to that shown in Fig. 3 was formed in all samples analysed. Centrifugation for 72 h did not change the shape or final position of the optical density peak of carrier ribosomes showing that adequate equilibrium was achieved in the shorter run.

Human lymphocytic leukemia cells, line 1301, EBV negative, growing in suspension were labeled for 8 h with [5,6-^{3}H]uridine (33 μCi/ml in MEM plus 15 % fetal calf serum) and chased for 20 h. They were collected by centrifugation, suspended in 0·05 M-KCl buffer, incubated 30 min at 37 °C, and lysed by gentle pipetting. Whole cells were removed by centrifugation at 100 *g* for 10 min (centrifugation of labeled lysed ova under these conditions removed negligible labeled material). Aliquots of the lysate were applied to CsCl gradients.

RESULTS

Sedimentation of ribosomes from mouse ova lysed and dispersed under various salt conditions

Ovulated mouse ova were labeled during their growth phase by injection of [^{3}H]uridine into the ovarian bursa 8–19 days before ovulation. The radioactivity in ova is distributed as follows: 11 % in acid soluble material, 18 % in transfer RNA, 10 % in poly (A)+RNA, and additional 5–10 % in heterogeneous RNA, and about 55 % in ribosomal RNA (Bachvarova, 1974; Jahn, Baran & Bachvarova, 1976; Bachvarova & De Leon, 1980). When ova lysed by freezing are suspended in 0·1 M ammonium chloride buffer, 20–25 % of the ribosomes sediment as polysomes, and about 70 % as monomers and subunits (Bachvarova & De Leon, 1977). The distribution of label in egg RNA is similar regardless of the stage of oocyte growth at which incorporation occurs; thus measurements at any one point are representative of total RNA.

If ribosomes are stored in lattices, it seemed likely that conditions for dispersal of the cytoplasm could be found such that a large portion of labeled RNA would sediment more rapidly than monomers and polysomes of moderate size. For these experiments, ova were not frozen since it was possible that freezing itself would disrupt the lattices. Zona-free ova were lysed and the cytoplasm dispersed in buffers of decreasing monovalent cation concentration. The lysate was subjected to two cycles of centrifugation to obtain a low speed pellet and a pellet containing membrane-bound organelles. About 30 % of the radioactivity, representing acid soluble and transfer RNA, was expected to appear in the supernatant in all cases. As shown in Table 1, 70–81 % of the radioactivity of ova lysed in isotonic medium appeared in the final supernatant,

Table 1. *Distribution of radioactivity of [^3H]uridine-labeled ova in pellets and supernatant*

Conditions for lysis and dispersal of ova	Percent of total label* in Pellets 650 *g*	Pellets 9000 *g*	Supernatant 9000 *g*
MEM + complement	12 ± 4	7 ± 2	81 ± 4
0·14 M-KCl buffer	17 ± 4	13 ± 1	70 ± 5
0·09 M-KCl buffer	20 ± 4	22 ± 1	58 ± 4
0·05 M-KCl buffer + complement	30 ± 1	14 ± 2	56 ± 1
0·05 M-KCl buffer	30 ± 3	23 ± 2	47 ± 3
0·05 M-KCl buffer + 0·2 M-sucrose	57 ± 3	14 ± 1	29 ± 2
0·05 M-KCl buffer + 0·5 % Triton X-100	11 ± 4	22 ± 4	67 ± 8
0·01 M-KCl or NaCl buffer	17 ± 2	19 ± 2	64 ± 2

* ± S.E.M.

When disrupted in 0·05 M-KCl buffer, only 47 % of the radioactivity was found in the supernatant, and more than half of the pelleted material was recovered in the 650 *g* pellet. An even higher proportion of the label was found in the low speed pellet when 0·2 M sucrose was added to the dispersal medium. Addition of 0·5 % Triton X-100 or reduction of the salt concentration to 0·01 M promoted release of labeled material.

To follow the ribosomes during the fractionation procedure, 9000 *g* supernatants from ova dispersed in MEM plus complement and from ova dispersed in 0·05 M-KCl buffer plus complement were centrifuged on sucrose gradients (Fig. 1). The material remaining at the top of the gradient is presumably acid soluble label and transfer RNA. The 0·05 M-KCl buffer supernatant contained negligible amounts of ribosomal subunits and monomers, while the additional material released by isotonic salt was clearly ribosomal. When 0·05 M-KCl buffer dispersed ova were divided into supernatant and 9000 *g* pellet, and the RNA released by treatment with 1 % LDS and run on SDS gradients, typical 18S and 28S RNA were found in both fractions with 75 % of the 28S RNA in the pellet.

Taken together, the results indicate that at least 75 % of the ribosomes of 0·5 M-KCl dispersed ova can be sedimented at 9000 *g* or less, and suggest that stored nonpolysomal ribosomes are contained in unusually large structures whose stability is promoted by 0·2 M sucrose but sensitive to isotonic salt, particularly isotonic NaCl.

Electron microscopy of the 9000 g pellet from ova dispersed in 0·05 M-KCl buffer

To obtain more information on the nature of the rapidly sedimenting particles containing ribosomes, the 9000 *g* pellet from unlabeled eggs was examined

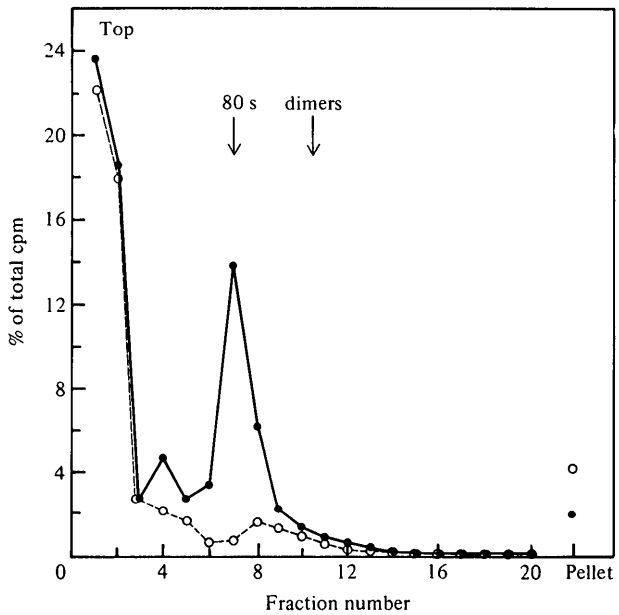

Fig. 1. Sucrose gradient analysis of the 9000*g* supernatant of [^{3}H]uridine-labeled ova lysed and dispersed in MEM plus complement (closed circles) or 0·05 M-KCl buffer plus complement (open circles). Gradients were made up in 0·1 M-NaCl buffer and 0·05 M-KCl buffer respectively. Optical density of the carrier polysomes is not shown. The balance of the total radioactivity was found in the 9000 *g* pellet.

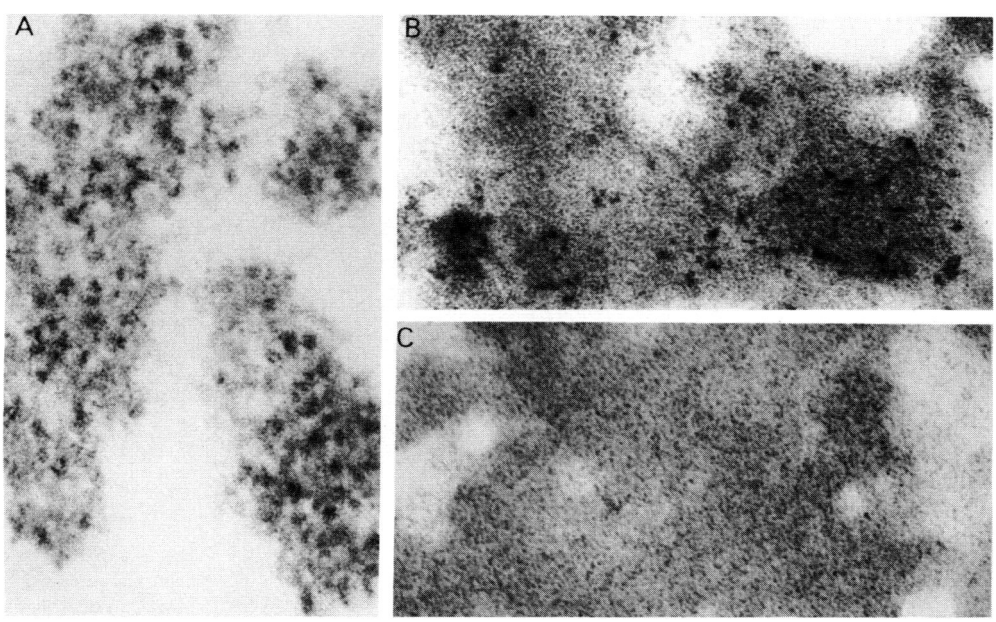

Fig. 2. Electron micrographs of 9000 *g* pellets from ova lysed and dispersed in 0·05 M-KCl buffer, illustrating material believed to be disordered lattices containing ribosomal particles. (A) Embedded in Araldite. (B) Embedded in HPMA. The particles appear smaller probably because OsO_4 fixation was omitted and because greater shrinkage occurs during HPMA embedding. (C) Embedded in HPMA and treated with RNase. All micrographs × 130 000.

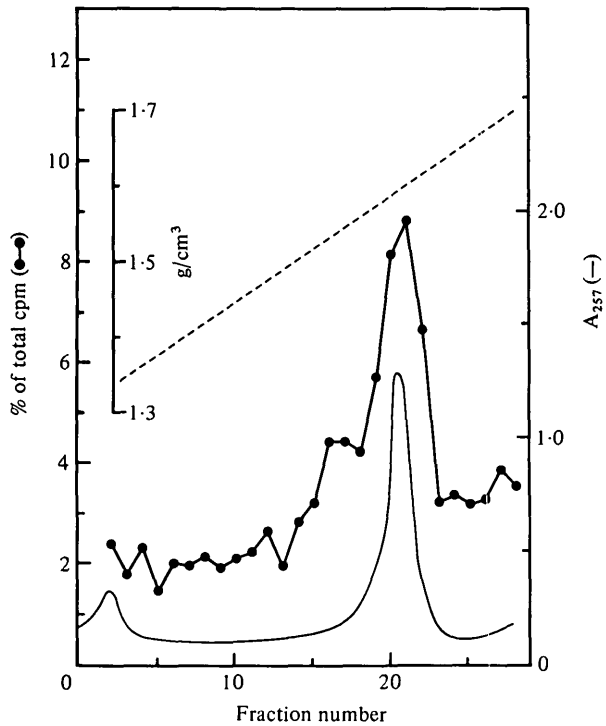

Fig. 3. CsCl density gradient analysis of [^{3}H]uridine-labeled frozen lysed ova suspended in 0·12 M-KCl buffer. The top of the gradient is at the left. Optical density of the carrier liver polysomes is shown.

with the electron microscope. The main constitutents of the pellet were smooth membrane vesicles, mitochondria, and masses of interlacing fibrils forming a feltwork in which electron-dense particles 16–20 nm in diameter were enmeshed. These masses and particles are illustrated in Fig. 2A. Structures similar to those of Fig. 2A were observed in samples embedded in HPMA (Fig. 2B) and the electron-dense particles were almost entirely removed by treatment with RNase (Fig. 2C). From their dimensions and RNase sensitivity, we conclude that the electron-dense particles are ribosomes. These observations suggest that upon exposure to 0·05 M-KCl buffer and centrifugation, the ordered structure of the lattices is lost, and the proteinaceous superstructure becomes a dense fibrillar network in which ribosomal particles are entrapped.

Analysis of mouse egg ribosomes on CsCl density gradients

To obtain further evidence for the association of ribosomes with a proteinaceous superstructure, the buoyant density of fixed egg RNP particles was examined on CsCl density gradients. In this case, the final position of the RNA-containing particle shifts to a lighter density as the protein content increases.

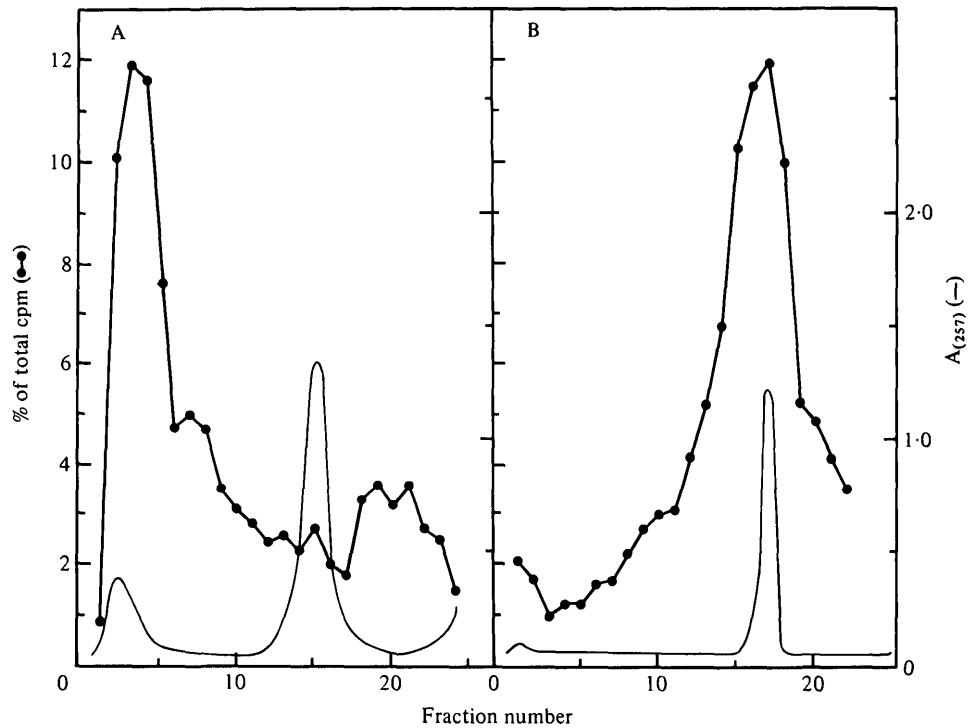

Fig. 4. CsCl density gradient analyses of fresh [^{3}H]uridine-labeled ova lysed and dispersed in 0·05 M-KCl buffer. (A) No additional treatment. (B) The sample was exposed to 0·01 % pronase for 5 min at room temperature before fixation.

The results for a sample of frozen lysed ova suspended in 0·12 M-KCl buffer are shown in Fig. 3. The main peak of radioactivity coincides with the optical density peak at a buoyant density of 1·58 g/cm^3, typical of ribosomes (Henshaw, 1968; Perry & Kelley, 1968). The radioactivity between the ribosomes and the bottom of the gradient is accounted for by tRNA, since labeled isolated tRNA centrifuged under these conditions peaks in the third fraction from the bottom (data not shown). Separate experiments showed that acid soluble label appears primarily in the upper half of the gradient, but it accounts for less than 1/4 of the radioactivity observed in fractions 1–15 of Fig. 3. The light shoulder on the side of the main peak of radioactivity falls at 1·52 g/cm^3, typical of 40S subunits. We conclude that under these conditions egg ribosomes have a density similar to that of liver ribosomes, and that the main peak of radioactivity in the CsCl gradient includes egg polysomes, monomers, and 60S subunits while the lighter shoulder represents 40S subunits. Pulse-labeled mRNP dissociated from liver polysomes has a density of 1·36–1·50 g/cm^3 (Henshaw, 1968; our own results). The material in this density range in Fig. 3 may represent stored egg mRNP particles.

Since most of the ribosomes of fresh ova dispersed in 0·05 M-KCl sedimented

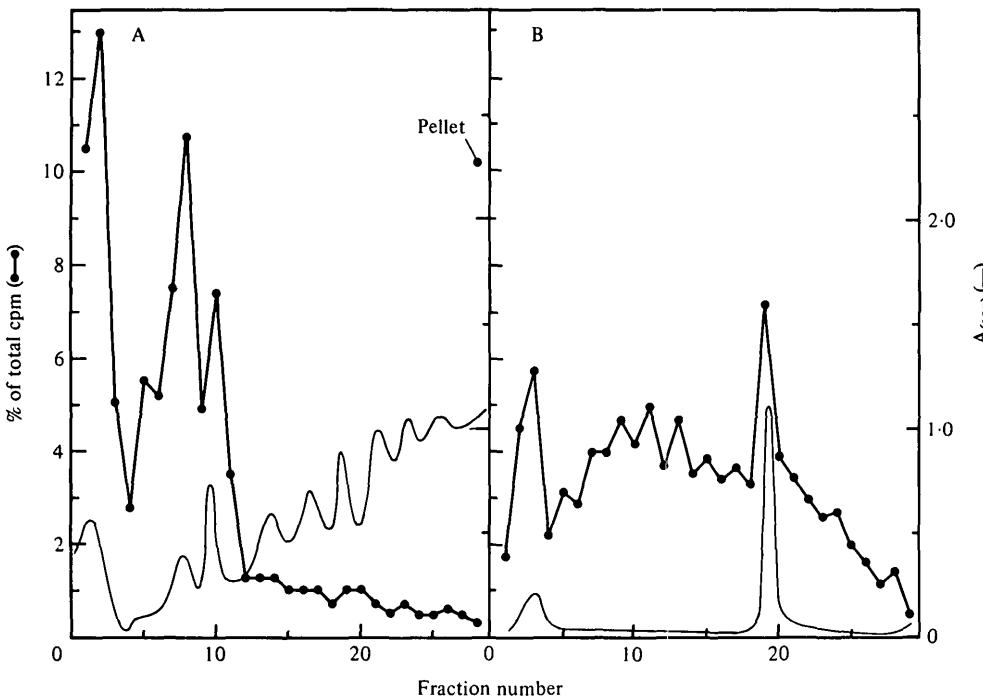

Fig. 5. (A) Sucrose gradient analyses of frozen lysed ova suspended in 0·01 M-NaCl buffer, fixed, and run on a 15–40% sucrose gradient in 0·01 M-NaCl buffer plus 4% formaldehyde and 0·8 Brij. (B) CsCl density gradient analysis of frozen ova suspended in 0·01 M-NaCl buffer.

at low *g* forces, such a sample was prepared, fixed, and centrifuged on a CsCl gradient (Fig. 4A). Most of the radioactivity was found near the top of the gradient far removed from the carrier ribosome peak. Its buoyant density of 1·33–1·38 g/cm^3 indicates an RNA content of roughly 5–15% (calculated according to Hamilton, 1971, using a value of 1·32 g/cm^3 for pure protein), and a ribosome to extra protein ratio of approximately 1:3. The material coinciding with the carrier polysomes or banding at higher density is accounted for by polysomes and tRNA. The data of Fig. 4A were obtained from ova labeled 8 days before ovulation when oocyte growth is almost complete. Very similar profiles were obtained from egg samples labeled at mid or early oocyte growth collected 14 or 19 days after exposure to [^{3}H]uridine. Also, very similar CsCl gradient profiles were obtained when ova were disrupted in 0·05 M-KCl buffer containing 0·5% Triton X-100 (data not shown).

As a control, somatic cells (a human lymphocytic leukemia line) pulse-chased with [^{3}H]uridine were dispersed in 0·05 M-KCl buffer under the same conditions used for labeled ova. On CsCl density gradients, essentially all the labeled material coincided with the optical density carrier ribosome peak or moved to a higher density (data not shown). These results indicate that ribosomes of

somatic cells dispersed under our relatively low salt conditions do not aggregate non-specifically with general cellular proteins.

To demonstrate that the unusually low density of mouse egg ribosomes prepared in 0·05 M-KCl buffer was due to binding of extra protein, a sample was treated with pronase before fixation. As shown in Fig. 4B, this treatment shifted the main peak of radioactivity to a higher density which coincided with the optical density carrier ribosome peak.

Evidence for extra protein associated with ribosomes was obtained under one more set of conditions. Samples of *frozen* ova were suspended in 0·01 M-NaCl buffer, fixed and centrifuged. On sucrose velocity gradients, most of the radioactivity in material larger than tRNA sedimented as ribosomal monomers or subunits (Fig. 5A). On CsCl density gradients, the radioactivity was spread over a broad buoyant density range of 1·3–1·6 g/cm^3 (Fig. 5B). The lattice structure has apparently fragmented into single ribosomes each carrying variable amounts of associated extra protein. Again, light pronase treatment resulted in a shift of the radioactivity to a higher density coinciding with the ribosomal carrier (data not shown).

DISCUSSION

The results presented here taken in conjunction with those of Burkholder *et al.* (1971) provide strong evidence that mouse egg ribosomes are stored in the prevalent lattice-like arrays seen throughout the cytoplasm in the electron microscope. We have found that when ovulated mouse eggs are dispersed in a moderately hypotonic buffer (0·05 M-KCl), ribosomes are contained in structures which sediment rapidly and have a low buoyant density indicating a high protein content. Thus the biochemical data demonstrate that egg ribosomes are contained within a proteinaceous superstructure. The difficulty of identifying another ultrastructural component which could represent the superstructure and Burkholder *et al.*'s evidence that the lattices contain ribosomes lead us to conclude that the fast sedimenting particles represent at least partially intact lattices.

Our data have defined several properties of the lattices. They are increasingly dispersed as the monovalent cation concentration is raised from 0·05 to 0·14 M in the presence of 2 mM-$MgCl_2$. The volume occupied by a set of closely associated elements of the lattices is large and variable in size, since the ribosome containing particles sediment at low and moderate g forces. (The lattice elements seen in the electron microscope may be interconnected in three dimensions to form large particles.) The regular structure of the lattices is collapsed upon dispersal and centrifugation in 0·05 M-KCl buffer. The lattices are roughly 25 % ribosomes and 75 % protein by weight, but the calculation depends heavily on the appropriate value to use for the density of pure protein which is unknown in this case (Hamilton, 1971). The protein scaffolding of the lattice is easily digested by pronase to release ribosomes of typical density. Exposure to 0·5 % Triton X-100 does not significantly change the buoyant density of the ribosome-

containing particles, confirming the electron microscopic evidence that ribosomes are not associated with membranes.

Other cases are known in which cells with an excess of ribosomes not used in translation form unusual structures. In cooled chick embryo cells (and other cell types), ribosomes form crystalline tetramers which in turn associate in a precise array to form large sheets of ribosomes occupying a significant fraction of the cytoplasm (Byers, 1966, 1967). The intertetramer bonds are salt-labile and protease-labile (Dondi & Barker, 1974). In cooled lizard eggs, ribosomes also form tetramers which in this case associate with parallel flat cisternae of the endoplasmic reticulum (Taddei, 1972). In both cases, the ribosomes contained in crystalline aggregates are capable of protein synthesis (Morimoto, Blobel & Sabatini, 1972; Taddei, Gambino, Metafora & Monroy, 1973). The ribosome-containing structures of rodent eggs are of a different type: they are not as precisely ordered, they are present at physiological temperature, they probably do not involve ribosome–ribosome interaction, and they clearly include significant amounts of extra protein.

Presumably, some active mechanism is required in the developing embryo for release of stored ribosomes from the lattice to permit their participation in protein synthesis. Such a situation may be quite unusual since ribosomes in the eggs of lower organisms such as frogs and sea urchins are apparently free and capable of protein synthesis (Cox *et al.*, 1970; Clegg & Denny, 1974).

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