PROTEINS OF THE MAMMALIAN MITOCHONDRIAL RIBOSOME

by

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ABBREVIATIONS USED

ATP	adenosine-5'-triphosphate
bisacrylamide	N, N'-methylenebisacrylamide
C	cytosine
cytoribosome	cytoplasmic ribosome
d	daltons
DNA	deoxyribonucleic acid
EDTA	(ethylenedinitrilo)tetraacetic acid
g	gravity
G	guanine
GTP	guanosine-5'-triphosphate
leu	leucine
mitoribosome	mitochondrial ribosome
MgAc ₂	magnesium acetate
ML	mitoribosomal large-subunit protein
MS	mitoribosomal small-subunit protein
mRNA	messenger RNA
phe	phenylalanine
poly U	polyuridylic acid
POPOP	1, 4-bis[2-(5-phenyloxazolyl)]benzene
PPO	2, 5-diphenyloxazole
RNA	ribonucleic acid
rRNA	ribosomal RNA
S	Svedberg unit
SDS	sodium dodecyl sulfate
TEA	triethanolamine
TEMED	N, N, N', N'-tetramethyl-ethylenediamine
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
β-ME	β-mercaptoethanol

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For some years it was widely believed that all ribosomes were either of the 70S type found in bacteria or of the 80S type occurring in the cytoplasm of eukaryotic cells. With the discovery and subsequent characterization of ribosomes in the mitochondria of eukaryotes, this simple generalization had to be rejected. The mitochondrial ribosomes of various organisms display a great diversity of structural properties, even though they all show considerable functional homology with the 70S prokaryotic ribosome. Animal species possess mitochondrial ribosomes with particularly interesting physical-chemical properties, by virtue of their high protein content relative to the quantity of RNA they contain.

The present research is an investigation into the nature of the individual proteins which make up the large complement of total protein

found in mammalian mitochondrial ribosomes. Two-dimensional electrophoresis in polyacrylamide gels was used to separate, identify, and characterize the proteins of mitochondrial ribosomes from bovine liver. It was found that these ribosomes contain a relatively large number of proteins: 52 are present in the large subunit and 41 in the small subunit. Several kinds of criteria and experimental evidence argue that these proteins are true ribosomal components rather than contaminants in the ribosome preparations. However, only 81 electrophoretically distinguishable proteins are present in a mixture of large and small subunits; the possibility that some of the large-subunit proteins are identical to proteins found in the small subunit is discussed.

The molecular weights of these proteins are similar to those of bovine cytoplasmic ribosomal proteins, and considerably larger than those of Escherichia coli ribosomal proteins. Comparisons of electrophoretic properties show that the mitochondrial proteins are more acidic than those of mammalian cytoplasmic or most bacterial ribosomes. The sum of the molecular weights of the 93 mitochondrial ribosomal proteins is somewhat greater than predicted by physical-chemical measurements of the total mass of protein in this ribosome; to account for this difference it is suggested that a proportion of the ribosomes as they are isolated may be lacking some of the ribosomal proteins.

Other experiments demonstrated that exposure of the ribosomes to certain ionic conditions could remove a small number of the proteins, with a corresponding loss of the peptidyl transferase activity of the

ribosomes. One or more of the proteins removed may therefore be involved in this particular ribosomal function. Finally, comparisons between the proteins of bovine and rat mitochondrial ribosomes revealed a large number of differences in their electrophoretic properties. The level of divergence in these properties is greater than that reported in similar comparisons of cytoplasmic ribosomal proteins. This observation parallels the great phylogenetic diversity observed in the overall structural properties of mitochondrial ribosomes from more distantly related organisms, and leads to the conclusion that these ribosomes have diverged more widely during evolutionary history than have their extra-mitochondrial counterparts located only a few Angstroms away.

The large number and low isoelectric points of the proteins of mammalian mitochondrial ribosomes are discussed with reference to their implications for the structural organization of these particles. Some mechanisms which may account for the rapid evolutionary divergence of mitochondrial ribosomes are proposed.

INTRODUCTION

Mitochondria are multifunctional organelles found in all eukaryotic cells, their primary function being the aerobic production of ATP. In addition to their important role in cellular energy metabolism, mitochondria make an essential contribution to their own biogenesis. Several of the multi-subunit enzymes of oxidative phosphorylation contain one or more subunits synthesized within the mitochondrion (Schatz and Mason, 1974). For this purpose mitochondria possess a large complement of biosynthetic enzymes and other macromolecules, distinct from their analogs in the nucleus and extramitochondrial cytoplasm. These components of the mitochondrial biogenetic system include DNA, DNA and RNA polymerases, messenger RNA, ribosomes, translation factors, transfer RNA species and aminoacyl-tRNA synthetases. Mitochondrial DNA codes for the ribosomal RNA and at least some of the tRNA and mRNA species found in mitochondria, and these macromolecules are indispensable in the biogenesis of functionally active mitochondria. On the other hand most of the (equally essential) protein components of the mitochondrial biogenetic system --- ribosomal proteins, factors, and enzymes --- appear to be coded by nuclear DNA and synthesized on cytoplasmic ribosomes. Thus the mitochondrial and nuclear-cytoplasmic macromolecule-synthesizing systems must cooperate as intimately in the production of the mitochon-

drial biogenetic system itself as they do in the synthesis of the enzymes of oxidative phosphorylation.

The ribosomes in a given organism's mitochondria are generally distinguishable from its cytoplasmic ribosomes on the basis of several functional or physical-chemical criteria. Indeed, especially in their functional properties, mitochondrial ribosomes have been found to be more like bacterial ribosomes than cytoplasmic ones. Some structural similarities between mitochondrial and Moneran ribosomes were noted early (Küntzel and Noll, 1967), and complemented reports of other biochemical homologies between organelles and prokaryotes that had already aroused considerable interest in the question of the evolutionary origin of mitochondria and chloroplasts. More recent comparisons of the structural parameters of bacterial and mitochondrial ribosomes have shown some similarities but also a surprising number of differences, both between the two groups and among mitochondrial ribosomes from different species. Thus, it is true that the mitochondrial ribosomes of most organisms studied to date sediment more slowly than the corresponding cytoplasmic ribosomes, and that some of them have sedimentation coefficients close to that of prokaryotic ribosomes (70S). However, mitoribosomes from various species range in sedimentation rates from 55S to 80S, a much wider variation than is found among bacterial, eukaryotic-cytoplasmic, or chloroplast ribosomes obtained from different organisms. Indeed, when all ribosomal attributes are considered, it seems that only in mitochondria do so many different kinds of ribosomes occur.

I. STRUCTURAL PROPERTIES OF NON-MITOCHONDRIAL RIBOSOMES

Before proceeding to the description of the physical and chemical characteristics of mitochondrial ribosomes, it will be useful to summarize the characteristics of the other kinds of ribosomes that exist. This task is simplified by the fact that the characteristics of non-mitochondrial ribosomes are not as divergent as might be thought. They all fit reasonably well into two large categories within which the members seem to share more similarities than differences. Table I shows the relative homogeneity of the properties of cytoplasmic ribosomes, whatever eukaryotic organism they are obtained from---protists, fungi, plants or animals. Despite small differences among the cytoplasmic ribosomes from these four taxonomic kingdoms, all of them appear to be members of a single structural class typified by a sedimentation coefficient of 80S, a buoyant density of 1.57 g/cc, and rRNA molecules of 0.7 and 1.3 million daltons containing 50 percent G + C.

The second category of non-mitochondrial ribosomes is also relatively uniform in physical and chemical properties. Described in Table II are several prokaryotic ribosomes---from E. coli, a mycoplasma, and a blue-green alga---and those of various chloroplasts. The prokaryotic particles can all be adequately described by the values 70S, 1.64 g/cc, $(0.56 + 1.10) \times 10^6$ daltons, and 50% for the structural parameters tabulated. Chloroplast ribosomes are similar in all respects except that some of them appear to be significantly lower in buoyant density. Perhaps it should be mentioned that this homogeneity of

Table 1. Properties of cytoplasmic ribosomes of eukaryotes.

Organism	Sedimentation Coefficient	Buoyant Density in CsCl, g/cc	Molecular Weight of rRNA ($\times 10^6$)	Base Composition of rRNA, % G + C
PROTISTS				
<u>Euglena</u>	87S ¹	[1.53] ²	(0.85 + 1.20) ¹	54% ³
<u>Tetrahymena</u>	80S ⁴	1.56 ⁴	0.52 + 1.18 ⁵	47% ⁴
<u>Chlamydomonas</u>	83S ⁶	[1.52] ⁷	(0.70 + 1.28) ⁶	
FUNGI				
<u>Neurospora</u>	77S ⁸	1.58 ⁹	0.67 + 1.28 ¹⁰	51% ⁸
<u>Saccharomyces</u>	80S ¹¹	1.55 ¹²	0.72 + 1.21 ⁵	53% ¹³
PLANTS				
Pea	80S ¹⁴	1.58 ⁹	0.7 + 1.3 ¹⁵	52% ¹⁶
ANIMALS				
Toad	87S ¹⁷	1.59 ¹⁸	0.7 + 1.5 ¹⁵	59% ¹⁹
Rat	83S ²⁰	1.55 ²¹	0.66 + 1.48 ⁵	64% ²²

[] Calculated from the data published in the cited reference, by the formula Buoyant Density = $\frac{1.89}{1 + 0.0040 (\% \text{ Protein})}$ (Hamilton, 1971).

() The data were published as ultracentrifugal or electrophoretic S values relative to *Escherichia coli* rRNA standards taken as 16S and 23S. To simplify the tabulation, these S values have been converted to molecular weights according to the formula $M = KS^a$; the constants K and a were evaluated from the values 0.56×10^6 and 1.10×10^6 for the molecular weights of 16S and 23S RNA.

¹Avadhani and Buetow, 1972.

¹²Grivell et al., 1971.

²Avadhani and Buetow, 1974.

¹³Morimoto and Halvorson, 1971.

- ³Krawiec and Eisenstadt, 1970.
- ⁴Chi and Suyama, 1970.
- ⁵Reijnders et al., 1973.
- ⁶Bourque et al., 1971.
- ⁷Sager and Hamilton, 1967.
- ⁸Küntzel and Noll, 1967.
- ⁹Cammarano et al., 1973.
- ¹⁰Neupert et al., 1969.
- ¹¹Cooper and Avers, 1974.
- ¹⁴Ts'o et al., 1958.
- ¹⁵Loening et al., 1969.
- ¹⁶Bonner and Varner, 1965.
- ¹⁷Swanson and Dawid, 1970.
- ¹⁸Leister and Dawid, 1974.
- ¹⁹Dawid et al., 1970.
- ²⁰Hamilton and Petermann, 1959.
- ²¹de Vries and Kroon, 1974.
- ²²Kirby, 1965.

Table II. Properties of Moneran and chloroplast ribosomes.

Source	Sedimentation Coefficient	Buoyant Density in CsCl, g/cc	Molecular Weight of rRNA (x 10 ⁶)	Base Composition of rRNA, % G + C
BACTERIA				
<u>Escherichia coli</u>	69S ¹	1.64 ²	0.56 + 1.10 ³	52% ⁴
<u>Mycoplasma hominis</u>	71S ⁵	[1.63] ⁵	(0.56 + 1.01) ⁵	46% ⁵
BLUE-GREEN ALGAE				
<u>Anabaena</u>	72S ⁶	1.63 ⁷	0.55 + 1.07 ⁸	
CHLOROPLASTS				
<u>Euglena</u>	69S ⁹	[1.67] ¹⁰	(0.63 + 1.01) ⁹	52% ¹⁰
<u>Chlorella</u>	67S ⁷	1.57 ⁷	0.56 + 1.10 ⁸	
Pea	70S ⁷	1.58 ⁷	0.56 + 1.10 ⁸	
Spinach	66S ¹¹		0.56 + 1.05 ¹²	54% ¹¹

Parenthesized and bracketed entries as in Table I.

¹Tissières et al., 1959.

²Sacchi et al., 1973.

³Kurland, 1960.

⁴Horimoto and Halvorson, 1971.

⁵Johnson and Horowitz, 1971.

⁶Taylor and Storck, 1964.

⁷Yurina and Odintsova, 1974.

⁸Loening, 1968.

⁹Avadhani and Buetow, 1972.

¹⁰Rawson and Stutz, 1969.

¹¹Lyttleton, 1962.

¹²Hartley and Ellis, 1973.

gross structural properties almost certainly conceals a great deal of diversity in fine structure. Differences in such properties as ribosomal protein electrophoretic mobilities and immunologic identities are the rule even for prokaryotic ribosomes related to each other more closely than are the entries of Table II (Geisser et al., 1973; Wittmann et al., 1970).

II. STRUCTURAL PROPERTIES OF MITOCHONDRIAL RIBOSOMES

In contrast to the rather simple classification scheme possible for ribosomes from all other sources, mitochondrial ribosomes do not seem to fall into one or even a few structural categories. Reference to Table III shows the degree of diversity found in mitochondrial ribosomes from different species. Many of the individual ribosome species in this table are as distinct in physical and chemical properties from each other as the 80S (Table I) and 70S (Table II) classes are. Furthermore, among the protists and fungi no two genera have yet been shown to contain similar mitoribosomes, so it seems likely that many more structurally different ribosomes will be found as other species are investigated. On the other hand, considerable homology is seen among the mitoribosomes of several species of higher animals, from locust to man.

A. Mitochondrial Ribosomes of Protists, Fungi, and Plants

The best-characterized protist mitochondrial ribosomes are those of Euglena gracilis and Tetrahymena pyriformis. The Euglena mitoribosome is unusual in that it is the only mitochondrial ribosome yet described that shows a large degree of structural homology with the

Table III. Properties of mitochondrial ribosomes.

Source	Sedimentation Coefficient	Buoyant Density in CsCl, g/cc	Molecular Weight of rRNA (x 10 ⁶)	Base Composition of rRNA, % G + C
PROTISTS				
<u>Euglena</u>	71S ¹	[1.61] ²	(0.56 + 0.93) ¹	27% ³
<u>Tetrahymena</u>	80S ⁴	1.46 ⁴	0.47 + 0.90 ⁵	29% ⁴
FUNGI				
<u>Neurospora</u>	73S ⁶ , 80S ⁷	1.52 ⁸	0.72 + 1.28 ⁹	38% ⁶
<u>Saccharomyces</u>	72S ¹⁰ , 75S ¹¹ , 80S ¹²	1.64 ¹³	0.70 + 1.30 ⁵	30% ¹⁴
<u>Candida utilis</u>	72S ¹⁵	1.48 ¹⁵	0.71 + 1.21 ¹⁵	34% ¹⁵
<u>Aspergillus</u>			0.66 + 1.27 ¹⁶	32% ¹⁷
PLANTS				
Maize	77S ¹⁸	1.56 ¹⁹	0.76 + 1.25 ²⁰	
Bean	78S ²¹		0.78 + 1.15 ²¹	
ANIMALS				
Locust	60S ²²		0.28 + 0.52 ²³	
Shrimp			0.35 + 0.50 ²⁴	43% ²⁴
Toad	60S ²⁵	1.45 ²⁶	0.32 + 0.58 ²⁶	41% ²⁷
Rat	55S ²⁸	1.43 ²⁹	0.30 + 0.50 ³⁰	47% ³¹
HeLa Cells	55S ³²	1.40 ³²	0.35 + 0.54 ³³	43% ³⁴

Parenthesized and bracketed entries as in Table I.

¹Avadhani and Buetow, 1972.

¹⁸Pring, 1974.

²Avadhani and Buetow, 1974.

¹⁹Pring and O'Brien, 1974,

³Krawiec and Eisenstadt, 1970.

private communication.

- ⁴Chi and Suyama, 1970.
- ⁵Reijnders et al., 1973.
- ⁶Kiintzel and Noll, 1967.
- ⁷Agsteribbe et al., 1974.
- ⁸Agsteribbe and Kroon, 1974,
private communication
- ⁹Neupert et al., 1969.
- ¹⁰Schmitt, 1970.
- ¹¹Cooper and Avers, 1974.
- ¹²Norimoto et al., 1971.
- ¹³Grivell et al., 1971.
- ¹⁴Norimoto and Halvorson, 1971.
- ¹⁵Vignais et al., 1972.
- ¹⁶Verma et al., 1970.
- ¹⁷Edelman et al., 1970.
- ²⁰Pring and Thornbury, 1975.
- ²¹Leaver and Harmey, 1973.
- ²²Kleinow et al., 1971.
- ²³Kleinow, 1974.
- ²⁴Schmitt et al., 1974.
- ²⁵Swanson and Dawid, 1970.
- ²⁶Leister and Dawid, 1974.
- ²⁷Dawid and Chase, 1972.
- ²⁸O'Brien and Kalf, 1967.
- ²⁹de Vries and Kroon, 1974.
- ³⁰Sacchi et al., 1973.
- ³¹Bartoov et al., 1970.
- ³²Perlman and Penman, 1970.
- ³³Robberson et al., 1971.
- ³⁴Vesco and Penman, 1969.

ribosomes of prokaryotes. The fact that only one of the many mitoribosomes shown in Table III is similar to bacterial ribosomes in its physical-chemical properties is quite remarkable, in view of the extensive functional homologies between mitochondrial and bacterial ribosomes that have been found in every case tested. And even in this instance the structural similarity is by no means complete. Although the mitoribosome of Euglena is similar to the E. coli ribosome in its sedimentation coefficient, the molecular weights of its rRNA, and its high buoyant density, the base composition of its rRNA (27% G + C) is very different.

Tetrahymena mitoribosomes are quite dissimilar from those of Euglena, and indeed from any other ribosome yet studied. Although the Tetrahymena mitoribosome shares with that of Euglena an exceptionally low rRNA GC content, it more nearly resembles cytoplasmic ribosomes with respect to its sedimentation coefficient. And its buoyant density and rRNA molecular weight values are smaller than those of any other ribosome except the mitoribosomes of animals.

The mitochondrial ribosomes of various fungi also show a rather large diversity in sedimentation coefficients and buoyant densities. Unfortunately, a nearly equal diversity in these parameters has been reported by different research groups studying the same fungal species. Thus the reported sedimentation coefficients range from 72S to 80S for Saccharomyces mitoribosomes, and from 73S to 80S for those of Neurospora. Although the buoyant density of a 74S form of the Saccharomyces

mitoribosome was found to be 1.64 g/cc (Grivell et al., 1971), the composition of the 80S version (Morimoto and Halvorson, 1971) corresponds to a buoyant density of 1.56 g/cc. There is reasonably good evidence that the 80S ribosomes described in these species really are mitochondrial ribosomes rather than cytoplasmic contaminants (O'Brien and Matthews, 1976). It therefore seems most likely that the contradictory reports are due to artifactual alterations of the structure of these ribosomes by some of the preparative procedures used. However it is not yet clear which of the results are artifactual, so it is difficult to compare the sedimentation coefficients and buoyant densities of fungal mitoribosomes with those of other kinds of ribosomes at present.

There is much less controversy about the properties of the rRNA of fungal mitoribosomes. Indeed, the molecular weights and GC contents of these molecules are quite similar in all the fungal species tested. The GC contents are rather low, nearly as low as those found in prokaryotic mitoribosomal RNA. The molecular weights, on the other hand, are quite high: these molecules are of about the same size as the rRNAs found in cytoplasmic ribosomes.

The properties of mitochondrial ribosomes from higher plants are not firmly established. The best-documented results are those shown in Table III. It may be seen that the physical-chemical characteristics of these ribosomes are quite similar to those of cytoplasmic ribosomes in general. In fact, they differ only slightly or not at

all from those of the cytoplasmic ribosomes found in these same organisms (Leaver and Harmey, 1973; Pring and Thornbury, 1975; Pring and O'Brien, 1974, private communication). Such results raise the possibility that these ribosomes are actually of cytoplasmic rather than mitochondrial origin. This interpretation is supported by the observation that these ribosomes are inhibited by anisomycin and not by chloramphenicol, like cytoplasmic ribosomes and unlike any other known mitochondrial ribosomes (Pring, Denslow, and O'Brien, 1975, private communication).

There have been isolated reports of plant mitoribosomes with properties different from those described above. A 70S particle was obtained from mung bean mitochondria (Vasconcelos and Bogorad, 1971), and a 66S mitoribosome from maize (Wilson et al., 1968). Mitochondrial RNA from Virginia creeper was found to be relatively small, 0.42×10^6 and 0.84×10^6 in molecular weight. However, the functional properties of these ribosomes have not been described, and the uncertainty about the nature of mitochondrial ribosomes in higher plants remains unresolved.

B. Mitochondrial Ribosomes of Animals

In contrast to the fascinating and perplexing diversity of mitochondrial ribosomes from protist, fungal and plant species, Metazoan mitoribosomes appear strikingly uniform in structure. Although most of the animal species investigated have been mammalian, the mitoribosomes of the toad Xenopus have also been thoroughly characterized, and enough data have been presented for several invertebrates to justify

a tentative conclusion that the mitoribosomes of all multi-cellular animals may be quite similar. Furthermore, this relatively homogeneous group of ribosomes is distinctly different in structural properties from any other ribosomes yet described.

The first difference to be noticed was the low sedimentation coefficient of animal mitoribosomes. Values within the range 54S to 61S have been obtained for these particles from rat (O'Brien and Kalf, 1967), HeLa cells (Perlman and Penman, 1970), rabbit, pig, cow (O'Brien, 1971), hamster (Coote et al., 1971), chicken (Rabbitts and Work, 1971), toad (Swanson and Dawid, 1970), shark (O'Brien, 1972), and locust (Kleinow et al., 1971). The early inference drawn from these low sedimentation coefficients was that animal mitoribosomes were smaller (lower in molecular weight) than other ribosomes (Borst and Grivell, 1971), and this inference was reinforced by the later observations of unusually small rRNA in these particles. An alternate possibility, that the 55S particle is actually a subunit of the functional monoribosome, has been excluded by the dissociation of the 55S structure into two subunits and the demonstration that either the 55S particle or a mixture of both subunits is competent for poly U-dependent phenylalanine incorporation (Leister and Dawid, 1974; O'Brien et al., 1974).

But there is a third interpretation of the low sedimentation coefficient of animal mitoribosomes, because in point of fact these 55S particles are not exceptionally small, either in molecular weight

or in physical dimensions. The particle weight of the bovine mitoribosome as determined by high-speed equilibrium centrifugation is 2.8 million daltons (Hamilton and O'Brien, 1974), slightly greater than the value reported for the ribosome of E. coli (Hill et al., 1969). De Vries and Kroon (1974) have presented evidence that rat mitoribosomes are even larger in volume than the E. coli particles, though smaller than rat cytoribosomes. These investigators electrophoresed ribosomes into gels composed of a gradient of polyacrylamide concentration until the particles could make no further progress through the decreasing pore size of the gel matrix. The mitoribosomes penetrated farther into the gels than cytoribosomes but not as far as bacterial ribosomes. The physical dimensions of ribosomes can also be determined by electron-microscopic measurements. The results confirm the conclusion that mitoribosomes are smaller than cytoribosomes in rat (O'Brien and Kalf, 1967; Aaij et al., 1972) and locust (Kleinow et al., 1974), but direct comparisons of mitochondrial and bacterial ribosomes (under the same conditions of fixation and staining) have not been performed.

How can two particles of the same molecular weight sediment respectively at 55S and 70S? The significant difference appears to be the much lower buoyant density of the animal mitochondrial ribosome. Buoyant density values from 1.40 to 1.46 g/cc have been found for mitoribosomes of HeLa cells (Perlman and Penman, 1970; Wengler et al., 1972), rat (Sacchi et al., 1973; de Vries and Kroon, 1974), cow

(Hamilton and O'Brien, 1974), and toad (Leister and Dawid, 1974). The buoyant density of a ribosome can be used to calculate the relative proportions of RNA and protein in the particle (Hamilton, 1971); from the relationship given in the notes to Table I an RNA content of about 30 percent can be calculated for animal mitoribosomes, as contrasted with 63 percent for bacterial ribosomes (Tissières et al., 1959). From the RNA content and the sum of the molecular weights of the rRNA molecules, the particle weight of the ribosome may be calculated. By this means molecular weight estimates equal to or greater than those for bacterial ribosomes were obtained for mitoribosomes from rat (Sacchi et al., 1973; de Vries and Kroon, 1974), cow (O'Brien et al., 1974), and toad (Leister and Dawid, 1974) even before the molecular weight was determined directly by sedimentation equilibrium (Hamilton and O'Brien, 1974).

Doubts have been raised about the validity of buoyant density values as measures of the protein content of ribosomes (McConkey, 1974). Others have suggested that the low buoyant densities of animal and Tetrahymena mitoribosomes might be due to membrane fragments adhering to these particles (specifically, due to membrane lipids, which are assumed to be absent in the calculation of protein content from buoyant density) (Borst and Grivell, 1971). Several lines of evidence indicate that these factors do not represent significant objections to the description of animal mitoribosomes presented above. Determinations of the protein content by either chemical analysis or ultra-violet

absorption spectra of the ribosomes are in agreement with estimates from buoyant density, yielding values of 70-80 percent protein in rat (O'Brien and Kalf, 1967) and toad (Leister and Dawid, 1974). No phospholipids were detectable in rat mitoribosomes (de Vries and Kroon, 1974). The molecular weight estimate for bovine mitoribosomes based on their buoyant density and the size of their rRNA agrees quite closely with the molecular weight determined by sedimentation equilibrium (Hamilton and O'Brien, 1974). Finally, similar values for the total protein content of Xenopus mitoribosomes have been obtained directly by summing the molecular weights of the individual ribosomal proteins (Leister and Dawid, 1974).

Besides their unusual sedimentation behavior and high protein content, another unusual characteristic of animal mitoribosomes is the small size of their rRNA molecules. Values of 0.35 and 0.54 million daltons have been obtained for HeLa mitoribosomal RNA by electronmicroscopic length measurements (Robberson et al., 1971), and these numbers are in good agreement with those found by other methods for rat (Sacchi et al., 1973), toad (Dawid and Chase, 1972; Leister and Dawid, 1974), shrimp (Schmitt et al., 1974), and locust (Kleinow, 1974). Thus animal mitoribosomes contain scarcely more than half as much RNA as any non-mitochondrial ribosome known. To a first approximation, these particles may be pictured as E. coli ribosomes modified by converting half of the RNA into an equal mass of protein.

The base composition of the rRNA of animal mitoribosomes is distinctly higher in guanine and cytosine than that of protist or fungal mitoribosomes, though still lower than that of animal cytoplasmic ribosomes. G + C contents of 40-47 percent have been found for mitochondrial rRNA of HeLa (Vesco and Penman, 1969), rat (Bartoov et al., 1970), toad (Dawid and Chase, 1972), and shrimp (Schmitt et al., 1974). The degree of methylation is also lower for animal mitoribosomal RNAs than for either cytoplasmic or prokaryotic rRNAs (Dubin, 1974).

Although mitochondrial ribosomes from all animal species examined thus far appear quite similar in their physical and chemical properties, it may be anticipated that they will differ in their detailed structure. In fact, detectable non-homology has already been found between the base sequences of mitoribosomal RNA from two species of toad, Xenopus laevis and X. mulleri (Dawid, 1972). Electrophoretic differences have also been demonstrated in several of the mitoribosomal proteins of these two species (Leister and Dawid, 1975).

III. PHYLOGENETIC RELATIONSHIPS IN RIBOSOME STRUCTURE

The comparisons presented above permit some conclusions about the variation in structural properties among mitochondrial ribosomes of different organisms, and the differences between the ribosomes of mitochondria and those of prokaryotes, eukaryotic cytoplasm, and chloroplasts. The most obvious generalization is that mitochondrial ribosomes show more diversity in all of their structural properties than do any other kinds of ribosomes. Cytoplasmic ribosomes of all four eukaryotic kingdoms

are relatively similar with respect to sedimentation coefficient, buoyant density, rRNA size, and guanine plus cytosine content. Prokaryotic ribosomes display even more uniformity in these characteristics, while chloroplast ribosomes, whether obtained from protists or from higher plants, show remarkable homologies not only among themselves but to a large extent between themselves and prokaryotic ribosomes. Mitochondrial ribosomes, on the other hand, can vary in sedimentation coefficient even within a kingdom: such differences do exist between the protists Euglena and Tetrahymena, and almost certainly exist among the fungi. Similarly, differences in the buoyant densities of mitoribosomes are found within both the protist and the fungal groups. Mitoribosomal RNA molecular weights and G + C contents, in contrast, appear to vary significantly between kingdoms but not within them.

The correlation between these last two structural properties of mitochondrial ribosomes and the taxonomic kingdoms in which they are found is illustrated in Figure 1. Also plotted in Figure 1 are the data for chloroplast and prokaryotic ribosomes, which cluster together as expected, and for four kingdoms of cytoplasmic ribosomes. The latter group also forms a cluster, with the exception of the cytoribosome from Tetrahymena. Both of these groups are dissimilar from any of the three mitoribosome kingdoms plotted, and these are in turn distinct from each other. Thus the two parameters, rRNA size and G + C content, discriminate ribosomes along the lines of their phylogeny and intracellular location.

Figure 1. Phylogenetic relationships in ribosome structure.

● Mitochondrial Ribosomes

Fungi

S	<u>Saccharomyces</u>	Reijnders <u>et al.</u> , 1973; Morimoto and Halvorson, 1971.
A	<u>Aspergillus</u>	Verma <u>et al.</u> , 1970; Edelman <u>et al.</u> , 1970.
C	<u>Candida utilis</u>	Vignais <u>et al.</u> , 1972.
N	<u>Neurospora</u>	Neupert <u>et al.</u> , 1969; Küntzel and Noll, 1967.

Protists

Eu	<u>Euglena</u>	Krawiec and Eisenstadt, 1970; calculated from Avadhani and Buetow, 1972.
T	<u>Tetrahymena</u>	Reijnders <u>et al.</u> , 1973; Chi and Suyama, 1970.

Animals

X	<u>Xenopus</u>	Leister and Dawid, 1974; Dawid and Chase, 1972.
H	HeLa	Robberson <u>et al.</u> , 1971; Vesco and Penman, 1969.
R	Rat	Sacchi <u>et al.</u> , 1973; Bartoov <u>et al.</u> , 1970.

■ Prokaryotic Ribosomes

E	<u>Escherichia coli</u>	Kurland, 1960; Morimoto and Halvorson, 1971.
M	<u>Mycoplasma hominis</u>	Johnson and Horowitz, 1971.

Δ Chloroplast Ribosomes

Eu	<u>Euglena</u>	Rawson and Stutz, 1969; calculated from Avadhani and Buetow, 1972.
Sp	Spinach	Hartley and Ellis, 1973; Lyttleton, 1962.

○ Cytoplasmic Ribosomes

Fungi

S	<u>Saccharomyces</u>	Reijnders <u>et al.</u> , 1973; Morimoto and Halvorson, 1971.
A	<u>Aspergillus</u>	Verma <u>et al.</u> , 1970; Edelman <u>et al.</u> , 1970.
C	<u>Candida utilis</u>	Vignais <u>et al.</u> , 1972.
N	<u>Neurospora</u>	Neupert <u>et al.</u> , 1969; Küntzel and Noll, 1967.

Protists

Eu	<u>Euglena</u>	Krawiec and Eisenstadt, 1970; calculated from Avadhani and Buetow, 1972.
T	<u>Tetrahymena</u>	Reijnders <u>et al.</u> , 1973; Chi and Suyama, 1970.

Animals

X	<u>Xenopus</u>	Loening <u>et al.</u> , 1969; Dawid <u>et al.</u> , 1970.
H	HeLa	Darnell, 1968; calculated from Vesco and Penman, 1969.
R	Rat	Reijnders <u>et al.</u> , 1973; Kirby, 1965.

Plants

P	Pea	Loening <u>et al.</u> , 1969; Bonner and Varner, 1965.

Sedimentation coefficients and buoyant densities, on the other hand, do not group mitoribosomes from the same kingdom together; nor do they differentiate them from the various non-mitochondrial ribosomes. This observation suggests that these two structural properties have been less conserved than rRNA size and G + C content in the course of ribosome evolution. It is noteworthy that buoyant density is the only one of these four structural characteristics which discriminates chloroplast from prokaryotic ribosomes (Stutz and Boschetti, 1976). However, some of the intrakingdom variability reported for sedimentation coefficients and buoyant densities may be due to the possibly greater sensitivity of these parameters to differing conditions used in the preparation of the mitoribosomes.

IV. SUMMARY

Perhaps the most remarkable structural property of mammalian mitochondrial ribosomes is the very high ratio of protein to RNA found in these particles. On the basis of various kinds of physical-chemical measurements it appears that this ratio is approximately 2:1, or 1.8×10^6 daltons of protein to 0.9×10^6 daltons of RNA. These ribosomes thus contain about twice as much protein as E. coli ribosomes, and about half as much RNA.

Such an unusual composition poses questions about the molecular architecture of these particles, and about the functional roles played by this large quantity of protein in the process of protein synthesis. Is the difference in protein content between the ribosomes of E. coli

and those of mammalian mitochondria due to a difference in the number of the ribosomal proteins or to a difference in their size? Do the different kinds of molecular interactions which must be involved in maintaining the structural integrity of such a protein-rich ribosome correlate with any identifiable differences in the properties of the proteins? On the other hand, might the high protein content found in this ribosome represent nothing more significant than the presence of large quantities of contaminating non-ribosomal proteins? A portion of the present research is directed toward these questions.

Also of interest is the phylogenetic diversity in structural properties which is found in comparisons of mitochondrial ribosomes from distantly related organisms. Only small differences in the overall physical and chemical properties of mitoribosomes are seen when species within the animal kingdom are compared. But if a high degree of evolutionary divergence is indeed the rule for mitochondrial ribosomes in general, it should be possible to detect significant differences between the mitoribosomes of more closely related species at a sufficiently detailed level of analysis. The rule further predicts that such differences will be greater between mitochondrial ribosomes than between the cytoplasmic ribosomes of the same species. The experiments presented below include a test of these predictions.

MATERIALS AND METHODS

I. MATERIALS

Sucrose (density gradient grade, ribonuclease free) was obtained from Schwarz/Mann. Urea (reagent-grade) was purchased from J. T. Baker or from Mallinckrodt. Stock solutions of 10 M urea were prepared, filtered, and stored at room temperature for no more than 24 hr before use; if the conductivity of the stock solution was greater than 50 μmho , it was deionized by stirring with AG501-X8 resin (Bio-Rad). Acrylamide and N, N'-methylene-bisacrylamide (Eastman) were recrystallized from chloroform and acetone, respectively; stock solutions were deionized with Rexyn-300 (Fisher) and stored at room temperature in the dark. Ampholine was obtained from LKB. Bovine serum albumin and human γ -globulin were obtained from Nutritional Biochemicals Corporation, and egg albumin, equine myoglobin and egg-white lysozyme were from Sigma Chemical Company. Puromycin dihydrochloride was from Nutritional Biochemicals Corporation. Na^{125}I (carrier-free, in 0.1 N NaOH) and [^{14}C] formaldehyde (44 mCi/mmol) were purchased from New England Nuclear; and [4,5- ^3H]L-leucine (55 Ci/mmol), [8- ^3H] GTP (12 Ci/mmol, tetrasodium salt), and [^3H]L-phenylalanine (7 Ci/mmol) from Schwarz/Mann.

Table IV. Compositions of buffers used in the preparation of ribosomes.

<u>Buffer</u>	<u>Composition</u>
A	0.1 M KCl, 20 mM MgCl ₂ , 5 mM β-ME, 20 mM TEA, pH 7.5
B	0.5 M KCl, 40 mM MgCl ₂ , 5 mM β-ME, 10 mM Tris, pH 7.5
C	0.5 M KCl, 10 mM MgCl ₂ , 5 mM β-ME, 10 mM Tris, pH 7.5
D	0.3 M KCl, 5 mM MgCl ₂ , 5 mM β-ME, 20 mM TEA, pH 7.5
E	0.5 M KCl, 5 mM MgCl ₂ , 5 mM β-ME, 20 mM TEA, pH 7.5
F	1.0 M KCl, 5 mM MgCl ₂ , 5 mM β-ME, 20 mM TEA, pH 7.5
G	1.0 M KCl, 1 mM MgCl ₂ , 5 mM β-ME, 20 mM TEA, pH 7.5
H	0.1 M KCl, 10 mM MgCl ₂ , 0.1 mM EDTA, 5 mM β-ME, 10 mM Tris, pH 7.5
I	0.2 M KCl, 40 mM MgCl ₂ , 1.6% Triton X-100, 5 mM β-ME, 10 mM Tris, pH 7.5
J	0.5 M KCl, 40 mM MgCl ₂ , 1.6% Triton X-100, 5 mM β-ME, 10 mM Tris, pH 7.5
K	0.34 M sucrose, 5 mM Tris, pH 7.4
L	0.25 M sucrose, 1 mM EDTA, 5 mM Tris, pH 7.5
M	0.1 M KCl, 5 mM MgCl ₂ , 5 mM β-ME, 20 mM TEA, pH 7.5
N	60 mM KCl, 14 mM MgAc ₂ , 6 mM β-ME, 10 mM Tris, pH 7.8
O	1.0 M NH ₄ Cl, 10 mM MgCl ₂ , 5 mM β-ME, 10 mM Tris, pH 7.5

II. PREPARATION OF RIBOSOMES

A. Bovine Mitochondrial Ribosomes

In the course of this research the procedure for the preparation of mitochondrial ribosomes from bovine liver was modified in several ways to improve the yield and purity of the ribosomes obtained. The major changes are discussed under Methods A, B, and C below. All of the preparative methods shared the following common features. Livers of freshly killed animals were obtained from a slaughterhouse and transported to the laboratory on ice. All subsequent procedures were performed in the cold unless otherwise noted. Four to 8 kg (fresh weight) of liver were passed through a meat grinder, diluted with 4 volumes of Buffer K or Buffer L, strained through a coarse-mesh cloth screen, and homogenized. In the earlier preparations homogenization was performed with a Potter-Elvehjem homogenizer modified so that the ground tissue could be pumped through it continuously. Increased cell breakage and increased final yields of mitochondria were obtained with the use of a high-frequency dispersion device (a Tekmar Company Super Dispax, Model SD-45K).

Unbroken cells and nuclei were removed by pumping the homogenate at 880 ml/min through a Vernitron CFR-2 continuous-flow rotor rotating at 11,000 rpm in a Vernitron LCA-2 centrifuge. Mitochondria were harvested from the supernatant by continuous-flow centrifugation in a Beckman JCF-Z rotor at 18,000 rpm and a flow rate of 440 ml/min. After the mitochondria had been washed as described below, they were

resuspended to a concentration of 20 mg protein/ml in buffer and lysed by the addition of non-ionic detergent. The lysate was clarified by centrifugation in a Beckman Type 35 rotor at 28,000 rpm for 10 min. The supernatant (after treatment with DEAE-cellulose, in the case of Method C below) was centrifuged in a Beckman Type 35 rotor at 35,000 rpm for 12 hr. The ribosome pellets obtained at this step are referred to as "crude ribosomes" below.

1. Method A

The buffer used for the homogenization of the liver and the preparation of mitochondria was Buffer K. The mitochondria were washed three times by resuspending in Buffer K and centrifuging in a Beckman JA-10 or Sorvall GS-3 rotor at 8,000 rpm for 10 min. Washed mitochondria were suspended in Buffer A containing 34% sucrose (ribonuclease-free), 50 $\mu\text{g/ml}$ heparin and 200 $\mu\text{g/ml}$ oligonucleotides (prepared by partial base hydrolysis of yeast tRNA according to Spencer and Poole (1965)). Triton X-100 and sodium deoxycholate were added to final concentrations of 2% and 0.5% respectively, and crude ribosomes were prepared from the mitochondrial lysate as described above.

The ribosome pellets were resuspended in Buffer A containing 50 $\mu\text{g/ml}$ heparin and 550 $\mu\text{g/ml}$ puromycin, and incubated at 37°C for 5 min. The ribosomes were then purified by sedimentation into a linear 10-30% sucrose density gradient made up in Buffer A, in a Beckman SW27 rotor. After centrifugation the gradient was pumped through the flow cell of a Gilford Model 2400 spectrophotometer, and its absorbance at 260 nm was recorded. One-ml fractions were collected.

2. Method B

Buffer L was used for the preparation and washing of the mitochondria. After two washes the mitochondria were resuspended to a concentration of 10 mg protein/ml in Buffer L containing 50 µg/ml digitonin. The suspension was stirred for 15 min, and the mitochondria pelleted at 8,000 rpm for 10 min. The mitochondria were washed once more with Buffer L, resuspended to 20 mg protein/ml in Buffer H, and lysed by the addition of Triton X-100 to a concentration of 1.6%. After clarification as described above, the lysate was layered onto 20 ml of Buffer H containing 34% sucrose and 1.6% Triton X-100, and centrifuged to prepare crude ribosomes. Purification of the ribosomes by sucrose-density-gradient centrifugation was as described above, except that Buffer H was used instead of Buffer A.

3. Method C

To the clarified mitochondrial lysate prepared by Method B was added a moist cake of DEAE-cellulose equilibrated in Buffer H containing 1.6% Triton X-100. The quantity of DEAE-cellulose used was about 1 g (dry weight) per 50 ml of lysate. The slurry was stirred for 30 min, placed in a large Buchner funnel, and filtered just until all excess liquid was removed. Ten ml of Buffer H/Triton per gram DEAE-cellulose was added and filtered off as before. The filtrates were discarded and the cake of DEAE-cellulose was stirred for 30 min in Buffer I (10 ml/g) to elute the ribosomes. The slurry was filtered and rinsed with 2.5 ml/g of Buffer I. The ribosomes in the filtrate were then

centrifuged through a layer of Buffer H/1.6% Triton/34% sucrose and purified as in Method B. By this procedure about 10 mg of ribosomes could be obtained from 6 kg of liver.

B. Rat Mitochondrial Ribosomes

The livers of 20 to 50 young (80-100 g) female Sprague-Dawley rats were homogenized in 4 volumes of Buffer L with a Potter-Elvehjem homogenizer. Rapidly sedimenting material was removed by centrifugation at 3,000 rpm for 10 min in a Beckman JA-10 rotor, and mitochondria were obtained from the supernatant by centrifugation at 8,000 rpm for 10 min in the same rotor. The mitochondria were washed as described for bovine mitochondria above (Method B) except that the concentration of digitonin used was 17 $\mu\text{g/ml}$. The washed mitochondria were resuspended to a concentration of 5 mg protein/ml in Buffer H and lysed by the addition of Triton X-100 to 1%. Ribosomes were then prepared and purified as in Method B above. The yield was about 50 μg of ribosomes per rat liver.

C. Bovine Cytoplasmic Ribosomes

Cytoplasmic ribosomes were prepared from a microsomal fraction of bovine liver. The liver was homogenized in 4 volumes of 25 mM KCl, 5 mM MgCl_2 , 0.34 M sucrose, 5 mM β -mercaptoethanol, 10 mM Tris, pH 7.5. Mitochondria and larger particles were removed at 8,000 rpm for 10 min in a Beckman JA-10 rotor, and microsomes were pelleted from the supernatant at 9,000 rpm for 45 min in the same rotor. The pellet was suspended in Buffer M and Triton X-100 was added to 2%. Ribosomes

were then prepared as described above except that Buffer M was used instead of Buffer A or H.

D. E. coli Ribosomes

Escherichia coli K-12, strain 1200F⁻ end A 1100 rns A Su⁻ or strain Hfr D10 RNase⁻, were grown in nutrient broth at 37°C. The cells were harvested by centrifugation at 6,000 rpm for 10 min in a Beckman JA-10 rotor, suspended in a small volume of cold Buffer N, and ruptured by sonication. After centrifugation in a Beckman Type 65 rotor at 15,000 rpm for 10 min, the supernatant was centrifuged again at 60,000 rpm for 2 hr in the same rotor to pellet the ribosomes. These were then washed once by resuspending and recentrifuging in either Buffer N (low-salt ribosomes) or Buffer O (salt-washed ribosomes).

III. EXTRACTION OF RIBOSOMAL PROTEINS

Proteins were prepared for electrophoresis by a modification of the method described by Leister and Dawid (1974). For each gel sample, a pellet of ribosomes containing 200-300 µg of protein was suspended in 40 µl of 0.1 M KCl, 10 mM MgCl₂. 40 µl of 10 M urea, 4 M LiCl, HCl, pH 3.5 was added and the mixture was stirred at 5°C for 12 hr. The RNA-containing precipitate was removed by centrifugation in a Beckman Type 65 rotor at 50,000 rpm for 1 hr, and re-extracted by stirring with 80 µl of 6 M urea, 3 M LiCl, HCl, pH 3.5 for 2 hr. The supernatants from the two extractions were combined, and disulfide bonds were reduced by the addition of 40 µl of 7.5 M urea, 0.25 M

dithiothreitol, 0.5 M EDTA, 1 M Tris, pH 8.8 and incubation at 37°C for 1 hr. The proteins were then dialyzed against Sample Buffer (8 M urea, 60 mM potassium acetate, 0.01% aminoethanethiol, pH 6.7).

IV. RADIOACTIVE LABELLING OF RIBOSOMAL PROTEINS

A. Radioiodination

Chemical labelling of ribosomal proteins with Na^{125}I and Chloramine T was performed according to Leister and Dawid (1974). A 15 μl aliquot (20 to 30 μg protein) of the ribosomal protein extract described above was set aside before the disulfide reduction step. The pH was adjusted with an equal volume of 6 M urea, 3 M LiCl, 100 mM Tris, pH 7.5. 100 μCi of Na^{125}I (100 Ci/mmol) was added, followed immediately by 30 μg of Chloramine T. The reaction (total volume = 55 μl) proceeded at room temperature for 10 minutes, and was stopped with 1 μl of 1 M β -mercaptoethanol. Disulfide bonds were then reduced as described above, and the radioactive protein was separated from unreacted ^{125}I by chromatography on Sephadex G-25 equilibrated in Sample Buffer.

Control experiments were performed to determine whether the labelling reaction affected the electrophoretic properties of the proteins. Radioiodinated ribosomal proteins were added back to a large non-radioactive sample of the same proteins, and the mixture was subjected to electrophoresis, staining and autoradiography as described below. No alteration in the electrophoretic mobilities of the proteins could be detected. However some of the stained proteins were not detectably labelled, and vice versa. Radioactive spots which did not correspond

to proteins regularly seen by staining were ignored. The electrophoretic positions of the proteins that could not be radiolabelled were determined by interpolation between the nearest spots that were labelled.

B. Reductive Methylation

A pellet of ribosomes containing 5-10 μg of protein was suspended in 2 μl of 4 M guanidine hydrochloride, 100 mM sodium borate, 10 mM MgCl_2 , 20 mM KCl, 6 mM β -ME, pH 8.5. Thirty nmol of [^{14}C] formaldehyde (44 Ci/mol) in 1 μl of water were added, and the mixture was incubated on ice for 30 sec. One μl of 30 nmol/ μl sodium borohydride was added, and this addition was repeated after one minute. The reaction mixture was left standing in the cold for 5 hr. A resuspended pellet of ribosomes containing 200-300 μg of protein in 40 μl of 0.1 M KCl, 10 mM MgCl_2 was then added, and the proteins were extracted for electrophoresis as described above.

V. TWO-DIMENSIONAL ELECTROPHORESIS

The procedure was modified from that of Leister and Dawid (1974). The first dimension was essentially the discontinuous-buffer electrophoretic system of Reisfeld et al. (1962), providing electrophoretic stacking of the protein sample. The composition of the first-dimension separation gel (1.5 mm in diameter, 11 cm long) was 7.5% acrylamide, 0.023% bisacrylamide, 0.063% TEMED, 8 M urea, 60 mM potassium acetate, pH 4.3. It was polymerized by adding ammonium persulfate to a concentration of 0.1%, and pre-electrophoresed before use for 12 hr at 0.2 ma

per gel, in a tank buffer of 0.01% aminoethanethiol, 8 M urea, 60 mM potassium acetate, pH 4.3. The stacking gel (2 cm long) was 3.86% acrylamide, 0.14% bisacrylamide, 0.05% TEMED, 8 M urea, 60 mM potassium acetate, pH 6.7, polymerized by the addition of ammonium persulfate to 0.02% and riboflavin to 0.001%. The tank buffer was 0.01% aminoethanethiol, 35 mM β -alanine acetate, pH 5.0.

The dialyzed protein samples (about 200 μ l) were loaded onto the gels and electrophoresed at 0.1 ma per gel. When the tracking dye (0.001% Pyronin Y in 200 μ l of Sample Buffer) in a parallel gel tube had reached the top of the separation gel, the current was increased to 0.2 ma per gel. Electrophoresis was continued until the tracking dye reached the bottom of the gel. The gels were extruded from the glass tubes with a 6-inch, 22 gauge needle through which water was flowing under pressure.

The second-dimension gel slabs were 18 cm high, 20 cm wide, and 1.5 mm thick. Each gel was poured between a pair of glass plates separated by two 1.5 mm-thick plexiglass strips, sealed at the bottom and sides with a piece of silicone-rubber tubing, and held together with spring clamps. After the gel was polymerized, the silicone tubing was removed. The gel composition was 9.65% acrylamide, 0.35% bisacrylamide, 0.1% TEMED, 5 M urea, 0.5% SDS, 0.1 M sodium phosphate, pH 7.2, polymerized with ammonium persulfate (0.025%).

The extruded first-dimension gels were laid on top of the second-dimension gel slabs and overlaid with 0.05% mercaptoacetic acid, 5 M

urea, 1% SDS. At each end of the first-dimension gel was placed a small piece of agarose (1.5 mm in diameter, 5 mm long) containing several marker proteins. The composition of the marker mixture was 0.5 mg/ml bovine serum albumin, 1 mg/ml human γ -globulin, 0.4 mg/ml ovalbumin, 0.33 mg/ml equine myoglobin, 0.4 mg/ml egg-white lysozyme, 0.33% SDS, 0.33% β -ME, 0.016% mercaptoacetic acid, 2% agarose. Electrophoresis was at 30 ma/gel with a tank buffer of 0.024% mercaptoacetic acid, 0.5% SDS, 0.1 M sodium phosphate, pH 7.2, until the dye marker (Bromophenol Blue) reached the bottom of the gel. The total time for electrophoresis in both dimensions was about 24 hr.

After electrophoresis the gels were soaked in the following solutions with continuous agitation: 25% isopropanol, 10% acetic acid (18 hr); 0.25% Coomassie Brilliant Blue R, 50% ethanol, 7.5% acetic acid (6 hr); and 5% ethanol, 10% acetic acid (several changes, until the gels were adequately destained). For convenient storage and for autoradiography, the gels were soaked in 3% glycerol for 30 min and then dried onto Whatman #3MM filter under vacuum on a steam bath, by the method of Maizel (1971).

For autoradiography the dried gel was placed in contact with Kodak RP14 medical X-ray film in an X-ray film cassette. Two small holes had previously been drilled through the cassette. After the gel and film were loaded and the cassette was closed, a needle was inserted through these holes to mark the alignment of the gel with the film.

Gels containing reductively methylated proteins were prepared for autoradiography as described by Bonner and Laskey (1974). They were shaken in two changes of dimethyl sulfoxide, for 30 min each time, then in 4 volumes of a 20% solution of 2,5-diphenyloxazole in dimethylsulfoxide for 3 hr, and in water for 1 hr. The gels were then dried onto filter paper as described above. The film used for autoradiography was Kodak RP/R-54 medical X-ray film, pre-exposed with a flash from a photographic strobe light according to Laskey and Mills (1975). Exposure at -70°C for 5 weeks was sufficient for gels containing 10,000 cpm of reductively methylated protein.

VI. ISOELECTRIC FOCUSING

A previously published procedure (Czempiel et al., 1976; Klose, 1975) was followed closely. The gel composition was 4.8% acrylamide, 0.2% bisacrylamide, 0.065% TEMED, 1.0% Ampholine (pH 3.5 - 10), 5% sucrose, 8 M urea, polymerized by the addition of ammonium persulfate to 0.019%. The gels were 0.4 cm in diameter and 7 cm long. The upper tank buffer was 5% phosphoric acid and the lower tank buffer was 5% ethylenediamine, with the anode in the upper tank. The protein sample for each gel was dissolved in 20 λ of 8 M urea and then mixed with 30 λ of Sephadex G-200 (superfine) swollen in 20% sucrose, 8 M urea, 10% β -ME, 1% Ampholine (pH 3.5 - 10).

The details of the procedure listed above differed from those of the published procedure only in three minor respects. In the latter procedure the ammonium persulfate concentration was slightly greater

(0.023%), the gel was longer (8 cm), and the upper tank buffer contained urea at a concentration of 3 M. Besides these insignificant differences, there was one further modification which was significant. Czempiel et al. (1976) used an Ortec 4100 Pulsed Constant Power Supply, which has power output characteristics different from those of the constant-voltage power supply used in the present experiments. The published procedure involved a program of increasing voltage (50V for 1 hr, 100V for 1 hr, 150V for 1 hr, 200V for 2 hr, 300V for 2 min, and 400V for 2 min). In the present experiments several parallel samples were electrofocused according to this schedule but for different lengths of time. The first sample was run for a total of 3 hr (the first 3 hr of the schedule), the second sample for the whole 5 hr of the schedule, and the third sample for the whole 5 hr plus an additional 2 hr at 200V.

After electrofocusing, the gels were extruded and subjected to a second dimension of electrophoresis in SDS as described in Section V.

VII. ASSAYS OF RIBOSOME FUNCTIONS

Peptidyl transferase activity was assayed by the modified fragment reaction (Denslow and O'Brien, 1974; de Vries et al., 1971). The reaction mixture contained 0.1 mg ribosomes, 83 nM (10,000 cpm) N-acetyl-[³H] leucyl-tRNA, 0.66 mM puromycin, 267 mM KCl, 13.3 mM MgAc₂, 33% ethanol, 33 mM Tris-HCl, pH 7.5 in a total volume of 0.15 ml. After incubation at 25°C for 10 min, KOH was added to 0.6 M and the mixture warmed to 40°C for 3 min. The N-acetyl-[³H]leucyl-puromycin synthesized in the reaction was extracted into 1.5 ml of ethyl acetate, and

radioactivity was determined by liquid scintillation counting in Triton X-100/toluene (1:1) containing 0.5% PPO and 0.05% POPOP.

[³H] GTP binding was measured by the Millipore filter assay of Bodley et al. (1970). Fifty μ l of reaction mixture containing 15 μ g of ribosomes, 42 pmol (0.5 μ Ci) of [8-³H] GTP, 10 mM NH₄Cl, 20 mM MgAc₂, 5 mM β -ME, 10 mM Tris-HCl, pH 7.4 was incubated at 0°C for 5 min and then filtered thru a Millipore filter. The filter was washed with 10 mM NH₄Cl, 10 mM MgAc₂, 10 mM Tris-HCl, pH 7.4, and the ribosome-bound radioactivity determined by liquid scintillation counting.

Poly U-dependent polyphenylalanine synthesis was assayed according to Hosokawa et al. (1966). The composition of the 0.25 ml reaction mixture was 0.64 mg/ml poly U, 5.4 μ M [³H]phenylalanine (1.82 Ci/mmol), 25 mM tyrosine, 50 mM of each of the other 18 amino acids, 0.5 mg/ml tRNA, 32 μ M GTP, 1 mM ATP, 5 mM phosphoenolpyruvate, 0.1 mg/ml pyruvate kinase, 1 mg/ml E. coli factors, 0.4 mg/ml ribosomes, 50 mM KCl, 20 mM MgAc₂, 6 mM β -ME, 1 mM dithiothreitol, 10 mM Tris-HCl, pH 7.8. Aliquots of 50 μ l were withdrawn at 5 min intervals, and radioactivity insoluble in hot trichloroacetic acid was determined by the method of Mans and Novelli (1960).

VIII. BUOYANT DENSITY DETERMINATIONS

Ribosomes were dialyzed in 50 mM KCl, 5 mM MgCl₂, 20 mM TEA, pH 7.5, and then fixed by the addition of formaldehyde to a concentration of 5%. They were analyzed by equilibrium centrifugation in gradients of CsCl containing 50 mM KCl, 5 mM MgCl, 20 mM TEA, pH 7.5, 0.3%

formaldehyde in a Beckman SW39 rotor, as described by Brunk and Leick (1969). The gradients were pumped through the flow cell of an Isco UA-2 absorbance monitor, and 0.25 ml fractions were collected. The refractive index of each fraction was measured with a Bausch and Lomb refractometer, and used to calculate the concentration of CsCl and the buoyant density in the fractions.

IX. QUANTITATIVE MEASUREMENTS

Quantities of mitochondria were estimated by absorbance at 550 nm. Samples of mitochondria of known 550 nm absorbance were analyzed by the method of Lowry et al. (1951), to establish a calibration curve relating the absorbance at this wavelength to the concentration of mitochondrial protein in mg/ml.

Ribosome quantities were determined by absorbance at 260 nm, using extinction coefficients ($E_{1\text{ cm}}^{1\%}$) of 110 for mitochondrial ribosomes, 135 for cytoplasmic ribosomes, and 160 for E. coli ribosomes.

RESULTS

The purpose of this research was to characterize the proteins of mammalian mitochondrial ribosomes, especially in comparison with the proteins of other kinds of ribosomes. The major characteristics to be investigated were the number of ribosomal proteins, their molecular weights and their electrophoretic properties. For this purpose it was necessary to establish and apply a number of criteria to distinguish the ribosomal proteins from the contaminating non-ribosomal proteins which might be present in the mitochondrial ribosome preparation.

The results will be presented in five sections. First, the means of preparing mitochondrial ribosomes in adequate quantity and purity for these experiments will be described. The two-dimensional electrophoretic patterns of bovine mitochondrial ribosomal proteins will be presented and analyzed in terms of criteria for the identification of the proteins which are most likely to be true components of the ribosome in vivo; the effect of the ribosome preparation procedure (particularly the effect of high salt treatment) on the electrophoretic pattern of ribosomal proteins will be evaluated, with a view to the possibility of various kinds of artifacts. The electrophoretic properties of the proteins will be compared with those of mammalian cytoplasmic and bacterial ribosomal proteins. The number and sizes of the mitochondrial

ribosomal proteins will be used to estimate the total mass of protein in the ribosome. Finally, the proteins of bovine mitochondrial ribosomes will be compared with those of other mammalian species to assess the level of evolutionary divergence in these proteins.

I. PREPARATION AND CHARACTERIZATION OF MITOCHONDRIAL RIBOSOMES

At the time this research was begun, the method used for the preparation of mitochondrial ribosomes (Method A in MATERIALS AND METHODS) was not capable of producing adequate quantities of material for the electrophoretic studies to be described below. Three separate preparations were required to produce enough ribosomes for a single electrophoretic analysis, each preparation being a week-long procedure. Moreover, the crude mitochondrial ribosome preparation was heavily contaminated with cytoplasmic ribosomes, as evidenced by the large amount of material sedimenting at 80S when the ribosomes were subjected to sucrose density gradient centrifugation (Figure 2A). The presence of such quantities of 80S ribosomes suggested that even the 55S mitochondrial ribosome region of the gradient contained significant amounts of cytoplasmic ribosomal subunits, as well as possibly other unknown structures with similar sedimentation coefficients. For this reason the intact 55S ribosomes were not suitable as samples for electrophoretic analysis.

Rather, a further purification step was performed by pooling the material in the 55S peak, treating it with a higher concentration of KCl and a lower concentration of $MgCl_2$ to dissociate the mitochondrial

Figure 2. Preparation of mitochondrial ribosomes by Method A.

(A) Crude mitochondrial ribosomes were prepared from bovine liver according to Method A in MATERIALS AND METHODS. The ribosomes were suspended in 2 ml. of Buffer A and layered onto a linear 10-30% sucrose density gradient made up in Buffer A. After centrifugation in a Beckman SW27 rotor at 27,000 rpm for 5 hours, the gradient was pumped through the flow cell of a spectrophotometer and its absorbance at 260 nm was recorded. One-ml fractions were collected. The direction of sedimentation is from left to right.

(B) Fractions corresponding to the 55S absorbance peak of the sucrose density gradient shown in Figure 2A were pooled and centrifuged in a Beckman Type 65 rotor at 65,000 rpm for 3 hours. The pellet of mitochondrial ribosomes was suspended in 2 ml of Buffer E and layered onto a sucrose density gradient made up in the same buffer. Centrifugation was at 20,000 rpm for 13.5 hours.

ribosomes to their subunits, and re-centrifuging on a second sucrose density gradient. The only contaminants in the preparation of subunits obtained in this manner, besides those that might be bound directly to the subunits, would be those which sedimented near 55S under the ionic conditions of the first centrifugation and in the 25S to 45S region under the second conditions. The kinds of possible contaminants which might behave in this manner seem intuitively to be few, and in particular do not include cytoplasmic ribosomal subunits. In fact, the discrete peaks of mitochondrial small and large ribosomal subunits were found to be by far the major species visible on the absorbance profile of the second centrifugation (Figure 2B), suggesting that the preparation was indeed reasonably free of co-sedimenting material.

Later improvements in the procedure increased both the yield and the purity of the mitochondrial ribosomes. The yield was approximately tripled by the use of a more efficient homogenizer for the disruption of the cells. Treatment of the mitochondrial preparation with digitonin (Method B) dramatically reduced the quantity of cytoplasmic ribosomes in the crude mitochondrial ribosome preparation (Figure 3). This detergent has been used previously to remove cytoplasmic ribosomal RNA (Malkin, 1971) and cytoplasmic ribosomes (de Vries and van der Koogh-Schuuring, 1973) from rat liver mitochondrial preparations. In addition, treatment under these conditions has been reported to solubilize latent lysosomal enzymes (Schnaitman and Greenawalt, 1968; Lowenstein *et al.*, 1970), thereby diminishing the possibility of degradation of the mitochondrial ribosomes during the preparation.

Figure 3. Preparation of mitochondrial ribosomes by Method B.

Crude bovine mitochondrial ribosomes obtained by Method B (MATERIALS AND METHODS) were suspended in Buffer H and analyzed by sucrose density gradient centrifugation in this buffer as described in Figure 2A, except that centrifugation was for 4 hours at 27,000 rpm. (No differences have been observed between Buffer H and Buffer A with respect to their effects on the mitochondrial ribosomes.)

At this point the maximum yield of mitochondrial ribosomes from a single preparation was limited by the volume of the largest preparative ultracentrifuge rotors available. After preparation and lysis of the mitochondria, the best means available for concentrating the ribosomes for purification on sucrose density gradients was to centrifuge them to a pellet at about 100,000xg. The volume of an average yield of mitochondria (about 35g protein), suspended in buffer to a concentration low enough for efficient detergent lysis, is far greater than the capacity of three Beckman Type 35 rotors. Therefore, a method was developed to preconcentrate the ribosomes before harvesting them by centrifugation (Method C).

The mitochondrial lysate was stirred with DEAE-cellulose as described in MATERIALS AND METHODS to adsorb out the mitochondrial ribosomes, and then the ribosomes were eluted by stirring with a smaller volume of buffer at a higher ionic strength. This procedure is very rapid and effects a fourfold concentration of the ribosomes, thereby quadrupling the yield of each preparation. Furthermore, any remaining traces of cytoplasmic ribosomes, as well as a considerable proportion of the other contaminants of the mitochondrial ribosome preparation, are removed by this means.

An assessment of the degree of purification achieved by this method is shown in Figure 4. A sample of purified, digitonin-treated mitochondria was split into two equal aliquots. Ribosomes were prepared from the first aliquot without DEAE-cellulose treatment and analyzed by

Figure 4. Preparation of mitochondrial ribosomes by Method C.

All samples were analyzed by sucrose density gradient centrifugation in Buffer H.

(A) Ribosomes from 1.4 g (protein) of bovine liver mitochondria prepared by Method B. The 55S absorbance peak contains 5.7 A_{260} units of ribosomes.

(B) Cytoplasmic ribosomes from 5 ml of a preparation of bovine liver microsomes. The 80S peak contains 5.6 A_{260} units of ribosomes.

(C) 1.4 g of mitochondria and 5 ml of microsomes in Buffer H were mixed together, lysed with Triton X-100, and stirred with 2 g (dry weight) of DEAE-cellulose equilibrated in Buffer H. The slurry was filtered and the filtrate discarded. Mitochondrial ribosomes were then eluted by stirring the DEAE-cellulose with Buffer I, and were prepared for sucrose density gradient analysis according to Method B. The 55S peak contains 6.1 A_{260} units of ribosomes.

(D) The once-eluted DEAE-cellulose from (C) was stirred with Buffer J to elute the cytoplasmic ribosomes. The 80S peak contains 4.6 A_{260} units, or 82% of the input cytoplasmic ribosomes.

sucrose density gradient centrifugation, giving the absorbance profile shown in Figure 4A. A preparation of bovine cytoplasmic ribosomes (having the sucrose density gradient profile shown in Figure 4B) was added to the second aliquot of mitochondria, to provide a more stringent test of the method. The mitochondria were then lysed and the ribosomes prepared by the DEAE-cellulose procedure described above. As seen in Figure 4C, the product consists primarily of mitochondrial ribosomes and subunits, much of the slowly-sedimenting material and all detectable 80S cytoplasmic ribosomes having been removed. Probably because of the larger proportion of rRNA in cytoplasmic ribosomes and their consequent higher density of negative charges, these particles remain bound to the DEAE-cellulose under the conditions used for elution of the mitochondrial ribosomes, as shown by re-elution of the DEAE-cellulose with a buffer of higher ionic strength (Figure 4D).

As may be seen in Figure 4, the yield of mitochondrial ribosomes per gram of mitochondria obtained with the DEAE-cellulose procedure is as great as or slightly greater than that obtained without this step. Since this method permits the processing of a four-fold greater quantity of mitochondria, and gives a cleaner preparation of ribosomes as well, it is clearly the method of choice unless it adversely affects the structural or functional integrity of the ribosomes. Of particular concern is the possibility that some of the more acidic ribosomal proteins might bind more strongly to the DEAE-cellulose than to the ribosome itself and thus might be stripped off when the ribosomes are eluted.

The most acidic proteins of E. coli ribosomes, L7 and L12, have been found to be relatively loosely bound to the ribosomes (Hamel et al., 1972).

To answer this question, the ribosomes were characterized by a number of functional and physical tests. The peptidyl transferase and GTP-binding activities of the ribosome were essentially unaltered by treatment with DEAE-cellulose (Table V). DEAE-cellulose-prepared ribosomes were also found to be active in the translation of poly U (400 pmoles phe incorporated/mg rRNA/15 min). The sedimentation coefficients and buoyant densities of mitochondrial ribosomes and the subunits derived from them were essentially the same as those obtained from previous preparations. The effect of this procedure on the two-dimensional electrophoretic pattern of the ribosomal proteins will be evaluated in Section IIC below.

II. ELECTROPHORETIC ANALYSIS OF MITOCHONDRIAL RIBOSOMAL PROTEINS

The very high proportion of protein which physical-chemical measurements have shown to be present in bovine mitochondrial ribosomes (see INTRODUCTION) predicts that a rather large number of individual proteins are to be found in these ribosomes. If the 1.8×10^6 d. of total protein per particle were made up of proteins similar in size to E. coli ribosomal proteins (average molecular weight about 17,000), for example, each ribosome would contain more than a hundred protein molecules.

For this reason it seemed unlikely that electrophoresis in one dimension would separate all the proteins sufficiently for the purposes

Table V. Functional activity of mitochondrial ribosomes prepared with or without DEAE-cellulose.

<u>Preparation</u>	<u>^3H-Leu-Puromycin Synthesis (cpm/nmole ribs/10')</u>	<u>^3H-GTP Binding (moles GTP/mole ribs)</u>
No DEAE	33,600	1.12
DEAE	36,200	1.09

of this study. Several two-dimensional electrophoretic systems have been devised to provide adequate resolution for the analysis of ribosomal proteins. The system used for most of the experiments to be described below was that of Leister and Dawid (1974), employing a separation partially on the basis of charge at pH 4.3 in the first dimension and on the basis of size in the second (SDS) dimension. For the purposes of this research, this electrophoretic system has several advantages over the one that is most widely used for ribosomal protein studies at present (Kaltshmidt and Wittmann, 1970). It permits a direct determination of the molecular weights of the proteins. It is more sensitive, requiring only about 5 μ g of each protein. And it has been used for the analysis of Xenopus mitochondrial ribosomal proteins (Leister and Dawid, 1974), making it possible to compare these published results with those obtained in the present experiments on mammalian mitochondrial ribosomes.

The results of one of the early electrophoretic analyses (shown in Figures 5 and 6) amply confirmed the expectation that a large number of proteins would be found in mitochondrial ribosomes. 40 to 50 reasonably intense and distinct spots may be discerned in the photograph of the electrophoretic pattern of proteins from each subunit. Somewhat larger numbers could be seen on the original gels, since some of the resolution of closely adjoining spots has been lost in the photographs.

It is evident in these figures that the description "reasonably intense" involves a rather arbitrary judgment. In fact, particularly

Figure 5. Electrophoretic pattern of large-subunit proteins from bovine mitochondrial ribosomes.

Mitochondrial ribosomes were dissociated to subunits by sucrose density gradient centrifugation in Buffer E. Gradient fractions corresponding to the large (39S) subunits were pooled and centrifuged in a Beckman Type 65 rotor at 65,000 rpm for 5.5 hours. The proteins were extracted from the ribosome pellet and analyzed by two-dimensional polyacrylamide gel electrophoresis. The first dimension (left to right) was run in urea at pH 4.3, and the second dimension (top to bottom) in SDS.

Just before the second dimension was begun, pieces of agarose gel containing a mixture of several proteins (bovine serum albumin, human γ -globulin heavy and light chains, ovalbumin, myoglobin, and lysozyme) were placed on top of the gel slab, immediately adjacent to each end of the first-dimension gel. These proteins may be seen at the left side of the photograph.

Figure 6. Electrophoretic pattern of small-subunit proteins from bovine mitochondrial ribosomes.

Small (28S) subunits were prepared by sucrose density gradient centrifugation in Buffer E, and their proteins were subjected to two-dimensional electrophoresis as described in Figure 5.

in the small-subunit pattern, there is a large range of spot intensities with a more or less continuous variation from the most intense to the very faintest. This result was not what had been anticipated. Since nearly all ribosomal proteins probably are present in one copy per ribosome, at least in vivo (Hardy, 1975), it had been expected that the gels would show a discrete group of ribosomal protein spots of roughly similar staining intensities. In addition there might be some other spots of varying intensity, corresponding to contaminating proteins. The results obtained indicated that there were considerable differences in the relative quantities of the ribosomal proteins present in the sample, making it impossible to distinguish them from contaminating proteins on the basis of their staining intensity. Some possible explanations for this phenomenon will be considered below (Section IV).

A. Criteria for the Identification of Ribosomal Proteins

For this reason it seemed necessary to seek other criteria for the purpose of identifying which of the spots appearing on the gels actually represented ribosomal proteins. Several possible criteria were considered, including reproducibility of occurrence, subunit-specific localization, and resistance to removal from the ribosomes by high salt treatment. It seemed reasonable to expect that the ribosomal proteins would be found reproducibly in separate preparations of the ribosomes, whereas the non-ribosomal contaminants might be more variable in occurrence. In fact, it was found that many of the proteins seen in individual

gels such as those of Figures 5 and 6, particularly some of the fainter ones, could be disregarded on the basis that they were not seen in the majority of the electrophoretic analyses. This rule was thus found to be a useful one, and the application of it will be described below.

The idea that some contamination might arise from the non-specific binding of some proteins to the ribosomes suggested that contaminants of this type might be found nearly equally in both ribosomal subunits. However, the existence of some plausible mechanisms by which ribosomal proteins might also show an apparent lack of subunit-specific localization (Section IIB3) made the application of this criterion difficult, and it was used only in conjunction with other kinds of evidence.

Yet another way to discriminate the ribosomal and non-ribosomal proteins, and the most unambiguous way, would be to purify all the proteins and then reconstitute the ribosome, withholding each protein in turn, to establish which proteins were essential for the generation of a structurally and functionally normal ribosome. Unfortunately, this approach is not practical in the case of ribosomes such as those of mammalian mitochondria, which can be obtained only in relatively small quantities. However the observations (Olsnes, 1971; Kurland, 1966; Hardy and Kurland, 1966) that contaminating proteins are in general more loosely bound to ribosomes than are the ribosomal proteins themselves, suggested that an analogous approach might be feasible. Specifically, it has been found that treatment of ribosomes with

moderately high concentrations (about 0.5 M) of monovalent salts dislodges adventitiously bound proteins from the particles, while affecting the binding of the ribosomal proteins to a lesser extent. Of course, higher salt concentrations do remove ribosomal proteins (Delaunay *et al.*, 1974; Gesteland and Staehelin, 1967) and the optimum concentration range, that which removes the largest quantity of contaminants and the smallest amount of ribosomal protein, may differ from one type of ribosome to another.

The effect of increasing salt concentration on the content of individual proteins in the bovine mitochondrial ribosome was assessed by centrifuging the ribosomes through various high-salt buffers and then analyzing the proteins remaining in the treated particles by two-dimensional electrophoresis. To establish the point at which these treatments began to remove ribosomal proteins, the peptidyl transferase activity of the ribosomes prepared under each condition was assayed. The electrophoretic protein patterns and specific activities obtained were then correlated, and any proteins which were found to be removed from the ribosome by a given treatment, without diminishing its functional integrity, were tentatively regarded as non-ribosomal contaminants. Since the peptidyl transferase activity is a property of the large subunit and is not dependent on the presence of the small subunit, this criterion was directly applicable only to the large-subunit proteins.

Figure 7 shows the effect of treatment with buffers containing increasing ratios of KCl concentration to $MgCl_2$ concentration on the

Figure 7. Effects of treatment with buffers of various ionic compositions on the peptidyl transferase activity and buoyant density of bovine mitochondrial large subunits.

Large subunits were prepared by sucrose density gradient centrifugation in various buffers. Peptidyl transferase activity was assayed as described in Materials and Methods, and expressed as a percentage of the activity found in Buffer A treated subunits. The buoyant densities of the formaldehyde-fixed subunits in CsCl were determined by isopycnic centrifugation.

The buffer conditions used are plotted along the horizontal axis according to their ratio of KCl to MgCl₂ concentrations. The actual concentrations of these two salts were:

<u>Buffer</u>	<u>KCl (M)</u>	<u>MgCl₂ (mM)</u>
A	0.1	20
C	0.5	10
D	0.3	5
E	0.5	5
F	1	5
G	1	1

The complete compositions of these buffers are given in Table IV.

activity and buoyant density of the large subunit. In this series, buffers containing increasing concentrations of KCl and decreasing concentrations of $MgCl_2$ were used to wash the ribosomes with increasing stringency. For other ribosomes monovalent and divalent cations have been found to produce opposing and competitive effects on the structure of the particle, with respect to the association of the two subunits with each other (Hamilton and Petermann, 1959; Zitomer and Flaks, 1962) and the binding of proteins to the ribosome (Spitnik-Elson and Atsmon, 1969; Staehelin et al., 1969). Such effects on subunit association have also been found in mammalian mitochondrial ribosomes (O'Brien, 1971), and Figure 7 indicates that either increasing KCl or decreasing $MgCl_2$ can diminish both the functional activity and the protein content (as measured by the increase in buoyant density) of these ribosomes.

The upper curve of Figure 7 shows that the large subunit retained most of its peptidyl transferase activity after treatment with either Buffer C or Buffer D. The activity was significantly reduced by treatment with Buffer E, and higher KCl/ $MgCl_2$ ratios yielded particles with negligible activity. Therefore in the analysis of the electrophoretic results presented below, proteins which were found to be absent from large subunits treated with either Buffer C or Buffer D are considered to be dispensable for this particular large-subunit function. These proteins are regarded as possible non-ribosomal contaminants, and are discussed separately.

B. Identification of the Ribosomal Proteins

1. Large Subunit

In order to apply the criteria of reproducibility and resistance to high-salt treatment, several electrophoretic analyses were performed on large-subunit proteins taken from separate preparations of ribosomes, after treatment with various high-salt buffers. The gels were scored for the presence or absence of individual proteins, and the results are given in Table VI. The electrophoretic positions of the proteins designated in the table are shown in Figure 8. The 52 proteins which were seen reproducibly and which were present both in large subunits treated with Buffer C and in those treated with Buffer D are shown as numbered spots. Lettered spots represent some of the proteins which did not satisfy these criteria.

For the purposes of Table VI the relatively subjective question of the intensities of the protein spots was not considered, and a "+" indicates only that the protein was clearly visible in the gel, whether it was very intense or quite faint. Proteins which were near the limit of visible detection, however, are marked with a "-"; some of these are not visible in the photographs of the gels. In a few cases ("nr") the electrophoretic resolution in a given experiment was inadequate to determine whether a protein was present or not. The three proteins which move fastest in the first dimension of electrophoresis (ML45, ML51, and ML52) could have been run off the end of the first-dimension gel in some experiments, and these cases are marked "ro".

Table VI. Appearance of large-subunit proteins in separate experiments.

Large subunits were prepared by sucrose density gradient centrifugation in the buffers specified. The proteins were then analyzed by 2-dimensional electrophoresis and the gels were scored for the presence of the proteins shown in Figure 8. +, present; -, trace; 0, absent; nr, not resolved; ro, run off the first dimension gel.

Protein	Buffer B		Buffer C		Buffer D		Buffer E	
	Expt. 1	Expt. 2 (Fig. 9)	Expt. 3 (Fig. 14)	Expt. 4 (Fig. 10)	Expt. 5	Expt. 6 (Fig. 11)	Expt. 7	Expt. 8 (Fig. 5)
1	+	+	+	+	+	-	+	+
2	+	+	+	+	+	-	+	+
3	-	-	-	+	+	-	+	-
4	+	+	+	+	+	-	nr	+
5	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+
7	+	+	+	+	+	0	nr	+
8	+	+	+	+	+	0	+	+
9	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+
11	+	+	+	+	+	+	+	+
12	+	+	+	+	+	0	+	+
13	+	+	+	+	+	+	+	+
14	+	+	+	+	+	+	+	+
15	+	+	+	+	+	-	nr	+
16	+	+	+	+	+	-	+	+
17	+	+	+	+	+	+	+	+
18	+	+	+	+	+	+	+	+
19	+	+	+	+	0	+	+	+
20	+	+	+	+	+	+	+	+
21	+	+	nr	0	+	+	+	+
22	+	+	+	+	+	+	+	+
23	+	+	+	+	+	+	+	+
24	+	+	+	+	+	+	+	+
25	+	+	+	+	+	+	+	+
26	+	+	+	+	+	+	+	nr
27	+	+	+	+	+	+	+	+

- - + + + + - + + - + + + + + + + + + + - - - - 0 + 0

+ + + + + + - + + + + - + + + 0 + 0 - + + - + 0 0 + - 0 + - +

- nr nr + + - + + + + + + + + 0 + + 0 + + ro ro + + 0 + + nr + +

+ + + + + + 0 0 + + + + + + + 0 + + - + + 0 - + + 0 0 0 + 0 -

+ + + + + + + + + + + + + + ro + + 0 - + ro ro + + 0 + + 0 +

0 - + + + + - + + - + + - + + + - + + - + + - ro - 0 - 0 - 0 - 0

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Figure 8. Schematic diagram of bovine mitochondrial large-subunit proteins.

The origin of electrophoresis is at the upper left corner of the figure. The second-dimension electrophoretic positions of bovine serum albumin (molecular weight 68,000), ovalbumin (44,000), human γ -globulin light chain (23,500), horse myoglobin (17,200), and egg-white lysozyme (14,400) are indicated at the left of the figure.

Proteins which were reproducibly present in functionally active large subunits are shown as numbered spots. Lettered spots designate some of the other proteins which were seen in some of the electrophoretic analyses.

a. Reproducibility

In order to discriminate ribosomal proteins from contaminants, an arbitrary level of reproducibility of 50% was taken as the cut-off. That is, a protein was considered to be non-ribosomal unless it was present in over half of the ribosome preparations analyzed. In fact, however, very few of the proteins in large-subunit preparations were near this borderline. Most of the proteins which were found in reasonable quantity, and many of the faint ones, in any one gel proved to be quite reproducible. A small number of proteins in the unreproducible group were seen so rarely that it appears most unlikely that they represent ribosomal proteins: the spot seen between ML16 and ML17 in Figure 5, for example, was not found in any other experiment. Such proteins are not listed in Table VI. The proteins designated by letters in the table were seen more frequently, especially in ribosomes prepared in certain buffers. Because the presence or absence of these proteins was correlated with the ionic conditions with which the ribosomes had been treated, they are considered below under "Resistance to salt-washing".

Among the numbered proteins in Table VI, many appeared in every ribosome preparation, and with reasonable staining intensity. Some faint spots, like ML29 and ML41, were likewise very regular in occurrence although sometimes present only in trace quantities. Occasionally a protein which was usually present in moderate quantity would be unaccountably lacking in a particular experiment: ML47, for example,

can be clearly seen in experiments 2 (Figure 9), 4 (Figure 10), and 6 (Figure 11) and is clearly absent from experiment 8 (Figure 5). Similarly, ML1 and ML2 are always found to have about the same staining intensity seen in Figures 5, 9, and 10, except that in experiment 6 (Figure 11) they were both reduced to barely detectable traces. Although such results are quite surprising, the overall reproducibility of these proteins was considered adequate to satisfy the criterion.

Predictably, the greatest variability was found among the faintest spots, including the only two real borderline cases. ML48 was seen with low but clearly visible intensity in three experiments (as in Figure 9), as only a trace on three other occasions, and three times not at all. ML45 was found even less often, again was sometimes only a trace when it was present, but was sometimes quite intense (Figure 9).

Overall, relatively little variability was observed in the pattern of large-subunit proteins from one experiment to another. Most of the proteins seen on any one gel (including the faintly-staining ones) were regularly found in separate preparations of ribosomes, and thus probably represent ribosomal proteins, according to this criterion. Some possible explanations for those variations that were obtained in these experiments will be discussed below in the analysis of the small-subunit proteins, for which variability is a more serious problem.

Figure 9. Electrophoretic pattern of proteins from large subunits prepared in Buffer C (Experiment 2).

Figure 10. Electrophoretic pattern of proteins from large subunits prepared in Buffer C (Experiment 4).

Figure 11. Electrophoretic pattern of proteins from large subunits prepared in Buffer D (Experiment 6).

b. Resistance to salt-washing

The salt-washing procedure used in the preparation of mitochondrial ribosomes was designed to remove two types of contaminants, those which were bound directly to the ribosomes and those which were not bound, but nonetheless accompanied the ribosomes through the early stages of the preparation. As mentioned in Section IIA, treatment of ribosomes with high concentrations of monovalent salts should preferentially release non-ribosomal material bound to them. Figure 12 shows the effect of washing E. coli ribosomes with 1 M NH_4Cl . It is evident that the major difference between the two protein patterns is the presence of many high-molecular-weight proteins in the unwashed ribosomes. This phenomenon is well-documented in the literature (Subramanian, 1974; Hardy, 1975; Brouwer and Planta, 1975), and the proteins removed by salt-washing are universally regarded as non-ribosomal for a number of reasons, including the essentially undiminished functional activity of the salt-washed ribosomes.

The fact that most high-salt buffers also dissociate mitochondrial ribosomes to their subunits permits the removal of non-ribosome-bound, co-sedimenting contaminants by the use of two cycles of sucrose-density-gradient centrifugation under different ionic conditions. As discussed above (Section I) the isolation of the ribosomes first at low ionic strength (0.1 M KCl) as intact 55S particles, and then a higher salt concentrations as subunits should separate them from most non-ribosome-bound contaminants.

Figure 12. Effect of high-salt treatment on the protein content of *E. coli* ribosomes. *E. coli* ribosomes were washed with Buffer N (60 mM KCl) or Buffer O (1 M NH_4Cl) as described in MATERIALS AND METHODS. The electrophoretic pattern of the proteins of Buffer N ribosomes is shown in the photograph on the left, and of Buffer O ribosomes on the right. (The complete compositions of these buffers are given in Table IV.)

Low-salt treatment. The effectiveness of this procedure can be evaluated by comparing subunits prepared in this way ("derived subunits") with the subunits which are present in small amounts in the first, low-salt sucrose density gradient ("native subunits"; see Figure 3). Figure 13 shows the electrophoretic patterns obtained from native large and small subunits, and Figure 14 shows the pattern of the derived large subunit from the same preparation of ribosomes. The most obvious difference is the presence of two high-molecular-weight proteins in large quantity in the native subunits. Several other proteins of similar and higher molecular weights are also present in native but not in derived subunits; many of these, like the two major proteins, are found equally in both the large and small subunits. Another native-subunit-specific protein, seen to the left of ML30, is also found in somewhat smaller quantity in the small subunit. Additionally, numerous proteins of various molecular weights and staining intensities appear in the native large subunit but not in either of the other two patterns.

It seems reasonable to conclude that most if not all of the proteins found in native but not derived subunits represent non-ribosomal contaminants. The similarity of these results to those obtained with E. coli ribosomes (Figure 12) is clear, particularly with respect to the high-molecular-weight proteins. The lack of subunit-specificity of many of these proteins is also incriminating. Most importantly, they are essentially absent from large subunits derived in Buffer C or Buffer D, and such subunits are functionally active. By visual comparison of the

Figure 13. Electrophoretic patterns of proteins from low-salt treated large and small subunits. The proteins of mitochondrial large (left-hand photograph) and small (right-hand photograph) subunits, prepared by sucrose density gradient centrifugation in Buffer H, were analyzed by two-dimensional electrophoresis.

Figure 14. Electrophoretic pattern of proteins from large subunits prepared in Buffer C (Experiment 3).

The irregular white line in the upper left is a crack in the gel.

relative staining intensities of these proteins in native and derived subunits most of them appear to be reduced in quantity by 90% or more in derived subunits. Yet the activity of Buffer C derived subunits is 75% of that found in native subunits (shown as Buffer A subunits in Figure 7). Thus none of these proteins can be essential for the peptidyl transferase activity of the ribosome.

The kinds of contaminants which have thus been identified are various, although some loose generalizations can be made about them. The contaminants may be present in a very wide range of staining intensities, have molecular weights ranging from values similar to those of the ribosomal proteins to considerably larger values, and may be present subunit-specifically or not. However, a major part of the contamination seems to be represented by a group of relatively intense, high-molecular-weight, non-subunit-specific proteins. It is of some interest to ask whether this contamination is due primarily to adherent proteins or to protein-containing structures that are not bound to the ribosomes but are large enough to sediment in the same region of the sucrose density gradient. The buoyant density of the native subunit is significantly lower than that of the Buffer C or Buffer D derived subunits (Figure 7), suggesting that at least some of the native-subunit-specific protein must be bound directly to the particle under these low-salt conditions. Further, in two experiments the subunits were derived directly from the crude ribosome pellet, rather than from the 55S fractions of a sucrose density gradient. Such subunits showed

only small quantities of these contaminants (Figure 10). Most of these proteins thus seem to be bound directly to the ribosomes under low-salt but not high-salt conditions, rather than components of separate structures which also sediment in the 25S to 45S region of the gradient.

High-salt treatment. In addition to these proteins which were absent from all derived large subunit preparations, several proteins were found to be removed when the ribosomes were dissociated under some ionic conditions but not others. These proteins are the ones designated by letters in Table VI. The buffer conditions of interest are Buffer C and Buffer D: since large subunits prepared in either of these conditions are functionally active (Figure 7), the absence of a protein from either kind of preparation indicates that that protein is not required for the function. Thus MLc and MLg are not essential for peptidyl transferase activity because Buffer D subunits lack them but possess the activity. MLb, d, e, f, and h are specifically absent from Buffer C subunits. MLa is found in Buffer C preparations, but only in much smaller quantity than in Buffer D subunits; since this large quantitative difference does not correlate with the similarity between the two kinds of subunits in functional activity, this protein too may be regarded as nonessential.

The fact that these proteins are dispensable for the peptidyl transferase activity of the ribosome does not necessarily imply that they are contaminating non-ribosomal proteins. It only indicates that

they are not components of the active site for this function, and are not structurally required to maintain the proper conformation of the active site. Peptidyl transferase is only one of many reactions and interactions involved in protein synthesis, including translocation, initiation, termination, and binding to factors and mRNA. And not all of the ribosomal proteins are required for each function. For example, the E. coli ribosomal protein L7/12, which is needed for the interaction of the elongation factors EF-T and EF-G with the ribosome, can be removed without significantly affecting the peptidyl transferase activity (Hamel et al., 1972). Therefore, in the absence of data demonstrating that both Buffer C and Buffer D ribosomes are active in a more demanding assay (such as the translation of poly U or a natural mRNA), it remains quite possible that all of the lettered spots in Figure 8 do represent ribosomal proteins.

Extreme-salt treatment. As an attempt to identify some proteins which were required for peptidyl transferase activity (and which therefore must clearly be ribosomal proteins), the electrophoretic patterns of large subunits prepared with Buffers E, F, and G were evaluated. Since Buffer F and Buffer G subunits are essentially inactive, it seemed likely that they would be missing one or more proteins, and that among these proteins at least one was required for the activity. One or several of these proteins might also be found in diminished quantities in Buffer E subunits, which have diminished activity.

For this purpose, two preparations of Buffer F subunits and two of Buffer G subunits were subjected to two-dimensional electrophoresis. One of the Buffer F analyses is shown in Figure 15, and one of the Buffer G gels in Figure 16. Some differences were observed between the Buffer F preparations and between the Buffer G preparations, but there were several proteins which were absent or significantly diminished in staining intensity in both of the analyses in each pair. Buffer F did not completely remove any of the numbered large-subunit proteins, but it did reduce five of them to trace-level staining intensity. ML1, 15, 16, and 18 were diminished to a tenth or less, and ML2 to less than half, of the quantity normally present in Buffer C or Buffer D subunits. In subunits treated with Buffer G, small quantities of ML16 and ML18 were still present, but ML1 and ML15 were not detectable. In addition ML23, 29, 30, 33, 43, and 48 were absent and the quantity of ML47 was considerably reduced.

Interestingly, the amount of ML2 found in Buffer G subunits did not appear to be significantly less than that in subunits treated with Buffer C or Buffer D, even though the less stringent treatment with 1 M KCl at 5 mM MgCl_2 (Buffer F) did remove some of this protein. A similar phenomenon was observed among the large-subunit proteins which are designated by letters. MLg, which is absent from subunits treated with the moderate-salt Buffer D and is present only in trace quantities after treatment with Buffer F, is not removed by Buffer G. Such results are at odds with the expectation that the set of proteins removed by a

Figure 15. Electrophoretic pattern of proteins from large subunits prepared in Buffer F.

Figure 16. Electrophoretic pattern of proteins from large subunits prepared in Buffer G.

given ionic medium should be a subset of the proteins removed by more stringent conditions. This expectation derives from the continuous decrease in the total protein content of the subunit which is observed as the $KCl/MgCl_2$ ratio in the medium is increased (Figure 7). Apparently the generalization that increasing $KCl/MgCl_2$ ratios remove increasing amounts of protein applies to the total protein content (as measured by the buoyant density) and to some of the individual proteins, but ML2 and MLg are exceptions. That a few such exceptions to this rule might exist, due to specific effects of particular buffer compositions on the binding of some of the proteins to the ribosome, is not too surprising.

The protein complement found in Buffer E treated large subunits, however, represents a more serious anomaly. None of the numbered proteins were found to be removed or significantly diminished in quantity by washing with this buffer, offering no explanation for the reduction in functional activity produced by this treatment. Furthermore, even the lettered proteins (which are all removed by either Buffer C or Buffer D) were each found at least once in preparations of Buffer E subunits (Table VI). Thus the buoyant density data (Figure 7) indicate that these particles must be lacking some proteins present in Buffer C or Buffer D subunits, but no missing proteins can be identified by electrophoretic analysis. The reasons for this discrepancy are not clear, although some considerations described below (Section IV) may be relevant to this question.

As mentioned above, several proteins are specifically missing or reduced in quantity in large subunits that have been treated with ionic conditions sufficiently stringent to inactivate them. In particular, the loss of ML1, 15, 16 and 18 correlates with the loss of functional activity. In order to prove that the inactivity of the stripped subunit is due to the loss of these proteins (and not, for example, due to a conformational alteration of the subunit induced by these salt conditions) it is necessary to demonstrate that the activity can be reconstituted by adding the proteins back. Such a partial reconstitution would also be useful as an assay to determine which of the missing proteins is (are) required for the activity. However, attempts to reconstitute the subunit were unsuccessful.

c. Summary of the large-subunit proteins

Electrophoretic analysis of the large subunit of the bovine mitochondrial ribosome revealed 52 protein components which were reproducibly present in functionally active large subunits and which therefore probably represent ribosomal proteins. Eight additional proteins were present in large subunits prepared only under certain ionic conditions; these may also be ribosomal proteins, but they are not required for the peptidyl transferase activity of the large subunit. Treatment with very-high-salt buffers removes a small number of the 52 proteins and destroys the peptidyl transferase activity, suggesting that one or more of the missing proteins is required for this function.

2. Small Subunit

As shown in Figure 13 above, native small subunits prepared in low-salt buffers contain a set of mainly high-molecular-weight proteins which are also found in the native large subunit. Although lesser quantities of these proteins are sometimes found in derived small subunits treated with moderate salt concentrations (Figures 6 and 17), they are usually much diminished relative to the quantities present in the native subunit. Figure 18, obtained from the derived small subunit from the same ribosome preparation whose native subunits are shown in Figure 13, is completely lacking in these proteins. For some of the same reasons given in the discussion of the large subunit, it seems unlikely that these proteins represent functional components of the small subunit either.

Table VII shows the results of several electrophoretic analyses of high-salt-washed small subunits. The proteins which were found regularly in separate ribosome preparations and thus satisfy the criterion of reproducibility are designated by numbers. Some of the proteins which were seen less frequently are also tabulated and are designated by letters. (To avoid confusion with the numerals 1 and 0, the letters l and o have been skipped.) The electrophoretic positions of these proteins are given in Figure 19.

The overall level of reproducibility of the small-subunit proteins was less than that of the large-subunit proteins. To some extent this variability is due to the absence of many of the proteins from a partic-

Figure 17. Electrophoretic pattern of proteins from small subunits prepared in Buffer C (Experiment 2).

Figure 18. Electrophoretic pattern of proteins from small subunits prepared in Buffer C (Experiment 3).

Table VII. Appearance of small-subunit proteins in separate experiments.

Small subunits were prepared by sucrose density gradient centrifugation in the buffers specified, and the proteins were analyzed by 2-dimensional electrophoresis. The electrophoretic positions of the proteins listed are shown in Figure 19. +, present; -, trace; nr, not resolved.

Protein	Buffer B		Buffer C		Buffer D		Buffer E		
	Expt. 1	Expt. 2
(Fig.17)	Expt. 3
(Fig.18)	Expt. 4	Expt. 5	Expt. 6	Expt. 7	Expt. 8
(Fig.6)	Expt. 9
(Fig.20)
1	-	+	-	+	0	0	+	+	+
2	nr	nr	+	nr	+	-	nr	+	+
3	nr	nr	+	nr	+	-	nr	+	+
4	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+
7	0	+	+	+	+	+	+	+	+
8	+	+	0	+	+	+	+	+	+
9	0	+	0	-	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+
11	+	-	+	+	+	+	+	+	-
12	+	+	0	+	+	+	+	+	+
13	+	+	+	+	0	0	0	+	+
14	0	+	0	+	+	+	+	+	+
15	+	+	nr	+	+	0	+	+	+
16	+	+	+	+	+	0	0	-	+
17	+	+	+	+	+	0	+	+	+
18	+	+	-	+	+	0	+	+	+
19	+	+	+	+	+	0	+	+	+
20	+	+	-	+	+	0	+	+	+
21	+	+	0	+	0	0	+	+	+
22	+	+	-	+	0	0	+	+	+
23	+	+	+	+	0	0	+	+	+
24	+	+	+	+	+	-	nr	+	-
25	+	+	+	+	+	+	+	+	+
26	-	+	+	+	+	0	+	+	+
27	+	nr	0	+	+	0	+	+	+

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ular experiment. In Experiment 6 for example, the quantity of protein in the sample was inadequate; as a result all of the spots obtained were faint, so the absence of many of the spots which are normally faint in adequately loaded gels is understandable. The absence of 6 of the numbered proteins from Experiment 3 (Figure 18), however, is not so simply explained. On the other hand, there was a subset of the experiments in which variability was not a problem at all: none of the numbered proteins were lacking in Experiments 2, 8, or 9, and only two were missing in Experiment 4.

The variability which was observed in the other experiments seemed to be random with respect to which proteins were found to be missing. That is, it was not due to a high level of variability in a few specific proteins. Only two of the numbered proteins, MS13 and MS21, were absent from as many as three of the nine experiments. In the case of these two proteins (as well as MS1), much of the lack of reproducibility was due to their absence from subunits prepared in Buffer D; thus there may be a specific ionic effect on the binding of these proteins to the ribosome, as was seen for MLc and MLg above. A complementary effect was found for MSa and MSb. These two proteins were always present in subunits prepared in Buffer C, but only once in any other preparation.

The general impression of unreproducibility in the small subunit pattern derives partly from the relatively large number of spots which appear in fewer than half of the analyses and thus are designated by letters in Table VII. Some of these, like MSc and MSn (Figure 6),

appeared in only one experiment and can be easily disregarded. Others (MSa, b, f, k, and p) were present in four of the nine experiments and thus represent borderline cases which are not clearly excluded from the ribosomal protein category.

Another factor which contributes to this impression of unreproducibility is the variation in staining intensity among the different spots on a given gel. In most experiments (Figure 17 is a conspicuous exception) the electrophoretic pattern was found to consist of a minority of heavily-stained spots, usually including MS4, 5, 6, 10, 17, 19, 24, 28, 31, 37, and 38, and a majority of fainter spots. Furthermore the relative intensity of a given spot, particularly among the fainter group, varied considerably among different gels. (Compare MS13, 15, and 16 in Figures 6 and 20, for example.) Some differences in the stain-binding abilities of different proteins have been reported (Fazekas de St. Groth et al., 1963; Bickle and Traut, 1971), but the differences are not of the magnitude required to explain the present results if all of the small-subunit proteins were present in equimolar quantities in these gels. And in any case this phenomenon could not account for the variations observed for a particular protein in separate experiments. It is therefore necessary to conclude that the small-subunit proteins are present in differing molar quantities in these preparations. It follows that the individual subunits in each preparation are heterogeneous with respect to the set of proteins they contain.

Figure 20. Electrophoretic pattern of proteins from small subunits prepared in Buffer E (Experiment 9).

This heterogeneity could be due to the non-specific binding of contaminating proteins to some of the subunits. However, in view of the small number of heavily-staining proteins found in these particles, it seems unlikely that all of the fainter spots represent contaminants. A more plausible hypothesis is that a proportion of the subunits are lacking some of the ribosomal proteins, as a result of an in vitro artifact. This hypothesis is supported by the similar results which have been obtained (Kaltshmidt and Wittmann, 1970) and explained (Hardy, 1975) for E. coli ribosomal proteins. Some possible causes of such an artifact will be considered below (Section IV); but whatever the cause, it is probably responsible for the variability between ribosome preparations as well as the heterogeneity within each preparation. Similar considerations also apply (to a lesser extent) to the proteins of the large subunit, which show a lower level of variability and non-uniformity of staining intensities.

The effect of extreme-salt treatment (Buffer G) on the protein content of the small subunit was also investigated. One of the gels obtained is shown in Figure 21. The most noticeable result was a general reduction in the quantity of the low-molecular-weight proteins, those below MS31. Several proteins (MS13, 16, and 35) were completely absent from all three preparations of Buffer G subunits, and MS27, 28, 33, 36, 37, and 41 were diminished to negligible levels. Some proteins, on the other hand, were increased relative to the quantities present in high-salt-treated (Buffers C and D) subunits: this was particularly

Figure 21. Electrophoretic pattern of proteins from small subunits prepared in Buffer G.

The irregular spots seen above and to the right of MS4 are artifacts due to precipitation of the stain on the surface of the gel.

true of MS8, 12, and 14. In addition, some of the lettered, unreproducible proteins were found reproducibly in Buffer G subunits. MSd, e, f, and m were each found in all three preparations. It was also observed that several of the high-molecular-weight proteins present in low-salt but not high-salt subunits are present in considerable quantity in these extreme-salt subunits. This rather confusing set of observations is further evidence for a conclusion reached earlier in the discussion of the large subunit. The general effect of treating these ribosomes with buffers of increasing KCl/MgCl₂ composition is to reduce both the total amount of protein as measured by the buoyant density of the particles and the number of proteins as detected by gel electrophoresis. But some of the individual proteins may show the opposite response to such treatments, being preferentially removed by buffers of lower KCl/MgCl₂ ratios.

As was the case with the large subunit, no proteins were found to be removed or significantly diminished by treatment with Buffer E (Table VII), even though the buoyant density of the Buffer E subunit is somewhat higher than that of Buffer C or Buffer D subunits. Thus buoyant density measurements appear to be a more sensitive means of detecting the loss of protein as a function of KCl/MgCl₂ than the electrophoretic analyses are. This lack of sensitivity may be due to the high background level of variability between electrophoretic experiments, especially in the case of the small subunit. However, it also seems likely that these treatments are acting non-selectively to some extent, removing small quantities of many of the proteins

(and thus increasing the heterogeneity of the subunits with respect to their protein content) rather than larger quantities of only a few proteins. Any such non-selective effects would not be detected by comparing the relative staining intensities of the proteins in the gel.

3. Comparison of Large and Small Subunits

In order to determine whether the proteins of the large subunit were all distinct from those of the small subunit, the two electrophoretic patterns were compared with each other. To obtain a sufficiently accurate relative positioning of the patterns, samples of large- and small-subunit proteins were mixed together and electrophoresed on the same gel: one sample was a trace quantity of protein which had been radioiodinated as described in MATERIALS AND METHODS, and the other sample was a larger (stainable) quantity of protein. After electrophoresis, staining, and autoradiography, the patterns of stained and radioactive proteins were compared. Control experiments in which a small aliquot of a protein sample was radioiodinated and then mixed with a large aliquot of the same sample before electrophoresis showed that the labelling reaction had no detectable effect on the electrophoretic mobilities of the proteins.

As shown in Figure 22, it was found that many of the small-subunit proteins overlapped at least partially with proteins of the large subunit, and there were twelve pairs of proteins which were electrophoretically indistinguishable. These were MS1 and ML2, MS4 and ML3,

Figure 22. Relative electrophoretic positions of bovine mitochondrial large-subunit and small-subunit proteins.

Filled spots, large-subunit proteins; open spots, small-subunit proteins; hatched spots, overlapping large-subunit and small-subunit proteins.

MS7 and ML5, MS11 and ML12, MS15 and ML17, MS23 and ML20, MS25 and ML25, MS26 and ML27, MS30 and ML32, MS31 and ML31, MS32 and ML35, and MS40 and ML46. Only the proteins which have been designated by numbers in the discussion above are shown in Figure 22. In addition the electrophoretic positions of MSk and ML38 overlap, as do those of MS37 and MLh.

There are several possible explanations for the relatively large number of electrophoretically identical proteins in the two subunits. First, it might be that two copies of some proteins are present in the ribosome, one in each subunit, even though such a double role for a single protein has not been demonstrated in any other ribosome to date. Alternatively it may be that the two proteins in each pair are non-identical but are not resolved by the electrophoretic system; in view of the generally crowded appearance of Figure 22 it would not be surprising if some proteins occupied the same electrophoretic position by coincidence. A third possibility is that some of these proteins are contaminants similar to the non-subunit-specific proteins found in native subunits (Figure 13), except that they have molecular weights similar to those of the ribosomal proteins and remain bound to the ribosomes even after washing with high-salt buffers.

Finally, it is possible that each of the protein pairs represents a single ribosomal protein which is present at the level of one copy per intact ribosome, but fails to bind exclusively to one subunit or the other when the ribosome is dissociated. Such a partitioning of

individual proteins between the dissociated subunits has been observed in other ribosomes. The E. coli small-subunit protein S20, which co-electrophoreses with the large-subunit protein L26, has been shown to be identical to it by immunochemical and genetic studies (Tischendorf et al., 1974; Wittmann et al., 1974). Significant quantities of S5 have been found in the large subunit as well (Tischendorf et al., 1974). Likewise, three proteins of rat cytoplasmic ribosomes appear to be shared between the two subunits upon dissociation (Sherton and Wool, 1974).

If this phenomenon is responsible for some of the overlapping protein spots observed, several related effects might also be seen. First, this hypothesis requires that the total quantity of protein present in each pair represent no more than one copy per ribosome. This requirement could be satisfied by almost all of the protein pairs (MS31/ML31 is the exception), since in each case one or both members of the pair are seen in quantities significantly smaller than the average of the proteins present on the gel. It might also be expected that even though these proteins do not bind exclusively to either of the subunits, they do bind somewhat more strongly to the subunit with which they are associated in the intact ribosome and thus are found preferentially in this subunit upon electrophoretic analysis. In fact, in six of the protein pairs, one protein of each pair does appear in much greater quantity than the corresponding protein in the other subunit. The overlapping proteins which are present in relatively

larger quantity on one subunit are ML2, 5, 17, 25, and 27, which are found in greater amounts than the corresponding small-subunit proteins, and MS4, which occurs in greater amounts than ML3.

Since the failure of such proteins to segregate exclusively to one subunit or the other upon dissociation probably represents an artifact of the method used to effect the dissociation, it seems likely that differences in the ionic composition of the buffer used for this purpose could alter the extent of the protein partitioning. In fact it was reported (Sherton and Wool, 1974) that the subunit localization of one of the three partitioning proteins in rat cytoplasmic ribosomes was determined by the ratio of potassium to magnesium ions in the dissociating buffer. In the present results such a quantitative shift of a protein from presence only in one subunit to presence only in the other, due to differences in the ionic medium, was not observed. However MS1 was found to be specifically absent from small subunits prepared in Buffer D, and MS23 and 25 were significantly reduced in quantity in this ionic condition as well.

Clearly, this phenomenon could contribute to the heterogeneity in protein content observed in these subunits, although it could not completely account for it. In any case more direct evidence to test this hypothesis will not be easy to obtain. Whether the proteins in each pair are in fact identical can perhaps be determined by chemical or immunochemical analysis of the isolated proteins. If they are identical, however, the question whether they are ribosomal proteins

or non-ribosomal contaminants still rests primarily on the criteria discussed above. Some additional evidence that they are ribosomal proteins would be obtained if they could be shown to be located at the interface between the two subunits in the intact ribosome; if these proteins are responsible for the binding of the subunits to each other, their partitioning behavior is more easily understood. The E. coli S20/L26 pair has been found to be a subunit-interface protein (Morrison et al., 1973).

C. Experimental Evaluation of Possible Artifacts

A total of 93 proteins (the numbered spots in Figures 8 and 19) were found reproducibly in high-salt-treated mitochondrial ribosome subunits. Eighty-one of these proteins were distinguishable from each other on the basis of their electrophoretic mobilities. Although neither of these numbers is greater than had been expected on the basis of measurements of the total protein content of this ribosome, they are considerably in excess of the numbers of proteins which have been reported for any other kind of ribosome. For this reason it was necessary to consider the possibility of various artifacts which might generate an unrealistically large number of apparent ribosomal proteins. One such possibility, the presence of adventitiously bound contaminants in the ribosome preparation, has been discussed above.

Another possibility is that some single proteins may be represented by more than one spot on a gel. This could occur if a protein had been partially modified as the result of either a normal in vivo process or

an artifact of the isolation or electrophoretic procedure. Thus E. coli L7 is actually identical to L12, except that it has been acetylated at the N-terminus in vivo (Terhorst et al., 1972); similarly, the rat liver cytoplasmic ribosomal protein S6 sometimes appears as multiple electrophoretic species due to in vivo phosphorylation (Gressner and Wool, 1974). Two electrophoretically distinct forms, thought to represent different states of oxidation, have been observed for each of the E. coli proteins S11, S12, and S17 (Wittmann, 1974). Carbamylation by cyanate ions formed spontaneously in urea solutions can alter the electrophoretic mobility of proteins (Gerding et al., 1971). In the electrophoretic system used in the present experiments, these kinds of chemical modifications could result in a small alteration of a protein's mobility in the first electrophoretic dimension (in urea at pH 4.5), but would not affect the migration in the second dimension (in SDS). Some groups of spots which do show this electrophoretic pattern include ML42, 43, and 44; MS30 and 31; and MS37 and 38.

Extra spots might also be produced by proteolytic degradation of some of the proteins to discrete polypeptide products. Alternatively, some of the proteins may be covalently crosslinked to give specific aggregates. Disulfide crosslinks have been reported to form during two-dimensional electrophoresis (Kaltshmidt and Wittmann, 1970). Peroxidation of lipids in the crude ribosome preparation could also lead to protein crosslinking (Tappel, 1973).

Some precautions against these possible artifacts were taken in the experiments presented above. BHT was added to all solutions used in the ribosome preparation to prevent peroxidation-induced crosslinks. Disulfide bonds were reduced with dithiothreitol, and the proteins were maintained in the reduced state by the inclusion of reducing agents throughout the electrophoretic procedure. In addition, the electrophoretic results themselves argue against the likelihood of some of these artifacts. Carbamylation, for example, would be expected to affect most or all of the proteins to a similar extent; thus the presence of many proteins which do not show the peculiar electrophoretic pattern represented by ML42, 43, and 44 argues that such patterns are not produced by a non-specific chemical modification.

To obtain more evidence about the possibilities of proteolytic, crosslinking, or other chemical alterations, as well as the non-specific binding of contaminants to the ribosomes, the effect of the ribosome isolation procedure on the proteins of purified E. coli ribosomes was assessed. To a preparation of bovine liver mitochondria was added a small quantity of salt-washed E. coli ribosomes, equal to the quantity of mitochondrial ribosomes the mitochondria were estimated to contain. The mitochondria were then lysed with Triton X-100 and ribosomes pelleted at 100,000 x g as usual. After sucrose density gradient centrifugation in Buffer H, the ribosomes in the 70S (E. coli ribosome) peak were dissociated to subunits by a second centrifugation in a sucrose density gradient in Buffer E. The

proteins extracted from these subunits were then analyzed by two-dimensional electrophoresis.

The results suggested that the types of artifacts mentioned above are not a problem in these experiments. The electrophoretic pattern of the proteins of the E. coli 50S subunit showed no significant difference from that obtained from ribosomes subjected to the same re-isolation procedure in the absence of mitochondria, or from freshly prepared ribosomes. The 30S subunits were apparently slightly contaminated with mitochondrial large (39S) subunits, since traces of all of the most intensely staining 39S proteins were found on the gel of 30S proteins; with this exception, the 30S subunit protein pattern was likewise unaltered in the number and electrophoretic mobilities of the proteins. Thus incubation with components of the mitochondrial lysate under the conditions of our normal preparation procedure does not cause any increase in the apparent number of ribosomal proteins of exogenously added ribosomes. This result argues especially against the possibilities that the number of proteins found in mitochondrial ribosomes is overestimated due to proteolysis, crosslinking, or the adsorption of non-ribosomal proteins.

Another possible artifact of the ribosome isolation procedure was mentioned in Section I above: the binding of the ribosomes to DEAE-cellulose, or the subsequent elution, during the preparation of ribosomes by Method C might dislodge some of the ribosomal proteins. This possibility seems unlikely in view of the undiminished functional

activity of ribosomes prepared in this way. However, the electrophoretic results presented above include analyses of subunits prepared both with and without the use of DEAE-cellulose, so a comparison of the protein patterns produced by these two methods is easily made. In Tables VI and VII the samples for Experiments 2, 3, and 4 were DEAE-cellulose prepared ribosomes. Comparing these three experiments with the other experiments tabulated shows no proteins which are clearly removed by treatment with DEAE-cellulose, although some possibly significant effects can be seen. They most likely involve proteins MS9 and MS14, which are both absent from two of the DEAE-cellulose preparations whereas they are otherwise quite reproducible. Somewhat weaker correlations are observed for MS30, MS33, and ML21. Thus it remains possible that a few proteins, particularly in the small subunit, are specifically removed by treatment with DEAE-cellulose. It may also be that MS9 and MS14 are unnecessary for the translation of poly U; however, a more direct comparison between the activities of ribosome preparations known to contain and to lack these proteins will be necessary to establish this point.

III. COMPARISON WITH PROTEINS OF NON-MITOCHONDRIAL RIBOSOMES

Yet another way in which the number of proteins in mitochondrial ribosomes could be over-estimated by the results of these experiments would be the possible contamination of the preparations by significant quantities of cytoplasmic ribosomes. Although the more purified preparations described in Section I contain no detectable cytoplasmic

ribosomes, it seemed worthwhile to demonstrate directly the absence of cytoplasmic ribosomal proteins from the electrophoretic patterns of mitochondrial ribosomes. It was expected that the results would also provide a comparative basis for the description of the properties of mitochondrial ribosomal proteins.

The relative electrophoretic positions of the two sets of ribosomal proteins were determined by co-electrophoresing radioiodinated and stainable protein samples as described in Section IIB3. The results for the large subunits of the two ribosomes are shown in Figure 23, and for the small subunits in Figure 24. With the exception of one protein which comigrates with MS25, all of the mitoribosomal proteins are electrophoretically distinct from those of the corresponding subunit of the cytoplasmic ribosome. Indeed most of the cytoribosomal proteins are found in a different region of the gel from that which contains most of the mitoribosomal proteins. Comparing proteins of similar molecular weights (similar positions in the second electrophoretic dimension), it is evident that most of the cytoribosomal proteins migrate more rapidly in the first dimension than do the mitoribosomal proteins. This tendency, which is most evident in the comparison of the large subunits, implies that the cytoplasmic ribosomal proteins bear a greater positive charge at pH 4.3 than the mitoribosomal proteins. It also suggests that the isoelectric points of many of the cytoribosomal proteins may be higher than those of the mitoribosomal proteins.

Figure 23. Relative electrophoretic positions of bovine mitoribosomal and cytoribosomal large-subunit proteins.

Filled spots, mitoribosomal proteins; open spots, cytoribosomal proteins.

Figure 24. Relative electrophoretic positions of mitoribosomal and cytoribosomal small-subunit proteins.

Filled spots, mitoribosomal proteins; open spots, cytoribosomal proteins; hatched spot, overlapping mitoribosomal and cytoribosomal proteins.

Comparing the mobilities of the two sets of proteins in the second electrophoretic dimension shows that the distributions of molecular weights of the mitoribosomal and cytoribosomal proteins are similar. The large-subunit proteins of the mitochondrial ribosome are slightly smaller on the average than those of the cytoplasmic ribosome, and the reverse is true for the small-subunit proteins.

The major difference in electrophoretic characteristics between mitochondrial and bacterial ribosomal proteins lies in the much greater second-dimension mobility of the latter. As seen in Figure 12, over half of the ribosomal proteins of E. coli have molecular weights less than that of myoglobin (17,200), whereas relatively few mitoribosomal proteins are this small. A more detailed description of the molecular weight properties of the mitoribosomal proteins is given in Section IV.

The suggestion above that mitoribosomal proteins may have isoelectric points significantly lower than those of other kinds of ribosomal proteins has some support in the literature. The relatively slow first-dimension migration of mitoribosomal proteins can be seen in the comparison of the large subunits of Xenopus laevis ribosomes (Leister and Dawid, 1974, Figure 7). About half of the proteins of rat mitochondrial ribosomes are anionic at pH 8.6 (van den Bogert and de Vries, 1976), whereas nearly all eukaryotic cytoplasmic and bacterial ribosomal proteins are cations at this pH. Indeed, while most ribosomal proteins have isoelectric points that are too high to permit analysis by isoelectric focusing, it has been reported that rat mitoribosomal proteins

can be analyzed by this technique (Czempiel et al., 1976). The isoelectric points of most of the proteins were reported to lie below a pI of 8.5, with none above a pI of 10.

Since the two-dimensional electrofocusing/electrophoresis system described in the latter report should be capable of much greater resolution than the system used in the experiments described above, an attempt was made to reproduce these results. The methods given for the electrofocusing procedure (Czempiel et al., 1976; Klose, 1975) were followed as closely as possible; the only significant exception was the use of a constant-voltage power source instead of a pulsed-power source. The electrofocusing was performed on parallel samples for various periods of time in order to determine the length of time required to focus the proteins. This question is of considerable importance in isoelectric focusing experiments. Short periods of time may be adequate for the carrier ampholytes to reach their equilibrium positions and establish the pH gradient, but not for the proteins, which are retarded by the gel due to their higher molecular weights, to reach their equilibrium positions. Thus false estimates of the isoelectric points of the proteins would be obtained. On the other hand after long times the pH gradient begins to break down, probably due to the migration of the carrier ampholytes out of the gel (Chrambach et al., 1973).

The results of this experiment are shown in Figure 25. After 3 hours of electrofocusing the pattern of protein spots looked similar to

Figure 25. Isoelectric focusing of mitoribosomal proteins.

Proteins from bovine mitochondrial ribosomes prepared in Buffer A were electrofocused as described in MATERIALS AND METHODS for 3 hr (upper photograph), 5 hr (middle photograph), or 7 hr (lower photograph). The electrofocusing gels were then extruded and placed at the top of SDS-gel slabs for electrophoresis in the second dimension. The isoelectric focusing dimension is horizontal in the photographs, and the SDS-electrophoresis dimension is from top to bottom. The point of application of the protein sample is at the upper left-hand corner of each photograph.

that shown by Czempiel et al. (1976). It may be seen that the spots tend to be arranged diagonally, especially on the right-hand side of the pattern, rather than randomly. This diagonal tendency, which is seen to a greater extent in the standard two-dimensional system used in Section II, is an indication that the proteins are being differentially retarded according to their molecular weights as they move through the first-dimension gel. Electrofocusing/SDS-electrophoresis patterns obtained after the proteins have reached their equilibrium positions in the first dimension show an apparently random distribution of the spots (O'Farrell, 1975).

The pattern observed after 5 hours of electrofocusing confirms the conclusion that most of the proteins were not focused at the 3-hour time point. Many of the proteins continued to move until they were caught at the basic end of the gel. Furthermore several proteins of relatively high molecular weight, seen in the upper right-hand corner of this pattern, also moved to the basic end of the gel after 7 hours. In the 7-hour pattern, those proteins which remained in the body of the first-dimension gel showed the randomly-dispersed appearance expected of focused proteins. It may be noted (Figure 26) that the pH gradient was already formed at 3 hours and remained stable through the 7-hour time point, although it had deteriorated by 10 hours.

The results thus seem to contradict those of Czempiel et al. (1976), and to suggest that the very low isoelectric points reported by these authors are in error due to the failure of the proteins to reach their

Figure 26. The pH gradient formed during isoelectric focusing of mitochondrial proteins. Measurements of pH were made on gels run in parallel with the first-dimension gels of the experiment shown in Figure 25. After electrofocusing for the periods of time indicated, the gels were sliced into 5 mm sections and each section placed in 1 ml of water. After 12 hours of soaking at room temperature, the pH of each solution was measured with a Fisher pH microelectrode.

isoelectric positions in the gel. It also appears that considerably fewer than half of the mitoribosomal proteins have isoelectric points below 8.5, in contrast to the results of van den Bogert and de Vries (1976). Although the latter results were obtained by electrophoresis at pH 8.6 rather than by isoelectric focusing, it would be surprising if these methodological differences were the source of the discrepancy. Some preliminary electrophoretic experiments, however, appear to confirm that about half of the large-subunit proteins are anions at pH 8.6 (Robert Hessler, 1976, private communication). Thus the question of the actual isoelectric points of the mitoribosomal proteins remains somewhat uncertain. But even the electrofocusing experiments presented above show that a relatively large proportion of these proteins are isoelectric below pH 8.5. All of the evidence therefore agrees that these proteins are significantly more acidic than the proteins of E. coli or mammalian cytoplasmic ribosomes, over half of which have isoelectric points above 10 (Kaltshmidt, 1971; Huynh-van-Tan et al., 1974).

IV. MOLECULAR WEIGHTS OF MITOCHONDRIAL RIBOSOMAL PROTEINS

The molecular weights of the mitoribosomal proteins were determined from their mobilities in the second electrophoretic dimension and are given in Table VIII (large-subunit proteins) and Table IX (small-subunit proteins). The molecular weights of the large-subunit proteins range from 11,100 to 45,500 (average: 21,200); the small subunit contains proteins from 12,300 to 43,000, averaging 22,600. For comparison, the average molecular weights of E. coli large-subunit and small-subunit

Table VIII. Molecular weights of mitochondrial large-subunit proteins.

<u>Protein</u>	<u>MW x 10⁻³</u>	<u>Protein</u>	<u>MW x 10⁻³</u>
1	45.5	27	18.3
2	42.5	28	18.2
3	38.5	29	18.2
4	32	30	18.0
5	31.5	31	17.8
6	31.5	32	17.6
7	31	33	17.4
8	29.2	34	17.1
9	28.1	35	16.8
10	27.6	36	17.0
11	26.3	37	16.5
12	26.0	38	16.2
13	26.0	39	15.8
14	25.7	40	15.9
15	25.0	41	15.3
16	23.6	42	15.2
17	23.5	43	15.2
18	22.2	44	15.4
19	21.6	45	14.7
20	20.6	46	14.0
21	20.4	47	13.6
22	19.9	48	13.4
23	19.5	49	13.5
24	19.2	50	13.3
25	19.0	51	11.2
26	18.8	52	11.1

Table IX. Molecular weights of mitochondrial small-subunit proteins.

<u>Protein</u>	<u>MW x 10⁻³</u>	<u>Protein</u>	<u>MW x 10⁻³</u>
1	43	21	21.2
2	40	22	21.0
3	38	23	20.3
4	38	24	19.7
5	33.5	25	18.9
6	32	26	18.3
7	32	27	18.3
8	29	28	17.8
9	28.1	29	17.6
10	26.7	30	17.5
11	25.6	31	17.5
12	24.8	32	16.9
13	24.1	33	16.3
14	23.7	34	15.9
15	23.5	35	15.5
16	23.3	36	15.3
17	22.0	37	14.6
18	21.8	38	14.7
19	21.6	39	14.0
20	21.1	40	13.7
		41	12.3

proteins are 16,300 and 19,000 (Dzionara et al., 1970). This rather large difference in the molecular weight distributions of mitochondrial and bacterial ribosomal proteins can be easily observed by visual comparison of their electrophoretic patterns (Figures 5 and 6 vs. Figure 12). Reported average molecular weights of mammalian cytoplasmic ribosomal proteins range from values similar to those of bovine mitoribosomal proteins (Martini and Gould, 1971; Creusot et al., 1975) to significantly larger values (Howard et al., 1975; Lin and Wool, 1974).

The data in Tables VIII and IX, with an assumption, permit a calculation of the total content of protein in the mitochondrial ribosome. If one copy of each of these proteins is present per ribosome, the sum of their molecular weights should equal the total protein content in daltons. This sum is 2.03×10^6 . The sum of the molecular weights of the E. coli ribosomal proteins, for comparison, is only 0.94×10^6 (Dzionara et al., 1970). This two-fold difference is due partially to the larger size of the mitoribosomal proteins, but mainly to their greater number. In fact, mitochondrial ribosomes appear to contain at least as much protein as 80S mammalian cytoplasmic ribosomes. As mentioned above, there is some uncertainty about the molecular weights of the cytoribosomal proteins; but because of the smaller number of proteins in these ribosomes, even the larger values reported for their molecular weights lead to estimates for the total protein content of 1.73×10^6 to 2.05×10^6 daltons (Howard et al., 1975; Lin and Wool, 1974), about the same as that of bovine mitoribosomes.

This relatively large protein content had been expected on the basis of physical-chemical measurements on the mitochondrial ribosomes. Since these measurements provided quantitative predictions, it was of interest to determine how closely the different kinds of estimates for the total protein content agreed. The protein content can be calculated from the buoyant density, ρ , of the ribosome in CsCl and the molecular weight of the ribosomal RNA, M_{RNA} , by the equations

$$\% \text{ RNA} = 309.8 - \frac{396.6}{\rho} \quad (\text{Hamilton and O'Brien, 1974})$$

and Protein content = $\frac{M_{\text{RNA}}}{\% \text{ RNA}} \times 100 - M_{\text{RNA}}$. The results of such a calculation, using a value of 1.43 g/ml for the buoyant density of either subunit and 0.54×10^6 and 0.35×10^6 for the molecular weights of the large and small ribosomal RNA's (Robberson et al., 1971), are given in the first line of Table X.

A second, independent measurement of the protein content can be obtained by determining the molecular weight of the ribosome by high-speed equilibrium centrifugation, and subtracting M_{RNA} . The second line of Table X is based on the values 1.65×10^6 and 1.10×10^6 for the molecular weights of the large and small subunits, respectively (Hamilton and O'Brien, 1974).

The results obtained from the electrophoretic analysis are given in the last line of the table. The sum of the molecular weights of the 52 proteins in the large subunit is 1.10×10^6 , a value which agrees well with the protein content as calculated from the buoyant density of this subunit or from its particle weight. However, the total of the 41

Table X. Calculation of the protein content of bovine
mitochondrial ribosomes.

<u>Data used in calculation</u>	<u>Protein content $\times 10^{-6}$ (daltons)</u>		
	<u>Large subunit</u>	<u>Small subunit</u>	<u>Total</u>
Buoyant density of subunit,
molecular weight of rRNA	1.10	0.71	1.81
Molecular weight of subunit,
molecular weight of rRNA	1.11	0.75	1.86
Molecular weights of
individual proteins	1.10	0.93	2.03

small-subunit proteins, 0.93×10^6 daltons, is somewhat greater than the protein content determined by other methods.

One interpretation of this discrepancy is that it indicates an overestimate of the number of proteins in the small subunit by about 20%. An argument in favor of this interpretation can be found in the results presented in Section IIB3. Several of the proteins in the large and small subunits were found to be electrophoretically indistinguishable from each other, possibly because each pair represents a single protein which partitions between the two subunits when the ribosome is dissociated. Five of these proteins are present in much greater quantity in the large subunit than in the small, suggesting that they are actually large-subunit proteins and thus should not be included in the small-subunit list. If MS1, 7, 15, 25, and 26 were disregarded for this reason, the total of the small-subunit protein molecular weights would be 0.79×10^6 daltons rather than 0.93×10^6 daltons, agreeing more closely with the total protein content determined by physical-chemical measurements (Table X).

A more convincing interpretation, however, is that the original assumption (that each ribosome contains one copy of each of the proteins) is false. If, on the average, the proteins are present in less than unit quantity in each ribosome, the total protein content would be overestimated by simply adding up the proteins' molecular weights. It is clear in the electrophoretic patterns shown above that the proteins are not all present in equal quantities, particularly in the

small subunit, but there is no direct evidence whether the average spot intensity in these patterns represents one copy per ribosome or less than one. However the results which have been obtained with other ribosomes indicate that ribosomal proteins are more likely to be deficient than to be found in greater than unit quantities when ribosomes are prepared according to the methods used in the present experiments.

Although E. coli ribosomes prepared under very gentle mechanical and ionic conditions appear to contain unit amounts of nearly all of the ribosomal proteins, the more usual procedures (particularly the salt-washing steps used to remove non-ribosomal proteins) remove varying quantities of many of the proteins (Hardy, 1975). As a result, several ribosomal proteins appeared on two-dimensional gels as spots that stained only faintly (Kaltshmidt and Wittmann, 1970), quantitative analyses showed that many of the proteins were present in less than unit stoichiometry (Voynow and Kurland, 1971; Weber, 1972), and the sum of the molecular weights of the E. coli small-subunit proteins was found to exceed the measured total protein content of the subunit by 25% (Dzionara et al., 1970).

It seems likely that this partial removal of ribosomal proteins by salt treatment is responsible for the finding that the mitochondrial ribosomes in each preparation appear to be heterogeneous with respect to their content of the individual ribosomal proteins, and for the variations observed among separate ribosome preparations. Such effects

may also explain the observation that none of the proteins are found to be removed by treatment with Buffer E, even though the buoyant densities of the subunits are increased by this treatment (Section IIB). The action of Buffer E may be relatively nonspecific, removing some proteins from all of the ribosomes but not always the same set of proteins. In this case none of the proteins would be found to be absent in the electrophoretic analyses, and even reductions in the relative quantities of some of the proteins might be undetectable.

Unfortunately no method has yet been developed for preparing ribosomes free of contaminating proteins but containing stoichiometric quantities of all the ribosomal proteins. But if this were done, the results presented above indicate that the measured protein content of mitochondrial ribosomes would be as given in the bottom line of Table X. Since functional ribosomes in vivo probably do contain unit copies of nearly all the proteins (Hardy, 1975), the comparison shown in Table X corresponds to some extent to the difference between the mitochondrial ribosome in vivo and in vitro. Thus one useful result of the electrophoretic experiments is that they provide information about the nature and degree of the differences between the ribosomes as isolated and as they probably exist in vivo. The table, and the electrophoretic patterns themselves, permit the conclusion that the isolated ribosomes are lacking considerable quantities of many of the proteins; further, it appears that most of the difference is localized in the small subunit, whereas the large subunit is more nearly intact.

V. COMPARISON WITH PROTEINS OF RAT MITOCHONDRIAL RIBOSOMES

The proteins of eukaryotic cytoplasmic ribosomes show a relatively low degree of evolutionary divergence. No differences were found among mammals, birds, or reptiles in the two-dimensional electrophoretic pattern of their cytoribosomal proteins by Delaunay et al. (1973). Other laboratories (Martini and Gould, 1975; Kuter and Rodgers, 1974) have been able to detect some electrophoretic differences among these species, but in all cases the number of such differences has been quite small. Two proteins were found to differ in comparisons of rat, mouse, and hamster cytoribosomal proteins, and one further protein was altered in HeLa cells (Kuter and Rodgers, 1974). However, the mitochondrial ribosomes of various distantly related organisms show greater differences in their physical-chemical properties than do the cytoplasmic ribosomes of the same organisms (Figure 1). This diversity at the level of the overall structure of the ribosome suggested that differences might be found between more closely related organisms at the level of the electrophoretic properties of their ribosomal proteins.

To test this possibility the mitoribosomal proteins of another mammal, the rat, were analysed and compared with the proteins found in bovine mitoribosomes. The electrophoretic results from one of the two preparations of rat mitoribosomes which were analyzed as described above for bovine mitoribosomes are shown in Figures 27 (large subunit) and 28 (small subunit). The large-subunit pattern was similar in overall appearance to that obtained from bovine mitoribosomes. In

Figure 27. Electrophoretic pattern of proteins from rat mitochondrial large subunits.

Figure 28. Electrophoretic pattern of proteins from rat mitochondrial small subunits.

comparisons of such complex patterns, it is easiest to recognize correspondences between proteins that have unusual electrophoretic properties. Thus a very acidic protein (that is, a protein which migrates more slowly in the first dimension than other proteins of the same molecular weight) is seen in the rat pattern at a position near that of the bovine protein ML30, and a faintly-stained protein corresponding to ML33 is present as well. Spots corresponding to the very basic protein ML13 and the low-molecular-weight proteins ML49 and 50 can also be identified. Some obvious differences between the two sets of proteins are also evident, such as the very basic rat protein just to the right of the spot corresponding to ML20, which was never found in bovine mitoribosomes. A detailed comparison shows many other differences. The small-subunit pattern showed no obvious similarity to that of bovine small-subunit proteins, and no individual rat proteins clearly corresponded to any bovine proteins.

In order to compare the rat and bovine patterns more accurately, samples of proteins from both sources were mixed together and co-electrophoresed. The bovine sample was a large (stainable) quantity of protein and the rat sample was a trace quantity (from a third preparation of ribosomes) radioactively labelled by reductive methylation as described in MATERIALS AND METHODS. The results are shown in Figures 29 and 30. In these figures only those proteins which were found in at least two of the three rat mitoribosome preparations are given; most of these appeared in all three. Rat proteins which have

Figure 29. Schematic diagram of rat mitochondrial large-subunit proteins.

Figure 30. Schematic diagram of rat mitochondrial small-subunit proteins.

electrophoretic mobilities identical to some of the bovine proteins are marked with the number of the bovine protein, as given in Figure 8 and Figure 19. In addition some of the rat proteins were found in positions very close to those of some bovine proteins and are designated by parenthesized numbers. Some of these near-correspondences occur among the proteins with unusual electrophoretic properties which appeared to represent clear similarities between rat and bovine large-subunit proteins. In these cases (ML13, 30, 33, 49, and 50) it seems very likely that the rat protein is closely related to that of the cow even though some difference is detectable.

Bovine mitoribosomal proteins are clearly more similar to the mitoribosomal proteins of rat than they are to the proteins of bovine cytoplasmic or bacterial ribosomes. Both kinds of mitoribosomal proteins have similar molecular weight and charge properties, which are different from those of cytoplasmic or E. coli ribosomal proteins (Section III). It seems likely that the numbers of proteins in both mitoribosomes are also about the same. Forty-eight proteins are seen in the rat large subunit as compared with 51 for the bovine large subunit. The small number of proteins found in rat small subunits (24) is rather puzzling. However, the quantity of protein used in these experiments may have been too small to permit the detection of faintly staining proteins. The similar physical-chemical properties of rat and bovine mitoribosomal subunits (O'Brien, 1971; O'Brien et al., 1974; de Vries and Kroon, 1974) indicate that the protein contents of the two should be nearly the same.

At a more detailed level of analysis, the electrophoretic positions of the individual proteins show more differences than similarities between these two mammalian species. A convenient means of quantitating the degree of similarity between the electrophoretic patterns of two sets of ribosomal proteins has been suggested by Delaunay and Schapira (1974), using the formula

$$\text{degree of similarity, } P = \frac{(a + b) - n}{\frac{1}{2}(a + b)}$$

where a is the number of proteins in one of the ribosomes, b is the number in the other ribosome, and n is the total number of electrophoretically different proteins found in a mixture of the two samples. If all of the proteins are electrophoretically distinct from each other, $P = 0$. When no differences at all are found, as reported by Delaunay et al. (1973) for mammals, birds, and reptiles, a value of $P = 1$ is obtained. The three protein differences seen between rat and HeLa cytoribosomal proteins (Kuter and Rodgers, 1974) correspond to $P = 0.95$.

When this formula is applied to the proteins of mitochondrial large subunits, in which only 13 of the 48 rat proteins are electrophoretically identical to bovine proteins, it is found that $P = 0.26$. A similar calculation for the small subunits, in which there are only 8 electrophoretic identities, gives a value of 0.25. The formula slightly underestimates the actual degree of similarity between the small subunits, since even if all 24 of the rat proteins detected were identical to cow proteins, a value of $P = 1$ would not be obtained.

However if a correction is made for the apparent failure of these experiments to detect all of the rat small-subunit proteins, the value of P is still only 0.33.

These results are consistent with the finding of at least seven mitochondrial large-subunit proteins which differ even between two species of the frog genus Xenopus ($P = 0.83$) (Leister and Dawid, 1975). The evidence thus indicates that the degree of evolutionary divergence among mitoribosomal proteins is much higher than among the cytoribosomal proteins of the same organisms. This conclusion extends to more closely-related species the generalization (see INTRODUCTION) that most of the physical and chemical properties of ribosomes appear to diverge more rapidly during evolution for the ribosomes of mitochondria than for their extramitochondrial counterparts. Some speculations about the molecular and genetic basis of this higher evolutionary rate will be discussed below.

DISCUSSION

The results presented above are relevant to questions about the structure and the evolution of mammalian mitochondrial ribosomes. It is found that the large complement of protein observed in these ribosomes by physical-chemical measurements can be accounted for by the number and sizes of the individual ribosomal proteins. The molecular weights of these proteins are not exceptionally high; rather, it is the large number of proteins present in these ribosomes which is primarily responsible for their high protein content. The proteins are found to be somewhat more acidic than those of bacterial or eukaryotic cytoplasmic ribosomes, a property which may be related to differences in the kinds of intermolecular bonding interactions which prevail in such a protein-rich structure. Finally, the electrophoretic properties of mammalian mitoribosomal proteins, like many of the other structural properties of mitochondrial ribosomes in general, show a greater degree of phylogenetic variation than is found among cytoplasmic ribosomes.

Ninety-three proteins are found in bovine mitochondrial ribosomes, as compared with about 70 in mammalian cytoplasmic ribosomes (Wool and Stöffler, 1974) and 53 in *E. coli* ribosomes (Wittmann, 1974; Pettersson *et al.*, 1976). Similar relatively large numbers of ribosomal proteins have been reported for the mitochondrial ribosomes of other vertebrate species. Leister and Dawid (1974) found 84 mitoribosomal proteins in

the frog Xenopus laevis. Recently the proteins of rat mitochondrial ribosomes were analyzed, and numbers ranging from 70 (van den Bogert and de Vries, 1976) to 107 (Czempiel et al., 1976) were reported.

The large number of proteins found in bovine mitochondrial ribosomes does not seem to be attributable to contamination of the ribosomes by non-ribosomal proteins, on the basis of a variety of evidence. These proteins are reproducibly found in separate preparations of ribosomes, and they are not removed by treatments that have been found to remove contaminating proteins from other kinds of ribosomes. Indeed, only a few of them are removed by even more stringent treatments that result in complete inactivation of the peptidyl transferase function of this ribosome. Furthermore, when heterologous ribosomes are added to the mitochondria and then re-purified by the same method used for the preparation of mitochondrial ribosomes, they are not contaminated by adsorbed proteins.

There is a possibility that the present results overestimate the number of bovine mitoribosomal proteins to some extent, due to a partitioning of some proteins between the two subunits. Although the total number of proteins found in analyses of the large and small subunits separately is 93, only 81 of these can be observed in mixtures of the two sets of proteins. That is, 12 of the large-subunit proteins are electrophoretically indistinguishable from proteins of the small subunit. It is therefore possible that the two members of all or some of these overlapping protein pairs are in fact identical, and that

each pair should be counted only once in the enumeration of the bovine mitoribosomal proteins. The evidence in favor of this possibility is only suggestive, and further characterization of the individual proteins involved will be required to resolve this question.

The proteins of bovine mitoribosomes are found to have molecular weights averaging 21,200 in the large subunit and 22,600 in the small subunit, values similar to those of mammalian cytoribosomal proteins. Much higher molecular weights were reported for the mitoribosomal proteins of Xenopus, with averages of 27,000 and 33,300 for the large and small subunits respectively (Leister and Dawid, 1974). Molecular weights were not determined in the published studies of rat mitoribosomal proteins, but the present experiments indicate that these are about the same size as bovine mitoribosomal proteins. The sum of the molecular weights of the bovine mitoribosomal proteins is somewhat greater than the total quantity of protein calculated to be present in these ribosomes on the basis of their buoyant density, particularly in the case of the small subunit. A similar result was obtained with Xenopus mitoribosomes (Leister and Dawid, 1974). The simplest interpretation of this phenomenon is that a proportion of the ribosomes in a given preparation are lacking some of the proteins, so that on the average there is less than one copy of each of these proteins per ribosome. This interpretation is consistent with results that have been obtained for E. coli ribosomes.

The finding that bovine mitoribosomal proteins are relatively acidic, compared to bacterial or mammalian cytoplasmic ribosomal proteins, is in qualitative agreement with the reported results of van den Bogert and de Vries (1976) and Czempiel et al. (1976) on rat mitoribosomal proteins. The proteins of Xenopus mitochondrial ribosomes also appear to be more acidic than those of the cytoplasmic ribosomes, at least in comparisons between the large subunits (Leister and Dawid, 1974). Quantitatively, there are some differences between the results of the present experiments and those which have been published for rat mitoribosomal proteins. The cause of these discrepancies is unclear. It does not seem to be related to differences between the rat and bovine proteins: the direct comparisons of these two sets of proteins by electrophoresis show that they have similar charge properties at pH 4.3.

A large number of electrophoretic differences are found between bovine and rat mitoribosomal proteins. This observation is consistent with comparisons which have been made between species related both more closely and more distantly than rat and cow. Xenopus laevis and Xenopus mulleri differed from each other in at least seven of the proteins of the large subunit alone (Leister and Dawid, 1975). Rat mitoribosomal proteins showed no similarity with those of the fungus Neurospora (van den Bogert and de Vries, 1976). No obvious similarities can be seen when the patterns of bovine or rat mitoribosomes are compared with those published for Xenopus laevis (Leister and Dawid, 1974), obtained in an

essentially identical electrophoretic system. Indeed even the molecular weights of the frog proteins are quite different from those of the mammalian proteins.

The significance of these results lies in their contribution to studies on the structure of ribosomes and on the evolution of mitochondrial genes. First, it has been suggested that the low buoyant density of animal mitochondrial ribosomes might be due to the presence of adherent membrane fragments (Borst and Grivell, 1971). The present results do not support the hypothesis that a third component of lower density than protein (i.e., lipid) makes any significant contribution to the low buoyant density of these ribosomes: the quantity of protein alone required to account for the observed density does not exceed the quantity which actually appears to be present by electrophoretic analysis.

The remarkably large number (and total quantity) of proteins in these ribosomes raises some interesting questions about the similarities and differences that must exist between these particles and other structural kinds of ribosomes with respect to their biosynthesis, assembly, and detailed functional activities. For example, it seems possible that these ribosomes, in which the ratio of RNA to protein is only 1:2, are held together predominantly by different kinds of intermolecular bonding interactions than are found in *E. coli* ribosomes, which have an RNA:protein ratio of about 1:0.6. Clearly the structure of the mitoribosome must involve more protein-protein interactions and

fewer protein-RNA interactions than that of the E. coli ribosome. Proteins which bind directly to polynucleotides, such as histones and many ribosomal proteins, frequently have exceedingly high isoelectric points; presumably a large part of the energy of binding in these cases is due to electrostatic attraction (and lack of electrostatic repulsion) between charged residues of the proteins and the phosphate backbone of the nucleic acid. Ionic bonding between two proteins, on the other hand, requires the interaction of both acidic and basic amino acids in the proteins. Thus the high protein content of animal mitochondrial ribosomes may be related to the lower basicity which is observed in their proteins. It may be predicted that other very protein-rich ribosomes, like the mitochondrial ribosomes of Tetrahymena (Chi and Suyama, 1970) and Candida utilis (Vignais et al., 1972), will likewise contain relatively acidic ribosomal proteins.

Since hydrophobic interactions are probably more important in stabilizing protein-protein associations than protein-RNA binding, it seems reasonable that this kind of interaction plays a larger role in the fundamental structural organization of very protein-rich ribosomes. One characteristic of hydrophobic interactions is that they are resistant to disruption by high salt concentrations, whereas electrostatic interactions are highly susceptible. Thus it is of interest that most of the proteins of bovine mitochondrial ribosomes are found to be much more resistant to salt-stripping than those of the cytoplasmic ribosomes (O'Brien et al., 1976). These mitoribosomes thus share some properties

(though to a lesser extent) with the ribosomes of the extreme halophile Halobacterium cutirubrum: the latter are structurally and functionally stable at the normal intracellular salt concentration of 4 molar, and their proteins have isoelectric points much lower than even those of animal mitoribosomes (Bayley, 1966).

The large number of electrophoretic differences observed between the mitoribosomal proteins of two mammalian species complements the previous reports of even larger differences in the structural properties of mitoribosomes from more distantly related organisms. Taken together, these observations clearly indicate a relatively high rate of evolutionary divergence among these ribosomes, and invite a consideration of the possible cellular, genetic, or molecular bases of this high evolutionary rate.

Perhaps the simplest explanation for this phenomenon is the possibility that mitochondrial ribosomes are not subject to very strong selective pressures in nature. Relative to the cytoplasmic ribosomes, mitoribosomes make only a very few different proteins. Although these proteins are major (and essential) components of the mitochondrion, their total quantity is also much less than the total quantity of cellular proteins synthesized on cytoplasmic ribosomes. Only a small number of ribosomes in each mitochondrion are required to keep up with the work load of protein synthesis for mitochondrial growth and turnover. Thus it would seem that a diminished efficiency due to the accumulation of slightly disadvantageous mutations might be more

tolerable in the mitochondrial ribosomes; the lower efficiency could be compensated by increasing the number of ribosomes, at only a modest expense in terms of the metabolic energy required to synthesize the extra ribosomes.

In this regard it is necessary to consider also the ribosomes of chloroplasts, since the role of these ribosomes in cellular protein synthesis is similar to that of mitochondrial ribosomes. It is noteworthy that the structural properties of these ribosomes indicate little evolutionary divergence among distantly related chloroplast-containing protists and plants, or even between these organisms and prokaryotes. This observation argues against the significance of a low level of selective pressure as a contributing factor in the high evolutionary rate of mitochondrial ribosomes. However the argument is weakened by the fact that chloroplast ribosomes are responsible for a somewhat larger proportion of the total protein synthesis in the cell than mitochondrial ribosomes are: one subunit of the very abundant protein ribulose-diphosphate carboxylase is made on chloroplast ribosomes. Moreover, the number of chloroplast ribosomes per cell is greater than that of mitoribosomes.

It is also possible that genetic mechanisms exist to effect the more rapid establishment of selectively neutral or advantageous mutations in mitochondrial ribosomes. No differences can be visualized in the genetic processes that operate directly on mitoribosomal versus cytoribosomal proteins, since both groups of proteins appear to be coded in

nuclear genes. But a rapid evolutionary rate in mitochondrial rRNA might force or encourage a complementary high rate in the proteins which must interact with this rRNA to form functional ribosomes. The mitochondrial rRNA is copied from mitochondrial genes, and several fundamental differences in the genetics of nuclear and mitochondrial DNA are known. Furthermore there is considerable experimental evidence demonstrating rapid evolutionary divergence not only of mitochondrial rRNA genes, but of the rest of the mitochondrial DNA as well.

Most of this evidence is based on DNA-DNA or DNA-RNA hybridization studies, usually including thermal denaturation analyses to evaluate the level of correspondence between the heterologous molecules hybridized. Such experiments measure differences in the primary sequence of the nucleic acids, and should be roughly comparable to electrophoretic comparisons of proteins with respect to their sensitivity in detecting interspecies variations. Unfortunately a direct comparison of the degree of variation found in mitochondrial DNA with that of nuclear DNA has only been reported in one instance. Groot et al. (1975) compared the nuclear and mitochondrial DNAs of the yeasts Saccharomyces carlsbergensis, Kluyveromyces lactis, and Candida utilis, finding that both mitochondrial rRNA and total mitochondrial DNA showed greater differences among these species than did the cytoplasmic rRNA and nuclear DNA.

A number of indirect comparisons can also be made. Sinclair and Brown (1971) found detectable homologies between the cytoplasmic rRNA

of Xenopus laevis and that of many distantly related organisms, including yeast; but no correspondence in base sequence was detectable between the mitochondrial DNAs of X. laevis and yeast (Dawid and Wolstenholme, 1968). In comparisons of rat, mouse, guinea pig, monkey and chicken, Jakovcic et al. (1975a and b) found a lower degree of homology among mitochondrial tRNA genes than had been reported for nuclear genes coding for rRNA or proteins; most of the rest of the mitochondrial DNA showed even less homology.

No significant differences were detected between the nuclear rRNA genes of X. laevis and X. mulleri, although the spacer DNA interspersed among these rRNA genes showed extensive non-homology (Brown et al., 1972). Mitochondrial rRNA genes, on the other hand, were distinguishable in hybridization comparisons between these two species, and most of the remaining mitochondrial DNA showed a significantly higher degree of evolutionary divergence (Dawid, 1972). Indeed, the high level of non-homology found between these two closely-related species in the bulk of the mitochondrial DNA sequences led Dawid (1972) to propose that these sequences represent nothing but spacer DNA, analogous to the spacer regions found in nuclear ribosomal DNA. But the weight of several other kinds of evidence contradicts this proposal, indicating that most of this mitochondrial DNA represents the structural genes for enzyme proteins (Schatz and Mason, 1974). Thus it must be concluded that all kinds of mitochondrial genes can show considerable divergence between closely related species. All these results are in contrast to

those obtained in chloroplast studies, in which hybridization experiments revealed little difference in the base sequences of chloroplast rRNA from various monocotyledonous and dicotyledonous plants (Thomas and Tewari, 1974).

What kinds of genetic mechanisms are known that might account for the high evolutionary rate of mitochondrial DNA? Among the known differences in the genetic processes acting on the mitochondria and nuclei of eukaryotic cells is the phenomenon of vegetative segregation of mitochondrial genes. Yeast zygotes "heteroplasmic" for a mitochondrial gene (that is, containing both mutant and wild-type mitochondrial DNA molecules) give rise to some homoplasmic progeny within one or two mitotic cycles, and all descendants are homoplasmic within 10-20 cell divisions (Birky, 1976). Groot et al. (1975) have pointed out that this process would lead to the rapid phenotypic expression of mitochondrial mutations, thereby promoting the establishment of these mutations if they were positively selective.

The latter authors have also postulated a mechanism for a relatively rapid establishment of selectively neutral mutations in mitochondrial genes. The presence of many copies of a gene per cell increases the probability of mutation in the gene. Multiple copies of genes for both mitochondrial and cytoplasmic rRNA are found in eukaryotic cells, but a master-slave correction process acting on the cytoplasmic rRNA genes has been proposed (Brown et al., 1972). If this process does act on the nuclear but not on the mitochondrial genes, it is argued, the latter

would be expected to show a higher mutation rate. Some objections may be raised to this line of reasoning, however. First, the spacer regions present in the nuclear rDNA are considered to be subject to the same correction process as the rRNA genes, because both are part of the same repeating DNA sequence and both are essentially identical in all of the copies present in a given cell; but the spacer regions show very rapid evolutionary divergence, as noted above (Brown et al., 1972). Second, all of the mitochondrial DNA molecules in a given organism are found to be identical to each other (David, 1972; Potter et al., 1975), suggesting that some kind of correction mechanism may operate on the mitochondrial genes too (Birky, 1976).

Another unique feature of mitochondrial genetics is the high rate of recombination observed between mitochondrial DNA molecules. In yeast the rate is high enough that no linkage can be observed among four gene loci, all of which are physically located on the same 5×10^7 d circular DNA molecule (Dujon et al., 1974). These results were interpreted to mean that the mitochondrial genomes undergo many repeated rounds of pairing and recombination. These events do not even appear to be limited in time to a short interval in the life cycle of the organism, such as meiosis. Such high rates of recombination should promote the spread of mutations through the population, and permit the combination of separate mutations into a single genome.

Mitochondrial recombination can also occur in mammalian cells: Coon et al. (1973) have presented biochemical evidence for the forma-

tion of recombinant mitochondrial DNA molecules in somatic cell hybrids of mouse and human origin. But the importance of this phenomenon in the exchange of mitochondrial genetic information between individuals in nature is questionable for mammals and most other organisms. In most species (unlike the case in yeast), mitochondrial genes are inherited uniparentally, affording little opportunity for recombination between the DNAs of the two parents. However some recombination can be detected between the parental chloroplast genomes in Chlamydomonas crosses, despite the fact that chloroplast genes in this organism are also uniparentally inherited (Sager, 1972).

The high recombination rate of mitochondrial DNA may be more significant as a possible inducer of mutations. The model of Dujon et al. (1974) includes a suggestion that mitochondrial recombination could account for the properties of the petite mutants which arise spontaneously at a rather high frequency in yeast. Faulty alignment of the DNA molecules during recombination might also lead to less dramatic mutations on occasion. In addition, mitochondrial DNA may be more mutable than nuclear DNA because of differences in repair processes. Yeast mitochondria are reported to lack an excision-repair system for thymidine dimers induced by ultraviolet radiation (Prakash, 1975). Mammalian mitochondria lack both this system and the photoreactivation system (Clayton et al., 1974).

There is certainly no shortage of possible mechanisms to account for the rapid evolutionary divergence of mitochondrial DNA and mito-

chondrial ribosomes. The question is which of these possibilities actually represent significant factors contributing to this divergence. Answers to this question can only come from further studies on the cellular and molecular processes acting on cytoplasmic genes. Particularly instructive would be a comparison of such processes between mitochondria and chloroplasts, since the latter show a much lower rate of evolutionary divergence.

Whatever the causes, the phylogenetic diversity of mitochondrial ribosomes should prove to be a useful tool for the detailed analysis of the biosynthesis, assembly, and mechanism of action of ribosomes in general. Important information about the relationships between structure and function in biological systems can frequently be derived from comparisons between naturally occurring systems which have the same function but differ structurally. Mitochondrial ribosomes offer a great variety of structural differences with considerable similarity in the details of their functional activity, and thus should be most valuable for such comparative studies.

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I certify that I have read this study and that, in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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