The General Occurrence of 55 S Ribosomes in Mammalian Liver Mitochondria

THOMAS W. O'BRIEN

SUMMARY

Ribosomes isolated from highly purified rat, cow, pig, or rabbit liver mitochondria occur predominantly as 55 to 56 S particles under conditions that stabilize 70 S Escherichia coli monosomes. These ribosomes are of mitochondrial origin since they incorporate 3H-leucine when mitochondria are incubated in vitro, under conditions in which extramitochondrial ribosomes are inactive. They are actively involved in mitochondrial protein synthesis, since puromycin strips 55 S ribosomes of their nascent peptide chains while inhibiting mitochondrial protein synthesis. Dissociation studies show that the 55 S ribosome is a mitochondrial monosome, composed of a 28 S small subunit and a 39 S subunit. These mammalian 55 S mitochondrial ribosomes are the smallest monosomes known, and they differ in several respects from extramitochondrial ribosomes, and even from the mitochondrial ribosomes of Neurospora, yeast, and Tetrahymena. They appear to constitute a unique class of miniature ribosomes of the prokaryotic type that are peculiar to animal mitochondria.

We have reported previously the isolation of 55 S ribonucleoprotein particles from highly purified rat liver mitochondria (1, 2). They occur as the major ribosomal component of rat liver mitochondria when isolated and analyzed under conditions which maintain 78 S rat liver microsomal ribosomes as well as 70 S Escherichia coli ribosomes in their monomeric forms. Although appreciably smaller than ribosomes described in other systems, these 55 S particles have the characteristics of ribosomes, and clearly reside within mitochondria. They are essentially the only ribosomes to incorporate radioactive amino acids when intact mitochondria are incubated in vitro under conditions in which protein synthesis is necessarily dependent upon intramitochondrial factors (2).

After these 55 S ribosomes were first described in rat liver mitochondria, other laboratories reported the isolation of significantly larger ribosomes from mitochondria of Neurospora (3, 4), yeast (5–7), and Tetrahymena (8). Neurospora mitochondrial ribosomes exist as 73 S monosomes which dissociate to subunits of approximately 51 and 30 S (3), while mitochondria of yeast and Tetrahymena appear to contain even larger ribosomes (5, 8). Nearly all ribosomes characterized, and even mitochondrial ribosomes of Neurospora, yeast, and Tetrahymena, fall into two main size classes of 70 or 80 S. One would therefore not expect the 55 S particle to be a monosome, but rather a ribosome subunit, or perhaps an altered or degraded form of a larger mitochondrial ribosome. As a possible exception to this general phenomenon, it is noteworthy that the 55 S particles have properties of mitochondrial monosomes.

Swanson and David (9) recently described a 60 S ribosome that appears to be the mitochondrial monosome of amphibian (Xenopus) oocytes. In recent studies of mitochondrial ribosomes from rat liver, Ashwell and Work (10) were unable to ascertain whether the 55 S mitochondrial ribosome represented a monosome or a subunit. The present study of mitochondrial ribosomes from other mammals was undertaken to learn whether the 55 S ribosome is an atypical species occurring only in rat liver, and more importantly, to determine whether the 55 S ribosome is a monosome or a subunit. In representative experiments with rat, pig, cow, and rabbit liver, it will be shown that mitochondria from these different mammalian species contain 55 S ribosomes of remarkably similar properties. Pulse-labeling experiments, with 3H-leucine, and dissociation studies identify the 55 S ribosome as a mitochondrial monosome comprised of two dissimilar subunits. These 55 S monosomes appear to constitute a unique class of miniature ribosomes common to mitochondria of mammals, and probably to mitochondria of other higher animals as well.

EXPERIMENTAL PROCEDURE

Materials

Bovine serum albumin was obtained from Nutritional Biochemicals. Schwarz BioResearch was the source of [4,5-3H]leucine, 15 Ci per mmole, and Soluene was obtained from Packard Instrument Company. RNase-free sucrose was obtained from Mann, and AMP, ADP, and bicine were obtained from Sigma.

Preparative Methods

Source of Mitochondria—Young Sprague-Dawley rats and New Zealand white rabbits used in this study were fasted overnight
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Ribosomes of Rat Liver Mitochondria—The earlier studies on rat liver mitochondrial ribosomes (1, 2) were performed at a constant Mg\(^{2+}\) concentration of 5 mM. Since ribosomal stability, subunit association, and sedimentation properties are influenced markedly both by the Mg\(^{2+}\) ion concentration and the ionic strength of the medium, it is important to determine the influence of these factors on the yield and sedimentation profile of ribosomes isolated from rat liver mitochondria. Furthermore, if the 55 S ribosomes arise from the dissociation of larger mitochondrial ribosomes, such as occur in yeast or Neurospora, this could be determined by isolating and analyzing the ribosomal fraction in buffers of higher Mg\(^{2+}\) concentration.

To minimize manipulations which may effect redistribution of nutrient broth were resuspended in standard buffer containing 1, 10, or 20 mM Mg\(^{2+}\) and disrupted by sonic treatment at 0-4°C. Undisrupted cells and cell debris were removed from the ribosome extract by centrifugation at 60,000 × g for 10 min. In some experiments, this extract was reconstituted directly onto sucrose gradients of corresponding ionic composition; in others, the ribosomes were first concentrated by centrifugation at 230,000 × g for 1 to 3 hours.

Sucrose Density Gradient Analysis of Ribosomes—Ribosome pellets were reconstituted to 2.0 ml in standard buffer and layered onto linear 10 to 30% sucrose gradients of the same ionic composition. The gradients were made with RNase-free sucrose and were centrifuged for 131 hours at 52,000 × g (20,000 rpm) in a Spinco type SW-27 rotor at 4°C.

Sedimentation coefficients of ribosomes were determined with E. coli ribosomes and subunits as sedimentation standards. Standard values of 31.8, 50.2, and 70.5 S (12) for the sedimentation coefficient of the small subunit, large subunit, and monomer E. coli ribosome, respectively, were used to construct a curve relating sedimentation coefficient and gradient position, which allowed the assignment of reproducible sedimentation coefficients to ribosome peaks.

Analysis and Fractionation of Sucrose Gradients—Sucrose density gradients were fractionated as described before (2), except that a 34% sucrose solution was introduced into the centrifuge tube bottom to allow monitoring the 260 nm absorbance with a Gilford model 2400 spectrophotometer as the contents were pumped upward through a modified Gilford flow cell of 10-mm light path.

Determination of Bacterial Contamination—The mitochondria were sampled routinely during the preliminary incubation to assess the level of contamination by bacteria. Bacterial colonies were counted after 24-hour growth on blood plates incubated at 37°C.

Analytical Methods—Protein was determined by the method of Lowry et al. (13) with bovine serum albumin as the standard. Radioactivity determinations were made as before (1), except that the protein samples for specific activity determinations were solubilized in Soluene, and 100 μg of bovine serum albumin were added to gradient fractions before recovery of the acid-insoluble radioactivity on Millipore filters. Samples in 10 ml of toluene scintillation fluid (containing 2,5-diphenyloxazole (PPO), 5 μl per liter, and 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (dimethylPOP), 0.5 g per liter) were counted at efficiencies (HE) of 30 to 45% in a Packard Tri-Carb model 3375 liquid scintillation spectrometer.

RESULTS

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prior to being killed by decapitation. Their livers were excised quickly and immersed in isolation medium at 0°C. Pig and cow livers, obtained at a slaughterhouse within minutes after death of the animal, were transported in ice to the laboratory. Subsequent operations were performed at 0-4°C.

Preparation of Mitochondria—The isolation medium and glassware used in the isolation and incubation of mitochondria were autoclaved to reduce bacterial contamination. Liver mitochondria were prepared and purified as previously described (1), except the isolation medium used was 0.34 M sucrose, 5 mM Tris, pH 7.4, and livers were passed through a meat grinder before the initial homogenization in 40-ml glass homogenizers with motor-driven teflon pestles. Traces of red blood cells were sometimes observed in the mitochondrial pellet during the early stages of mitochondrial purification by differential centrifugation. In these cases, the pellets were resuspended carefully, leaving behind the central core of contaminated mitochondria. Alternatively, the 1000 × g centrifugation was repeated, and removal of these contaminants was verified by phase microscopy. After the final centrifugation, purified mitochondria were resuspended in isolation medium and incubated immediately.

Pulse Labeling of Mitochondrial Ribosomes by Incubation in Vitro with 3H-Leucine—Mitochondria were incubated at concentrations of 1 to 5 mg of protein per ml in a final volume of 100 ml. The standard incubation medium contained 50 mM KCl, 10 mM MgCl\(_2\), 2.8 mM Na\(_2\)EDTA, 15 mM KH\(_2\)PO\(_4\), 174 mM sucrose, 45 mM bicine, 10 mM α-ketoglutarate, 2 mM AMP, 1.5 mM ADP, and 30 μg per ml of mixed amino acids (11) minus leucine, modified to contain proline and serine in molar ratios of 1.0 and 0.75, respectively. Final pH of the medium was 7.2. In this medium, amino acid incorporation is dependent upon mitochondrial respiration and microsomal ribosomes are inactive. Only those ribosomes are labeled that have immediate access to an entire complement of tRNA's, aminoacyl-tRNA synthetases, and accessory factors, such as occur in mitochondria. This functional criterion provides a convenient means of identifying ribosomes that reside within mitochondria. The flask was gassed with 95% O\(_2\)-5% CO\(_2\) after addition of the mitochondria. After preliminary incubation at 37°C for 5 min, 3H-leucine (15 Ci per mmole) was added to a final concentration of 2.5 μCi per ml, and the mitochondria were incubated for 5 min at 37°C. To terminate the incubation, the flask contents were usually cooled rapidly to 2°C by swirling the flask in a Dry Ice-acetone bath. The mitochondria were collected by centrifugation and used to isolate ribosomes immediately.

Isolation of Mitochondrial Ribosomes—Purified mitochondria resuspended to a concentration of 1 to 5 mg of protein per ml in standard buffer (50 mM KCl, 50 mM NH\(_4\)Cl, 5 mM Tris, pH 7.0, containing specified concentrations of MgCl\(_2\)) were usually lysed by addition of 0.1 volume of 10 or 20% Triton X-100. This suspension was centrifuged for 10 min at 60,000 × g in a Spineco type 30 rotor to sediment a “membrane residue” fraction. The resulting supernatant fluid, containing released ribosomes, was layered over 2 ml of standard buffer containing 24% sucrose and centrifuged for 1 to 3 hours at 230,000 × g in the Spineco type 65 rotor to sediment the crude mitochondrial ribosome fraction. Variations from this procedure will be described below.

Preparation of E. coli Ribosomes Used as Sedimentation Standards—E. coli cells (strain B) collected from log phase cultures in

\(^1\) All centrifugal forces reported are average values computed for the center of the sample tube.
FIG. 1. Effect of Mg\(^{2+}\) concentration on sedimentation profile and yield of ribosomes from rat liver mitochondria. Aliquots of 38 mg of mitochondria (protein) in standard buffer containing specified concentrations of Mg\(^{2+}\) were made l% in Triton X-100 (final volume of 2 ml) and layered directly onto linear sucrose gradients of the corresponding ionic composition. NH\(_4\)Cl was omitted from the 100 mm Mg\(^{2+}\) buffer.

Ribosomal particles, aliquots of mitochondria were layered onto sucrose gradients immediately after lysis. Because so few ribosomes occur in rat liver mitochondria (2), their detection by ultraviolet absorbance with this procedure requires samples of such volume and high concentration that the particles are often poorly resolved. Yet, this approach does show that rat liver mitochondrial ribosomes exist predominately as 55 S particles over a wide range of Mg\(^{2+}\) concentrations (Fig. 1). Stabilization of the 55 S particle requires relatively high Mg\(^{2+}\) concentrations. Maximum yields of these ribosomes are obtained over the range of 20 to 50 mm Mg\(^{2+}\). At Mg\(^{2+}\) concentrations of 5 mm and lower, the 55 S form dissociates, and more material appears in the 20 to 40 S subunit region of the gradient. Reduced yields of 55 S ribosomes in the presence of 100 mm Mg\(^{2+}\) buffer presumably results from incomplete extraction, as well as aggregation of the particles. Although only small amounts of 55 S ribosomes are observed in the low Mg\(^{2+}\) buffer, no discrete subunit peaks are resolved amidst the high background of other lysate components which sediment into the subunit region of the gradient. In fact, it has not been possible to distinguish the subunits of mitochondrial ribosomes in any adult mammalian system, by analysis of mitochondrial lysates directly. As reported below, however, subunits are detected in these systems when the ribosome fraction is concentrated by prior high speed centrifugation.

The absence of any 80 S ribosomes in the experiment of Fig. 1 reflects the thorough removal of microsomal contaminants from these mitochondria. Moreover, failure to detect ribosomes larger than 50 S over the entire range of Mg\(^{2+}\) concentrations used, makes it improbable that 70 to 80 S ribosomes exist in rat liver mitochondria.

Ribosomes of Pig Liver Mitochondria—Fig. 2 shows that detergent treatment of pig liver mitochondria releases 52 and 56 S ribosomes, as well as two smaller particles presumed to be subunits of the 56 S ribosomes. For reasons discussed below, the 82 S ribosomes are thought to originate from contaminating microsomes.

From a comparison of the sedimentation profiles obtained in high and low Mg\(^{2+}\) buffers it appears that the relative distribution of the three smaller ribosomal components depends on the Mg\(^{2+}\) concentration. Although the combined yield of 28, 39, and 56 S ribosomes is the same in both buffers, proportionately more of these ribosomes exist as 28 and 39 S subunits in the 5 mm Mg\(^{2+}\) buffer (Table I). This behavior suggests the 28 and 39 S particles are subunits of the 56 S ribosome. In this respect, pig liver mitochondrial ribosomes resemble those of the rat in requiring relatively high Mg\(^{2+}\) levels for stabilization of the 56 S form.

The 82 S ribosomes are probably derived from microsomes which coexist with pig liver mitochondria. Similar 82 S ribosomes are obtained if the postmitochondrial (microsomal) fraction is treated with detergent. Like the microsomal ribo-
Ribosomes of Mammalian Liver Mitochondria

FIG. 3. Sucrose density gradient analysis of ribosomes isolated from cow liver mitochondria. The mitochondria were incubated for 5 min with 3H-leucine as described under "Preparative Methods," except the concentrations of AMP and ADP were 1.5 and 1.1 mm, respectively, and 3H-leucine was added to 1.1 μCi per ml in a final incubation volume of 150 ml. The pulse-labeled mitochondria were divided into two portions of 240 mg (protein) each, one for fractionation and analysis in standard buffer containing 5 mM Mg2+ (a), and the other, in the presence of 20 mM Mg2+ (b). The detergent to protein ratio was 1.0, and ribosomes were collected by centrifugation at 240,000 × g for 2.6 hours.

TABLE II
Distribution of incorporated 3H-leucine in subfractions of pulse-labeled cow liver mitochondria

Mitochondria were incubated 5 min with 3H-leucine and fractionated as described in Fig. 3. The relative specific activity of a subfraction is given by the ratio of its specific radioactivity to that of the unfractionated mitochondria. The absorbance and radioactivity profiles in Fig. 3 were used to determine the specific radioactivity (counts per min per mg of protein) of 55 S ribosomes, assuming they are 75% protein, and have an E260 nm of 50.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Fractionated in 5 mM Mg2+ buffer</th>
<th>Fractionated in 20 mM Mg2+ buffer</th>
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<tr>
<td></td>
<td>Percentage of total radioactivity</td>
<td>Relative specific activity</td>
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<tr>
<td>Intact mitochondria</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>60,000 × g pellet</td>
<td>32</td>
<td>3.6</td>
</tr>
<tr>
<td>100,000 × g supernatant</td>
<td>25</td>
<td>0.6</td>
</tr>
<tr>
<td>Sucrose gradient pellet</td>
<td>23</td>
<td>13.1</td>
</tr>
<tr>
<td>55 S ribosomes</td>
<td>5.1</td>
<td>125.0</td>
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which extramitochondrial ribosomes are inactive. Under these conditions amino acid incorporation proceeds in nearly linear fashion for at least 30 min. After incubation for 5 min, the mitochondria were cooled rapidly and divided into equal portions, one for fractionation in standard buffer containing 5 mM Mg2+ and the other, in 20 mM Mg2+. Fig. 3 shows these mitochondria contain three prominent ribosomal particles, a major 55 to 60 S component and two smaller species of approximately 28 and 39 S. The absence of any significant peak in the 80 S region of the gradient indicates the procedure developed for purifying rat liver mitochondria also reduced microsomal contamination of bovine liver mitochondria to acceptable levels.

Although the combined yield of 28, 39, and 55 S particles is approximately the same (Table I), whether isolated in the 5 mM Mg2+ buffer (Fig. 3a) or in the presence of 20 mM Mg2+ (Fig. 3b), the relative distribution of these particles depends upon the ionic conditions. In 20 mM Mg2+, the 55 S ribosomes comprise 60% of the total mitochondrial ribosome fraction. The proportion of 55 S ribosomes drops to 40% with a corresponding increase in the amount of 28 and 39 S particles, when isolated in 5 mM Mg2+, suggesting the two smaller particles are subunits of the 55 S ribosome.

During the 5-min incubation with 3H-leucine, the mitochondria attained a specific radioactivity of 180 cpm per mg of protein. Approximately 5% of this incorporated amino acid was associated with the 55 S ribosome. The radioactivity peaks at 56 to 57 S in Fig. 3 are believed to represent nascent polypeptides bound to 55 S ribosomes that are active in mitochondrial protein synthesis. The sucrose gradient pellets contained 8200 cpm (Fig. 3a) and 7000 cpm (Fig. 3b). Some of this radioactivity, as that of the 90,000 × g pellet (Table II), undoubtedly corresponds to released nascent protein, present possibly in small aggregates or assembled into miscellar complexes or insoluble membranous elements. However, in view of the high relative specific radioactivity of the gradient pellets, some of the radioactivity may accompany a small portion of the mitochondrial ribosomes occurring as mitochondrial polysomes. This possibility is also suggested by the presence of small amounts of radioactivity in the polysomal region of Fig. 3.

Ribosomes of Bovine Liver Mitochondria—As an aid in the unequivocal identification of intramitochondrial ribosomes in bovine liver, they were pulse labeled by incubation of mitochondria washed five times with 3H-leucine in vitro, under conditions in...
Fetal calf liver mitochondria were used to study the effects of puromycin on amino acid incorporation by mitochondrial ribosomes. When prepared and analyzed in standard buffer containing 20 mM Mg²⁺, ribosomes of fetal calf liver mitochondria exist predominantly as 55 to 56 S particles which, as in the adult system, are essentially the only ribosomes to become labeled when incubated in vitro with ³H-leucine for 5 min (Fig. 4). A 30-s exposure of mitochondria to puromycin (100 μg per ml) is sufficient to strip the incorporated radioactivity from these 55 S ribosomes (Fig. 4b). In view of the known mechanism of puromycin action (14), this observation indicates that the ribosome-associated radioactivity is probably in the form of nascent peptidyl-tRNA, as in other ribosomal systems. Puromycin treatment also caused nearly complete inhibition of mitochondrial amino acid incorporation (Fig. 5), establishing an active role for the 55 S ribosome in mitochondrial protein synthesis.

For this study, rabbit liver mitochondria were incubated 5 min as usual and cooled rapidly to 2° before the ribosome fraction was prepared and analyzed in the presence of standard buffer containing 50 mM Mg²⁺ and no NH₄Cl (Fig. 6). This preparation yielded major amounts of 39 and 55 S particles, and a small peak at 28 S with only a trace of material in the 60 to 70 S region, showing the major components of the mitochondrial ribosome fraction are similar to those of other mammals studied (Figs. 1, 2, and 3). As shown in Fig. 6b, native 39 S particles present in high Mg²⁺ preparations of mitochondrial ribosomes are stable in the 1 mM Mg²⁺ buffer, and still sediment as a discrete species. However, 55 S ribosomes dissociate in the 1 mM Mg²⁺ buffer (Fig. 6c), giving rise to subunits resembling the native 28 and 39 S particles that normally occur in preparations of mitochondrial ribosomes.

The broad peak in the 55 to 65 S region of the gradient probably contains some 55 S ribosomes which have not dissociated, and perhaps aggregated material, as well as any ferritin or large subunits of microsomal ribosomes present in the leading portion of the 55 S peak in Fig. 6a. The ultraviolet absorbance near the top of the low Mg²⁺ gradients is thought to represent ribonucleotide products of RNAse action on the ribosomes, especially the 28 S subunits, and also some soluble mitochondrial proteins associated with the ribosome fraction. The mitochondrial proteins normally adsorbed to ribosomes, as well as those occurring in aggregate complexes which sediment with the ribosome fraction, impart an ochre hue to the transparent ribosome pellets. Most of these extraneous proteins are removed when ribosomes are
present in approximately equal amounts (Fig. 7a), but the nascent polypeptides are associated almost exclusively with 55 S ribosomes pulse labeled with 3H-leucine, it should be possible to determine whether ribosomes bearing nascent peptidyl-tRNA are in the presence of 1 mM Mg2+ standard buffer, containing 1 mM Mg2+ standard buffer, and reanalyzed in sucrose gradients containing 1 mM Mg2+ standard buffer. The profile in a was monitored at 280 nm, and those in b and c at 260 nm.

washed or reanalyzed in sucrose gradients, and only traces of colored material accompany ribosome subunits recovered from dissociated monosomes.

These results show that 28 and 39 S particles arise during the dissociation of isolated 55 S ribosomes, strongly suggesting that these particles are indeed subunits of the mitochondrial ribosome. Additional studies on the reversible dissociation of 55 S ribosomes are in progress. Preliminary results of these experiments confirm the above findings and discount the possibility that the 39 S subunit is merely an unfolded form of the 55 S ribosome.

Dissociation of Pulse-labeled Mitochondrial Ribosomes—The proportion of mitochondrial ribosomes obtained as monosomes depends primarily upon the Mg2+ concentration and ionic strength of the medium. Having shown that 55 S ribosomes are largely stable in 5 mM Mg2+ standard buffer (Figs. 2 and 3), but dissociate in the 1 mM Mg2+ buffer (Fig. 6), it was of interest to study the Mg2+-dependent dissociation of mitochondrial ribosomes at intermediate Mg2+ levels. Moreover, by using ribosomes pulse labeled with 3H-leucine, it should be possible to determine whether ribosomes bearing nascent peptidyl-tRNA are more resistant to dissociation, as is known for E. coli (15, 16) and extramitochondrial ribosomes (17).

For this study, rabbit liver mitochondria were pulse labeled with 3H-leucine and split into four aliquots for extraction and analysis of ribosomes under different ionic conditions. In 10 mM Mg2+ (standard buffer), 55 S monosomes and 39 S subunits are present in approximately equal amounts (Fig. 7a), but the nascent polypeptides are associated almost exclusively with 55 S ribosomes. However, at 3 mM Mg2+ (Fig. 7b), most of the ribosomes have dissociated and, whereas the bulk of the radioactivity is still associated with 55 S ribosomes, about 20% appears in the 39 S peak. The absorbance and radioactivity profiles seen in the presence of 2 mM Mg2+ generally resemble those described in 3 mM Mg2+. Only a trace of 55 S ribosomes remain undissociated in 1 mM Mg2+ (Fig. 7d), but they contain over half the incorporated radioactivity. Interpreted in reference to "stuck" monosomes described in other systems (15-17), these results indicate that active mitochondrial ribosomes are likewise stabilized against dissociation under low Mg2+ conditions.

Very few 28 S subunits were observed in this experiment. It should be emphasized that 28 S subunits are normally quite unstable and are obtained in variable yields, depending generally on the conditions of their preparation. These properties are probably a consequence of RNase action, primarily on dissociated or unprotected subunits, after mitochondria are lysed in low Mg2+ buffers. The nuclease implicated may be associated typically with the 28 S subunit, maybe one of the known mitochondrial endonucleases (18, 19), or may even originate from lysosomes contained in the mitochondrial fraction. As no special measures were taken to inhibit nuclease activity in the present example, 28 S subunits undoubtedly received excessive exposure during the prolonged processing of multiple aliquots, and also during the extended centrifugation. Conditions are presently being sought which will stabilize ribosome subunits and allow their further characterization.

In Fig. 8, data from the above experiment is included with that obtained in other studies of mitochondrial ribosomes of rabbits, cows, and pigs, conducted over a wide range of ionic conditions.
Fig. 8. Mg\(^{2+}\) dependence of physical state and nascent peptide chain content of pulse-labeled mammalian mitochondrial ribosomes. a, distribution of 55 S monosomes and 39 S and 28 S subunits as a function of the ratio of Mg\(^{2+}\) concentration to ionic strength of the medium (\(\mu\)). Particle distributions were determined from area measurements of the respective peaks in sucrose gradient analyses of mitochondrial ribosomes from rabbits (O), cows (U), and pigs (A). Solid symbols refer to 55 S monosomes; open symbols, 28 S subunits; and open symbols with dots, 39 S subunits. b, partition of nascent peptide chains between 55 S monosomes and 39 S subunits as a function of medium composition. Mitochondria were incubated for 5 min with \(^3\)H-leucine before extraction and analysis of ribosomes in standard buffer containing different Mg\(^{2+}\) concentrations. No significant radioactivity was associated with 28 S subunits. Symbols as in a.

The identification and physical characterization of 55 S ribosomes from adult mammalian cells is difficult because of restrictions imposed both by low yields of these ribosomes, and also the necessity of reducing contamination by extramitochondrial ribosomes to acceptable levels. Their detection in mitochondrial lysates by 260-nm absorption alone requires relatively concentrated extracts of highly purified mitochondria. Only trace amounts of ribosomes are detected in lysates of adult mammalian mitochondria (Fig. 1), in contrast to the well-defined profiles obtained under similar conditions with less concentrated extracts of Neurospora mitochondria (3). On this basis, and from data in Table 1, it is estimated that the ribosome yield of liver mitochondria from adult mammals is only 5 to 10% that of Neurospora mitochondria.

Because they are detected in small amounts, and usually in the presence of significant quantities of extramitochondrial ribosomes, positive identification of mitochondrial ribosomes requires the application of functional criteria. The most direct and unequivocal approach is pulse labeling intramitochondrial ribosomes by \textit{in vitro} incubation of isolated mitochondria with the incorporated \(^3\)H-leucine are detected in the 39 S subunit fraction. The 28 S subunits are not represented as they contained no significant radioactivity. The reciprocal nature of the curves in Fig. 8b follows from the retention of nascent peptide chains on the large subunit of the mitochondrial ribosomes as the 55 S monosome dissociates.

**Bacterial Contamination**—Bacterial contamination of the purified mitochondria was routinely determined and ranged from 100 to 15,000 viable cells per mg of mitochondrial protein. \textit{E. coli} cells grown in nutrient broth and stored overnight at 4°, were used to assess the possible contribution, by contaminating bacteria, to the ribosome fraction isolated from mitochondrial preparations. Fifteen milligrams (protein) of cells were washed with isolation medium, pulse labeled with \(^3\)H-leucine and detergent treated under the same conditions used for mitochondria. Under these conditions, and even when sodium deoxycholate (0.5%) was used in conjunction with Triton X-100, the bacteria are not lysed. Only traces of 66 to 69 S particles were released from the bacteria, and these had no associated radioactivity. Sonic disruption of these bacteria, however, released large quantities of highly labeled 70 S ribosomes. On the basis of these results, it is extremely unlikely that bacterial contaminants contribute significantly to the mitochondrial ribosome fraction.

**DISCUSSION**

This report confirms the existence of unique 55 S ribosomes in rat liver mitochondria, and establishes that these ribosomes are the major component of the mitochondrial ribosome fraction over a wide range of ionic conditions. In addition, it is shown that ribosomes of similar size and properties occur in mitochondria of pig, cow, and rabbit liver. From these studies, and other recent reports (9, 10, 20, 21), it appears that the basic unit of the protein synthetic apparatus in mitochondria of higher animals is a 55 S ribosome. From the standpoint of countless studies of other ribosomes, it is unexpected that a ribosome as small as 55 S should be identified as a monosome since it is approximately the size of a ribosome subunit. The presence of 55 S ribosomes in these mitochondria is especially intriguing, for in other organisms where mitochondrial ribosomes have been characterized, \textit{Neurospora} (3, 4), yeast (57), and \textit{Tetrahymena} (8), they are in the 70 to 80 S size range.

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TABLE III
Sedimentation coefficients of ribosomes and ribosomal subunits of mammalian liver mitochondria

Sedimentation coefficients were determined relative to E. coli ribosomes as described under "Experimental Procedure." Mean values are listed with standard deviations. The number in parentheses gives the number of different ribosome preparations analyzed.

<table>
<thead>
<tr>
<th>Source of mitochondrial ribosomes</th>
<th>Relative sedimentation coefficient</th>
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<tr>
<td></td>
<td>Small subunit</td>
</tr>
<tr>
<td>Rabbit</td>
<td>28.3 ± 1.1 (9)</td>
</tr>
<tr>
<td>Cow</td>
<td>27.9 ± 0.8 (6)</td>
</tr>
<tr>
<td>Pig</td>
<td>27.8 ± 0.6 (5)</td>
</tr>
</tbody>
</table>

Labeled amino acids, with conditions under which extramitochondrial ribosomes are completely inactive. By this means, 55 S mitochondrial ribosomes were identified in rat (2, 10), cow (Fig. 3), and rabbit liver (Fig. 6). As an alternative to in vitro incubation, pulse labeling of mitochondrial ribosomes specifically may be achieved in vivo or in cell culture with inhibitors of protein synthesis such as cycloheximide or emetine which act only upon extramitochondrial ribosomes (10, 20-22).

These mitochondrial ribosomes also have characteristic biochemical properties distinguishing them from extramitochondrial ribosomes. In addition to the markedly smaller size of mitochondrial monosomes and subunits (Table III), mitochondrial monosomes require higher Mg\(^{2+}\) levels to prevent dissociation. This property was exploited in showing 56 S monosomes and ribosome subunits in pig liver mitochondria (Fig. 2). In addition, they contain about 23% RNA (1), considerably less than that of extramitochondrial ribosomes and their buoyant density in CsCl gradients is correspondingly less (20).

Quite naturally, their unusually small size and low RNA content lead one to suspect that the 55 S particles may arise from the degradation of somewhat larger mitochondrial monosomes, perhaps through the combined action of nucleases and proteases of the mitochondrial fraction. To minimize the possibility of degradation in this study, mitochondria were routinely isolated, purified, incubated, and fractionated as rapidly as possible, and without interruption. If their small size resulted from degradation, one would not expect to obtain discrete peaks of similar ribosomes reproducibly from different mammalian sources, whether ribosome extracts were concentrated before analysis (Figs. 2, 3, and 6) or layered directly onto sucrose gradients (Fig. 4). Moreover, appreciable degradation is not likely to occur during the isolation and purification of mitochondria, since such mitochondria readily incorporate amino acids when incubated under appropriate conditions (Fig. 5). At least some of the 55 S ribosomes remain active during this procedure, for they become labeled rapidly during the incubation. Except for the presence of nascent peptidyl-tRNA stabilizing ribosomes against dissociation at reduced Mg\(^{2+}\) levels (Fig. 8), these "active" ribosomes are virtually indistinguishable from "inactive" ribosomes not labeled during the incubation (Fig. 7). Furthermore, ribosomes isolated from purified fetal calf liver mitochondria incorporate "H-phenylalanine when incubated with polyuridylic acid and homologous mitochondrial soluble factors,\(^{a}\)

\(^{a}\) T. W. O'Brien, unpublished observation.

Laboratory Research. Preparations of 55 S ribosomes were obtained from mammalian mitochondria. In sucrose gradients varying Mg\(^{2+}\) concentration likewise shows the 55 S ribosomes are not subunits of larger mitochondrial ribosomes, but are themselves monosomes. As for bacterial ribosomes (15), and those of Neurospora mitochondria (3, 4), stabilization of the monosome requires high Mg\(^{2+}\) levels. In standard buffer the 55 S ribosomes comprise approximately 50% of the ribosome fraction obtained from Triton X-100 lysates of mammalian mitochondria (Fig. 8), provided the Mg\(^{2+}\):p ratio is maintained above approximately 0.1. The remainder of the ribosomes exist largely as 28 and 39 S subunits that cannot be caused to associate merely by raising the Mg\(^{2+}\):p ratio. At Mg\(^{2+}\):p values less than 0.1, proportionately more ribosomes occur as 28 and 39 S subunits. Of these, the small subunit is less stable, preventing the recovery of subunits in 1:1 amounts.

Dissociation studies of pulse labeled ribosomes (Figs. 7 and 8) disclose the existence of "stuck" 55 S ribosomes, similar to the "stuck" monosomes described in E. coli (15, 16) and hepatic extramitochondrial ribosomes (17). In contrast to the bulk of the 55 S ribosomes, these active ribosomes are stabilized against dissociation under low Mg\(^{2+}\):p conditions, presumably by attached nascent peptidyl-tRNA. This demonstration of "stuck" 55 S mitochondrial ribosomes as well as binding of nascent peptide chains to the large subunits provides further evidence that the 55 S ribosome is a monosome, and also shows that these ribosomes bear a general structural and functional resemblance to E. coli and extramitochondrial ribosomes.

The mitochondrial ribosomes of the four mammalian species studied in this investigation are of remarkably uniform size. Because no significant differences were noted in relative sedimentation coefficients (Table III) of monosomes (55 S), large subunits (39 S), and small subunits (28 S), these forms undoubtedly typify mitochondrial ribosomes of mammals in general. In view of these results, the 56 S "protein-synthesizing structure" described in HeLa cells by Perlman and Penman (20) probably corresponds to a mitochondrial polysome. Accordingly, brief treatment of these structures with RNase converts them to the 55 S form. Under the conditions of the present study, the mitochondrial ribosome fraction consists almost entirely of monosomes and subunits. Only occasional evidence of polysomes is seen in preparations of liver mitochondria from adult mammals (Fig. 3). This may indicate a low incidence of polysomes in these mitochondria, as a consequence of low ribosome concentration, as well as disruption of existing polysomes by endogenous nuclease and shear forces during preparative manipulations.

As a consequence of the small size of the mammalian mitochondrial ribosome, it might be anticipated that its constituent ribosomal RNA (rRNA) molecules would be correspondingly smaller than those from other sources. Indeed, recent reports have described two unusually small, discrete molecules of rRNA from mammalian mitochondria. In sucrose gradients the small and large rRNA molecules have sedimentation coefficients of 12 to 13 S and 15 to 17 S respectively (23-25), whereas their corresponding mobilities in acrylamide gel electrophoresis are characteristic of 12 S and "21 S" RNA (24-26). Such mito-
chondrial rRNA has been found in rat liver (25, 27, 28), human (HoLa) cells (23, 26, 29), and hamster and mouse cells (24, 25) again showing the 55 S type of ribosome is characteristic of mammalian mitochondria.

While present in all mammalian mitochondria examined, ribosomes of the 55 S class are not restricted to these species, as similar ribosomes occur in the mitochondria of the toad, Xenopus laevis (9). On the basis of both ribosome and rRNA size, these ribosomes appear to belong to the same class. Like mammalian 55 S ribosomes, the 80 S amphibian mitochondrial ribosomes contain 13 S and “21 S” rRNA (9). Studies underway in this laboratory indicate ribosomes of the 55 S class are distributed widely, occurring in mitochondria of other animals as well. As expected from studies of mitochondrial ribosomes of lower forms, 55 S mitochondrial ribosomes have properties of prokaryote ribosomes.

Ribosomes of the prokaryotic type are generally distinguished by their small size, requirement of high Mg$^{2+}$ concentrations for stability, and sensitivity to chloramphenicol. With the notable exception of 55 S ribosomes, however, prokaryotic ribosomes range in size from the 65 to 68 S variety in photosynthetic bacteria (30) and chloroplasts (3, 31, 32) to mitochondrial ribosomes of Neurospora, yeast and Tetrahymena at the upper size extreme of 73 to 80 S (3, 5, 8). Thus, the 55 S class of ribosomes represents a singular example of ribosome evolution in which the ancestral prokaryote ribosome (putative 70 S monosome) lost several components during its evolution to a highly differentiated form within animal mitochondria.

As might be expected from its marked size difference, ribosomes of mammalian mitochondria also exhibit significant functional differences from mitochondrial ribosomes of lower forms. Protein synthesis in yeast mitochondria and bacteria is inhibited by the aminoglycoside antibiotics, neomycin B and C, and paromomycin, on one hand, and by lincomycin and erythromycin, on the other (33, 34). These antibiotics have little effect on protein synthesis in mammalian mitochondria (33), providing a basis for further distinction between mitochondrial ribosomes of mammals and those of lower forms. As the former group of antibiotics acts on the small subunit (35, 36), and the latter, on the large subunit of the bacterial ribosome (37-39), these inhibitor studies suggest loss or modification of components has occurred in both the 28 and 39 S subunits of the mammalian mitochondrial ribosome.

The emergence of 55 S ribosomes in animal mitochondria represents a striking departure from the general pattern in which evolution to higher forms is accompanied by increased size of ribosomes and rRNA (3, 30, 40-42). It will be especially interesting to discern additional functional properties underlying the unusually small size of these ribosomes.

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The General Occurrence of 55 S Ribosomes in Mammalian Liver Mitochondria
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