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Composition of Mammalian Ribosomal Subunits: A Re-Evaluation

(RNA/protein/CsCl/aldehyde fixation)

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ABSTRACT **A method for preparation of highly active mammalian ribosomal subunits is described, which yields 60S subunits containing no more than 33% protein. It is suggested that the composition of these subunits corresponds closely to that of *Escherichia coli* 50S subunits. Data on the composition of bacterial and mammalian ribosomal subunits recovered from CsCl are given. It is shown that the commonly employed assumptions about the relationship between the buoyant density of a particle in CsCl and its protein content are in error. The composition of ribosomal subunits cannot ordinarily be calculated from the buoyant density in CsCl.**

The fundamental aspects of protein synthesis in eukaryotes appear to be virtually the same as in prokaryotes; but, surprisingly, eukaryotic ribosomes have often been reported to contain significantly more proteins than bacterial ribosomes (1–3). In this report I shall show that it is possible to prepare active mammalian ribosomal subunits in such a way that the large (60S) subunits contain the same number of proteins as the homologous 50S subunits of *Escherichia coli*. This leads to the suggestion that there is no fundamental difference in the composition of large ribosomal subunits of bacteria and mammals, although there is a marked difference in size.

Many studies on eukaryotic ribosomes and their derivatives have relied on the assumption that RNA/protein ratios can be calculated from equilibrium buoyant densities of the particles in CsCl (4–7). I demonstrate here that this assumption, as generally used, is wrong. Mammalian and bacterial ribosomal subunits retain less protein in CsCl than has been assumed by others, and subunits with widely different buoyant densities may have the same overall composition.

MATERIALS AND METHODS

Preparation of Ribosomal Subunits. Livers were obtained from male black C57 mice that had been starved overnight. HeLa cells were grown in Eagle's Minimum Essential Medium (8) in spinner culture containing 5% calf serum. The standard "high-salt" isolation procedure for ribosomal subunits was based on the method of Blobel and Sabatini (9). Livers or HeLa cells were homogenized in 10 mM Tris·HCl, pH 7.4, 10 mM KCl, 1 mM $MgCl_2$, 1 mM dithiothreitol, using 10 ml of buffer per gram of tissue. One-tenth volume of 3 M KCl, 20 mM $MgCl_2$ was added slowly to disrupt cytoplasmic aggregates and improve yield of ribosomes. Nuclei and mitochondria were removed by centrifugation at 15,000 × *g* for 15 min. The supernatant was then adjusted to 0.5% Brij 58 and 0.5% sodium deoxycholate. Ribosomes were sedimented at 40–50,000 rpm overnight in the Type 65 Beckman rotor through a cushion consisting of 1.75 M sucrose, 50 mM Tris·HCl, pH 7.4, 100 mM KCl, 1 mM $MgCl_2$, 1 mM dithiothreitol. Ribosome pellets were suspended in cushion buffer lacking sucrose and treated with 5×10^{-4} M puromycin for 15 min at 37°. Subunits were then separated on 10–30% sucrose gradients containing 500 mM KCl, 5 mM $MgCl_2$, 10 mM Tris·HCl, pH 7.4, and 1 mM dithiothreitol. Subunits were harvested by centrifugation, if *in vitro* activity was to be measured, or by precipitation with one volume ethanol (after raising the Mg^{2+} concentration to 10 mM) or 10% trichloroacetic acid, depending on the analyses to be done subsequently.

Mouse liver ribosomes and EDTA-treated subunits were also prepared by the technique of Hamilton and Ruth (5). *E. coli* 70S ribosomes were prepared by Larry Gold, as described by Gold and Schweiger (10). Subunits from these ribosomes were separated on sucrose gradients containing 500 mM KCl as detailed above.

RNA and protein were measured by the orcinol (11) and Lowry (12) techniques, respectively. Purified yeast RNA, calibrated against ribose, served as a standard for the orcinol assay; corrections were made for differences in the purine content of the various ribosomal RNAs that were measured. Bovine-serum albumin was the standard for the Lowry protein assay; it was assumed that 1 mg/ml gives $A_{279} = 0.677$.

Poly(U)-dependent activity of ribosomal subunits *in vitro* was assayed as described by Falvey and Staehelin (13). Endogenous phenylalanine concentration in the assay mixture was estimated by isotope dilution experiments. The formation of salt-stable 80S monomers was measured on sucrose gradients containing 0.3 M KCl (13).

Radioactive ribosomes were prepared by labeling HeLa cells for 4 doubling times with [^{3}H]uridine (New England Nuclear no. 174) and a mixture of 15 [^{14}C]amino acids (New England Nuclear no. 445).

CsCl Gradients. Subunits were fixed in 6% formaldehyde, 50 mM triethanolamine·HCl, pH 7.6, 100 mM KCl, 1 mM $MgCl_2$ for 2–3 hr, then dialyzed overnight against 1% formaldehyde, 25 mM triethanolamine·HCl, pH 7.6, 50 mM KCl (7). CsCl (Harshaw optical grade) was dissolved in the latter buffer, and preformed linear gradients were prepared with the sample contained in the lighter CsCl solution. Gradients were usually made from solutions having densities (ρ) of 1.5 and 1.7 g/cm^3 in the case of mammalian subunits; for *E. coli* subunits the density range was 1.55–1.75 g/cm^3. Gradients of approximately 6 ml total volume were overlaid with mineral oil and centrifuged in the Beckman SW41 rotor at 30,000 rpm for 16–20 hr at 25°. Fractions were collected through a no. 22 gauge needle inserted into the bottom of the centrifuge tube. Refractive indices were read on every fourth fraction;

TABLE 1. *Composition of ribosomal subunits from sucrose gradients*

Source	Ribosomal particle	Preparation	% Protein
E. coli	Small subunit	0.5 M KCl	34
E. coli	Large subunit	0.5 M KCl	31
E. coli	70S	Gold & Schweiger (10)	33
Mouse liver	Small subunit	0.5 M KCl	44
Mouse liver	Small subunit	EDTA	49
HeLa	Small subunit	0.5 M KCl	44
HeLa	Small subunit	1.0 M KCl	44
Mouse liver	Large subunit	0.5 M KCl	33
Mouse liver	Large subunit	EDTA	45
HeLa	Large subunit	0.5 M KCl	33
HeLa	Large subunit	1.0 M KCl	33

TABLE 2. *Activity of subunits in poly(U)-directed polyphenylalanine synthesis*

Source	pmol of subunits 40S	pmol of subunits 60S	pmol of Phe incorporated	Phe polymerized/limiting subunit
HeLa	16	32	293	18
	32	16	316	20
	16	0	12	<1
	0	16	26	1.6
Mouse liver	16	32	510	32
	32	16	483	31
	16	0	14	<1
	0	16	47	2.9

Assays were carried out according to Falvey and Staehelin (13) using supernatant and pH 5 fraction from mouse liver in all cases.

in converting refractive indices to densities, account was taken of the refractive index contribution of the buffer.

RESULTS

Composition of Subunits from Sucrose Gradients. Ribosomal subunits from various preparations were separated on sucrose gradients, collected by ethanol precipitation and assayed for RNA and protein as described in *Methods*. Table 1 summarizes the data obtained in this study. Some of the measurements in Table 1 serve as controls, to validate the colorimetric assays. The EDTA subunits from mouse liver are the most pertinent. The methods described by Hamilton and Ruth (5) for the preparation of EDTA subunits from rat-liver ribosomes were followed in detail; the composition of mouse-liver EDTA subunits was found to be almost identical to the published values for rat-liver subunits. Measurements were also made on *E. coli* ribosomes. The protein content of 70S ribosomes was found to be identical to that reported by Kurland (14); the protein content of 30S and 50S subunits departed slightly from published values (15, 16). The differences probably reflect the different preparation techniques employed.

The principal conclusion to be drawn from Table 1 is that the large subunit of HeLa and mouse-liver ribosomes can be prepared with significantly lower protein content than has been previously reported. This is achieved with the modified Blobel and Sabatini (9) procedure detailed in *Methods*, which employs 0.5 M KCl in the sucrose gradients. The use of 1.0 M KCl does not further reduce the protein content. The difference between the HeLa subunits described here and the active rat liver subunits prepared with urea by Hamilton *et al.* (1) is particularly striking (33% protein versus 45% protein). As explained in the *Discussion*, the lower value leads to a totally different view of the composition of these subunits.

The small subunits from HeLa cells and mouse liver reported in Table 1 have significantly less protein than the urea-treated small subunits from rat liver described by Hamilton *et al.* (1) which had 55% protein; but they are probably not significantly different from the reticulocyte subunits with 48% protein described by King *et al.* (17), who also used 0.5 M KCl to separate subunits.

Activity of Subunits. Ribosomal subunits from HeLa cells or mouse liver, prepared with 0.5 M KCl or 1.0 M KCl as described above, actively support the poly(U)-dependent polymerization of phenylalanine. The highest activities reported in Table 2 are actually somewhat higher than the best activities thus far reported for mammalian ribosomes (13). In addition, it can be shown that at least 50% of each subunit population is capable of forming active 80S ribosomes in the presence of excess subunits of the complementary class (Fig. 1). This is comparable to the results of Falvey and Staehelin (13). These observations provide assurance that the relatively low protein content I report here for 60S subunits is not due to inactivation of the particles.

Buoyant Densities of Subunits. Ribosomal subunits from *E. coli*, mouse liver and HeLa cells were fixed with formaldehyde and centrifuged to equilibrium in CsCl density gradients. These gradients showed that each subunit was a homogeneous density class. It follows that inactive subunits, if present, either constituted a negligible proportion of the total, or did not differ significantly from active subunits in buoyant density. The CsCl gradients also showed that contamination of 60S subunit preparations by 40S subunits, and vice versa, was insignificant.

Table 3 summarizes the buoyant densities measured at band center, ρ_0, for the various subunits. *E. coli* and mouse-liver EDTA subunits again serve as controls; the observed densities agree closely with previous reports (5, 7), except for the small EDTA-treated liver subunit. Hamilton and Ruth (5) reported $\rho_0 = 1.59$ for the comparable rat-liver subunit, whereas I find $\rho_0 = 1.56$. The buoyant densities of the large subunits from HeLa cells and mouse liver prepared with high salt agree with one another, and differ from the buoyant densities of *E. coli* subunits and EDTA-treated liver subunits. In all cases, the small subunits have a markedly different buoyant density from the corresponding large subunits. The small subunits of EDTA-treated liver ribosomes, however, do not band at a density significantly different from KCl-treated liver subunits.

TABLE 3. *Composition and buoyant densities of ribosomal subunits in CsCl*

Ribosomal particle	Treatment*	% protein† Before CsCl	% protein† After CsCl	ρ_0†, g/cm³
E. coli small subunit	0.5 M KCl	34	26	1.63
E. coli large subunit	0.5 M KCl	31	23	1.67
Mouse liver small subunit	0.5 M KCl	44	39	1.55
Mouse liver large subunit	0.5 M KCl	33	23	1.64
Mouse liver small subunit	EDTA	49	30	1.56
Mouse liver large subunit	EDTA	45	23	1.60
HeLa small subunit	0.5 M KCl	44	39	1.55
HeLa large subunit	0.5 M KCl	33	22	1.64

* See *Methods* for full details.

† All entries represent the average of three or more determinations. Values for percent protein have been rounded off to the nearest whole number; values for ρ_0 have been rounded off to the nearest 0.01 g/cm³.

Composition of Subunits Recovered from CsCl. Subunits were recovered from CsCl gradients by precipitation with ethanol or trichloroacetic acid and analyzed by colorimetry (see *Methods*). It was necessary to establish that aldehyde fixation did not alter the reactivity of RNA or ribosomal protein in the orcinol or Lowry assays, respectively. Lerman *et al.* (18) had already stated that "the fixation of protein by formaldehyde did not significantly interfere with the Lowry determinations." Table 4 confirms and extends their observation. It is shown for both *E. coli* and HeLa ribosomes that formaldehyde treatment, either alone or in combination with exposure to CsCl, has no effect on the measurements of RNA and protein content. It was also observed that the ratio of orcinol color to A_{260} was the same for fixed and unfixed ribosomes.

The results of the colorimetry on ribosomal subunits recovered from CsCl were surprising. As shown in Table 3, all subunits recovered from CsCl had less protein per unit of RNA than the corresponding inputs, i.e., the pooled sucrose gradient peak fractions. This was true for *E. coli*, for EDTA-treated mouse-liver ribosomes, for KCl-treated mouse-liver ribosomes and for KCl-treated HeLa ribosomes. In no case was the protein content as high as would be expected from the literature. The reasons for this apparent anomaly are considered in the *Discussion*.

At this point, it is essential to establish that the results are not in error. It was demonstrated above that colorimetric measurements for RNA and protein are done correctly in this laboratory, as shown in Table 1, and that these measurements can be applied to formaldehyde-fixed ribosomes. Table 4 shows the results of an additional control. Lines 7 and 8 compare the composition of HeLa 60S ribosomes determined in two ways: (*1*) by colorimetry on ribosomes recovered from CsCl, and (*2*) by calculation of RNA and protein contents from the isotope ratio of a CsCl gradient peak containing doubly-labeled ribosomes. In the latter case, RNA was uniformly labeled with [³H]uridine and proteins were uniformly labeled with [¹⁴C]amino acids. The cpm/μg of RNA and cpm/μg of protein were determined on unfixed ribosomes. Since the RNA/protein ratio determined by isotope ratio agrees with that determined by direct colorimetry, it is further established that none of the operations involved in fixation, centrifugation or recovery of subunits from CsCl affects the results of the orcinol or Lowry assays.

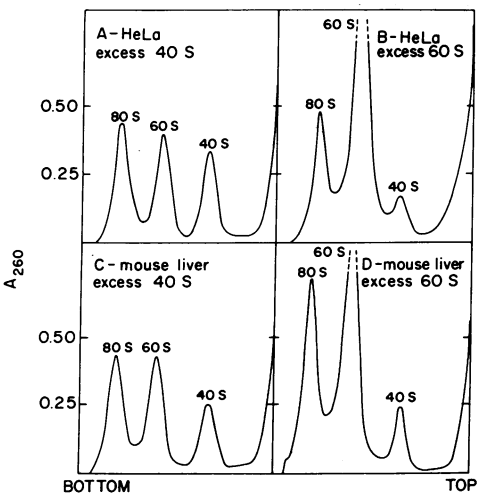

FIG. 1. Ribosomal subunits were prepared as described in *Methods*, using 0.5 M KCl. Subunits were mixed at a ratio of 2 40S: 1 60S (*A* & *C*) or 1 60S: 2 40S (*B* & *D*) and assayed for the formation of stable 80S monomers on sucrose gradients containing 0.3 M KCl (13).

TABLE 4. *Protein content of ribosomes after various treatments*

Ribosomal particle	Treatment	% Protein	Method of analysis
E. coli 70S	Unfixed	33.4	Colorimetry
E. coli 70S	Fixed 24 hr	34.2	Colorimetry
E. coli 70S	Fixed 24 hr, then CsCl 24 hr (not centrifuged)	33.6	Colorimetry
HeLa 60S	Unfixed	33.3	Colorimetry
HeLa 60S	Fixed 24 hr	33.9	Colorimetry
HeLa 60S	Fixed 24 hr, then CsCl 24 hr (not centrifuged)	33.0	Colorimetry
HeLa 60S	Fixed (HCHO, dialysis, no Mg^{2+}), recovered from CsCl	22.4	Colorimetry
HeLa 60S	Fixed (HCHO, dialysis, no Mg^{2+}), recovered from CsCl	22.8	Isotope ratio

Different laboratories have employed various methods for fixation of ribosomes and preparation for CsCl gradient centrifugation. Additional control experiments showed that the composition of HeLa ribosomes at equilibrium in CsCl was not affected by the presence of 1 mM Mg^{2+} during dialysis and centrifugation, nor by the use of glutaraldehyde instead of formaldehyde, nor by the omission of the dialysis step. The last case refers to the method of Baltimore and Huang (19).

Another significant result is documented in Table 3, where it is shown that different subunits with essentially the same RNA/protein ratio have different equilibrium buoyant densities. For example, the large subunits of *E. coli* ribosomes, EDTA-treated mouse-liver ribosomes, and KCl-treated liver or HeLa ribosomes all contain 22–23% protein at equilibrium in CsCl, but their buoyant densities are 1.67 g/cm^3, 1.60 g/cm^3 and 1.64 g/cm^3, respectively. The consequences of this fact are discussed below.

DISCUSSION

Comparison of Bacterial and Mammalian Ribosomal Subunits. There is excellent evidence that the large subunits of all eukaryotic ribosomes contain an RNA molecule significantly larger than the homologous 23S RNA of prokaryotes (20). The difference is maximal when *E. coli* and mammals are compared. The 23S RNA of *E. coli* is 1.05×10^6 daltons (21) whereas the 28S RNA of mammals is at least 1.7×10^6 daltons (22) and may range as high as 1.9×10^6 daltons (23). Estimates of the protein content of mammalian ribosomal subunits have seldom been based on direct analyses. The most widely quoted measurements are those of Hamilton *et al.* (1, 5) who found that rat-liver large subunits contain 45% protein, whether dissociation of ribosomes was achieved with urea or EDTA. (These data and the rest of this section refer only to subunits isolated from sucrose gradients.)

A particle consisting of 45% protein, with total RNA equivalent to 1.7×10^6 daltons, contains 1.4×10^6 daltons of protein. By contrast, the 60S subunits of mouse-liver and HeLa ribosomes reported in this communication contain only 33% protein, which implies 0.85 to 0.95×10^6 daltons of protein, corresponding to 28S RNA's of 1.7 to 1.9×10^6 daltons, respectively. Bickle and Traut (3) have published a number-average molecular weight ($\bar{M}_n$) of 27,000 for ribosomal proteins from mouse 60S subunits. Dividing the total daltons of protein per particle by 27,000 leads to the conclusion that the 60S subunits of Hamilton *et al.* (1, 5) contain, on the average, 52 proteins. The 60S subunits reported in this paper contail 32–35 proteins, on the average.

I have shown above that ribosomal subunits prepared as described herein are highly active, by the crude but conventional criterion of their ability to polymerize phenylalanine in the presence of poly(U). I therefore suggest that proteins in excess of a total of 900,000 daltons associated with the 60S subunit are not essential proteins, i.e., are not required for the formation of peptide bonds *per se*. These excess proteins probably have multiple origins: contaminants, initiation factors, modulators of unknown function, etc.

In view of the above, it is now permissible to entertain the hypothesis that the increase in size of eukaryotic large ribosomal subunits during evolution has *not* been accompanied by a major change in the composition of the subunits. The *E. coli* 50S subunit, when extensively salt-washed, contains at most 34 proteins (16, 24). The 60S subunit of mouse-liver and HeLa cell ribosomes contains 32–35 proteins. The RNA and protein components show a proportionate phylogenetic size change (mouse 28S RNA/*E. coli* 23S RNA = 1.62; mouse ribosomal proteins/*E. coli* ribosomal proteins = 1.59).

The 40S mouse and HeLa subunits reported here contain approximately 45% protein, or 570,000 daltons, taking the molecular weight of 18S RNA to be 0.7×10^6 (20, 23). This amount of protein is comparable to that reported by King *et al.* (17) for rabbit reticulocyte subunits, but less than the protein content of rat-liver subunits dissociated by EDTA or urea (1, 5). Bickle and Traut (3) give 29,000 for $\bar{M}_n$ of mouse proteins from 40S subunits. Using their number, I calculate that the HeLa and mouse-liver 40S subunits reported here contain an average of 20 proteins. This can be compared with an average of 13–15 proteins associated with each 30S subunit of *E. coli* (25). The two numbers appear to be significantly different.

It is important to recognize that the average number of proteins per particle need not be the same as the total number of different proteins associated with a population of subunits; for example, there are 21 proteins associated with *E. coli* subunits, but not all of them are present on every particle at a given moment (25). The calculations presented here for mammalian subunits are average numbers; they are not to be compared with the numbers of proteins separable by two-dimensional gel electrophoresis (e.g., ref 26, which contains an excellent summary of the earlier literature).

Ribosome Composition and CsCl. Spirin *et al.* (27) introduced the use of formaldehyde fixation to stabilize ribosomes against the dissociative effects of CsCl and showed that the buoyant densities of *E. coli* ribosomes and their derivatives were inversely correlated with protein content. Perry and Kelley (4) proposed a formula relating the RNA and protein content of ribosomal particles to their equilibrium buoyant density in CsCl, and Hamilton (6) has published a graph showing a linear relationship between percent protein and the reciprocal of the buoyant density. Both relationships have been widely used to calculate the protein content of ribosomal particles from diverse sources (e.g., 2, 7, 28).

I have shown above that the actual protein content of ribosomal subunits recovered from CsCl is much less than predicted by the formula of Perry and Kelley (4) or Hamilton's graph (6). The conflict implied by these observations actually does not exist. It is an astonishing fact that an extensive series of measurements on the composition of eukaryotic ribosomal subunits recovered from CsCl has never before been published! *E. coli* ribosomes have been subjected to such analysis (18, 27) and there is one recent report by Hirsh *et al.* (31) on Ehrlich ascites tumor cell ribosomes, in which the composition of the particles from CsCl gradients was measured by isotope ratios. Otherwise, it has apparently been universally assumed that the protein content of ribosomal particles purified on sucrose gradients would be completely conserved during aldehyde fixation and CsCl gradient centrifugation. That assumption is incorrect, as first shown by Lerman *et al.* (18) and documented here in Table 3.

Perry and Kelley's formula and the extrapolations in Hamilton's graph were apparently based on the expectation that the buoyant density of a ribonucleoprotein would be a simple additive function of the buoyant densities of the RNA and protein components. Ribosome investigators may be

dismayed to learn that the actual situation is much more complex, but physical chemists will not be surprised. The behavior of such well known proteins as bovine-serum albumin in CsCl is rather complicated (29), and the buoyant density of the bacteriophage lambda has been shown to be quite sensitive to the preferential hydration of the DNA (32), which varies with the water activity of the surrounding solution. Therefore, the buoyant densities of the separated components of a nucleoprotein do not necessarily correspond to the buoyant densities of those components when the nucleoprotein is at equilibrium in a salt gradient. The assumption of additivity of nucleic acid and protein buoyant densities is not strictly accurate unless the buoyant density of each component at the banding density of the particle is known (32, 33). In ribosomes the effects of macromolecular interactions on hydration, ion binding and other factors are virtually unknown. In addition, aldehyde fixation can be expected to affect the buoyant behavior of the particles; its significance has not been assessed.

Any or all of the factors mentioned above may be responsible for the fact that several subunits with equal RNA/protein ratios were found to have different buoyant densities in CsCl (Table 3). Although this places restrictions on the usefulness of CsCl as a tool for the study of ribosome composition, it need not mean that the technique should be abandoned. A simple relationship between ρ_0 and composition may exist for particles in a given preparation, as suggested by the data of Rosbash and Penman (30) for example. Each system will have to be calibrated empirically.

Several questions are raised by the observation that many ribosome-associated proteins cannot be fixed to the subunits in a CsCl-resistant condition. Space does not permit an adequate discussion, but the following points should be noted. First, there is no theoretical reason to assume that every protein in a particle should be so close to another protein that bridges can be formed between the appropriate reactive groups by formaldehyde or glutaraldehyde. It is therefore possible that some essential ribosomal proteins are lost during fixation. Second, there is no reason to assume that all contaminants of ribosomes cannot be fixed to the particles. Therefore, we do not yet know whether CsCl gradient centrifugation can be used as a test for purity, although this may turn out to be true in some situations. Finally, a large fraction of the published values for the composition of eukaryotic ribosomal subunits must be re-evaluated.

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