ELECTRON MICROSCOPIC OBSERVATIONS ON THE LARGE SUBUNIT OF THE RAT LIVER RIBOSOME

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ABSTRACT

Active large subunits obtained by urea treatment of rat liver ribosomes, 59S, were compared with large subunits in intact ribosomes and with the 50S subunits obtained by EDTA treatment. For electron microscopy the specimens were negatively stained or shadow cast. The negatively stained 59S subunits had a slightly ovoidal form; their average dimensions, 244 ± 17 × 207 ± 18 A, were very close to the dimensions of the large subunits in intact ribosomes, and lay between the theoretical dimensions for anhydrous and fully hydrated particles that were calculated from the physical properties of the subunits in solution. The shadow-cast preparations showed particles of similar shape. The 50S subunits, which had lost their 5S RNA, were shadow cast at the same time. They appeared to be more spread out than the 59S subunits and had threadlike extensions. In the positively stained regions of uranyl oxalate-stained preparations the 50S particles varied greatly in shape and size, with average dimensions of 330 ± 21 × 276 ± 33 A, and showed threadlike extensions like those of the shadow-cast particles. For 50S particles in solution the frictional drag of these extensions probably accounts for their low sedimentation coefficient.

INTRODUCTION

While isolated large subunits of bacterial ribosomes have been extensively studied by electron microscopy (1–6), except for the work of Dass and Bayley (7) few studies have been made on mammalian preparations. Only recently have active subunits been obtained from animal ribosomes, most often by a salt treatment (8–10). Active subunits can also be obtained from rat liver ribosomes by urea treatment (11). Some of the bound magnesium remains on the particles and, although the transfer RNA's are detached, the 5S RNA is not. The large subunits, which have a sedimentation coefficient of 59S, can reassociate with the small ones to give ribosomes that are active in protein synthesis.

A second form of the large subunit, obtained by complete removal of magnesium, has the same molecular weight as the 59S particle, but a sedimentation coefficient of only 50S (12). This paper is concerned chiefly with electron microscopic studies of the 59S and 50S subunits. The morphology of the two kinds of large subunit helps to explain their sedimentation behavior.

METHODS

Preparation of Ribosomes and Monosomes (82S)

The rat liver ribosomes used for the first preparation of 50S subunits, 50-1, were isolated as described

previously (13). For the other preparations the procedure was modified by the addition of 1 mM dithiothreitol (DTT) to all solutions. Since these preparations consisted of polysome fragments, and included a large proportion of disomes and larger particles, monosomes (82S) were sometimes prepared as follows. The ribosome solution was mixed with one-third volume of 0.9 M KCl, 6 mM $MgCl_2$, 1 mM potassium phosphate, pH 7.2, then dialyzed overnight against 0.3 M KCl, 1 mM $MgCl_2$, 1 mM DTT, 1 mM potassium phosphate, pH 7.2. It was then transferred to a beaker set in an ice bath, and cold absolute ethanol was added slowly, with good stirring, to a final concentration of 15%. The solution was warmed to 37°C for 20 min, then chilled in an ice bath, and dialyzed for 2 hr in a Zeineh Dialyzer (Biomed Instruments Inc., Chicago, Ill.) against 30 mM KCl, 1 mM potassium phosphate, pH 7.2 (buffer K), containing 1 mM DTT and 0.2 mM $MgCl_2$.

Compact Large Subunits (59S)

The 59S subunits were obtained by dissociating the ribosomes with urea. For the first preparation, 59-1, the polysome fragments were used directly. A sample containing 60 mg of ribosomes was dialyzed for 2 hr against buffer K containing 1 mM DTT and 0.2 mM $MgCl_2$ in the Zeineh Dialyzer. One-third volume of 8 M urea, freshly dissolved in water and treated with charcoal, was added at 5°C. After standing for 30 min at 5°C the material was dialyzed in the Zeineh Dialyzer against 10 mM KCl, 0.1 mM $MgCl_2$, 1 mM DTT, 1 mM potassium phosphate, pH 7.8, for 1 hr, and centrifuged in a Beckman B-XIV zonal rotor (Beckman Instruments, Inc., Fullerton, Calif.) for 17 hr at 26,000 rpm in a 5 to 20% sucrose gradient in the same buffer. A sample from the peak tube of the 59S fraction was fixed by dialysis for 1 day against 6% formaldehyde, 0.2 mM $MgCl_2$, 3 mM potassium phosphate, pH 7.4, and then by dialysis for a 2nd day against 0.3% formaldehyde, 0.1 mM $MgCl_2$, 1 mM potassium phosphate, pH 7.0.

The second sample, 59-2, was prepared from monosomes obtained by ethanol treatment. After the dialysis against the 0.2 mM $MgCl_2$ buffer the urea treatment, dialysis, and zonal fractionation were carried out as described above. To remove sucrose 1 ml of the peak fraction was passed through a 30 x 1.5 cm column of G-50 Sephadex in buffer K containing 0.5 mM $MgCl_2$.

Large Subunits Obtained by EDTA Treatment (50S)

Monodisperse samples of the large subunit were prepared as previously described (12) except that 0.2 mM $MgCl_2$ was added to the sucrose gradients to improve the stability of the particles. Sample 50-1 was fixed by dialysis for 1 day in the cold against 6% formaldehyde, 0.2 mM $MgCl_2$, 3 mM potassium phosphate, pH 7.4, followed by dialysis against 0.3% formaldehyde, 1 mM potassium phosphate, pH 7.3. Sample 50-2, which was not fixed, was passed through a G-50 Sephadex column in buffer K to free it of sucrose.

Ultracentrifugal Analysis of Ribosomes and Subunits

The monosome and large-subunit preparations were examined in the analytical ultracentrifuge in 12 mm cells at 5°C, at 44,000 or 48,000 rpm, with ultraviolet absorption optics. Before analysis the samples from the zonal separations were freed of sucrose by dialysis or passage through G-50 Sephadex. For the 59S large subunits the sedimentation coefficients were measured in buffer K containing 0.2 or 0.5 mM $MgCl_2$. To examine fraction 59-2 for small subunits EDTA was added to a concentration of 10 mM; for 59-1, which was still in the gradient buffer, 1 mM EDTA was used. The 50S particles were analyzed in the buffers in which they were freed of sucrose.

Electron Microscopy

The 59S and 50S subunits were prepared for electron microscopy by negative staining or shadow casting. The ribosomes were negatively stained. The concentration of subunits or ribosomes ranged from 40 to 60 μg/ml. The negative staining of preparations 59-2 and 50-2 was carried out according to Mellema and van Bruggen (14). A solution containing 12 mM uranyl acetate and 12 mM oxalic acid was adjusted to pH 6.5–6.8 by the addition of concentrated ammonium hydroxide. A drop of sample was placed on a Formvar-covered, carbon-coated grid. After 1 min the excess sample was washed from the vertically held grid with 2 drops of the staining solution. The liquid left on the grid was removed with filter paper, leaving the surface slightly wet. The grids were air dried. The samples for shadow casting (59-1 and 50-1) were deposited on grids in the same way except that double-distilled water was used in place of the staining solution. Single-angle and rotational shadow casting were performed with platinum–palladium (80:20)wire at an angle of approximately 5:1.

The micrographs were taken with an Elmiskop-1 electron microscope at a magnification of 36,700 at 80 kv. To measure the dimensions of the particles the pictures were printed at a total magnification of 202,000, and the diameters of the particles were read from the prints with a magnifying glass and a ruler marked in 0.5 mm divisions. With the single-angle shadow-cast pictures only particles whose long axes

appeared to be perpendicular to the direction of shadowing were used to calculate the mean dimensions. For each type of particle 50 measurements were averaged.

RESULTS

59S Subunits

On analytical ultracentrifugation sample 59-2 showed a main 59S boundary with very little faster or slower material. Analysis in the presence of EDTA, however, showed that 15% of the sample consisted of small subunits. In the electron micrographs, most of the unfixed, negatively stained subunits had almost round or slightly elongated polygonal forms (Fig. 1), with dimensions of 244 ± 17 × 207 ± 18 A (the mean ± 1 SD). The sizes fell within a fairly narrow range; 34% of the 50 measured particles had dimensions within 20 A of the mean. The preparation used for shadow

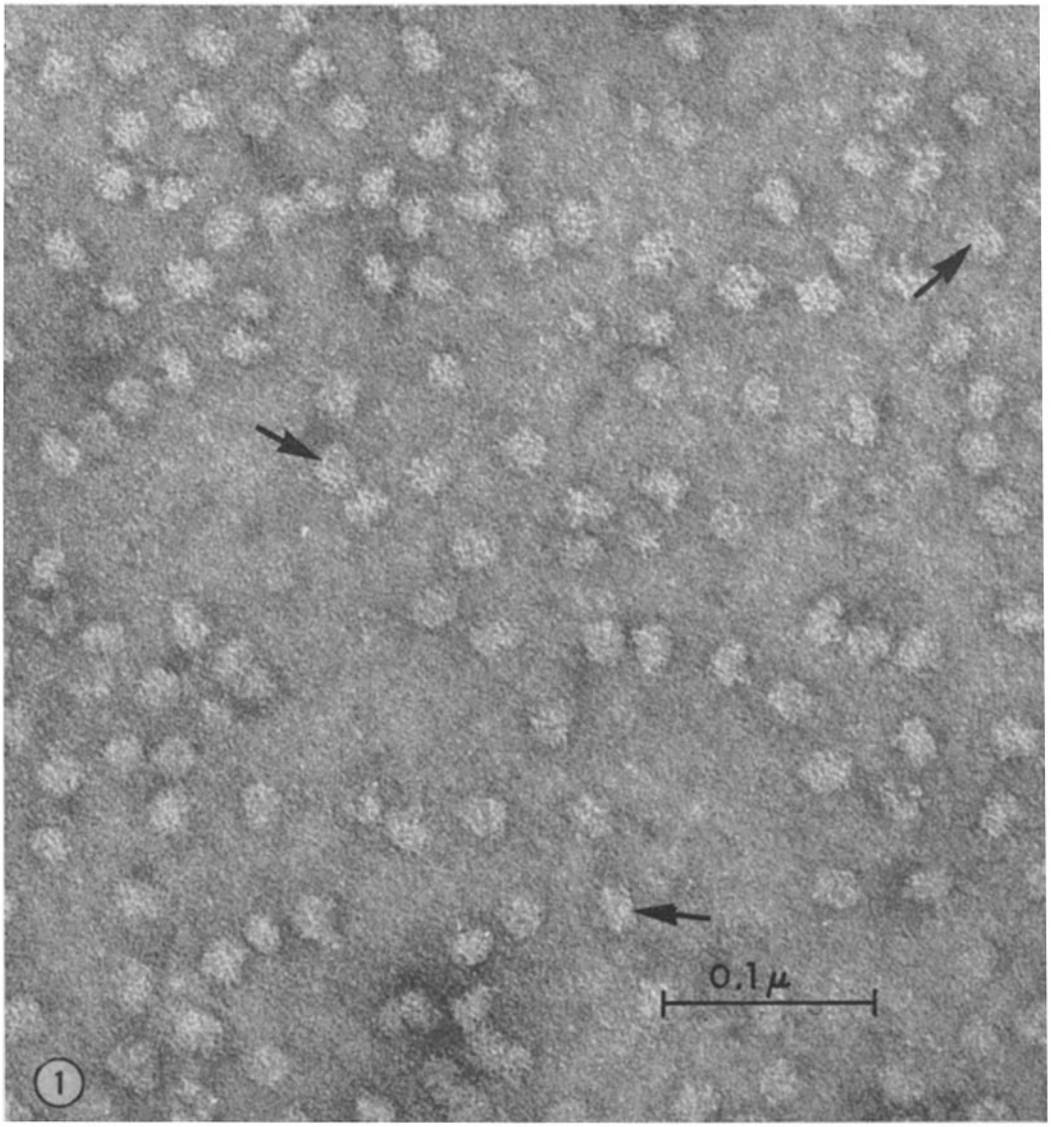

FIGURE 1 Unfixed 59S subunits, negatively stained with uranyl oxalate. Some particles (arrows) appear to show a small round structure in the center (see Discussion). × 280,000.

casting, 59-1, contained about 20% of small subunits. Its sedimentation coefficient was close to 59S and was not affected by fixation. Particles from the same fixed preparation were shadow cast rotationally (Fig. 2 *A*) and at a single angle (Fig. 2 *C*). Both the heavy metal deposits around the outlines of the particles in Fig. 2 *A* and the shapes of the shadows in Fig. 2 *C* suggested that the particles were relatively compact. The dimensions of the shadowed particles in Fig. 2 *A* were 370 ± 29 × 310 ± 31 A, and 42% of the 50 measured particles were close to the mean size. The unfixed, shadow-cast subunits (not shown) had similar shapes and dimensions. The average height of the particles, calculated from the lengths of the shadows in Fig. 2 *C*, was 95 ± 18 A. Some particles in Fig. 2 *A* had threadlike extensions. These particles were often smaller and more elongated than the usual 59S particles and the extensions were less sharply defined than those in Fig. 2 *B*. These particles may represent small subunits.

The Large Subunits in Whole Ribosomes

To study the large subunits further, whole ribosomes were examined. The monosome preparation consisted mainly of 82S ribosomes with 10% of faster and 10% of slower material. The unfixed, negatively stained, whole ribosomes are shown in Fig. 3. The variable size, shape, and location of the small subunits suggested that all the ribosomes might not be lying on the supporting film with the same side up. Some small subunits were also separated from the large ones. The large subunits in the whole ribosomes were in slightly elliptical or acorn forms and their shapes appeared somewhat more compact than those of the isolated 59S particles. One typical form showed the small subunit attached laterally or slightly tangentially to one of the long sides of the large subunit; the outline of the large subunit and the cleavage furrow between the small and large subunits were particularly noticeable (*t*). The average dimensions of the large subunits in these ribosomes were 242 ± 17 × 197 ± 17 A, close to the size of the isolated 59S subunits. The average dimensions of the whole ribosomes were 303 ± 18 × 256 ± 23 A.

50S Subunits

Preparation 50-1, which had been isolated and fixed in the presence of magnesium, had a sedimentation coefficient of 51S. After rotational shadow casting the particles showed vague outlines (Fig. 2 *B*) and appeared to be much less compact than the 59S subunits (Fig. 2 *A*) which had been shadowed at the same time. The most striking difference between the two forms was the appearance of threadlike extensions in the 50S particles. The shapes of the shadows obtained by single-angle shadow casting (Fig. 2 *D*) indicated that these particles were flattened; their calculated height was 77 ± 8 A, less than that of the 59S subunits (Fig. 2 *C*) which, again, had been shadowed simultaneously.

Unfixed 50S subunits prepared in the absence of magnesium (50-2) showed a single ultracentrifugal boundary with a sedimentation coefficient of 46S. Where all the excess uranyl oxalate was removed from the grids during the negative staining, the particles appeared to be positively stained. Fig. 4 *A* was taken from such an area. The particles showed a wide variety of sizes and irregularly outlined shapes with pointed corners. Again, the most striking feature of the 50S form was the appearance of the threadlike extensions. (When 59S subunits were positively stained in similar circumstances [Fig. 4 *B*], no such extensions appeared.) These extensions, which were stained positively, were of various lengths and widths, and the number of extensions per particle varied. The extensions of neighboring particles seemed to be easily tangled. Particles similar to the ones in Fig. 4 *A* had average dimensions (excluding the threads) of 330 ± 21 × 276 ± 33 A, with only 20% of the 50 measured particles being close to the average size.

No satisfactory negatively stained images of 50S particles were obtained. The negatively stained areas of the picture described above were unclear. A second sample of preparation 50-2 had a sedimentation coefficient of 52S in buffer containing 0.2 mM $MgCl_2$. Although it was monodisperse in the ultracentrifuge, it tended to aggregate and to break into fragments on the grid, and negative staining (not shown) revealed many deformed structures.

DISCUSSION

Our results with negative staining show that the 59S particles have a slightly elongated, ovoid form. The dimensions of the unfixed particles and the large subunits in the unfixed whole ribosomes were about the same. Furthermore, the average dimensions of the fixed subunits were similar, 245 ± 27

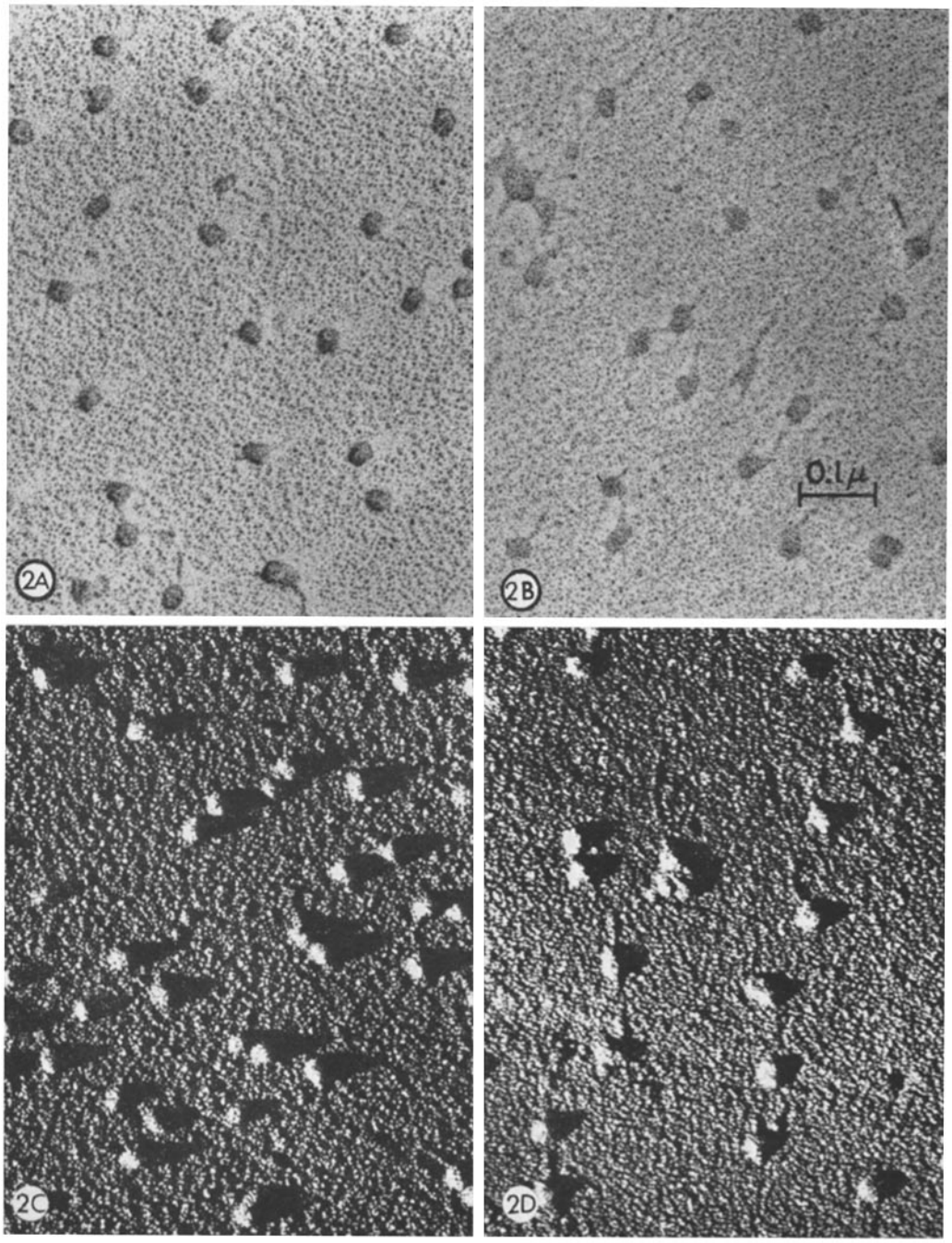

FIGURE 2 *A*, fixed 59S subunits; *B*, fixed 50S subunits, both with rotational shadow casting; *C*, fixed 59S subunits; *D*, fixed 50S subunits, both with single-angle shadow casting. Each pair, (*A* and *B*) or (*C* and *D*), was shadowed simultaneously. All × 110,000.

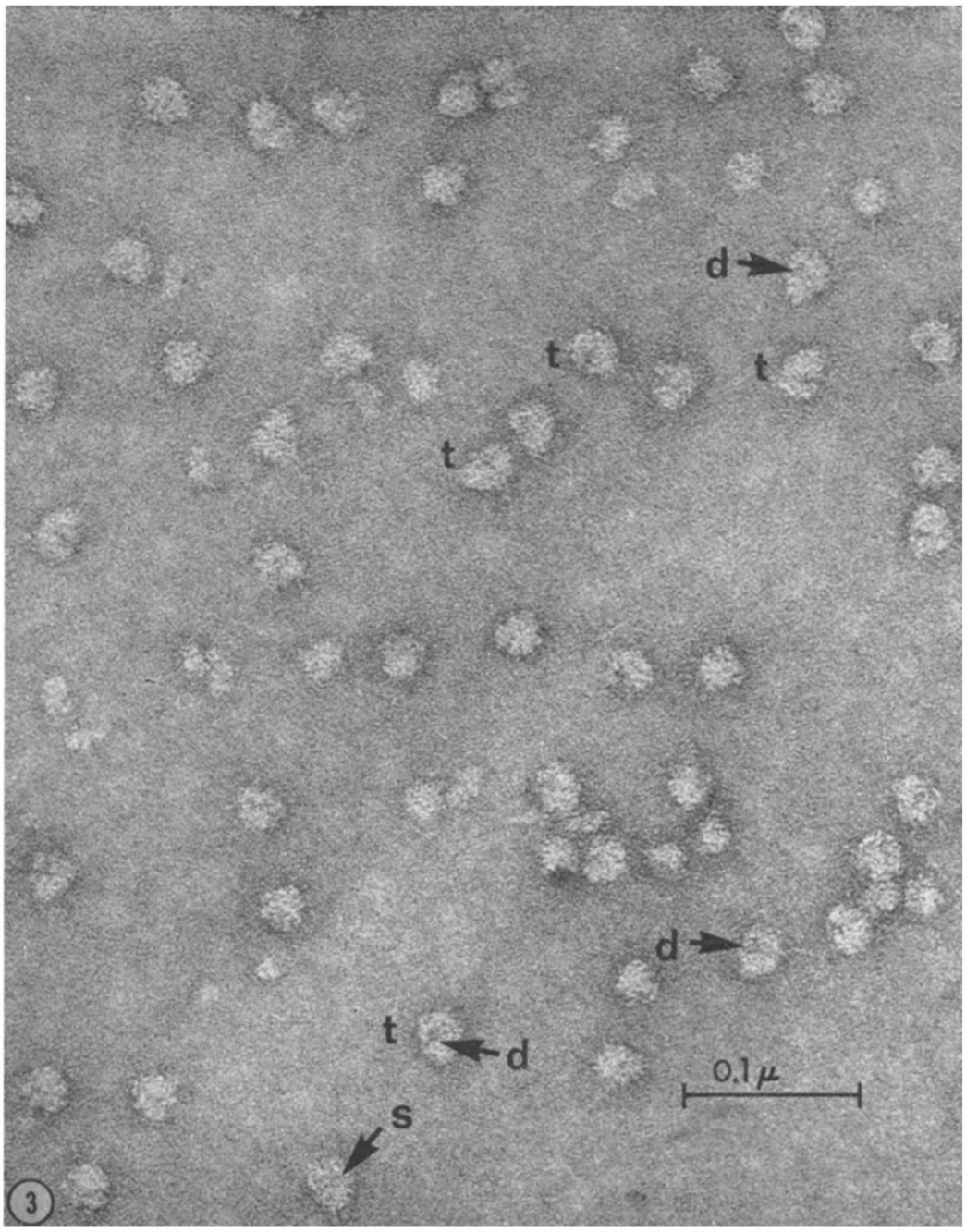

FIGURE 3 Unfixed whole ribosomes, negatively stained with uranyl oxalate. The particles designated by the letter *t* are one typical form, used for measuring the dimensions (see text). A small round structure in the central region of the large subunit (arrow-*s*) and a dark spot in the furrow (arrow-*d*) appear to be present (see Discussion). × 250,000.

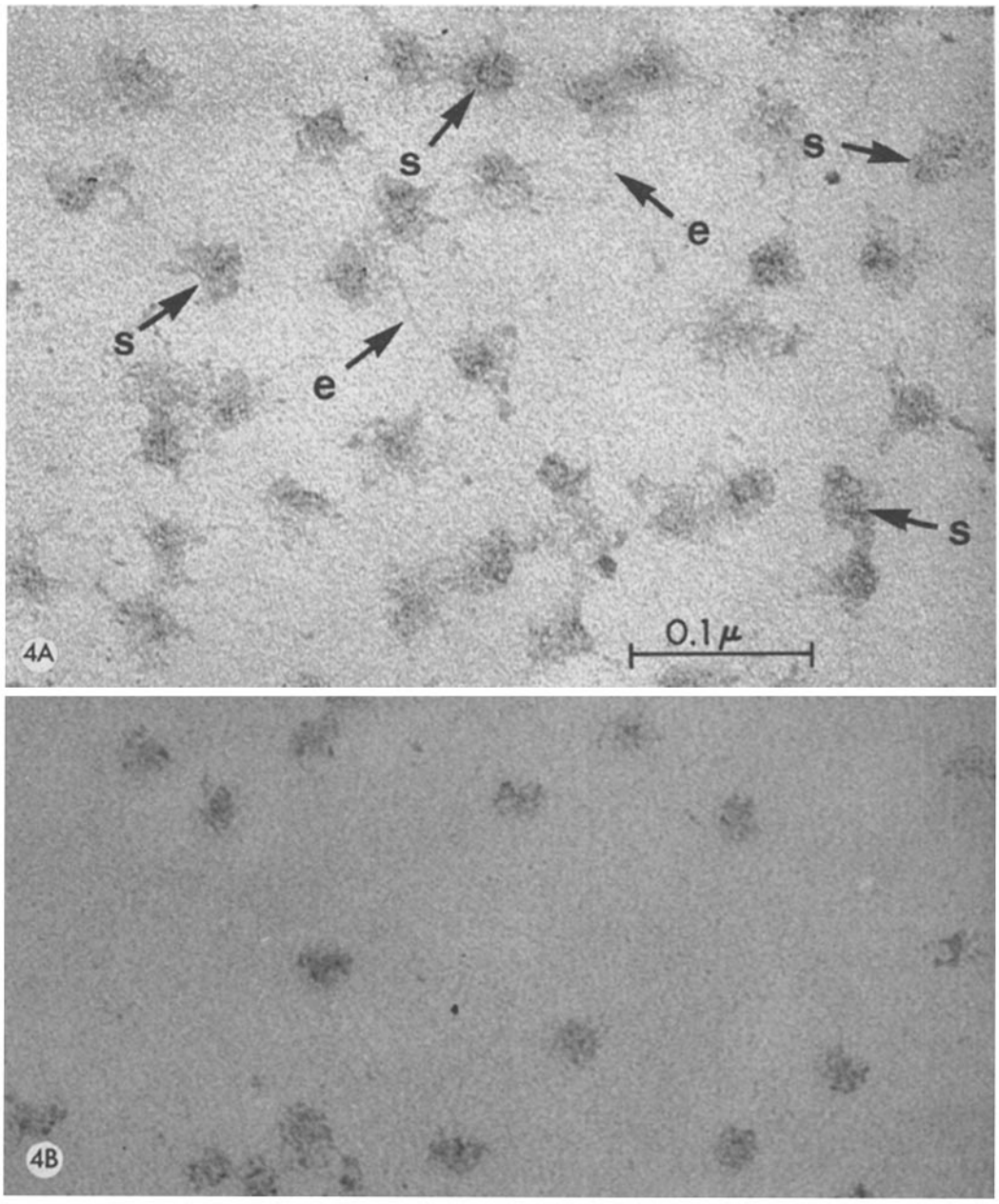

FIGURE 4 *A*, unfixed, magnesium-free 50S particles stained with uranyl oxalate. Only the positively stained areas of the negatively stained preparations showed threadlike extensions (letter *e*) and detailed structure. Some particles (arrow-*s*) appear to show a small round structure in the center (see Discussion). *B*, unfixed 59S subunits stained with uranyl oxalate (positively stained area). Both × 250,000.

× 206 ± 22 A (unpublished data). Thus the urea-treated particles are very close to the size and shape of the large subunits in whole ribosomes. That the isolated subunits are intact was also shown by their ability to incorporate phenylalanine in the presence of poly U (11).

The calculated height of the dried 59S subunit was low, approximately 95 A. This value was ob-

tained from the average lengths of the shadows in the single-angle shadowed pictures (Fig. 2 *C*). The distribution of these shadow lengths was very wide and discontinuous, with no discrete peak. Moreover, since it was difficult to distinguish the contaminating small subunits, some may have been included; this would decrease the average value. A more precise value might have been obtained if an internal standard had been used, and some correction had been introduced, to account for the penetration of the particles into the supporting film upon drying. Many shadows in Fig. 2*C* showed no curvature at the base. This suggests that the particles were lying flat on the supporting film and that they were not rounded, at least at the bottom. This flattening also contributed to the shortening of the shadow length. Hart (2) found that *E. coli* ribosomes that had been freeze-dried prior to shadow casting showed taller shadows than air-dried specimens.

The dimensions of the 59S subunit can also be calculated from the physical properties of the molecule in solution. From its molecular weight, 3.1×10^6 daltons (12), sedimentation coefficient, 58.7S (Petermann and Pavlovec, unpublished data), and partial specific volume, 0.63 cc/g (12), we calculate (15) a frictional ratio, f/f_0, of 1.86. When the ribosomes begin to dissociate the first subunit to appear is a 60S particle. If this represents a large subunit plus one or more transfer RNA's and a nascent peptide, its molecular weight would also be slightly higher, about 3.2×10^6 daltons, and its frictional ratio would be the same. The total frictional ratio, f/f_0, is the product of the frictional ratio due to asymmetry, $(f/f_0)_A$, and the frictional ratio due to hydration, $(f/f_0)_H$ (15). In a previous paper we assumed that the large subunit had an axial ratio of 1, and ascribed all of its high frictional ratio to hydration (16). The axial ratio obtained from the electron micrographs of the negatively stained particles, 1.18, corresponds to a frictional ratio of only 1.01, so $(f/f_0)_H$ is 1.85, and we must still assume that the hydration of the large subunit is unusually high, 3.3 g/g (Table I). Since the hydration of the small subunit seems to be much lower, 1.7 g/g, we suggested that the large subunit might contain a solvent-filled groove or channel (16). A channel has been postulated by Redman and Sabatini (17). Grooves have been found in electron micrographs of large subunits ("slit forms") from bacteria (6) and beans (18), but were not clearly visible in our liver particles.

From the hydrated volume of the particle in solution, and the axial ratio found by electron microscopy of negatively stained particles, the dimensions of possible models have been calculated. The height was set equal to the shorter dimension (Table I). The dimensions of an ellipsoid are the same as those of a half ellipsoid (beehive) shape, and 15% greater than those of an elliptic cylinder. Similar calculations have been made for an anhydrous particle.

From the dimensions of the negatively stained particle, and a height equal to that of the smaller dimension, the volumes of the corresponding cylinder and ellipsoid have also been calculated. Both sets of values, for cylinder and ellipsoid, are much closer to the anhydrous than to the hydrated volumes. Perhaps, during the drying process, the particle shrank before the uranium was completely deposited at the periphery. Further difficulties discussed by Shelton and Kuff (19), are flattening of the particles on the supporting film and variability in the amount of stain deposited around the individual particle. If the surface of the particle were irregular the proportion of the frictional ratio due to shape would be underestimated, and the calculated hydration and hydrated volume would be too high; but a similar computation for the whole ribosome, with $M = 4.7 \times 10^6$ and $\bar{v} = 0.63$ (calculated from unpublished data), an axial ratio of 1.29, and $s = 82S$, gave a hydration of 2.7 g/g, the same value as was found for the rabbit reticulocyte ribosome by a number of physical methods (20).

The anhydrous large subunit had a calculated volume of only 3.2×10^6 A^3 (Table I). The dried, shadow-cast particle had a volume, including the metal, almost three times as large as this; to get a volume of 3.2×10^6 A^3, one must assume that the metal is 65 A thick. The calculated dimensions (Table I) are still somewhat greater than those found for the large subunits of mouse liver ribosomes in osmium-fixed, dehydrated thin sections, 150–175 × 175–200 A (21).

The unfolding of the large subunits to 50S particles was accompanied by the formation of threadlike extensions. This observation was confirmed by two different techniques of shadow casting. Moreover, to insure that these extensions were not artifacts, blank grids were washed with the solvent and shadowed simultaneously with

TABLE I

The Size and Shape of the Large Subunit, as Calculated from its Physical Properties in Solution (Molecular Weight = 3.1 × 10⁶ daltons, $\bar{v}$ = 0.63, $s^0_{20,w}$ = 58.7S) or as Measured in the Electron Microscope.

Particle	Width/ length	Hydration	Volume	Length*	Width‡	Height	Shape
		g/g	$A^3 \times 10^{-6}$		A		
59S							
Hydrodynamic particle	(1.18)§	3.3‖	20.4	321	379	(321)¶	Ellipsoid
				280	330	(280)¶	Cylinder
Anhydrous particle	(1.18)§	0	3.2	173	204	(173)¶	Ellipsoid
				151	178	(151)¶	Cylinder
Electron micrograph, negative staining	1.18		5.5	207	244	(207)¶	Ellipsoid
			8.2	207	244	(207)¶	Cylinder
Electron micrograph, dried, shadowcast	1.19		8.6	310	370	95	Cylinder
			3.2	(180)**	(240)**	95	Cylinder
50S							
Electron micrograph, positively stained	1.20		8.2	276	330	(115)‡‡	Cylinder

* Parallel to the long axis of the ribosome.
‡ Parallel to the short axis of the ribosome.
§ From the dimensions of the negatively stained particles.
‖ Calculated from the frictional coefficient (see text).
¶ Assumed to be equal to the smaller lateral dimension.
** Assuming the metal deposit to be 65 A thick.
‡‡ Assumed thickness to give a volume of 8.2 × 10^{-6} A^3.

the specimens. The electron micrographs of the blank grids showed little background contamination and no threadlike forms. Structures of this type have occasionally been described by other workers. With rat-liver ribosomes threadlike extensions have been seen on both positively stained (7) and negatively stained (22) 50S particles. They have also been found on cesium chloride-treated large subunits of *B. subtilis* ribosomes (4), and Huxley and Zubay (1) have observed long threads, 10–20 A in diameter, on degraded 70S particles from *E. coli*.

The 50S particles have lost the 5S RNA but still have about the same molecular weight as the 59S subunits, 3.1 × 10^6 daltons (12). Since their sedimentation coefficients are low, 51S and 46S, their frictional ratios are correspondingly higher, 2.15 and 2.38. How much of these increases is due to hydration could not be determined. If $(f/f_0)_H$ were unchanged, 1.85, the $(f/f_0)_A$ values would be 1.16 and 1.29, corresponding to axial ratios of 4 and 6 for oblate ellipsoids. The uranium-stained 50S particles, however, have an axial ratio of less than 2; they are about 330 × 276 A, and are roughly similar to the 50S liver subunits of Dass and Bayley (7). Thus our 50S subunits do not resemble discs or rods, but show highly irregular structures with numerous projections that would greatly increase the frictional drag during ultracentrifugation. Starfish-shaped polymers also sediment very slowly (23).

Since the lateral dimensions of these particles are much greater than those of the 59S subunits, their height must be less. The shadows of the 50S particles (Fig. 2 *D*) appeared to be much shorter than those of the 59S subunits (Fig. 2 *C*), but the calculated difference between the heights was only 23%. If the volumes of the two stained particles are the same, the height of the 50S particles should be 115 A.

In some of the ribosomes the cleavage furrow between the subunits could be identified, but it was not as clearly visible as the furrow in the *E. coli* ribosome (1, 5). This may be because the liver ribosome contains more protein or, as shown in some of our electron micrographs, because the small subunit is wrapped closely around part of the large one. A similar suggestion was made by Shelton and Kuff (19) to explain their observations on tumor ribosomes.

Some additional fine structure was observed, but its interpretation proved difficult. Ribosomes stained with uranyl oxalate can be examined without formaldehyde fixation (6). This procedure reveals surface details such as small furrows, but may cause artifacts. Because of its affinity for RNA the uranyl salt may be concentrated in a region rich in RNA, and may give an electron-opaque area which could be interpreted as a negatively stained furrow or hole (24). There is no way to ascertain whether a furrow or hole penetrates the entire particle. Morever, since the electron microscope used in this study lacked an anticontamination device, deposits from contamination can not be ignored. Beam damage on the negatively stained preparations should also be considered in interpreting the electron micrographs. In every preparation described in this report (Figs. 1, 3, and 4), small round structures were observed in some of the large subunits. Because the background grains appeared similar, and because a limited range of magnification was used, it is difficult to ascertain if these structures are real. Similar forms have, however, been seen in several laboratories. Nanninga (4) described a small round area (40–60 A) in 50S subunits from *B. subtilis*. In the large subunits of *E. coli* ribosomes Lubin (6) found a central depression, and Bruskov and Kisselev (5) noted a 30–40 A hollow. Another small structure, described by Bruskow and Kisselev as a 30–40 A hollow, was found in the cleavage furrow of the *E. coli* ribosome (5). The same authors found a channel in the large subunit of the bean leaf ribosome (18). Miller et al. (24) observed an electron-opaque central dot in the furrow of the 70S chloroplast ribosome of tobacco leaves. Whether these forms are similar to the small round structure seen in our large subunit is not clear.

While electron microscopy is beginning to reveal the fine structure of the ribosome, many problems remain. With the 59S particles described in this paper, however, the electron microscopic observations support the incorporation studies and physical-chemical measurements in showing that the particles obtained by the urea method represent undamaged subunits.

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