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# Size Changes in Eukaryotic Ribosomes

**(diffusion constant/sedimentation constant/ribosomal dissociation/chick embryo)**

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**ABSTRACT Evidence is presented that ribosomes active in protein synthesis and attached to messenger RNA on polysomes have a smaller diameter than free cytoplasmic single ribosomes. Measurements have been made on these two types of ribosomes of differences in sedimentation velocity and diffusion constant. Differences in these quantities suggest about a 20-Å decrease in the diameter of the ribosomes from chick embryo muscles when they are attached to messenger RNA. Similar differences are also observed in rabbit reticulocytes and mouse ascites tumor cells. These two ribosomal states have different sensitivity to Pronase digestion and dissociate into ribosomal subunits at different KCl concentrations. This size difference is not associated with a significant difference in overall ribosomal mass and appears not to be dependent upon the presence of nascent polypeptide chains.**

The ribosome is a cellular organelle containing proteins and RNA that has the role of translating messenger RNA into polypeptide sequences during proteins synthesis. A great deal is known about many of the chemical steps that occur during protein synthesis. However, little is known about the actual changes in the ribosome that ultimately make this process possible. For some time it has been known that the ribosome engaged in protein synthesis on a polysome has somewhat different properties from the free single ribosome found in the tissue cytoplasm that is inactive in protein synthesis. For example, in a study of the effects of proteolytic digestion on polysomes it was shown that single ribosomes are more sensitive to proteases (1). Recently a number of observations have been made with *Escherichia coli* ribosomes that indicate that there are changes in the sedimentation and tritium-exchange properties of polysomal ribosomes depending upon their stage with respect to the translocation step in protein syntheses (2, 3). Further, it has been reported that *E. coli* single ribosomes sediment more slowly in sucrose gradients than do ribosomes obtained by ribonuclease digestion of polysomes (4). However, the recent demonstration of pressure-induced ribosomal dissociation in sucrose gradients suggests that one should be cautious in intepreting changes in sedimentation velocity in terms of ribosomal conformation (5, 6). It has been reported in yeast (7) that single ribosomes are more sensitive to dissociation by KCl than are polysomes. In the present paper, we show that there is a substantial difference in physical properties between ribosomes derived from eucaryotic polysomes and the free ribosomes of the cytoplasm. In chick-embryo muscle tissue, polysomal ribosomes sediment 9% more rapidly than free single ribosomes, and have a diffusion constant that is also 9% greater. Using these measurements, we show that the molecular weights of these two particles are similar. However, these changes suggest that when the ribosome is attached to messenger RNA it has a smaller diameter than is found for the free cytoplastic ribosome. This more compact form of the ribosome is maintained even when the nascent polypeptide chain is relased by puromycin. We thus infer that there are substantial differences in the interactions between the ribosomal subunits when they are attached to messenger RNA as compared to the free cytoplasmic single ribosome, which is inactive in protein synthesis.

## METHODS AND MATERIALS

*Preparation of Ribosomes.* Ribosomes were prepared from the leg and thigh muscles of 14-day-old chick embryos (White Leghorn; Spafas Inc., Norwich, Conn.). Cytoplasmic ribosomes were obtained by centrifugation of cytoplasmic extracts from embryonic muscle (8) through 35 ml of a 10–30% linear sucrose gradient. The buffer (MSB) contained 0.01 M Tris (pH 7.4)–0.25 M KCl–0.01 M $MgCl_2$. Centrifugation was generally for 11 hr at 26,500 rpm and 4°C with a Beckman SW 27 rotor in an L2-65 centrifuge. Particular ribosomal fractions were isolated by collecting them from the sucrose gradient and pelleting by centrifugation at 45,000 rpm for 6 hr at 4°C in a Beckman Ti50 rotor. The pellet was resuspended in MSB buffer by gentle shaking at 4°C and centrifuged through another 10–30% sucrose gradient. The twice-purified fraction was again pelleted, and then resuspended for physical studies. Ribosomes were prepared from polysomes either by digestion of polysomes with low concentrations of RNase, or by fractionation from cytoplasmic extracts ground with alumina. The polysomes for RNase digestion were routinely prepared by the method of Heywood *et al.* (9). Such preparations were typically free of cytoplasmic ribosomes. Ground cytoplasmic extracts were prepared by extensively grinding muscle tissue with an equal weight of alumina (Norton Co., Worcester, Mass.) at 4°C, suspending the mix in an equal weight of MSB buffer, and pelleting out the alumina by centrifugation at 5000 × *g* for 10 min. The ribosomes from both kinds of preparations were fractionated and prepared for study exactly like cytoplastic ribosomes.

Nascent polypeptide chains were labeled by injection of the chick-embryo blood vessels as described (10). A [$^{14}$C]-aminoacid mixture was used (New England Nuclear Co.) and 10 μCi was usually injected. Puromycin (Nutritional Biochemicals Co.) was sometimes added to the injection mixture. The materials were analyzed on sucrose gradients as described below. Fractions were collected and, after the addition of 50 μg of bovine serum albumin to each fraction, the material was precipitated with 3 ml of cold 10% trichloroace-

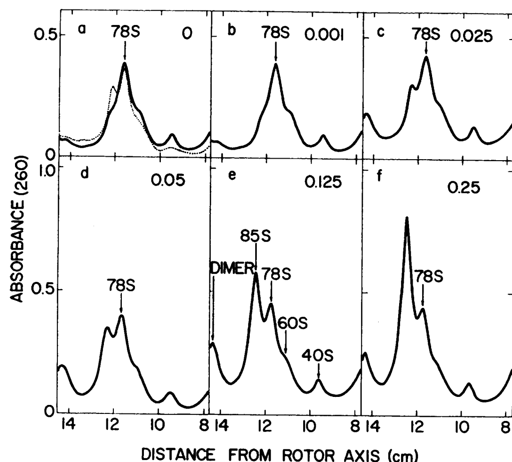


FIG. 1. Sucrose density gradient centrifugation of ribosomal particles showing the effect of ribonuclease digestion on cytoplasmic extracts of 14-day-old chick-embryo muscle. The cytoplasmic extracts were incubated for 10 min at 24°C with the amounts of RNase (in $\mu$g/ml) indicated in the *upper right corner* of each frame. 0.2-ml aliquots, containing 8 $A_{260}$ units, were layered onto 13 ml of 10–30% linear sucrose density gradients (MSB buffer), and centrifuged for 4 hr at 4°C in an SW41 rotor at 39,500 rpm. Fig. 1*a* has the sedimentation profile of an alumina-ground cytoplasmic extract superimposed as a *dotted line*.

tic acid. The samples were heated to 90°C for 20 min, cooled, and filtered onto Millipore filters. The dried samples were counted in a Nuclear Chicago low-background gas-flow counter with a background of 2.0 cpm. In some experiments, nascent polypeptide chains were digested with Pronase as described (1). Ribosomes and polysomes were also prepared from rabbit reticulocytes and from mouse ascites tumor tissue. The methods used were similar to those described for chick-embryo tissue.

CsCl banding studies were performed according to the method described by Leick *et al.* (11). Diffusion constants were measured in collaboration with L. Hocker and G. Benedek by the use of a self-beat laser spectrometer. The construction and the theory for operation of the self-beat laser spectrometer have been described (12). A 1-ml sample was generally used, containing 50 $\mu$g of ribosomes in MSB buffer. Sedimentation constant measurements were done on a Spinco model E analytical ultracentrifuge operating at 19,160 rpm with ultraviolet optics. The ribosome concentration was 50 $\mu$g/ml in MSB buffer. All sedimentation and diffusion-constant measurements were made at 24–26°C. Polyacrylamide gel analyses were performed on ribosomal RNA and ribosomal proteins as described (10, 13).

## RESULTS

In this paper we compare the properties of single ribosomes that are not active in protein synthesis with ribosomes obtained from polysomes. Two methods have been used for freeing ribosomes from polysomes. One involves alumina grinding, which is known to sever the messenger RNA strand by mechanical shearing. The other method involves the use of low concentrations of ribonuclease, which has great affinity for the exposed single-stranded messenger RNA and rapidly converts polysomes into ribosomes. The difference in sedimentation properties of these two types of ribosomes can be shown directly in a sucrose gradient analysis. Fig. 1 shows a gradient analysis of aliquots of a cytoplasmic extract of chick-embryo muscle tissue. The aliquots had added to them various amounts of ribonuclease and were then incubated at 24°C for 10 min. Without any addition of ribonuclease (Fig. 1*a*) it can be seen that there is a prominent 78S peak, with smaller amounts of 60S and 40S subunits seen to the right of the peak. To the left of the 78S peak, a smaller shoulder can be seen. The effect of shearing due to alumina grinding is shown in the dotted curve of Fig. 1*a*. There is little change in the 78S peak and the peaks of the two smaller subunits to the right, but there is a substantial increment in the more rapidly sedimenting peak to the left of the 78S peak. This peak, sedimenting at 85 S, is due to ribosomes that are derived from the mechanical breakdown of polysomes. Unbroken polysomes do not appear in the figure since they sediment to the left of the diagram. Panels *b* through *f* of Fig. 1 demonstrate the effect of adding ribonuclease to the extract. Very little effect is seen with the addition of 0.001 $\mu$g/ml (*b*); however, a distinct change is seen with the addition of 0.025 $\mu$g/ml (*c*). A more rapidly sedimenting 85S peak begins to appear to the left of the 78S ribosomes. As the concentration of ribonuclease is increased, the height of the 85S peak rises steadily until this peak is appreciably greater than the 78S peak of 0.125 $\mu$g/ml. In curves *c–f*, a peak can be seen at the left of the figure that represents an increase in ribosomal dimers. Electron microscope studies were performed in all of the peaks shown in Fig. 1; the peak labeled dimer in Fig. 1*e* is composed of pairs of ribosomes, while the 85S and 78S peaks appear as single ribosomes. The 60S and 40S peaks look like eukaryotic ribosomal subunits. The appearance of an increased number of ribosomal dimers would be expected in Fig. 1 since ribonuclease attacks the messenger RNA at random. In the course of digestion it generates large numbers of ribosomal dimers, together with a greater number of monomeric ribosomes freed from polysomes. The 85S peak ultimately overwhelms the 78S peak, since most of the ribosomes are present initially in polysomes rather than as free cytoplasmic single ribosomes. In previous studies these two species have not been clearly differentiated from each other.

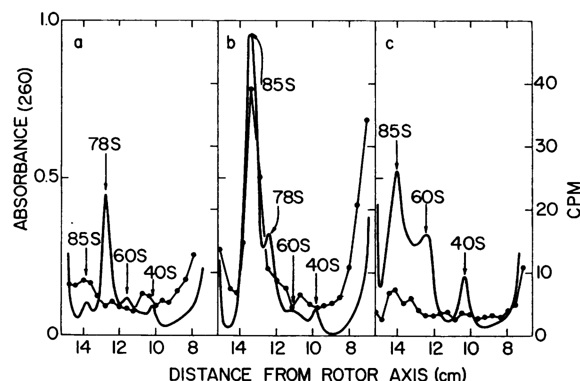


FIG. 2. *In vivo* labeling of nascent polypeptide chains. A mixture of $^{14}$C-labeled aminoacids (10 $\mu$Ci) was injected intramuscularly into 14-day-old chick embryos, and the embryos were incubated at 37°C for 15 min. Cytoplasmic extracts were centrifuged, (*a*) control; (*b*) treated with RNase alone (0.25 $\mu$g/ml, 10 min at 24°C), or (*c*) treated with Pronase (1) followed by RNase. Centrifugation was as described in Fig. 1, and 8-drop fractions were collected and analyzed. Radioactivity is shown as solid points.

TABLE 1. *Physical measurements on ribosomes*

| | $s_{20,w}$ ($\times 10^{13}$ sec) | $D_{20,w}$ ($\times 10^7$ cm$^2$/sec) | $M$ (daltons) ($\times 10^{-6}$) | $\rho$ 20°C (g/cm$^3$) |
|---|---|---|---|---|
| Free single ribosomes | 78.1 ± 0.8 | 1.04 ± 0.02 | 5.2 | 1.61 ± 0.01 |
| Polysomal-derived ribosomes | 85.1 ± 0.5 | 1.14 ± 0.02 | 5.2 | 1.60 ± 0.01 |

The course of this process can be followed directly by analysis of the gradients as a function of time of ribonuclease digestion. In this way it has been shown that there is a substantial and rapid rise in the amount of 85S ribosomes, while the amount of 78S ribosomes and 60S and 40S subunits do not increase with time.

Similar experiments were done with cytoplasmic extracts from rabbit reticulocytes and mouse ascites tumor cells. Similar differences were observed in the sedimentation properties of these two types of ribosomal particles.

The ribosomes freed from polysomes by alumina grinding or ribonuclease treatment appear to be identical in most respects; however, even with rather low concentrations of ribonuclease, there is some scission of the macromolecular ribosomal RNA, although the amount of digestion of ribosomal RNA at concentrations of ribonuclease less than 0.1 μg/ml is minimal.

A series of studies was performed to characterize the 78S and the 85S ribosomes generated either by alumina grinding or low concentrations of ribonuclease. Fig. 2 shows that the nascent polypeptide chain found in polysomal ribosomes is associated with the 85S peak, rather than with the 78S. A mixture of [$^{14}$C]aminoacids was injected into the thigh of 14-day-old chick embryos; 10 min later, the leg muscle was homogenized to prepare a cytoplasmic extract. With no additions, the sucrose gradient analysis was as shown in Fig. 2*a*. There are very few 85S particles, and there is no accumulation of radioactivity in the 78S ribosomal subunits, since they are not active in protein synthesis. A small accumulation of radioactivity in the 40S subunit peak may be due to the formation of initiation complexes. When 0.25 μg/ml of ribonuclease is added (Fig. 2*b*), there is a large accumulation of 85S ribosomes, which have radioactivity associated with them due to nascent chains. The smaller 78S shoulder on this peak does not contain radioactivity.

It was shown in studies with rabbit reticulocyte polysomes that Pronase digestion of ribosomes and polysomes results not only in digestion of the nascent polypeptide chain, but also brings about dissociation of the single ribosome into ribosomal subunits (1). This effect is illustrated in Fig. 2*c*, where Pronase has been added to an aliquot of the chick-embryo extract and incubated for 1 hr at 0°C, after which the extract was treated with ribonuclease at 24°C for 10 min. Most of the nascent-chain radioactivity has been removed from the 85S ribosomes. Furthermore, the 78S peak has entirely disappeared, and there is now a rise in the absorbance of the 60S and 40S subunits, in agreement with the reticulocyte experiments (1). This clearly demonstrates that the two kinds of single ribosomes differ, not only because one has a nascent polypeptide chain, but also in their sensitivity to proteolytic digestion. The total amount of absorbance in Fig. 2*c* is less than that seen in the aliquot found in Fig. 2*b*. The difference is probably due to the digestion of ribonuclease by Pronase. This would decrease the ribonuclease concentration and result in a smaller yield of 85S particles (as seen in Fig. 1). Analogous experiments to those shown in Fig. 2 were done with ribosomes obtained by alumina grinding. In these experiments also, the nascent chain was found in the 85S particle and not in the 78S single ribosome, and it had similar differences in sensitivity to Pronase.

A number of physical measurements were made on purified preparations of single cytoplasmic 78S and polysome-derived 85S ribosomes. The results of these measurements are listed in Table 1. Sedimentation velocity measurements in the analytical ultracentrifuge yielded sedimentation constants ($s_{20,w}$) of 78.1 Svedberg units for free, single cytoplasmic ribosomes and 85.1 S for the polysome-derived ribosomes. In view of the reported pressure-induced dissociation of ribosomes (5, 6), it is important to note that the sedimentation constants were determined in the model E analytical ultracentrifuge, with an average gravitational field near 30,000 × *g*. Pressure-induced ribosomal dissociation is seen in sucrose gradients in higher-speed centrifuges at gravitational fields near 200,000 × *g*, which generate substantially greater pressures. The translational diffusion constants were measured by helium–neon laser scattering in a self-beat spectrometer (12). The diffusion experiments were carried out in collaboration with Lon Hocker and George Benedek, and the results of these and other related experiments will be described in greater detail elsewhere. Here we list the diffusion measurements, which yield diffusion constants ($D_{20,w}$) of $1.04 \times 10^{-7}$ cm$^2$/sec for the 78S ribosome and $1.14 \times 10^{-7}$ cm$^2$/sec for the 85S ribosomes. The polysome-derived ribosome thus has a sedimentation constant 9% greater than the single free ribosome, and a diffusion constant that is similarly increased.

The density of these particles was measured in a CsCl density gradient after formaldehyde fixation (11). Both of these particles have about the same density, 1.60–1.61 g/cm$^3$. This figure is slightly higher than that reported elsewhere (11, 14), and it probably reflects the fact that the buffer contains 0.25 M KCl, a necessary ingredient in the preparation of muscle polysomes (8). It is likely that this salt releases some proteins. The ribosomal RNA in the purified 78S and 85S (alumina-ground) particles was analyzed by acrylamide gel electrophoresis. The ratio of 18S to 28S ribosomal RNA is the same in both particles, which shows that the differences are not due to a fortuitous association of a ribosome with an additional 60S or 40S subunit. The density measurement in CsCl is a sensitive function of the ratio of RNA to protein. The conclusions reached from the data presented in Table 1 are that these two particles are very similar, both in their RNA content and in the amount of protein found in the particles. This conclusion was further reinforced by acrylamide gel electrophoresis experiments on the pro-

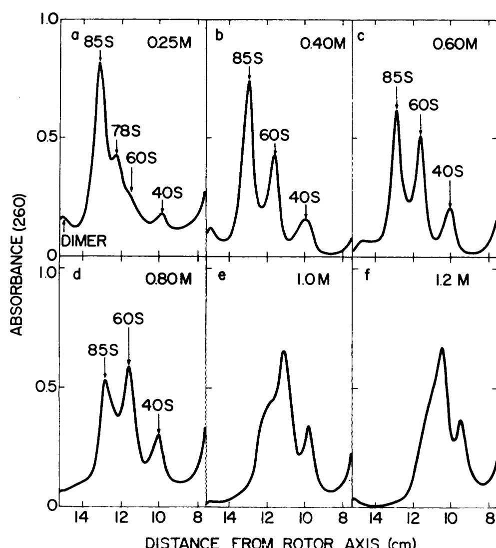


FIG. 3. The KCl-induced dissociation of ribosomal particles. Identical aliquots of an RNase-treated cytoplasmic extract were centrifuged at 39,500 rpm and 4°C for 4 hr through 13 ml of 10–30% linear sucrose gradients containing various concentrations of KCl. The KCl concentrations are indicated on the *upper right* of each frame.

teins found in the two types of particles. The gel patterns from the two particles were identical; however, because there is considerable overlap in the positions of various proteins on the gel, this analysis is not as sensitive an indication of the protein composition as is the CsCl gradient, since a loss of protein components would result in a modified density. The ultraviolet-absorption spectra of the 78S single cytoplasmic ribosome and the polysome-derived 85S ribosomes were identical.

Even though these particles have similar proteins and nucleic acids, they nonetheless behave very differently in KCl solutions. Fig. 3 shows the effect of putting the chick cytoplasmic extract into different concentrations of KCl after ribonuclease treatment. A 0.25 M KCl solution (Fig. 3*a*) gives results similar to those found in Fig. 1*f*, in which the 85S peak is much larger than the 78S peak. On raising the KCl concentration to 0.4 M, we see a dramatic decrease in the 78S particles, and a corresponding increase in the absorbance of the 60S and 40S subunits. At this KCl concentration, the 85S particles appear to be unaffected. However, when the concentration is raised to 0.6 or 0.8 M, a progressive decrease in the 85S particle results, while at 1 M KCl it has virtually disappeared. There is considerable asymmetry in the subunit peaks at 1.0 and 1.2 M KCl. This is probably related to a salt-induced release of some ribosomal proteins (15). This difference in sensitivity to KCl dissociation of polysomal-derived and single ribosomes is in agreement with similar observations in a temperature-sensitive mutant from yeast (7).

An experiment was performed to determine the role played by the nascent chain in maintaining the form of the 85S polysomal ribosome, as distinct from the 78S free single ribosome. Puromycin is known to release the nascent polypeptide chain by competing with aminoacyl-tRNA on the ribosome. Thus, we ask whether the polysomal ribosome deprived of the nascent chain, and presumably of aminoacyl-tRNA, is maintained as an 85S particle or as a 78S particle. In Fig. 4, a chick embryo was injected intravenously with 200 $\mu$g of puromycin plus 10 $\mu$Ci of a $^{14}$C-labeled amino-acid mixture. A control embryo received only the [$^{14}$C]aminoacids. After 15 min of incubation, the thigh muscle was homogenized and the cytoplasmic extract was treated with 0.25 $\mu$g/ml of ribonuclease (as in Fig. 2). Fig 4*a* shows that the control has 85S ribosomes labeled with amino acids due to the nascent polypeptide chain. The puromycin-treated embryo in Fig. 4*b* also has 85S particles, but without radioactivity, which indicates the absence of most of the nascent polypeptide chain. Thus, it appears that the nascent chain is not needed to maintain the 85S polysomal ribosome in its more compact configuration.

## DISCUSSION

Measurements of the sedimentation ($S$) and diffusion ($D$) constant of the two types of ribosomal particles allow us to calculate the particle weight ($M$) from the Svedberg equation, $M = SRT/1 - \bar{v}\rho)D$, where $\bar{v}$ is the partial specific volume and $\rho$ is the density of the solvent. As shown in Table 1, the calculated molecular weight is $5.2 \times 10^6$ for both particles if we use $\bar{v} = 0.65$ (16), and assume that it is the same for both particles. The calculated mass is the same due to the fact that the polysome-derived ribosome sediments about 9% faster than the free cytoplasmic ribosome, but it also has a diffusion constant that is about 9% larger. This result is in agreement with the measurements in the CsCl density gradient. Thus, there has not been a significant change in the molecular weight of these two particles, even in the face of significant changes in sedimentation and diffusion constants. It should be pointed out, however, that it is likely that there are some differences in the particle weight of the ribosomes, since the polysome-derived ribosome contains a fragment of messenger RNA, transfer RNA, and a nascent polypeptide chain, in addition to other possible protein factors. We can estimate the molecular weight contributions of these species. The incremental RNA due to the messenger fragment may consist of 40–50 nucleotides, and, including an additional transfer-RNA, it would make a mass contribution of about 50,000 daltons. We estimate that an average nascent polypeptide chain of chick-embryo muscle may have a molecular weight of about 20,000–30,000. The total mass increment may

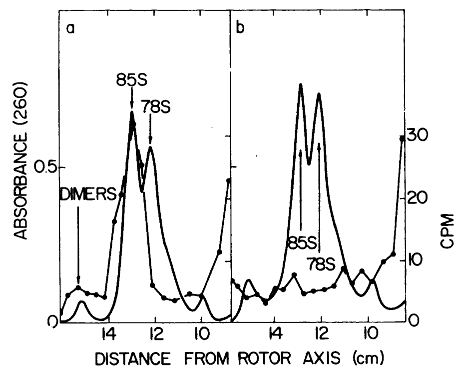


FIG. 4. The effect of puromycin on the sedimentation profile of labeled cytoplasmic extracts. 10 $\mu$Ci of [$^{14}$C]aminoacids plus and minus 200 $\mu$g of puromycin was injected intravenously into chick embryos, and cytoplasmic extracts were sedimented as described in Fig. 2. (*a*) ribonuclease alone: 0.1 ml of cytoplasmic extract contained 2284 cpm; (*b*) ribonuclease plus puromycin: 0.1 ml of cytoplasmic extract contained 3408 cpm.

thus be 70,000–80,000 daltons, which is about 1–2% of the molecular weight of the ribosome. This difference would not be detected by the molecular weight determination.

However, there is a large change in the sedimentation and diffusion properties between these two types of ribosomes that is directly attributable to an appreciable change in the ribosomal hydrodynamic dimensions. The diffusion constant ($D$) is inversely related to the frictional coefficient ($f$) by the relationship $D = RT/f$. The ribosome is approximately a spherical particle. For an ideal sphere, by Stoke's law the frictional coefficient ($f$) is equal to $6\pi\eta r$ where $\eta$ is the viscosity of the medium and $r$ is the radius of the particle. Thus, a 9% change in the diffusion constant should reflect a 9% change in the diameter of a spherical particle. In the electron microscope, the eukaryotic ribosome has a diameter of about 220–240 Å (16). In this approximation, we anticipate that the diameter of the polysome-derived ribosome should be about 20 Å smaller than the diameter of the single cytoplasmic ribosome. Changes in the frictional coefficient can also be brought about by changes in hydration (17). However, alterations in hydration are not likely to account for such a large change in the frictional coefficient without substantial modifications of shape as well. Since the ribosome is not a sphere, it is possible that the size difference may be concentrated in particular directions. For example, there might be a greater interpenetration between the two ribosomal subunits when they surround the messenger RNA. Comparative electron microscopic studies of these dimensional change are underway and will be reported elsewhere. These results, therefore, suggest that when two ribosomal subunits associate with each other around a messenger RNA to synthesize proteins, they form a more compact and solid structure with smaller dimensions than when these two subunits associate with themselves to form a ribosome that is free in the cytoplasm. This morphological transformation is associated with several functional changes, as seen in the altered sensitivity to proteolytic digestion or dissociation by KCl. Recognition of these two different ribosomal states may clarify other investigations. For example, in an experiment dealing with the inhibition of protein synthesis in HeLa cells by NaF, a single ribosomal peak labeled by its attached messenger-RNA could be seen that clearly sediments more rapidly than the inactive single ribosomes (18).

Several investigators have shown that the single ribosomes are not active in protein synthesis, and only become active by slowly dissociating into ribosomal subunits (19, 10). The cause of the slow dissociation may be related to the different manner in which the ribosomal subunits are held together to form the nonfunctional single ribosome in the cytoplasm. We infer from the present work that the mode of association is substantially different from that which occurs when the subunits associate with messenger RNA to synthesize protein.

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