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PURIFICATION AND PARTIAL CHARACTERIZATION OF MITOCHONDRIAL RIBOSOMES FROM A HELA CELL LINE RESISTANT TO CHLORAMPHENICOL

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Purification and partial characterization of mitochondrial ribosomes from a HeLa cell line resistant to chloramphenicol

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LIST OF ABBREVIATIONS

1. Buffers

NKMT: 0.14M NaCl; 0.001 M Tris-HCl, pH 7.4; 0.00015M $MgCl_2$; 0.01M KCl

TKM: 0.01M Tris-HCl, pH 7.4; 0.01M KCl; 0.00015M $MgCl_2$

TS: 2.5M sucrose; 0.01M Tris-HCl, pH 7.4

ST: 0.25M sucrose; 0.01M Tris-HCl, pH 7.4; 100 µg/mL bovine serum albumin

STE: 0.2M sucrose; 0.01M Tris-HCl, pH 7.4; 100 µg/mL bovine serum albumin; 0.002M ethylene diamine tetraacetic acid (EDTA)

ATP: 0.15M KCl; 0.01M $MgCl_2$; 0.01M KH_2PO_4, pH 6.7; 0.002M adenosine triphosphate; 0.005M 2-ketoglutarate; 2 mg/mL bovine serum albumin

KMEDT: 0.01M Tris-HCl, pH 7.4; 0.01M $MgCl_2$; 0.1M KCl; 0.002M dithiothreitol; 0.001M EDTA

TMA2: 0.01M Tris-HCl, pH 7.4; 0.01M $MgCl_2$; 0.06M NH_4Cl

2. Reagents

Tris: tris(hydroxymethyl)amino methane

DTT: dithiothreitol

SDS: sodium dodecylsulfate

TCA: trichloroacetic acid

tRNA: transfer ribonucleic acid

rRNA: ribosomal ribonucleic acid

mRNA: messenger ribonucleic acid

DNA: deoxyribonucleic acid

TEMED: N,N,N^1,N^1-tetramethylethylenediamine

EDTA: ethylene diamine tetraacetic acid

BSA: bovine serum albumin

ATP: adenosine triphosphate

GTP: guanosine triphosphate

PEP: phosphoenol pyruvic acid

poly(U): polyuridylic acid

poly(C,U): a copolymer of cytidylic and uridylic acid

PPO: 2,5-diphenyloxazole

3. Units

S: svedbergs

M: molar

mM: millimolar

mL: milliliter

mg: milligram

A_{260} That amount of material which when dissolved in 1 mL has an optical density of 1 when measured at 260 nanometers.

$E_{260}^{0.1\%}$: The optical density of a 0.1% solution when measured at 260 nanometers.

INTRODUCTION

Part of "the essence of being eukaryotic" is the possession of mitochondria, the cytoplasmic organelles responsible for the generation of most of the cellular ATP. The biological origin of these organelles has puzzled researchers for many years. Microscopic observations suggested that they multiplied by binary fission (Palade, 1953) much like bacteria. Their ellipsoidal shape and physical structure, e.g., possession of a double lipid bilayer, also suggested a relationship with the prokaryotes but the meaning of these similarities remained unclear. Recently investigators have begun to speculate that an early event on the ancestral cell's road to becoming eukaryotic was the capture and domestication of a bacterium capable of aerobic respiration (Margulis, 1971; Sagan, 1967). The organism was referred to as an aerobic, amitotic, amoeboid cell. This cell then underwent a profound modification of the symbiotic relationship between host and promitochondrial genomes, eventually resulting in the present day eukaryotic cell. One early impetus for this theory was the discovery of DNA in mitochondria.

Nass and Nass (1963) using electron microscopy were the first to demonstrate that mitochondria contained discrete pieces of DNA. Shortly after this, Luck and Reich (1964) showed that nuclear DNA and mitochondrial DNA from N. crassa had distinctly different base compositions. This was the first biochemical evidence that mitochondria contained DNA molecules distinct from those of the nucleus. Subsequently, mitochondrial DNA from numerous sources was characterized and the results suggest that the two genetic systems are undergoing a gradual but

essentially independent evolution of base composition (Rabinowitz and Swift, 1970).

Mitochondrial DNAs from the various kingdoms of eukaryotes are quite different from each other. The mitochondrial DNA of all animals thus far investigated (including humans, frogs, fruit flies and sea urchins) occurs as a 5 μm, supercoiled, covalently closed, circular duplex with a molecular weight of 8 to 10 million daltons (Borst, 1972). More complex forms such as catenated or circular dimers are also known to occur (Hudson et al., 1968). Yeast mitochondrial DNA occurs as a 25 μm circle of about 50 million daltons. It has not been isolated intact but the circular form has been visualized by electron microscopy (Hollenberg et al., 1970). Using even the gentlest of techniques, it is possible to isolate only randomly sheared molecules which are one-half to one-third the length of the intact genome (Locker et al., 1974). DNA from Neurospora is similar to that of yeast but somewhat smaller (20 μm) (Agsteribbe et al., 1972). The only linear mitochondrial genomes so far discovered occur in the ciliated protozoans--paramecium and tetrahymena (Cummings et al, 1976; Goldbach et al., 1976). In higher plants, the molecule is again circular but with a contour length of 30 μm making it the largest mitochondrial DNA molecule known (Koldner and Tewari, 1972). Mitochondrial DNA from most sources, including human, has a very low guanine plus cytosine content, ca. 20% to 30% (Borst and Kroon, 1969). In yeast, this low G plus C content reflects the occurrence of fairly long tracts of dA:dT and dTA:dAT as well as a preference for GC exclusive codons in the known coding regions of the genome

(Bernardi, 1976). There do not, however, seem to be any corresponding regions in the human mitochondrial genome, and codon selection is limited to a preference for G in the third position (Barrel et al., 1979). The complete DNA sequence of the human mitochondrial genome has been determined (Anderson et al., 1981); bovine mitochondrial DNA has also been sequenced (mentioned in Anderson et al., 1981).

Despite early reports by Swanson (1971) and Dawid and Chase (1972), it now seems clear that most, if not all, of the RNA found in mitochondria is transcribed from mitochondrial DNA (Borst, 1972; Fukahara, 1970; Mahler and Dawidowicz, 1973). In HeLa cells, both strands of the mitochondrial DNA are completely transcribed into high molecular weight RNA which are then processed into tRNA, rRNA, and mRNA (Murphy et al., 1975). The strand having the highest buoyant density in alkaline cesium chloride (the H-strand) codes for the two ribosomal RNAs, the majority of the known tRNAs and most of the poly(A) containing RNAs (Aloni and Attardi, 1971a; Angerer et al., 1976; Lynch and Attardi, 1976). The majority of the L-strand transcript is rapidly degraded and, during long term labeling, products of the H-strand predominate. L-strand products, which represent less than 2% of the total RNA hybridizable to mitochondrial DNA (Aloni and Attardi, 1971b; Attardi et al., 1970), comprise seven tRNAs and one small poly(A) containing RNA (Amalric et al., 1978).

A total of 19 mitochondrial tRNAs have been shown to hybridize to human mitochondrial DNA (Angerer et al., 1976; Lynch and Attardi, 1976). From DNA sequence data, an additional 3 tRNA genes have been identified, bringing the final count to 22 (Anderson et al., 1981). Transfer RNA

does not seem to be imported into mammalian mitochondria (Aujame and Freedman, 1979); therefore, 22 or 23 may be an upper limit to the number of tRNA species existent in the mitochondria. This amazing finding then raises the question of how these few tRNAs are able to read all the codons of the genetic code, a task which requires a minimum of 32 tRNAs according to the wobble hypothesis of Crick (1966). Two possibilities which present themselves are restricted codon usage by the mitochondrial mRNAs and unusual codon-reading abilities of the tRNAs. It seems the latter may be the correct explanation. In three papers published simultaneously, Barrel et al. (1980), Bonitz et al. (1980), and Heckman et al. (1980), suggested that in humans, Neurospora and yeast, some mitochondrial tRNAs are able to read all the codons of a four-codon family. For example, a single mitochondrial tRNA is able to decode UCN (where N is any nucleotide) as serine. The mitochondrial tRNAs apparently accomplish this by having an unmodified U in the first position of the anticodon. U is the only base which can form stable base pairs with either A, G, C, or U (Lagerkvist, 1978). When U occurs in the first position of a prokaryotic or eukaryotic cytoplasmic tRNA, it is always modified (Sprinzl et al., 1980) presumably to limit recognition to less than all four bases. The single known exception is yeast $tRNA^{Leu}_{UAG}$ which contains an unmodified U and can somehow recognize all six leucine codons (Randerath et al., 1979). In codon families where two different amino acids are specified, e.g., glutamate (GAG, GAA) and aspartate (GAU, GAC), the purines in the third position are read by an anticodon with a modified U in the first position, the nature of the

modification is unknown; the pyrimidines are read by a G in the first position. With this system, AUA would then code for methionine instead of isoleucine and UGA would code for tryptophan. These coding changes have been shown to occur in the mitochondrial tRNAs (Bonitz et al., 1980; Heckman et al., 1980; Martin et al., 1980). In the mammalian system, it has been suggested that AGA and AGG as well as UAA and UAG code for termination. This would reduce the number of tRNAs required for complete decoding of the mitochondrial code to 22 (Barrel et al., 1980).

It is interesting to note that the formation of the proposed U:C and U:U base pairs involves a significant conformational change in the codon (Grosjean et al., 1978) which may, in part, be accommodated by the unusual structures of some of the mitochondrial tRNAs, e.g., RNA and DNA sequence data has shown that a mitochondrial seryl-tRNA is completely devoid of the dihydrouridine loop found in all other tRNAs (Arcari and Brownlee, 1980; de Bruijn et al., 1980). This conformational change, not required in other systems, may also be facilitated by the structure of the mitochondrial ribosome (see discussion below). It is not known whether heterologous ribosomes could permit such changes. Studies using purified mitochondrial tRNAs to translate messages on homologous and heterologous ribosomes might reveal more restricted coding properties for the tRNAs under the latter conditions (Heckman et al., 1980).

Some mitochondrial mRNAs have been found to contain polyadenylic acid (poly(A)) at their 3'-OH end (Ojala and Attardi, 1974; Perlman et al., 1973). Unlike the longer (up to 170 nucleotides) terminal poly(A) sequences found at the 3' ends of cytoplasmic messengers (Darnell et al.,

1971), mitochondrial mRNAs have only 50 to 80 residues in their poly(A) tails. In this respect, the mitochondrial mRNAs may be intermediate between that of the prokaryote and the eukaryote. A 7MeG linked to the 5' terminal nucleotide through a 5' to 5' triphosphate is known to "cap" eukaryotic mRNA (Both et al., 1975). Shafritz et al. (1976) have reported that this eukaryotic cap participated directly in the interaction of the mRNA with eukaryotic initiation factor IF-M_3 during initiation of protein synthesis. Mitochondrial mRNAs seem to lack a 5' capping structure but in the light of the more "bacterial" nature (see below) of mitochondrial protein synthesis, this might have been expected (Grohmann et al., 1978).

Recently Attardi et al. (1976) have identified 18 different poly(A) containing RNAs from HeLa mitochondria. Of these, only the eight smallest can be fit into the presumed mRNA coding regions on the genetic map of the HeLa mitochondrial genome. The rest are presumably incompletely processed precursor molecules. There are, coincidently, only nine known products of mitochondrial translation. These are the three largest subunits of cytochrome c oxidase, one of the subunits of the cytochrome b complex and four separate subunits which constitute a specific membrane site for the integration of mitochondrial oligomycin-sensitive ATPase complex (Schatz and Mason, 1974; Tzagoloff et al., 1973). Rabinowitz and associates have shown that translation of yeast mitochondrial poly(A)-containing RNA in an E. coli in vitro system gives rise to products which include peptides precipitable by antibodies to authentic subunits of cytochrome c oxidase (Padmanaban et al., 1975;

Rabinowitz et al., 1976). At least one protein associated with the mitochondrial ribosomes of yeast (Var 1) and Neurospora (S-5) is also translated on mitochondrial ribosomes (Lambowitz et al., 1976; La Polla and Lambowitz, 1977; Terpstra et al., 1979). As the products of animal mitochondrial translation bear a qualitative and quantitative resemblance to those of yeast when analyzed on SDS gels, it seemed likely that few, if any, translation products remained to be identified (O'Brien and Matthews, 1976). However, eight other unidentified reading frames (URFs), presumably additional protein coding genes, have been located in the human and bovine mitochondrial DNA sequence (Anderson et al., 1981).

All the known products of mitochondrial translation are integrated into multimeric functional units to which both the nuclear and mitochondrial genomes make a contribution (Schatz and Mason, 1974). The best studied example of this interaction is the biosynthesis of cytochrome c oxidase in the yeast mitochondrion. Cytochrome c oxidase is a transmembrane protein (Eytan et al., 1975) composed of seven individual polypeptides. Subunits I, II, and III are synthesized in the mitochondria while subunits IV, V, VI, and VII are synthesized in the cytoplasm (Mason and Schatz, 1973; Rubin and Tzagoloff, 1973). The biosynthesis of these cytoplasmic and mitochondrial components is, as expected, interdependent. Poyton and Kavanaugh (1976) showed that synthesis of subunits I, II, and III but not other products of isolated yeast mitochondria was dependent upon a specific cytoplasmic stimulating factor. This factor was immunologically related to subunits IV and VI.

Tryptic peptide analysis of this 55,000 dalton precursor revealed that it had the same peptide composition as a mixture of all the subunits made by the cytoplasm. Interestingly, processing of the precursor takes place after it enters the membrane and entry is the signal for stimulation of synthesis of the mitochondrially made subunits. The precursor is able to enter the inner membrane in the absence of bound cytoplasmic ribosomes. It has since been shown that the cytoplasmically synthesized subunits of the ATPase complex and the cytochrome b complex are likewise made as larger precursors (Schatz, 1979) which may subsequently be processed after entry into the inner membrane.

Biosynthesis of mitochondrial ribosomes may be controlled in a manner similar to the one outlined above. In Neurospora, the mitochondrially synthesized small subunit protein S-5 has been shown to play a significant role in the assembly of the small subunit by facilitating processing of the 19S RNA and stabilizing the binding of several cytoplasmically synthesized proteins (Lambowitz et al., 1976; La Polla and Lambowitz, 1977). In the absence of functional S-5, e.g., in the poky mutation, or when its synthesis is inhibited by chloramphenicol, there are significant aberrations in processing the small subunit precursor (Lambowitz et al., 1979). A similar effect has been noted for the small subunit protein Var 1 in yeast (Terpstra et al., 1979).

McLean et al. (1958) were the first to demonstrate that isolated but intact rat liver mitochondria were able to incorporate radioactively labeled amino acids into high molecular weight polypeptides. The findings of Rendi (1959) and Mager (1960) that incorporation of amino

acids by this system could be inhibited by chloramphenicol, a drug known to act on bacterial ribosomes, suggested that a ribosome might be involved in the polymerization. Further evidence for the existence of a ribosome unique to the mitochondria was presented by Kroon (1963) and Kalf (1963) who showed that puromycin, a known analogue of the 3' end of aminoacyl-tRNA, would also inhibit mitochondrial protein synthesis. Andre and Marinozzi (1965), using electron microscopy, finally provided visual evidence with the observation of small ribonuclease sensitive particles inside the mitochondrion.

Unlike cytoplasmic ribosomes, mitochondrial ribosomes from the different kingdoms are structurally quite distinct. Among the fungi, mitocondrial ribosomes have been isolated from S. cerevisiae (Schmitt, 1971), Neurospora crassa (Kuntzel and Noll, 1967), and Candida utilis (Vignais et al., 1972). These ribosomes are all quite similar and possess sedimentation coefficients between 72S and 78S. They contain large and small subunit RNAs with sedimentation coefficients in the ranges 21S to 24S and 16S to 17S, respectively. No small RNAs such as the 5S and 5.8S RNAs of the cytoplasmic ribosome have been reported.

The mitochondrial ribosomes of the protozoa seem to differ considerably even between orders. The mitochondrial ribosomes of Euglena gracilis have a sedimentation coefficient of 71S with subunits of 50S and 32S (Avadhani and Buetow, 1972a) while the mitochondrial ribosomes of Crithedia luciliae sediment at 60S with subunits of 45S and 32S (Laub and Thirion, 1972). The difference is even greater when different phyla are compared. The mitochondrial ribosomes of the ciliated

protozoan Tetrahymena pyriformis sediment at 80S and dissociate into two subunits with identical sedimentation coefficients of 55S (Chi and Suyama, 1970).

Plant mitochondrial ribosomes sediment at 77S to 78S with subunits of 60S and 44S (Leaver and Harmey, 1972). The mitochondrial ribosomes of higher plants, in contrast to those of fungi, protozoa or animal cells, have been shown to contain a 5S RNA which is present in equimolar amounts with the large components of the mitochondrial rRNA (Leaver and Harmey, 1976). They do not contain a 5.8S RNA. In this respect, they resemble bacterial ribosomes.

Mitochondrial ribosomes from all animal sources so far investigated bear a remarkable structural and functional similarity (O'Brien and Matthews, 1976). They have a narrow range of sedimentation values (54S to 61S with subunits approximately 40S and 30S) which are considerably lower than those of previously studied bacterial or eukaryotic ribosomes (Kleinow et al., 1971; O'Brien, 1971; O'Brien and Kalf, 1967; Perlman and Penman, 1970; and Swanson and Dawid, 1970). An early conclusion (Borst and Grivell, 1971) that animal mitochondrial ribosomes were mini-ribosomes, e.g., of low molecular weight and/or small size, was supported by the small size of their rRNAs. The large subunit RNA weighs 5-5.8 x 10^5 and the small subunit RNA, 2.8-3.5 x 10^5 daltons (Dawid and Chase, 1972; Kleinow et al., 1974; Robberson et al., 1971; Sacchi et al., 1973). The miniribosome concept was put in question, however, by the findings that mitochondrial ribosomes had a slightly higher total molecular weight, 2.8 x 10^6 daltons, (Hamilton and O'Brien, 1974) than

did the bacterial ribosomes, 2.6 x 10^6 daltons, (Hill et al., 1969) and had a larger volume as well (DeVries and Van der Koogh-Schuuring, 1973). It now seems clear that the lower sedimentation coefficient of the animal mitochondrial ribosome is largely due to its lower boyant density, a reflection of a higher protein to RNA ratio (Hamilton and O'Brien, 1974; Leister and Dawid, 1974; Perlman and Penman, 1970). In fact, while E. coli ribosomes are known to have 54 different proteins (Stoffler and Wittmann, 1977), animal mitochondrial ribosomes have been shown to contain as many as 94 separate proteins (O'Brien and Matthews, 1976). Present evidence suggests that all of these proteins are required for functional activity (O'Brien et al., 1976).

There were enormous difficulties experienced in purifying mitochondrial ribosomes, as evidenced by the appreciable lag between their discovery (McLean et al., 1958) and the isolation of a reasonably pure ribosome preparation (Kuntzel and Noll, 1967; O'Brien and Kalf, 1967). These difficulties were caused, in part, by the lack of any clear-cut criteria to distinguish between authentic mitochondrial ribosomes, cytoplasmic ribosomes, and bacterial ribosomes originating from chance contamination of the isolation medium. The problem of bacterial contamination was remedied by assuring that all buffers and apparatus used in the isolation were sterile before and during use. The problem of contaminating cytoplasmic ribosomes was partially circumvented by using selective in vivo labeling in the presence of any one of a variety of specific inhibitors. For example, the mitochondrial ribosome can be labeled in its RNA component by growing cells in the presence of

[^{3}H]-uridine and low levels of actinomycin D (0.04-0.1 μg/mL). This technique was used by Attardi and Ojala (1971) and Brega and Vesco (1971) to demonstrate that HeLa cell mitochondria contain a ribonucleoprotein which sediments at 55S to 60S. This particle contained two stable RNAs of 16S and 12S and was able to incorporate [^{3}H] leucine into nascent polypeptide *in vivo* in the presence of cycloheximide but not chloramphenicol. These mitochondrial ribosome preparations were heavily contaminated with cytoplasmic ribosomes but because of differential labeling a distinction could be made.

Animal mitochondrial ribosomes were first isolated by O'Brien and Kalf (1967) from the livers of young rats. The preparations were free of cytoplasmic and bacterial contamination but were not active in *in vitro* polypeptide synthesis. In 1973, Ibrahim *et al.*, using a modification of the procedure of O'Brien and Kalf (1967), and Greco *et al.* (1973), were able to isolate rat liver mitochondrial ribosomes which were active in poly(U)-directed polyphenylalanine synthesis and which were more active than the preparation of O'Brien and Kalf in the peptidyl transferase assay. Both systems used *E. coli* supernatant factors and were inhibited by low levels of chloramphenicol. Greco *et al.* (1973) purified their mitochondria using three washes in 1 mM EDTA rather than the extensive washing through discontinuous gradients used by Ibrahim *et al.* (1973), but both preparations still required sucrose gradient purification to remove cytoplasmic ribosomes. In that same year, DeVries and Van der Koogh-Schuuring (1973) used digitonin washed mitochondria to prepare rat liver mitochondrial ribosomes free of

cytoplasmic contamination as judged by sucrose gradient centrifugation and gel electrophoresis. The preparations were not active in *in vitro* polypeptide synthesis. Then, in 1974, Avadhani and Rutman combined EDTA washing and digitonin treatment to prepare mitochondrial ribosomes from Ehrlich acites cell mitochondria. These preparations were active in poly(U)-directed polypeptide synthesis using either mitochondrial or bacterial supernatant factors and did not require further purification on sucrose gradients. Mitochondrial ribosomes active in protein synthesis have also been isolated from yeast (Ibrahim *et al.*, 1973), Xenopus (Swanson, 1973) and Euglena (Avadhani and Buetow, 1972b).

Although animal mitochondrial and bacterial ribosomes seem to be completely unrelated at the level of gross structure, as pointed out above, they do have certain functional similarities. Protein synthesis is initiated on mitochondrial ribosomes using formylated methionyl-tRNA (Schatz and Mason, 1974) just as in bacterial systems and the initiation and elongation factors are believed to be interchangeable with those from bacteria (Avadhani and Buetow, 1972b; Grandi *et al.*, 1971; Grivell *et al.*, 1971); however, recent studies by Denslow and O'Brien (1979) and Ulbrich *et al.* (1980) on the species specificity of elongation factor G suggest that the homology may not be as extensive as once thought. It is clear, however, that mitochondrial ribosomes are able to use bacterial tRNAs for translation of synthetic messages (Schatz and Mason, 1974).

In 1960, Mager pointed out that amino acid incorporation into isolated animal mitochondria was inhibited by the drug chloramphenicol, an antibiotic whose inhibition of bacterial ribosomes in general and the

E. coli peptidyl transferase center in particular has been well documented (Pestka, 1977; Vasquez, 1979). Subsequent studies have shown that mitochondrial ribosomes are sensitive to most of the inhibitors of bacterial ribosomes but that the degree of sensitivity may be quite different (Denslow and O'Brien, 1974).

Chloramphenicol has been shown to inhibit the peptidyl transferase center of bovine mitochondrial ribosomes (Denslow and O'Brien, 1978). This suggests but does not prove that the mode of action of chloramphenicol may be the same in mitochondrial as in bacterial systems. In bacterial systems chloramphenicol acts by binding to the 50S subunit in the vicinity of the acceptor site (A site) (Lessard and Pestka, 1972). Irradiation of this complex leads to covalent attachment of the chloramphenicol and irreversible inactivation of the peptidyl transferase center of the ribosome (Sonnenberg *et al.*, 1974). Many antibiotics which bind to the 50S subunit interfere, to one extent or another, with the binding of chloramphenicol (Pestka, 1977). Inhibition of peptidyl transferase activity is probably due to blockage of the ribosomal A site either sterically or allosterically by the drug. This is suggested by the observations that: 1) chloramphenicol is a competitive inhibitor of puromycin in the formation of peptidyl-puromycin (Pestka, 1972). As puromycin is an analogue of the 3' end of an aminoacyl-tRNA, this suggests that chloramphenicol may act by directly blocking the functional attachment of the 3' terminus of tRNA; 2) chloramphenicol blocks the binding of aminoacyl-oligonucleotides but not N-acetyl-aminoacyl-oligonucleotides to the ribosome (Celma *et al.*, 1970; Lessard and

Pestka, 1972). This demonstrates directly that chloramphenicol inhibits the binding of the 3' end of aminoacyl-tRNA to the A site but not the P site of the ribosome. Prevention of the functional attachment of an aminoacyl-tRNA easily accounts for the inhibition of peptide bond formation (Pestka, 1977; Vasquez, 1979).

Interestingly, chloramphenicol does not inhibit aminoacyl-oligonucleotide binding to ribosomes in all systems equally. Higher concentrations of the drug are required for 50% inhibition of the binding of phenylalanyl-oligonucleotides than are required for the same effect on lysine-, leucine-, or serine-containing oligonucleotide fragments (Hishizawa and Pestka, 1971; Pestka et al., 1970). Inhibition of polynucleotide-directed polypeptide synthesis by chloramphenicol has been found to be template dependent as well. Poly(U)-directed peptide synthesis is significantly more resistant to inhibition by the drug than is poly(C,U)- or poly(A)-directed synthesis (Kucan and Lipmann, 1964; Vasquez, 1966). Because poly(U) codes for polyphenylalanine, both effects may be the result of a greater affinity of phenylalanyl-tRNA for the ribosome (Pestka, 1977; Vasquez, 1979).

There have been numerous reports of acquired resistance to chloramphenicol by prokaryotic cells. Resistance usually occurs via one of two major biochemical mechanisms. The first involves an acquired impermeability of the cell membrane to the drug. This can occur as the results of an episomal transfer, or of multi-step mutations (Okamoto and Mizuro, 1962, 1964). Alternatively, transfer of an episome carrying the gene for a chloramphenicol acetyl-transferase, which acetylates

chloramphenicol to the inactive diacetyl derivative, can occur (Okamoto and Suzuki, 1965; Shaw et al., 1970). A third mechanism for resistance, mutation of a ribosomal constituent, seems to be a comparatively rare event (Pestka, 1977). The difficulty of obtaining ribosomal mutations to chloramphenicol resistance may be due to the occurrence of multiple gene copies of ribosomal RNA in most organisms (Smith, 1977). If mutation to chloramphenicol resistance involves a change in the rRNA sequence, then only genetic systems with single copy rRNA genes, e.g., cell organelles, would be easily mutable. By selection of a cell resistant to erythromycin, Cerna and Rychlik (1968) obtained a strain of E. coli with ribosomes which were resistant to inhibition by chloramphenicol as well. Later Osawa et al. (1973) isolated a chloramphenicol resistant mutant of B. subtilis and were able to show that the resistance was due to alterations of ribosomal proteins but no other examples of chloramphenicol resistant ribosomes have been reported in bacteria. In eukaryotic cells, resistance to chloramphenicol has been reported for yeast (Bunn et al., 1970), mouse (Bunn et al., 1974) and human (Mitchell et al., 1975; Spolsky and Eisenstadt, 1972) cell lines.

In 1972, Spolsky and Eisenstadt successfully isolated a fast growing, chloramphenicol resistant mutant of HeLa S_3 cells which they designated 296-1. They had induced the mutation with ethidium bromide, a mutagen known to preferentially affect mitochondrial DNA (Leibowitz, 1971); but this did not exclude the possibility that the mutation was of nuclear origin. In order to clarify this point, Wallace et al. (1975) fused cytochalasin produced cytoplasts (cells which have been

enucleated) prepared from the chloramphenicol resistant cell line with chloramphenicol sensitive cells containing a nuclear mutation which inactivated the enzyme thymidine kinase. The resulting cybrids (cytoplasmic hybrids) were then plated in medium containing chloramphenicol and the thymidine analog 5-bromodeoxyuridine, conditions which inhibited the growth of both parental lines. Finding the cybrids were able to grow in this medium provided substantial evidence that the mutation to chloramphenicol resistance resided on a mitochondrial gene. By measuring peptide synthesis in mitochondria permeabilized by treatment with a low concentration of Triton X-100, Spolsky and Eisenstadt (1972) had shown that resistance was not due to an alteration in the mitochondrial membrane rendering it impermeable to the drug. In a later paper, Kislev et al. (1973) reported that chloramphenicol induced large alterations in the mitochondrial ultra-structure of HeLa S_3 but not 296-1, again supporting the belief that the loss of cytotoxicity was due to an alteration in some aspect of mitochondrial protein synthesis.

The above mentioned results have three likely possibilities to account for drug resistance: induction or mutation of a preexisting, mitochondrially encoded enzyme capable of acting on the drug and directly rendering it inactive (the mitochondrial equivalent of the plasmid encoded bacterial transacetylase); induction or mutation of a mitochondrially encoded protein- or RNA-modifying enzyme capable of modifying some constituent of the protein synthetic machinery (a ribosomal protein, RNA or translation factor); an alteration in a structural gene encoding either a ribosomal protein, RNA or translation

factor. In order to decide among these possibilities, a regime for the isolation and purification of human mitochondrial ribosomes and a protein synthesis assay system that is sensitive to inhibition by chloramphenicol was established. Results with this system show that mitochondrial ribosomes from the chloramphenicol resistant line of HeLa cells are resistant to the drug *in vitro*, thus clearly demonstrating that antibiotic resistance is due to a functional modification of the ribosome itself. Recent sequence analysis of yeast (Dujon, 1980) and mouse and human (Blanc *et al.*, 1981; Kearsey and Craig, 1981) mitochondrial DNA have identified single nucleotide changes in the 3' region of the large (21S) rRNA gene which correlate with the antibiotic resistance. Similar findings were made with HeLa 296-1 (D. C. Wallace, Stanford University, unpublished observations).

In a larger context, the work described in this thesis is part of an ongoing effort to characterize the complex interactions required of the products of the nuclear and mitochondrial genetic systems during mitochondrial biogenesis. Central to these considerations is the description of an *in vitro* polypeptide synthesizing system which is totally of mitochondrial origin and in which mitochondrial mRNA is translated into polypeptide that is identical to the *in vivo* product. This work is a first step toward accomplishing that end with a system of human origin.

EXPERIMENTAL PROCEDURES

Origin of Cell Lines

Both HeLa cell lines used in these experiments were obtained from Dr. J. M. Eisenstadt of the Department of Human Genetics, Yale University School of Medicine. The chloramphenicol resistant line was isolated as a mutant of HeLa S_3 by treating the cells for one generation with ethidium bromide (0.5 μM), washing the cells free of mutagen and growing them for several generations in medium containing 50 μg/mL chloramphenicol. At the end of this time several clones were selected and one, a fast growing line designated 296-1, was employed in these studies (Spolsky and Eisenstadt, 1972).

Conditions for Cell Growth

Cell lines were maintained as stock cultures by growth in monolayers on 25 cm^2 tissue culture flasks in minimum essential medium (MEM) (Eagle, 1959) supplemented with 10% (v/v) serum; in the majority of experiments an equal mixture of horse and calf serum was used. The plates were incubated at 37°C in a humidity controlled atmosphere of 5% CO_2 to maintain the pH of the medium. The cells were fed every other day by replacing the spent medium with 5 mL of fresh medium. When the cells reached confluency, they were removed from the flask by trypsinization in phosphate buffered saline solution (PBS) containing 0.05% trypsin. The cells were then counted in a hemocytometer and replated after dilution with fresh medium to a density of 1×10^4 cells/mL. The medium used to maintain the

drug resistant cell line contained 50 μg/mL chloramphenicol to suppress revertants and minimize the possibility of cross contamination during passage. The sensitive cell line was assayed periodically for continued sensitivity to the drug by preparing growth curves in the presence of 50 μg/mL chloramphenicol (Spolsky and Eisenstadt, 1972).

Cell stocks were prepared for frozen storage by trypsinizing a plate as described earlier and collecting the cells by centrifugation at 300 x G_{ave} for 10 minutes. After washing with PBS the cells were resuspended at a concentration of 1 x 10^6 cells/mL in minimum essential medium supplemented with 10% dimethylsulfoxide and the suspension pipetted onto several small screw cap freezer vials. These were chilled rapidly in an ice water bath, wrapped in tissue paper, packed in a thick-walled polystyrene container and stored at -70°C. After 24 hours a sample of the frozen cells was checked for viability by thawing and plating out an appropriate dilution in fresh MEM containing serum. Viabilities of 90% or better were not unusual. Cells frozen by this procedure remained viable for up to 6 months (the longest period checked).

For preparative scale growths, cells were plated onto two 125 cm^2 flasks and these were grown to confluency as described above. The plates were then trypsinized and the entire culture diluted to a cell density not less than 5 x 10^5 cells/mL in a 250 mL spinner flask. In spinner culture, the cells were grown in RPMI 1640 supplemented with 6% serum (an equal mixture of horse and calf serum) and 0.03% glutamine. An aliquot of the suspension culture was counted daily in a hemocytometer

and fresh medium was added to maintain a cell density of not less than 4-5 x 10^5 cells/mL. Doubling time of the cells in spinner culture was usually 24-28 hours. Cells were harvested when the volume of culture was 8-16 liters and a small volume of cells (250-500 mL) was saved to initiate the next growth, thus avoiding restarting the spinner culture from stock.

Harvesting the Cells

When the cells had reached the desired volume and density, they were transferred, aseptically, into autoclaved 500 mL centrifuge bottles with O-ring seals and centrifuged at 440 x G_{ave} for 10 minutes in a Beckman J-21C centrifuge. The pelleted cells were then washed by resuspension in NKMT buffer (0.14M NaCl; 0.001M Tris-HCl, pH 7.4; 0.00015M $MgCl_2$; 0.01M KCl) and centrifugation at 440 x G_{ave}. This process usually yielded 2-3 g of cells/L.

Isolation of Mitochondria

Washed cells were resuspended in TKM buffer (0.01M Tris-HCl, pH 7.4; 0.01M KCl; 0.00015M $MgCl_2$), 6 mL/g of cells, and allowed to swell for 10 minutes at 4°C. At the end of this time, the cells were examined in a microscope to observe the effects of the hypotonic swelling. With well swollen cells a distinct nucleus surrounded by a ring of cytoplasm is visible; swollen cells are approximately twice the normal diameter. The cells were then broken by homogenization (10-15 strokes) in a Thomas tissue homogenizer with a motor driven Teflon pestle rotating at 1,500 rpm. One tenth volume of TS buffer (2.5M sucrose; 0.01M Tris-HCl, pH 7.4)

was quickly added and nuclei and unbroken cells were pelleted by centrifugation at 1,200 x G_{ave} for 3 minutes. The supernatant was saved and the pellet resuspended in one half the initial volume of TKM and again homogenized. After addition of one tenth volume of TS buffer the homogenate was centrifuged at 1,200 x G_{ave} for 3 minutes and the supernatants from the two centrifugations were combined. The remaining pellet, which contained mostly nuclei, was discarded. The combined supernatants were then centrifuged at 11,900 x G_{ave} for 10 minutes to sediment the mitochondria. The postmitochondrial supernatant was either discarded or used to prepare cytoplasmic ribosomes. Crude mitochondria were resuspended in 100 mL of ST buffer (0.25M sucrose; 0.01M Tris-HCl, pH 7.4; 100 µg/mL bovine serum albumin) or, in later experiments, in STE buffer (ST buffer plus 0.002M EDTA) and again centrifuged at 11,900 x G_{ave} for 10 minutes. This washing procedure was repeated 1 to 4 times. After being washed, the mitochondria were resuspended in 10 to 20 mL of ST buffer and the absorbance of a standard dilution was read at 550 nm. The amount of mitochondrial protein in the preparation was estimated from a standard curve which related the absorbance at 550 nm to total mitochondrial protein as determined by the method of Lowry et al. (1951).

Digitonin Treatment of Purified Mitochondria

The washed mitochondrial preparation was diluted to 20 mg mitochondrial protein/mL with ST buffer. An equal volume of ST buffer containing 3.0 mg/mL digitonin was added dropwise with stirring at 0°C. The digitonin solution, always freshly prepared, was made by dissolving a weighed

amount of digitonin in ST buffer heated in a boiling water bath; the solution was rapidly cooled on ice just before use. After the final addition of digitonin, the solution was stirred at 0°C for 2 minutes, then rapidly diluted with 3 volumes of ice cold ST buffer and centrifuged at 19,000 x G_{ave} for 5 minutes. The pellet was resuspended in one-half the above volume of ST buffer and centrifuged at 19,000 x G_{ave} for 10 minutes. This wash step was repeated once more and the digitonin treated mitochondria recovered by centrifugation at 19,000 x G_{ave} for 20 minutes (Loewenstein et al., 1970).

Incubation of Mitochondria wtih Puromycin and ATP

Digitonin treated mitochondria were resuspended in ATP buffer (0.15M KCl; 0.01M $MgCl_2$; 0.01M KH_2PO_4, pH 6.7; 0.002M ATP; 0.005M 2-ketoglutarate; 2 mg/mL BSA) at 20 mg/mL and were incubated 10 minutes at 37°C. Puromycin (0.1M) was then added to a final concentration of 0.1mM and incubation was continued for an additional three minutes. At the end of this time the mitochondria were pelleted by centrifugation at 20,000 x G_{ave} for 10 minutes.

Preparation of Ribosomes

Mitochondrial Ribosomes

The digitionin treated mitochondrial pellet was weighed and resuspended by homogenization in KMEDT buffer (0.1M Tris-CHl, pH 7.4; 0.01M $MgCl_2$; 0.1M KCl; 0.002M DTT; 0.0001M EDTA) to a final concentration of 35 mg/mL (wet weight). Mitochondria were then lysed by the addition of

one tenth volume of a detergent mixture containing 5% Brij-58 and 5% Nonidet P-40 (w/v). After homogenization with 5-6 strokes in a tissue homogenizer, the mixture was allowed to sit at $0^{o}C$ for 5 minutes before insoluble material was removed by centrifugation at 20,000 x G_{ave} for 20 minutes. The straw-colored supernatant was layered over a 2 mL pad of 1M sucrose in KMEDT buffer and centrifuged at 105,000 x G_{ave} for 16 hours in a Beckman Type 40 rotor. After centrifugation, the supernatant and half of the sucrose pad were removed with a pasteur pipette and the walls of the centrifuge bottles were washed with distilled water. The remaining portion of the sucrose pad was then removed and the bottles were drained by inverting them in an ice bucket. The small yellow pellets were resuspended by homogenization in 0.3 mL of KMEDT buffer. The ribosome solution was clarified by centrifugation at 20,000 x G_{ave} for 20 minutes and the supernatant was then divided into 0.05 mL aliquots, quick frozen and stored in liquid nitrogen until used. Ribosomes were thawed just prior to use and any unused portion was discarded as they could not be refrozen with retention of full activity.

Cytoplasmic Ribosomes

To be used as sedimentation markers and for the preparation of cytoplasmic ribosomal RNA, cytoplasmic ribosomes were prepared from the postmitochondrial supernatant. This fraction was layered over 3 mL of 1M sucrose in KMEDT buffer and centrifuged at 105,000 x G_{ave} for 16 hours in a Beckman Type 40 rotor. The supernatant and sucrose pad were discarded and the pellets were resuspended in 3-4 mL of KMEDT buffer.

Insoluble material was removed by centrifugation at 20,000 x G_{ave} for 20 minutes and the supernatant was divided into 0.2 mL aliquots, quick frozen and stored in liquid nitrogen until used.

Bacterial Ribosomes

Escherichia coli strain B (3/4 log phase), grown on enriched medium, was purchased from the Grain Processing Corp., Muscatine, Iowa as a frozen paste. Cells were broken by grinding with alumina (Alcoa A-305) in the cold for 30 minutes. The resulting paste was suspended in TMA2 buffer (0.01M Tris-HCl, pH 7.4; 0.01M $MgCl_2$; 0.06M NH_4Cl) containing 2 µg/mL deoxyribonuclease. Ribosomes were prepared by differential centrifugation as described by Tissieres et al. (1959). They were washed twice with TMA2 buffer, then once with TMA2 buffer containing 0.5M NH_4Cl. The resulting ribosomal pellet was resuspended in TMA2 buffer and dialyzed against the same buffer. Ribosomes were stored frozen at -70°C. The concentration of ribosomes was calculated from the absorbance at 260 nm using a value of $A_{260}^{0.1\%} = 16$.

Rate Zonal Centrifugation in Sucrose Gradients

Linear gradients of 10% to 30% sucrose were prepared in KMEDT buffer and samples, in volumes no greater than 200 µL, were layered directly on top. The gradients were then centrifuged at 40,000 rpm in a Beckman SW 40 rotor for 3 hours or at 27,000 rpm in a Beckman SW 27.1 rotor for 8 hours. Under these conditons a peak sedimenting at 55S migrated to the middle of the centrifuge tube. After centrifugation, fractions of

0.4-0.5 mL were collected from the top of the gradient using a Buchler Densi-Flo gradient fractionator. Fractions were analyzed for radioactivity either directly, when incorporation of [^{3}H]-uridine into ribosomes was determined, by addition of 5 mL of toluene-Triton-PPO scintillation cocktail (2 L toluene; 1 L Triton X-100; 19 g PPO) after dilution with 1 mL of H_2O or following TCA precipitation. In the latter procedure 0.5 mL of 10% TCA was added and the samples were heated at 90°C for 20 minutes. After cooling, the precipitate was collected on Millipore filters, dried and counted in toluene-PPO scintillation cocktail (3.6 L toluene; 19 g PPO; 0.32 g POPOP). The hot TCA treatment completely solubilized radioactivity present in ribosomal RNA, so that it did not interfere with the detection of labeled polypeptide.

Radioactive Labeling of Ribosomal RNA *in vivo*

Mitochondrial ribosomal RNA was labeled with [^{3}H]-uridine, in the absence of nuclear ribosomal RNA synthesis, by preincubating a liter of cells (5-7 x 10^5 cells/mL) with 0.05 µg/mL actinomycin D for 30 minutes, followed by addition of [^{3}H]-uridine (20-30 Ci/mMole; 0.5 mCi/mL (Dubin and Monteenecourt, 1970; Zylber *et al.*, 1969). The actinomycin D was prepared as a stock solution, 100 µg/mL, in 95% ethanol. Cells were harvested ca. 16 hours later, and the labeled cells were combined with a larger volume of unlabeled cells before further processing. When labeling of both cytoplasmic and mitochondrial ribosomal RNA was desired, cells were grown with [^{3}H]-uridine in the absence of actinomycin D.

Extraction of Mitochondrial and Cytoplasmic Ribosomal RNA

Ribosomal RNA from both cytoplasmic and mitochondrial ribosomes was prepared by the method of Lambowitz and Luck (1976). Ribosomes containing 50,000 to 70,000 cpm of [^{3}H]-uridine were diluted to 2 mL with extraction buffer (0.1M NaCl; 0.025M Tris-HCl, pH 7.4; 1% SDS; 0.002M EDTA; 10 µg/mL polyvinyl sulfate). E. coli tRNA (100 µg) was added as carrier and protein was degraded by incubation at 37°C for 20 minutes with 100 µg/mL pronase. The enzyme had been predigested by incubation at 37°C for 1 hour before use. The mixture was then shaken with an equal volume of phenol/chloroform/isoamyl alcohol (25:25:1) for 20 minutes at room temperature and the phases separated by centrifugation. The organic phase was discarded and the extraction process repeated. RNA in the combined aqueous phases was then precipitated by the addition of 2 volumes of ice cold 95% ethanol. The precipitate was stored at -20°C for at least 2 hours and was then collected by centrifugation at 20,000 x G_{ave} for 20 minutes; the pelleted RNA was dissolved in 2 mL of distilled H_2O. One tenth volume of 20% potassium acetate, pH 5 was added and the RNA was reprecipitated with 2 volumes of ethanol. After at least 2 hours at -20°C, the precipitate was collected by centrifugation and the final RNA pellet was resuspended in 50 µL of distilled, deionized H_2O, quick frozen, and stored in liquid nitrogen until used.

Electrophoresis of Mitochondrial and Cytoplasmic Ribosomal RNA

Electrophoresis of mitochondrial and cytoplasmic rRNA was performed according to the method of Gegenheimer et al. (1977) in 0.5 x 10 cm

cylindrical tubes. The 3% polyacrylamide gels (acrylamide:bis-acrylamide, 15:1) were prepared in a buffer containing: 0.04M Tris-HCl, pH 7.8; 3% glycerol; 0.001M EDTA; 0.2% SDS and were polymerized by the addition of 0.12% TEMED and 0.12% $(NH_4)_2S_2O_8$. [^{3}H]-ribosomal RNA samples (5-7 x 10^4 cpm) were diluted with an equal volume of 2X sample buffer (40% sucrose in 0.01M Tris-CHl, pH 7.4; 0.1M KCl; 0.01M $MgCl_2$; 0.1% bromphenol blue) and layered directly on top of the gel beneath the electrode buffer. Electrophoresis was usually carried out at 3 mA/gel for 4 hours. The electrode buffer contained 0.04M Tris-acetate, pH 7.4; 3% glycerol; and 0.001M EDTA. After electrophoresis, gels were removed from the tubes by means of a water filled bulb and prepared for counting by the procedure of Loening (1967). Each gel was sliced into 1 mm discs which were placed into individual glass scintillation vials. One half mL of 10% (v/v) piperidine was added and the vials were heated to dryness at 60°C for several hours. Then 0.5 mL of H_2O was added and the gels were allowed to swell to many times their original size. Five mL of toluene-Triton-PPO scintillation cocktail were added; the samples were mixed and allowed to stand in the dark at room temperature overnight. The following day they were mixed again and counted.

Preparation of Protein Synthesis Supernatant Factors (S-100)

Mitochondiral S-100 was prepared according to the procedure of Denslow and O'Brien, 1979. All operations were carried out at 0 to 4°C. Liver (rabbit or rat), excised from a freshly sacrificed animal, was chilled and washed by immersion in 300 mL of buffer A (0.34M sucrose;

0.005M Tris-HCl, pH 7.4). It was then blotted dry, weighed, and minced with scissors. The minced tissue was resuspended (5 mL/g) in buffer A and homogenized with 3 strokes of a tissue homogenizer fitted with a motor driven Teflon pestle rotating at 1,500 rpm. Unbroken cells and nuclei were removed by centrifugation at 1,100 x G_{ave} for 15 minutes. The mitochondria were then pelleted by centrifugation at 10,000 x G_{ave} for 10 minutes and washed repeatedly by resuspension in buffer A and centrifugation. Washed mitochondria were resuspended in 25 mL of buffer B (0.02M Tris-HCl, pH 7.6; 0.01M KCl; 0.015M $MgCl_2$; 0.006M 2-mercaptoethanol) and sonicated with a Branson Cell Disruptor Model 200 (4 minutes at 50% duty cycle, setting number 7). The sonicate was centrifuged 20,000 x G_{ave} for 20 minutes. The KCl concentration of the supernatant was adjusted to 0.5M, and it was layered on 10 mL of 1M sucrose in KMEDT buffer and centrifuged 9 hours at 105,000 x G_{ave} in a Beckman Type 70Ti rotor. The upper half of the supernatant was dialyzed against buffer B and then concentrated to 3 mL with Aquacide and stored frozen at -20°C.

An S-100 from E. coli was prepared by grinding cells with alumina as described for ribosome preparation. The cell paste was resuspended in TMA2 buffer containing 0.006M 2-mercaptoethanol and the alumina and cell debris were removed by low speed centrifugation (10,000 x G_{ave} for 10 minutes). The resulting supernatant was then centrifuged at 105,000 x G_{ave} for 4 hours. The upper 2/3 of this high speed supernatant was divided into aliquots and stored frozen at -70°C until used.

Polypeptide Synthesis Assays

Poly(U)-directed polypeptide synthesis

Assay mixtures contained, in a final volume of 0.1 mL, 0.01M Tris-HCl; pH 7.4; 0.05M KCl; 0.045M $(NH_4)_2SO_4$; 0.012M $MgCl_2$; 0.005M 2-mercaptoethanol; 0.005M dithiothreitol; 0.001M ATP; 0.0004M GTP; 0.004M PEP; 4.5 units pyruvate kinase; 30 µg *E. coli* S-100 (or variable amounts of mitochondrial S-100); 45 µg $tRNA^{mixed}_{E.\ coli}$; 50 µM of each amino acid except phenylalanine; 1.5 µM [^{3}H]-phenylalanine (50-60 Ci/mMole) and ribosomes as indicated. Reactions were started by immersing the tubes in a water bath at 37°C, and terminated by the addition of 3 mL 5% trichloroacetic acid (TCA). The incubation period was usually one hour. After precipitation with TCA, reaction mixes were heated to 90°C for 20 minutes and then cooled on ice for at least 20 minutes. The precipitate was collected on Millipore filters, washed 3 times with ice cold 5% TCA, dried and counted in 5 mL toluene-PPO scintillation cocktail.

Poly(C,U)-directed polypeptide synthesis

The assay mixture was formulated exactly as above, except that the nonradioactive amino acid mixture lacked phenylalanine, leucine, and proline and contained 2 µM serine. 1 µM each [^{3}H]-phenylalanine, [^{3}H]-proline, and [^{3}H]-leucine were added and 50 µg poly(C,U) was used in place of poly(U).

Kinetic studies

For studies of reaction rates, samples of at least 50 µL were removed from a scaled up incubation mixture. After taking the zero time sample,

the assay was begun by immersing the remaining incubation mixture in a water bath at 37°C. At the appropriate times, samples were removed and precipitated with TCA. After all samples were collected the tubes were heated and further processed for counting as described earlier.

RNase Assay

The procedure was that described by Gribnau et al. (1970), and measured the hyperchromicity at 260 nm resulting from the hydrolysis of an RNA sample. Each assay was made up to a final volume of 0.6 mL by adding to 0.2 mL of distilled, deionized H_2O: 0.1 mL 1M Tris-HCl, pH 7.4: 0.2 mL cytoplasmic ribosomal RNA (15 mg/mL); and 0.1 mL of the solution to be assayed. The mixture was incubated for 30 minutes at 37°C, an equal volume of 1N HCL in 76% ethanol was added to each assay tube before it was chilled on ice for 1 hour. After centrifugation at 2,000 x G_{ave} for 30 minutes, 0.5 mL of the supernatant was taken, diluted with 2.5 mL H_2O and the absorbance at 260 nm was read in a 0.2 cm cell blanked against H_2O. The assay was standardized by using pancreatic RNase dissolved in H_2O containing 0.1% bovine serum albumin at a concentration of 0.05 µg/mL.

Materials

All enzymes and E. coli tRNA were from Sigma Chemical Co., St. Louis, Missouri. Media for cell culture were obtained from Grand Island Biological Co., Grand Island, New York. The polynucleotides, poly(U) and poly(C,U) were obtained from P & L Biochemicals. Sephadex G-75 was purchased from Parmacia, Inc. DEAE-cellulose was Whatman microgranular

grade DE-32. [^{3}H]-labeled amino acids were from New England Nuclear. Ribonuclease free sucrose was from Schwarz/Mann. Chloramphenicol and cycloheximide were products of Sigma Chemical Co.; other antibiotics were a gift of Dr. Jerome Eisenstadt. All other reagents used were of analytical grade or higher.

Phenol was distilled before use to remove impurities and the distillate was stored at 4^{o}C in the dark. Dialysis tubing was treated by boiling in 0.0054M Na_2EDTA; 0.014M $NaHCO_3$ and 0.007M 2-mercaptoethanol for 30 minutes to remove heavy metal and enzyme contaminants. The tubing was then thoroughly washed with deionized water and stored at 4^{o}C. Acrylamide and N,N-methylene bisacrylamide were obtained from Eastman Kodak and were recrystallized before use according to the procedure of Loening (1967).

RESULTS

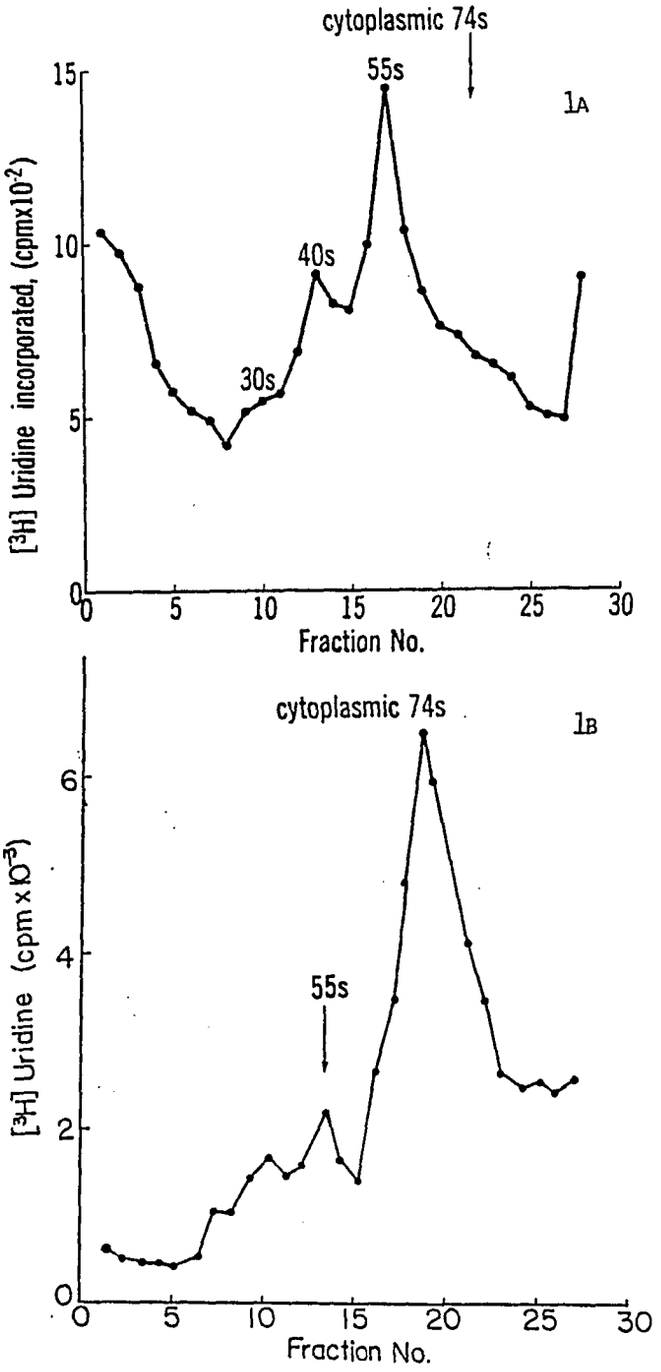
Isolation and Activity of Mitochondrial Ribosomes

Mitochondrial ribosomes, labeled in their RNA, were prepared from digitonin treated mitochondria of cells grown in the presence of [^{3}H]-uridine and actinomycin D as described in the Experimental Procedures Section. Under these conditions, only mitochondrial ribosomes become labeled (Dubin and Monteenecourt, 1970). When these ribosomes were analyzed by sucrose gradient centrifugation, three major peaks were seen (Figure 1A). Using cytoplasmic ribosomes (74S), centrifuged in a parallel tube as a standard, these peaks were assigned sedimentation coefficients as indicated in the figure, under the assumption that sedimentation through these gradients was isokinetic. Sedimentation coefficients of 55S for the monomer, and 40S and 30S for the large and small subunits, respectively, are in good agreement with published values for mammalian mitochondrial ribosomes (Attardi and Ojala, 1971; Avadhani et al., 1975; Brega and Vesco, 1971; Neupert, 1977; O'Brien and Matthews, 1976).

In order to estimate the level of cytoplasmic contamination, ribosomes were prepared from the mitochondria of cells grown in the presence of [^{3}H]-uridine but in the absence of actinomycin D. When these ribosomes were examined by sucrose gradient centrifugation, an additional peak sedimenting at 74S was seen (Figure 1B). The sedimentation behavior of this peak was indistinguishable from that of authentic cytoplasmic ribosomes under these conditions. Suppression of label incorporation into this peak by low levels of actinomycin D, was a strong indication

Figure 1. Centrifugation profile of mitochondrial ribosomes

Mitochondrial ribosomes were prepared from HeLa S_3 cells labeled *in vivo* with [^{3}H]-uridine in the presence (Figure 1A) or absence (Figure 1B) of 50 ng/mL actinomycin D. The ribosomes were separated by centrifugation through a 10-30% sucrose gradient for 8 hours at 27,000 rpm. Fractions were prepared for liquid scintillation counting as outlined in Experimental Procedures Section.


cytoplasmic 74s
55s
1A
40s
30s
[3H] Uridine incorporated, (cpmx10-2)
Fraction No.
cytoplasmic 74s
1B
55s
[3H] Uridine (cpm x 10-3)
Fraction No.

that this component represented contaminating cytoplasmic ribosomes (Dubin and Monteenecourt, 1970).

Mitochondrial ribosomes, prepared as described, were active in protein synthesis with either poly(U) or poly(C,U) as messenger. As shown in Table 1, synthesis of polypeptide was dependent upon supplementation with both synthetic message and mitochondrial ribosomes. Cycloheximide (2 mg/mL) was included in the reaction to suppress polypeptide synthesis by cytoplasmic ribosomes. To demonstrate that the product formed was synthesized on mitochondrial ribosomes, we took advantage of the observation by O'Brien and Kalf (1967) that nascent polypeptide remains associated with ribosomes during sucrose gradient centrifugation. Isolated mitochondrial ribosomes were incubated in the polyphenylalanine synthesizing system for 60 minutes at 37°C. The mixture was then layered directly on top of a 10-30% sucrose gradient and centrifuged for 8 hours at 27,000 rpm in a Beckman Type SW 27.1 rotor. Fractions were collected starting from the top of the gradient and were analyzed for [^{3}H]-polyphenylalanine by trichloroacetic acid precipitation and liquid scintillation counting as outlined in the Experimental Procedures Section. In a parallel reaction, cytoplasmic ribosomes were incubated in an assay mixture from which cycloheximide had been excluded. The results, shown in Figure 2, reveal that the product synthesized on mitochondrial ribosomes sedimented at 55S and could easily be distinguished from product synthesized by the cytoplasmic ribosomes which sedimented at 74S.

Table 1. Polypeptide synthesis by isolated mitochondrial ribosomes[a]

(A) Poly(U)-directed incorporation of [^{3}H]-phenylalanine

Mixture	CPM
Complete	48,600
-Ribosomes	14,100
-Poly(U)	16,300

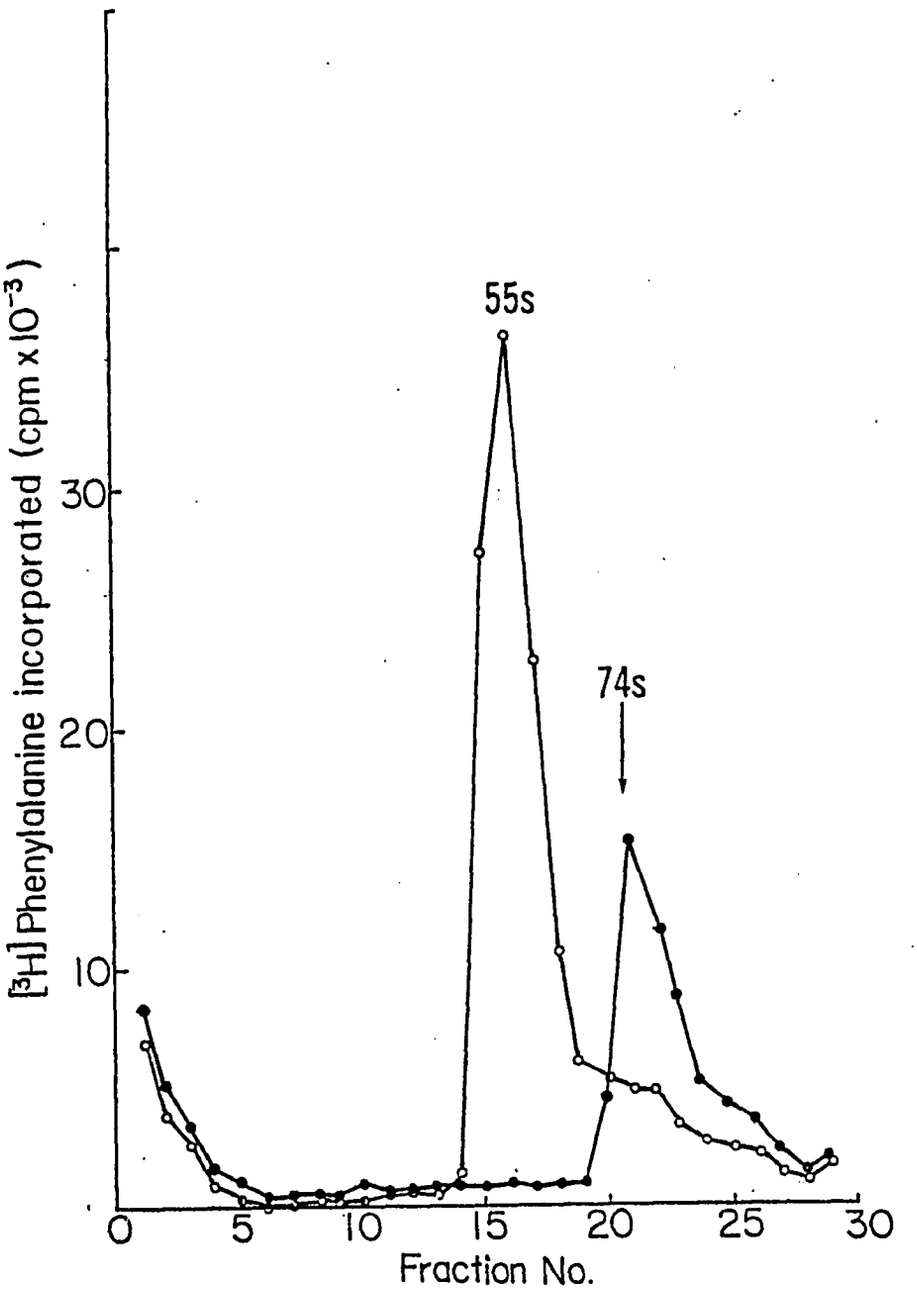
(B) Poly(C,U)-directed incorporation of [^{3}H]-leucine

Mixture	CPM
Complete	35,800
-Ribosomes	13,400
-Poly(C,U)	19,200

[a]Polypeptide synthesis assays were conducted as described in the Experimental Procedures Section, and included 2 mg/mL cycloheximide to inhibit polypeptide synthesis by cytoplasmic ribosomes.

Figure 2. Sucrose gradient centrifugation of nascent polypeptides associated with mitochondrial (55S) and cytoplasmic (74S) ribosomes

[^{3}H]-polyphenylalanine was synthesized in a poly(U)-directed reaction mixture which contained either mitochondrial (o-o) or cytoplasmic (●-●) ribosomes. After incubation, the nascent polypeptide was visualized by sucrose gradient centrifugation as described in Figure 1.


[3H] Phenylalanine incorporated (cpm x 10-3)
30
20
10
55s
74s
0
5
10
15
20
25
30
Fraction No.

These mitochondrial ribosome preparations were contaminated with cytoplasmic ribosomes as shown in Figure 3. In the absence of cycloheximide, a peak of nascent polypeptide was observed on sucrose gradients, sedimenting at the position expected for cytoplasmic ribosomes. This activity was suppressed by cycloheximide (Figure 3) with little or no inhibition of mitochondrial ribosome activity. High concentrations of cycloheximide were found necessary to obtain effective inhibition of cytoplasmic ribosome activity when assayed under the conditions outlined in the Experimental Procedures Section (Table 2). A more detailed examination of the protein synthetic activity of the mitochondrial ribosome will be presented later.

Purification of Mitochondrial Ribosomes from Contaminating Cytoplasmic Ribosomes

The degree of contamination with cytoplasmic ribosomes varied from one mitochondrial preparation to another. To minimize this contamination, ribosomes were prepared from mitochondria which had been treated with digitonin by a modification of the procedure of Loewenstein et al. (1970) (See Experimental Procedures Section). This produced mitoplasts by removing the outer mitochondrial membrane together with adhering cytoplasmic ribosomes. In an attempt to optimize the digitonin treatment for recovery and activity of HeLa cell mitochondrial ribosomes, as well as removal of contaminating cytoplasmic ribosomes, the effect of several different concentrations of the detergent was examined.

Figure 3. Sucrose gradient centrifugation of nascent polypeptides synthesized in the presence or absence of cycloheximide

Ribosomes from digitonin treated mitochondria were incubated in a poly(U) directed polypeptide synthesis mixture in the presence (●-●) or absence (o-o) of 1 mg/mL cycloheximide. After incubation, the nascent [^{3}H]-polyphenylalanine was visualized by sucrose gradient centrifugation (10 to 30% sucrose gradient centrifuged 8 hrs at 27,000 rpm in a Beckman SW27.1 rotor). Gradient fractions were prepared for liquid scintillation counting as outlined in Experimental Procedures Section.

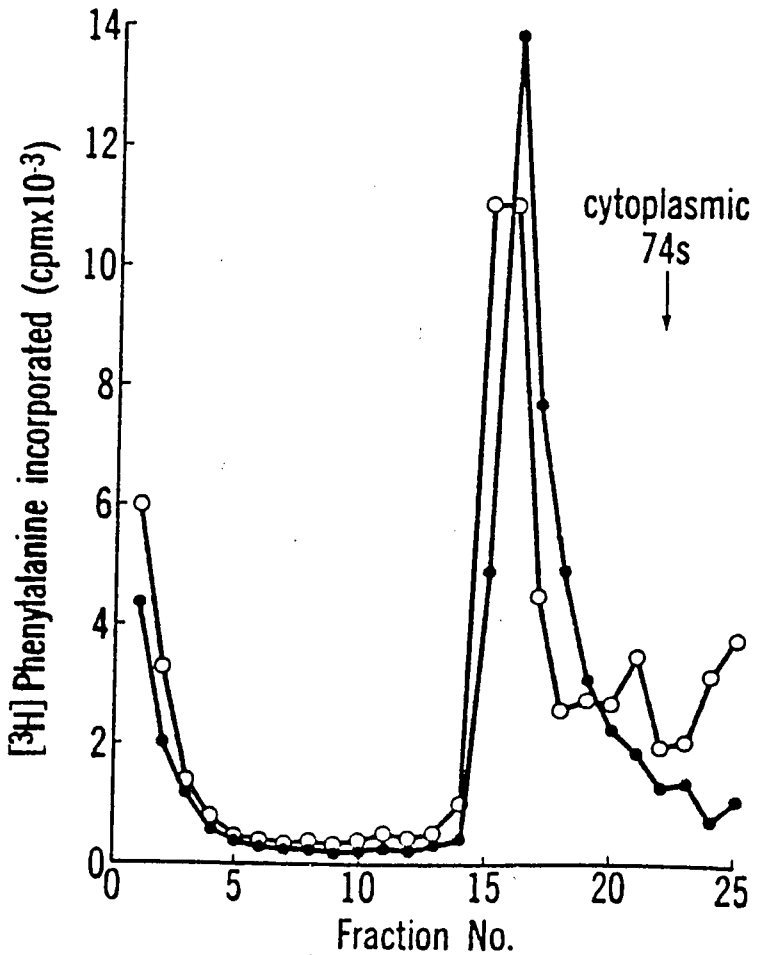

14
12
10
8
6
4
2
0
[3H] Phenylalanine incorporated (cpmx10-3)
cytoplasmic
74s
0
5
10
15
20
25
Fraction No.

Table 2. Effect of cycloheximide on poly(U)-directed polypeptide synthesis by 74S cytoplasmic ribosomes[a]

(Cycloheximide)	[^{3}H]-polyphenylalanine CPM (-zero time)	%
0	166,000	100
0.4 mg/mL	135,000	81
1.0 mg/mL	107,000	64
2.0 mg/mL	72,000	43

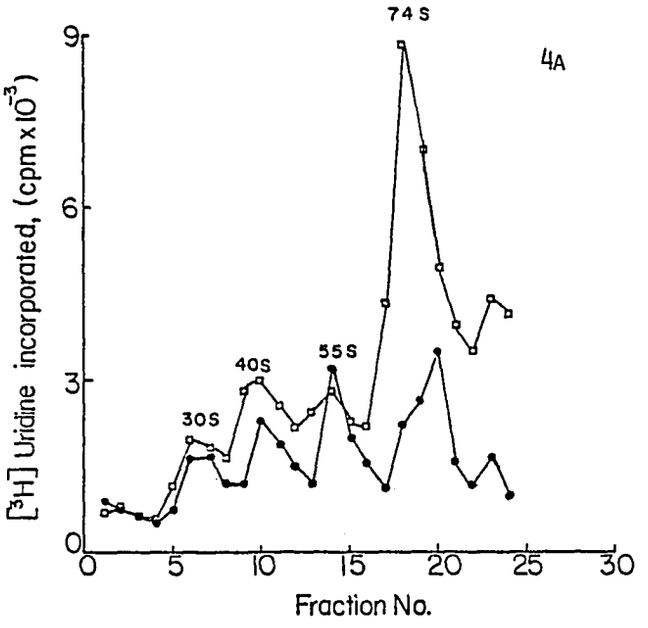
[a]Assay conditions were as noted in Experimental Procedures Section.

In these experiments, cells were grown in [^{3}H]-uridine without actinomycin D in order to label both mitochondrial and cytoplasmic ribosomes. Mitochondria were isolated as described under Experimental Procedures and treated with increasing amounts of digitonin. Ribosomes isolated from these mitochondria were then examined by centrifugation on 10-30% sucrose gradients. Figure 4 shows the results of treatment with 0, 0.15, 0.20 and 0.25 mg digitonin per mg of mitochondrial protein. Mitochondrial ribosome preparations from untreated mitochondria were heavily contaminated with cytoplasmic ribosomes. At a ratio of 0.15 mg digitonin per mg of mitochondrial protein, the extent of 74S ribosome contamination was greatly decreased with no concomitant loss of 55S ribosomes. Higher digitonin concentrations, however, resulted in a significant loss of 55S material. A concentration of digitonin of 15-20 mg per mg ribosomal protein gave optimal results.

The effectiveness of digitonin in removing cytoplasmic ribosomes can also be observed by examining the sedimentation behavior of nascent polypeptide synthesized by the mitochondrial ribosome preparations. Sucrose gradient profiles of poly(U)-directed polypeptide synthesis assays (without cycloheximide) using mitochondrial ribosomes prepared from mitochondria treated with varying concentrations of digitonin are shown in Figure 5. The heavy contamination with functionally active cytoplasmic ribosomes in the ribosome preparations made without detergent is apparent from the large peak of [^{3}H]-labeled polypeptide

Figure 4. Effect of treatment with varying concentrations of digitonin on the recovery of mitochondrial and cytoplasmic ribosomes

Mitochondria were prepared from HeLa cells labeled with [^{3}H]-uridine in the absence of actinomycin D. They were then treated with digitonin at varying ratios of detergent to mitochondrial protein. The ribosomes were subsequently isolated and examined by sucrose gradient centrifugation as described in Figure 1. Detergent to protein ratios were: Figure 4a, no digitonin (o-o), 0.15 mg digitonin/mg mitochondrial protein (●-●); Figure 4b, 0.20 mg/mg (▲-▲) and 0.25 mg/mg (o-o).


74 S
4A
30S
40S
55S
[3H] Uridine incorporated, (cpm x 10-3)
Fraction No.

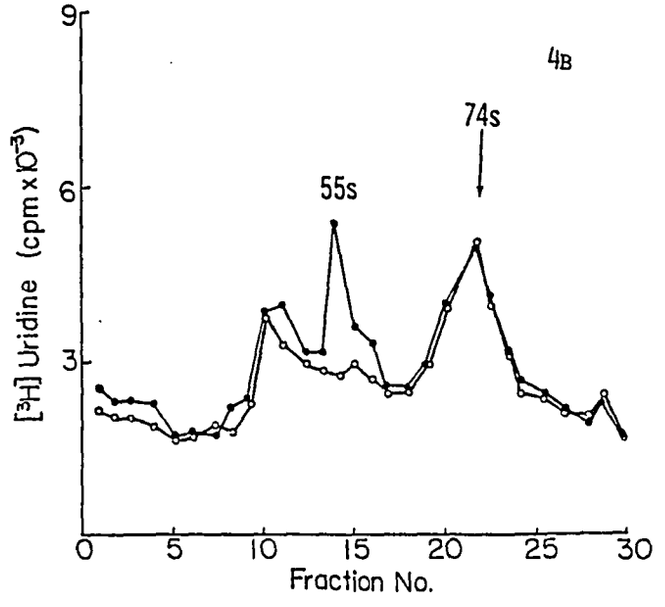
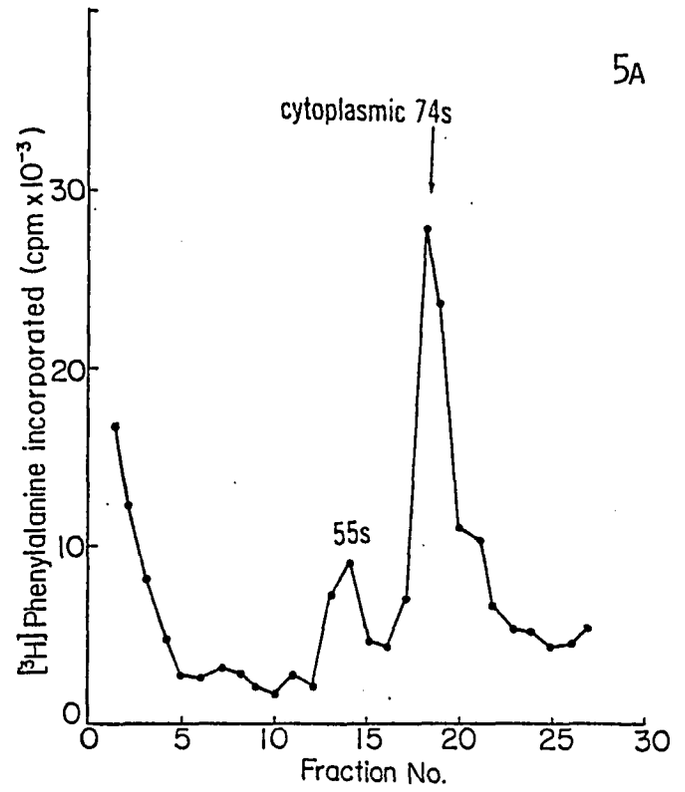
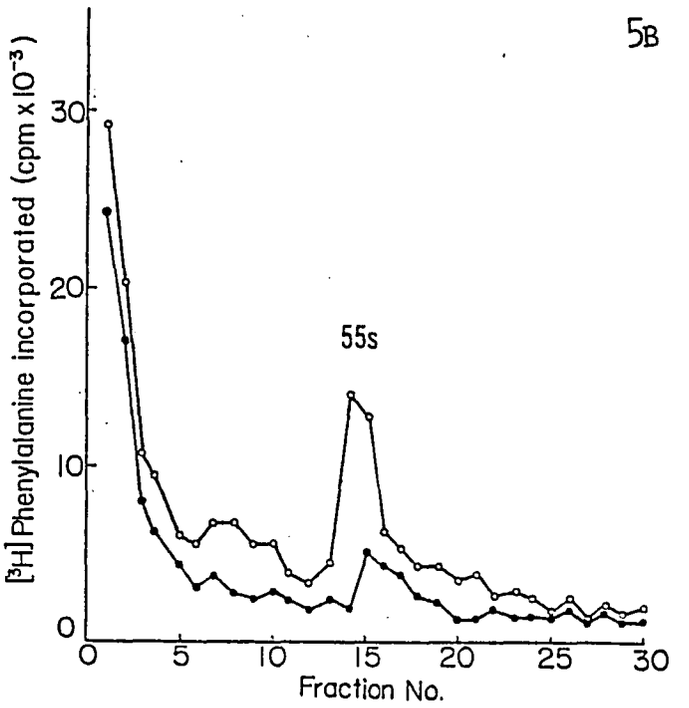

4B
74s
55s
[3H] Uridine (cpm x 10-3)
Fraction No.

Figure 5. Sucrose gradient centrifugation of nascent polypeptides synthesized on mitochondrial ribosomes prepared with and without digitonin

Ribosomes prepared from mitochondria untreated with digitonin (Figure 5a) or from mitochondria treated with digitonin (Figure 5b), at concentrations of 0.15 (o-o) or 0.25 (●-●) mg/mg of mitochondrial protein, were incubated in a poly(U)-directed polypeptide synthesis mixture containing no cycloheximide. After 60 minutes of incubation, the newly synthesized [^{3}H]-polyphenylalanine was visualized by centrifugation through a 10 to 30% sucrose gradient (3 hrs at 40,000 rpm in a Beckman SW 40Ti rotor). Gradient fractions were prepared for liquid scintillation counting as outlined in the Experimental Procedures Section.


5A
cytoplasmic 74s
55s
[3H] Phenylalanine incorporated (cpm x 10-3)
0
10
20
30
0
5
10
15
20
25
30
Fraction No.


5B
55s
[3H] Phenylalanine incorporated (cpm x 10-3)
0
10
20
30
0
5
10
15
20
25
30
Fraction No.

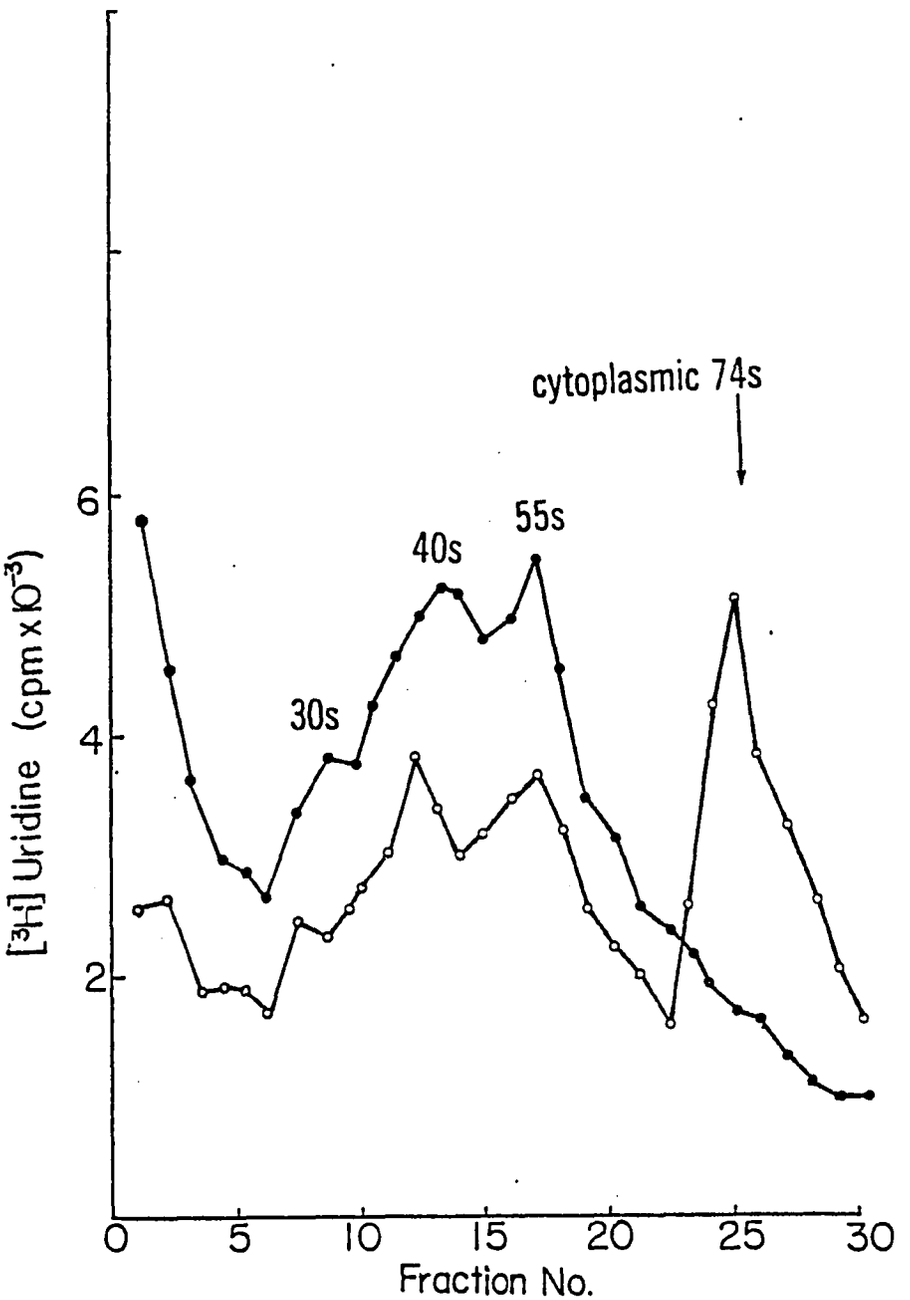
sedimenting at 74S (Figure 5A). Ribosomes isolated from mitochondria washed with 0.15 and 0.25 mg digitonin per mg mitochondrial protein had greatly reduced levels of active cytoplasmic ribosomes (Figure 5B). At the higher digitonin concentration, there was a significant decrease in the amount of polypeptide associated with mitochondrial ribosomes and sedimenting at 55S (Figure 5B).

It was concluded from these results that a digitonin to mitochondrial protein ratio of 0.15 represented the optimal conditions for isolation of mitochondrial ribosomes, and all subsequent mitochondrial preparations were treated with digitonin at this level.

Despite digitonin treatment, mitochondrial preparations were not entirely free from contamination by cytoplasmic ribosomes. Additional methods of purification were therefore sought. Lewis *et al.* (1976) washed ascites cell mitochondria with 2 mM EDTA (in addition to washing with digitonin) to remove cytoplasmic ribosomes from their preparations. In order to assess the effect of EDTA treatment of mitochondria on HeLa cell mitochondrial ribosomes, mitochondria were prepared from cells grown in [^{3}H]-uridine without actinomycin D (see Experimental Procedures Section). Before treatment with digitonin the mitochondrial preparation was divided into two portions. One was washed twice with ST buffer and the other with the same buffer containing 2 mM EDTA. When the *in vivo* labeled ribosomes prepared from these mitochondria were examined on sucrose gradients, it was clear that EDTA washing had greatly reduced the level of contaminating cytoplasmic ribosomes (Figure 6). The

Figure 6. Effect of EDTA treatment on the recovery of mitochondrial and cytoplasmic ribosomes

Mitochondria were prepared from HeLa cells labeled with [^{3}H]-uridine in the absence of Actinomycin D. The mitochondria were then washed twice with ST buffer alone (o-o) or with ST buffer containing 2 mM EDTA (●-●) and then treated with digitonin. Ribosomes were isolated and examined by centrifugation at 40,000 rpm for 3 hrs through 10-30% sucrose gradients (KMEDT buffer) in a Beckman SW-40Ti rotor. Gradient fractions were prepared for liquid scintillation counting as described in the Experiment Procedures Section.


cytoplasmic 74s
55s
40s
30s
[3H] Uridine (cpm x 10-3)
6
4
2
0
5
10
15
20
25
30
Fraction No.

effectiveness of washing with EDTA in removing cytoplasmic ribosomes was confirmed by examining the products of poly(U)-directed polypeptide synthesis by these ribosomes preparations on sucrose gradients. As shown in Figure 7, washing mitochondria with 2 mM EDTA resulted in a major reduction of the amount of polyphenylalanine associated with cytoplasmic ribosomes (74S), while no loss of polypeptide sedimenting with mitochondrial ribosomes (55S) was observed.

These results, while encouraging, did not conclusively prove that cytoplasmic ribosomes were actually absent from the mitochondrial ribosome preparations. It was possible that the EDTA caused cytoplasmic ribosomes to dissociate into subunits of 60S and 40S which would be indistinguishable from the mitochondrial ribosome monomer (55S) and large subunit (40S), on sucrose gradients. To rule out this possibility it was necessary to identify the ribosomal RNA species present in our preparations.

RNA was extracted from ribosomes isolated from mitochondria that had been prepared with and without EDTA and was examined by electrophoresis in 3% polyacrylamide gels (see Experimental Procedures Section). As a standard, authentic cytoplasmic rRNA was electrophoresed in a parallel gel. The 18S rRNA of the cytoplasmic small subunit and the 16S rRNA of the mitochondrial large subunit were poorly resolved under our conditions (results not shown); however, the presence of 28S rRNA from the large cytoplasmic ribosomal subunit could easily be detected. Figure 8 shows the results of such an experiment. Ribosomal RNA from

Figure 7. Sucrose gradient centrifugation of nascent polypeptides synthesized by ribosomes from mitochondria treated with digitonin and EDTA

Ribosomes from mitochondria prepared with (o-o) and without (●-●) EDTA treatment were incubated in a poly(U)-directed polypeptide synthesizing system. Nascent [^{3}H]-polyphenylalanine was visualized by sucrose gradient centrifugation as described in Figure 6. Gradient fractions were prepared for liquid scintillation counting as described in the Experimental Procedures Section.

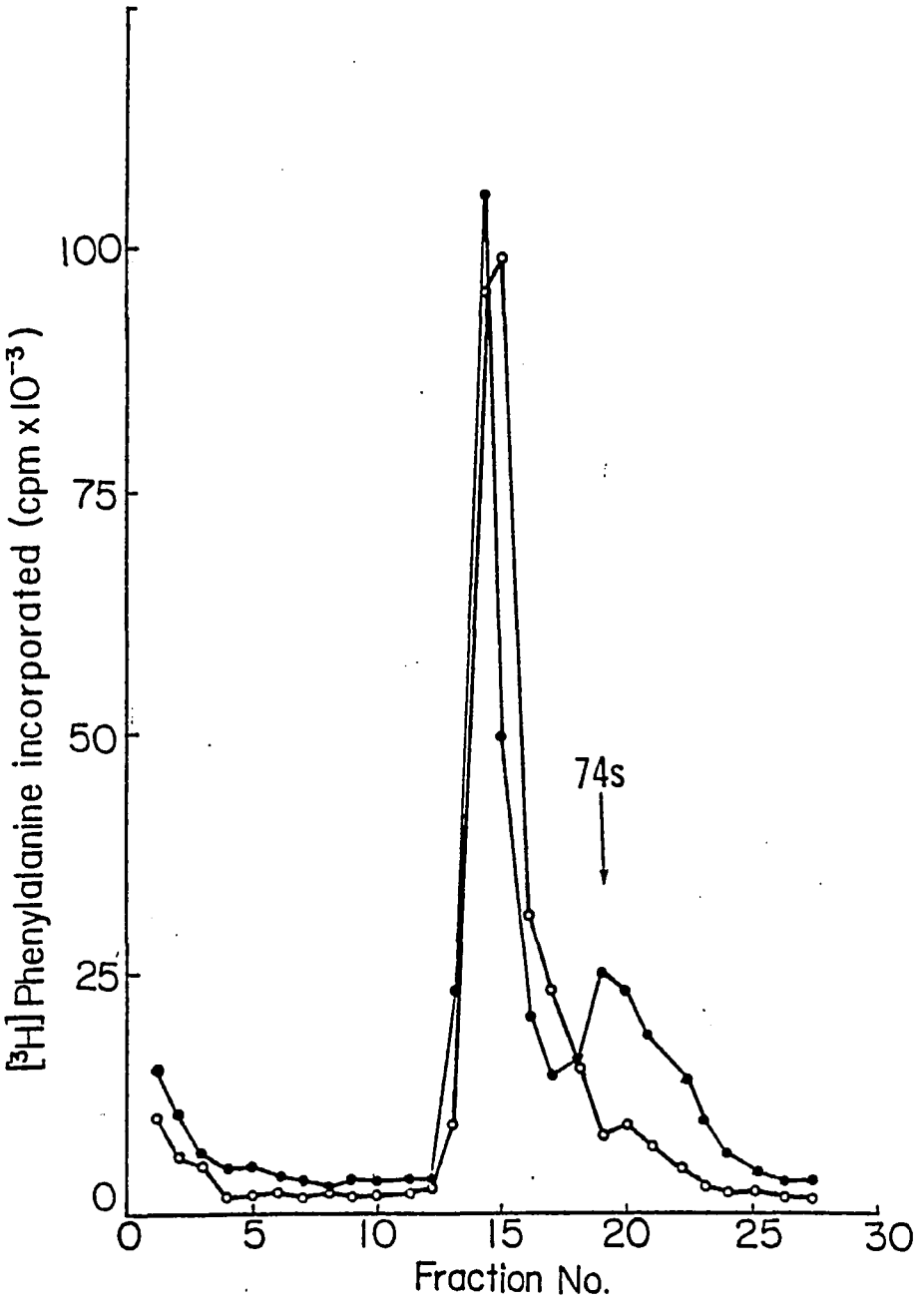
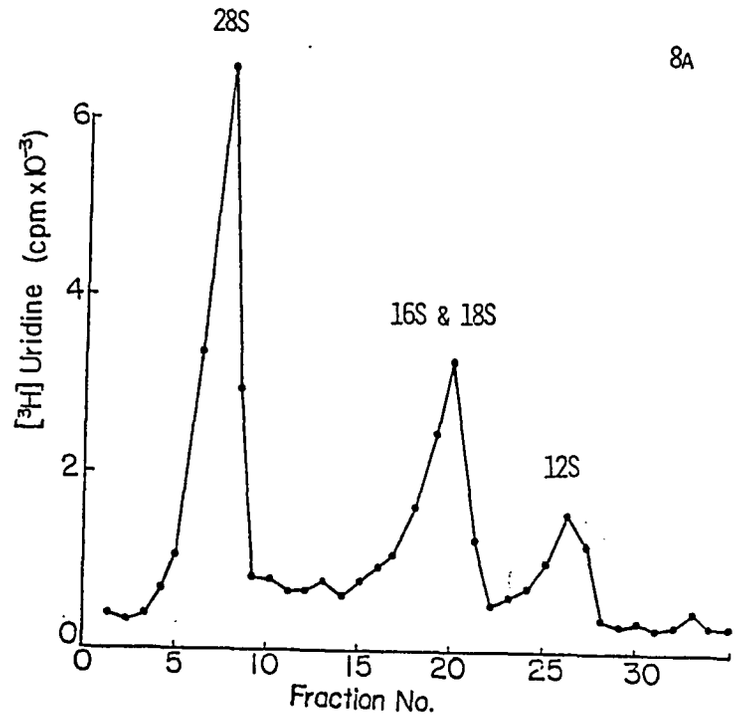
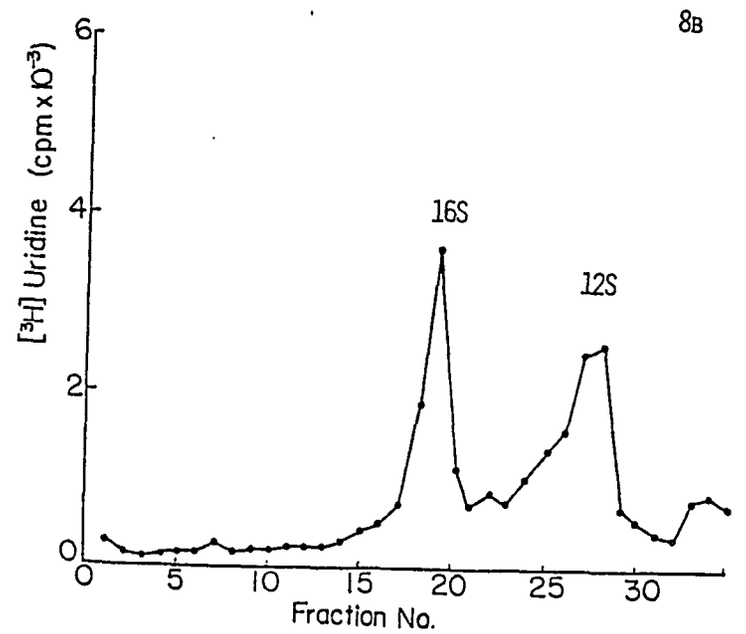

[3H] Phenylalanine incorporated (cpm x 10-3)
100
75
50
25
0
74s
0
5
10
15
20
25
30
Fraction No.

Figure 8. Polyacrylamide gel electrophoresis of RNA prepared from mitochondrial ribosomes

Mitochondria (digitonin treated) were prepared from HeLa cells labeled with [^{3}H]-uridine in the absence of actinomycin D. The mitochondria were washed 5 times with ST buffer (A) or with ST buffer containing 2 mM EDTA (B). Ribosomes were prepared and RNA extracted as described under Experimental Procedures. The RNA was separated by electrophoresis in 3% polyacrylamide at 3mA per gel for 4 hours. The gels were sliced and the fractions prepared for liquid scintillation counting. Authentic 28S and 18S rRNAs were electrophoresed in parallel gels as markers.


28S
8A
16S & 18S
12S
[3H] Uridine (cpm x 10-3)
0
2
4
6
0
5
10
15
20
25
30
Fraction No.


8B
16S
12S
[3H] Uridine (cpm x 10-3)
0
2
4
6
0
5
10
15
20
25
30
Fraction No.

the preparation made without EDTA shows a large 28S cytoplasmic rRNA component (Figure 8A) in addition to the peaks expected to result from the 16S and 12S mitochondrial constituents; the 16S peak may also contain 18S cytoplasmic rRNA. RNA from ribosomes prepared from mitochondria washed twice with EDTA shows only a minor 28S rRNA component (results not shown). When the number of EDTA washes was increased to five, 28S RNA was virtually eliminated from the preparation (Figure 8B). These data provide clear evidence that washing the mitochondria with EDTA prior to treatment with digitonin leads to the removal of the major portion of the contaminating cytoplasmic ribosomes while leaving the mitochondrial ribosomes functionally active.

Protein Synthetic Activity of Mitochondrial Ribosomes In Vitro

The requirements for poly(U)-dependent synthesis of polyphenylalanine by mitochondrial ribosome preparations are shown in Table 3. Full activity required an energy source (ATP and GTP), E. coli supernatant factors (factors from other sources were not examined, however, see Discussion), tRNA and ribosomes. Omission of any of these components led to a 70-80% reduction in activity. RNA but not DNA was required, as evidenced by the results of the assays in which RNase and DNase were added. Inclusion of puromycin (1 mM), a potent inhibitor of polypeptide synthesis on all known ribosomes (Peska, 1977), led to a 66% reduction in polypeptide formation.

Added messenger RNA was required by the system (Table 3 and Figure 9). In the absence of poly(U), no nascent polyphenylalanine was observed

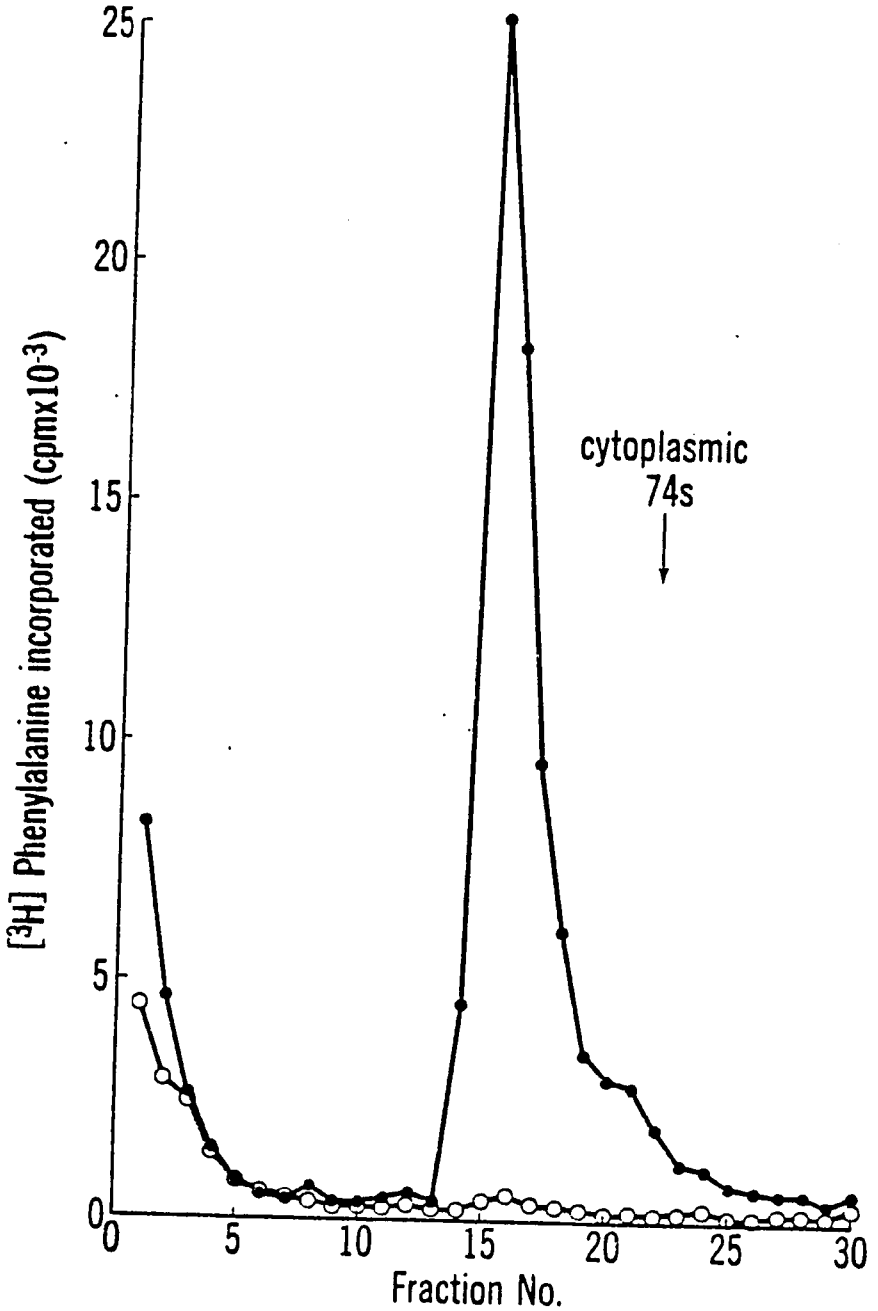
Table 3. Requirements for polypeptide synthesis by mitochondrial ribosomes *in vitro*

Reaction Mix[a]	CPM [^{3}H]-polyphenylalanine	%
Complete	76,000	(100)
-Energy	22,000	29
-S-100	12,000	16
-tRNA	16,000	21
-Poly(U)	19,000	25
-Ribosomes	22,000	29
Puromycin (1 mM)	26,000	34
DNase (100 µg/mL)	74,000	97
RNase (100 µg/mL)	21,000	28

[a]The complete reaction mixture contained, in a final volume of 100 µL: 10 M Tris-HCl, pH 7.4; 1 mM DTT; 5 mM mercaptoethanol; 0.1 mM GTP; 1 mM ATP; 4 mM PEP; 11 mM Mg $(CH_3COO)_2$; pyruvate kinase, 25 µg/mL; 30 mM $(NH_4)_2SO_4$; 50 mM KCl; *E. coli* tRNA, 1 mg/mL; *E. coli* S-100, 0.6 mg/mL; poly(U), 0.5 mg/mL; 8 µM [^{3}H]-phenylalanine, 54 Ci/mMole; cycloheximide, 1 mg/mL and digitonin treated mitochondrial ribosomes. After incubation for 1 hr at 37°C, the samples were prepared for liquid scintillation counting as outlined in the Experimental Procedures Section.

Figure 9. Sucrose gradient centrifugation of nascent polypeptides synthesized by mitochondrial ribosomes in the presence and absence of poly(U)

Ribosomes from digitonin prepared mitochondria were incubated under conditions for polypeptide synthesis with (●-●) or without (o-o) poly(U). At the end of 60 minutes, the newly synthesized [^{3}H]-polyphenylalanine which remained associated with the ribosomes was visualized by sucrose gradient centrifugation as described in Figure 5. The reaction mixture contained 2 mg/mL cycloheximide to inhibit cytoplasmic ribosome function. Fractions were prepared for liquid scintillation counting as outlined in the Experimental Procedures Section.


25
20
15
10
5
0
0
5
10
15
20
25
30
[3H] Phenylalanine incorporated (cpmx10-3)
Fraction No.
cytoplasmic
74s

to sediment with mitochondrial ribosomes on a 10-30% linear sucrose gradient (Figure 9). The slight shoulder on the leading edge of the mitochondrial ribosome peak was due to contamination by cytoplasmic ribosomes not completely removed by digitonin treatment. Experiments with poly(C,U) gave similar results; however, despite several attempts, we did not observe a poly(C) stimulated incorporation of [^{3}H]-proline into trichloroacetic acid insoluble material. The effect of other messengers was not examined.

Polypeptide synthesis by mitochondrial and cytoplasmic ribosomes had distinctly different magnesium optima. As shown in Figure 10, optimum activity with cytoplasmic ribosomes was obtained at 7 mM Mg^{2+} while mitochondrial ribosomes had a magnesium optimum at 12 mM. Although these results were obtained with poly(C,U), the results were similar regardless of the synthetic messenger RNA used. It is clear from these results that by assaying for polypeptide synthesis at magnesium levels above 10 mM, the contribution from contaminating cytoplasmic ribosomes is minimized.

The time course for poly(U) and poly(C,U)-directed polypeptide synthesis is shown in Figure 11. The reaction was linear for ca. 60 minutes, after which it reached a plateau. Higher backgrounds (incorporation without mRNA or ribosomes) were always observed with [^{3}H]-phenylalanine than with [^{3}H]-leucine.

Protein synthesis in this system was dependent on added mitochondrial ribosomes (Table 3); and, within the range of ribosome concen-

Figure 10. Effect of Mg^{2+} concentration on polypeptide synthesis by mitochondrial and cytoplasmic ribosomes

Mitochondrial (o-o) and cytoplasmic (●-●) ribosomes were assayed for poly(C,U)-directed polypeptide synthesis in reaction mixtures which contained varying concentrations of Mg^{2+}. After incubation the samples were processed for liquid scintillation counting as described in Experimental Procedures.

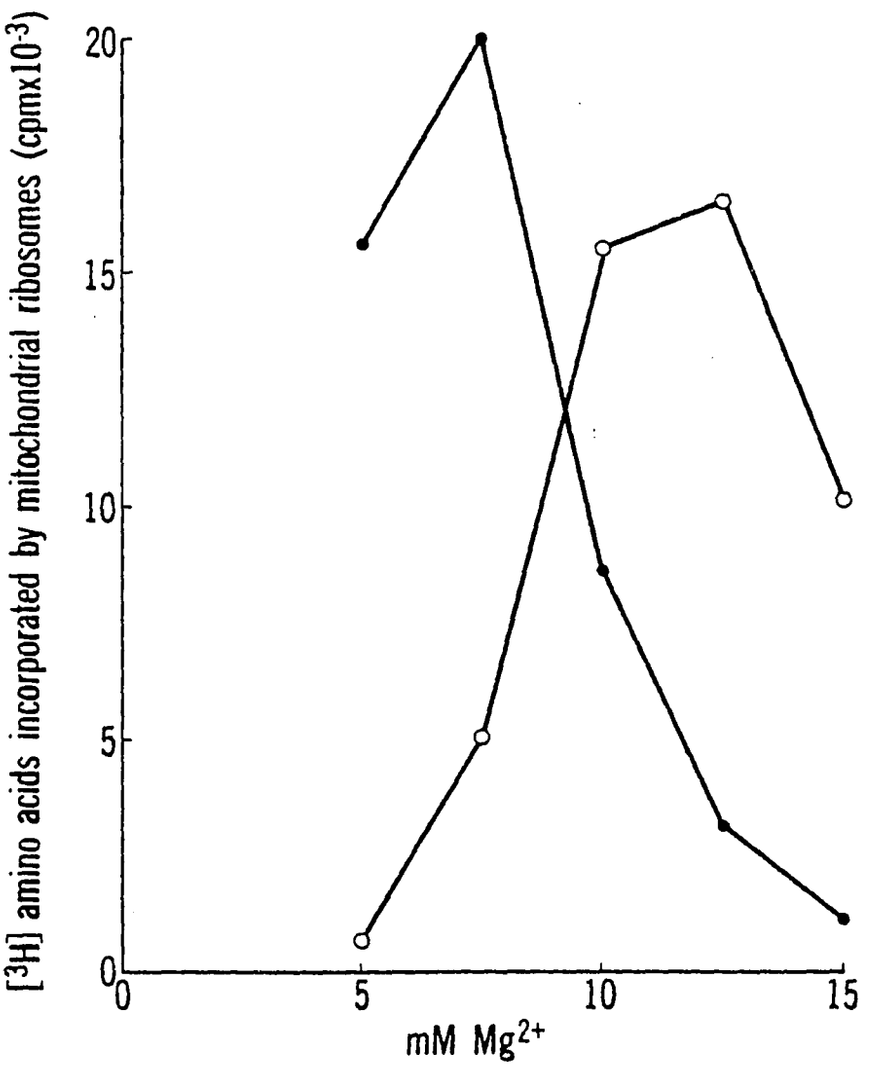
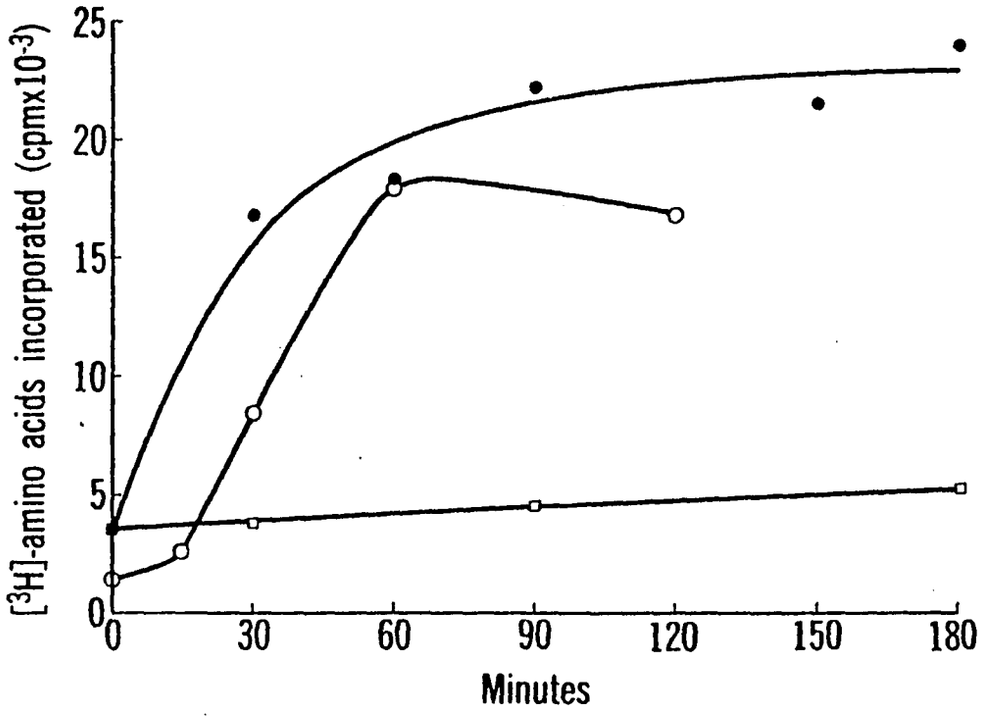

[3H] amino acids incorporated by mitochondrial ribosomes (cpmx10-3)
20
15
10
5
0
0
5
10
15
mM Mg2+

Figure 11. Time course of polypeptide synthesis by mitochondrial ribosomes

Ribosomes from digitonin treated mitochondria were incubated under conditions for polypeptide synthesis. The incorporation of [^{3}H]-phenylalanine into polypeptides was examined with (●-●) and without (□-□) poly(U) as messenger RNA. The incorporation of [^{3}H]-leucine using poly(C,U) as messenger RNA was also determined (o-o). Aliquots were taken at the times indicated and processed for liquid scintillation counting as outlined in the Experimental Procedures Section.


[3H]-amino acids incorporated (cpmx10-3)
25
20
15
10
5
0
0
30
60
90
120
150
180
Minutes

trations used in these assays (0.1 A_{260} to 0.5 A_{260}), the response was linear (Figure 12). Assuming $E_{260}^{0.1\%} = 8$ for mitochondrial ribosomes (O'Brien and Matthews, 1976), we can calculate that about 210 pmoles of [^{3}H]-phenylalanine are being incorporated into polyphenylalanine per milligram of ribosomes per hour.

Effect of Chloramphenicol and Other Antibiotics on In Vitro Polypeptide Synthesis by HeLa S_3 and 296-1 Mitochondrial Ribosomes

Because poly(U) was more efficiently translated than poly(C,U), in the mitochondrial system, first attempts at showing in vitro sensitivity to chloramphenicol used the poly(U)-directed polyphenylalanine synthesizing system. However, despite repeated attempts with both rat liver and HeLa S_3 mitochondrial ribosomes, in our hands poly(U)-directed polypeptide synthesis was never significantly inhibited by the drug (Table 4). Greco et al. (1973) and Ibrahim et al. (1974) reported that rat liver mitochondrial ribosomes were sensitive to chloramphenicol when assayed in a poly(U)-directed polyphenylalanine synthesizing system. The reasons for the different responses to chloramphenicol in their experiments and ours are not clear. However, a recent report by Ulbrich et al. (1980) using rat liver mitochondrial ribosomes, also finds no inhibition by chloramphenicol of poly(U)-directed polypeptide synthesis.

When poly(C,U) was used as a messenger, [^{3}H]-labeled leucine, proline and phenylalanine were incorporated into a polypeptide whose synthesis on HeLa S_3 mitochondrial ribosomes was inhibited by chloramphenicol. Table 5 shows the effect of increasing concentrations of

Figure 12. Effect of mitochondrial ribosome concentration on poly(U) directed [^{3}H]-polyphenylalanine synthesis

Varying amounts of ribosomes from digitonin treated mitochondria were assayed for their ability to stimulate polypeptide synthesis in the standard assay mixture (Experimental Procedures Section). After incubation, samples were processed for liquid scintillation counting as described in the Experimental Procedures Section.

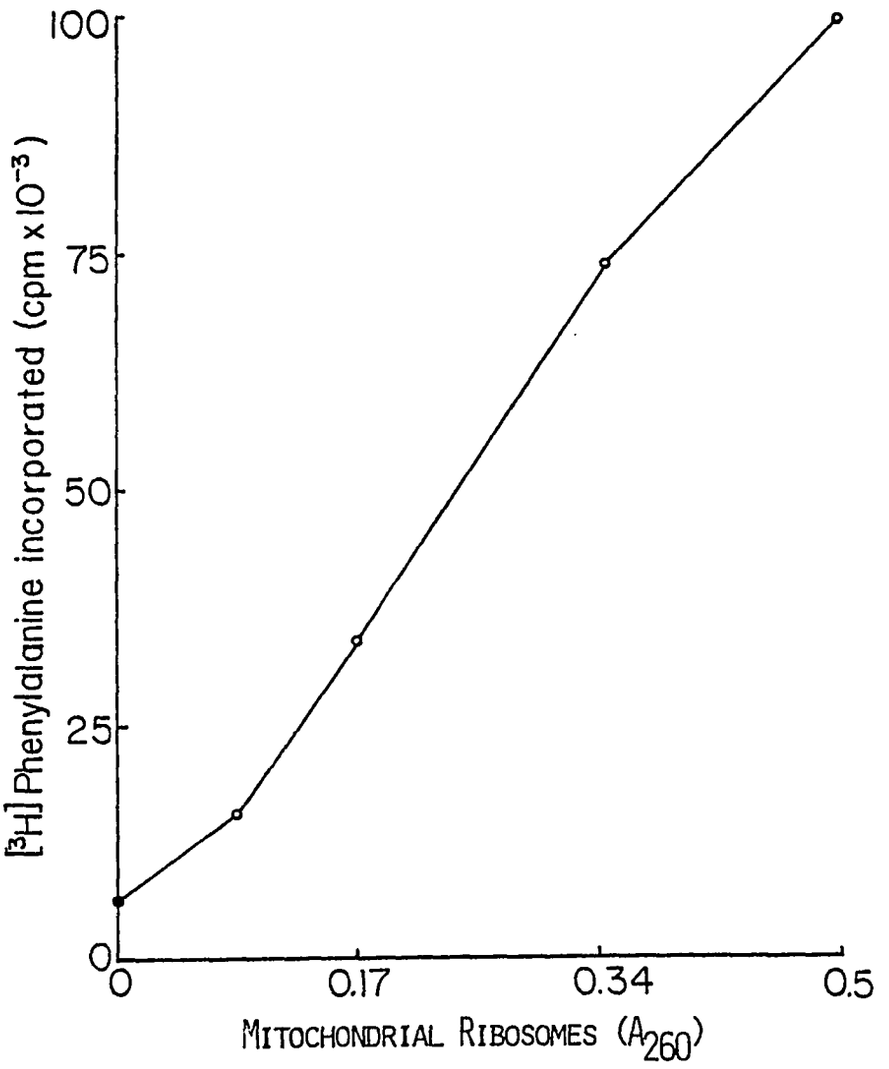

100
75
50
25
0
[3H] Phenylalanine incorporated (cpm x 10-3)
0
0.17
0.34
0.5
Mitochondrial Ribosomes (A260)

Table 4. Effect of chloramphenicol on poly(U)-directed polypeptide synthesis by mitochondrial ribosomes from HeLa S_3[a]

Chloramphenicol (μg/mL)	[^{3}H]-polyphenylalanine CPM[b]
0	1.3×10^5
100	1.3×10^5
200	1.4×10^5
400	1.3×10^5

[a]Assays were conducted as described in Table 3.

[b]The values reported were corrected for incorporation observed in an assay without poly(U) (2×10^4 cpm).

Table 5. Effect of chloramphenicol on poly(C,U)-directed polypeptide synthesis by mitochondrial ribosomes from HeLa S_3 and HeLa 296-1[a]

Chloramphenicol (μg/mL)	Polypeptide Synthesis (cpm)			
	S_3 Ribosomes	%	296-1 Ribosomes	%
0	10,300	(100)	21,200	(100)
5	9,400	(90)	22,900	(107)
10	8,900	(85)	23,600	(111)
20	7,400	(72)	26,000	(112)
50	6,800	(65)	24,100	(113)
100	6,300	(61)	21,900	(103)
200	5,700	(54)	21,400	(100)
400	4,100	(39)	22,200	(104)

[a]Assay was conducted as in Table 3, except that the [^{3}H]-amino acids included were leucine, proline and phenylalanine.

chloramphenicol on poly(C,U)-dependent polypeptide synthesis by mitochondrial ribosomes from both the sensitive (S_3) and the resistant (296-1) HeLa cell lines. At 400 µg/mL chloramphenicol polypeptide production on S_3 mitochondrial ribosomes was inhibited by 60%; at the same drug level, synthesis on mitochondrial ribosomes from HeLa 296-1 was unaffected. The level of inhibition by chloramphenicol varied somewhat from one experiment to another. In one experiment chloramphenicol (400 µg/mL) inhibited HeLa 296-1 mitochondrial ribosome function by 45% while S_3 mitochondrial ribosomes were inhibited 89%. In all cases where mitochondrial ribosomes from both resistant and sensitive cell lines were assayed in parallel, HeLa 296-1 mitochondrial ribosomes were significantly more resistant to the drug.

Because the level of inhibition of poly(C,U)-directed polypeptide synthesis by chloramphenicol might be influenced by the radioactive amino acid used, various combinations of amino acids were tested. With all amino acids, HeLa S_3 mitochondrial ribosomes were found to be sensitive to the drug (Table 6). The greatest inhibition was observed when radioactive leucine or leucine plus proline were used. It is not certain why significant incorporation was observed when only one amino acid was added since poly(C,U) is a random copolymer and should code for a mixed polypeptide. However, it may be that the level of amino acids present in the S-100 preparations used in the reaction was sufficient to supply the missing components. The choice of which labeled amino acid to use did not appear to be critical for the

Table 6. Inhibition by chloramphenicol of the poly(C,U)-directed incorporation of various amino acids into polypeptides[a]

(^{3}H)-amino acid	100 µg/mL Chloramphenicol	CPM	% of control
1. (^{3}H)-leucine	-	8,400	
	+	1,900	22
2. (^{3}H)-proline	-	6,400	
	+	2,200	34
3. (^{3}H)-phenylalanine	-	10,300	
	+	2,300	23
4. (^{3}H)-leucine + (^{3}H)-proline	-	9,300	
	+	2,700	22
5. (^{3}H)-leucine + (^{3}H)-proline + cold serine	-	9,500	
	+	3,700	49
6. (^{3}H)-leucine + (^{3}H)-proline + (^{3}H)-phenylalanine + cold serine	-	13,100	
	+	5,800	54
7. (^{3}H)-leucine + (^{3}H)-proline + cold phenylalanine + cold serine	-	13,900	
	+	3,600	26

[a]Assay was conducted as described in Table 3, using HeLa S_3 mitochondrial ribosomes prepared from digitonin treated mitochondria.

observation of chloramphenicol sensitivity when poly(C,U) was used as mRNA. Therefore, because higher levels of incorporation were observed when phenylalanine, leucine, and proline were all labeled, subsequent assays were carried out with this mixture of radioactive amino acids. Radioactive serine was not included in these experiments because it was not commercially available at the high specific activity used in our assays, and thus its contribution to observed incorporation would have been negligible.

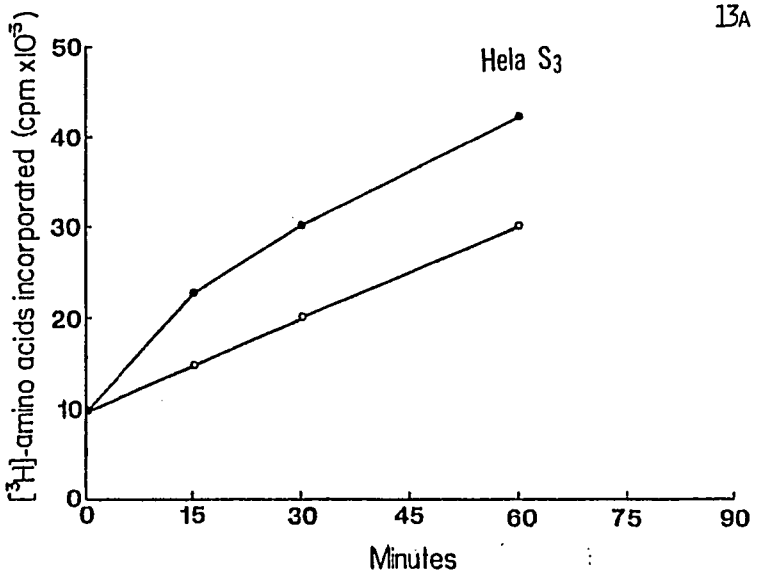
When present at a concentration of 100 μg/mL, chloramphenicol consistently inhibited polypeptide synthesis by HeLa S_3 mitochondrial ribosomes by about 40%, after one hour of incubation at 37°C. Under these conditions, no effect was seen on mitochondrial ribosomes from HeLa 296-1. To check for kinetic effects which might be masked by taking only a single time point in these assays, the time course of poly(C,U)-directed polypeptide synthesis in the presence and absence of 100 μg/mL chloramphenicol was determined. Figure 13 shows that while the rate of polypeptide synthesis by mitochondrial ribosomes from HeLa S_3 was inhibited rather uniformly throughout the assay period (40% inhibition after one hour at 37°C), the rate of synthesis by mitochondrial ribosomes from HeLa 296-1 was unaffected.

The resistance of ribosomes from HeLa 296-1 mitochondria to chloramphenicol was also evident when the reaction mixtures were analyzed on sucrose gradients (Figure 14). As expected, chloramphenicol (100 μg/mL) had no effect on the amount of nascent polypeptide associated with mito-

Figure 13. Time course of poly(C,U) directed polypeptide synthesis by mitochondrial ribosomes from HeLa S_3 and HeLa 296-1 cells in the presence and absence of chloramphenicol

Mitochondrial ribosomes from HeLa S_3 (A) and HeLa 296-1 (B) cells were incubated in a poly(C,U)-directed peptide synthesis mixture in the presence (o-o) or absence (●-●) of 100 µg/mL chloramphenicol. Aliquots were removed at the times indicated and were prepared for liquid scintillation counting as described in the Experimental Procedures Section.

13A


Hela S3
[3H]-amino acids incorporated (cpm x10-3)
50
40
30
20
10
0
0
15
30
45
60
75
90
Minutes

13B

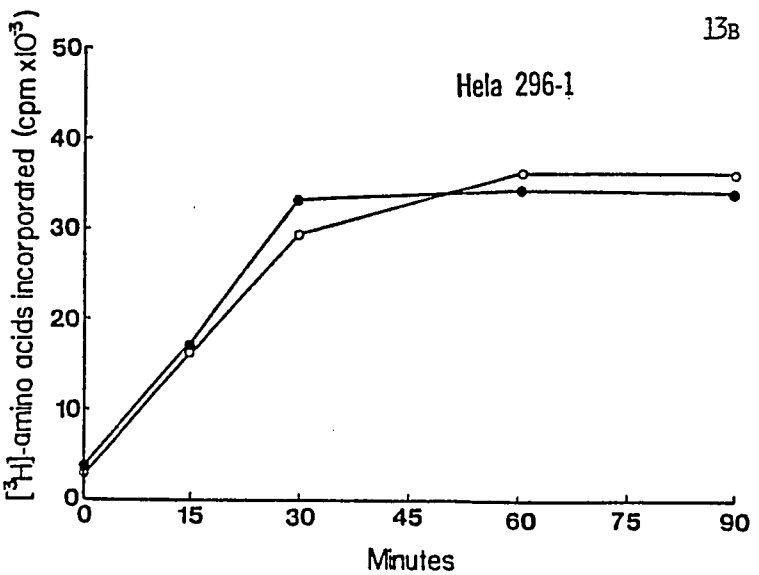
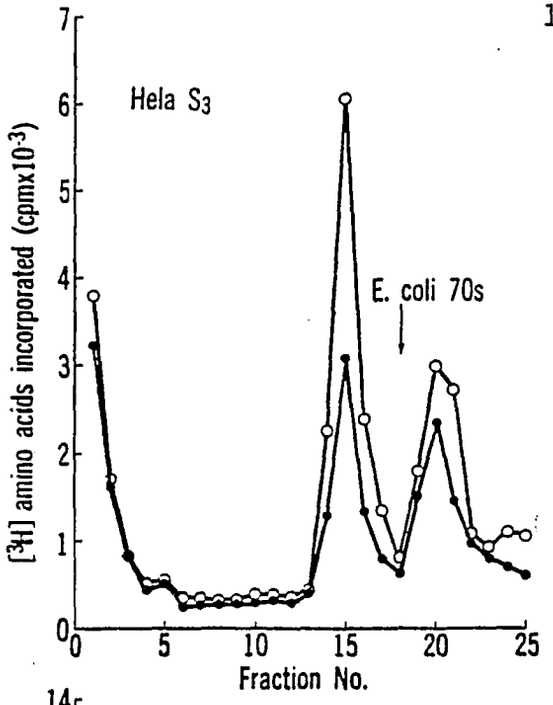

Hela 296-1
[3H]-amino acids incorporated (cpm x10-3)
50
40
30
20
10
0
0
15
30
45
60
75
90
Minutes

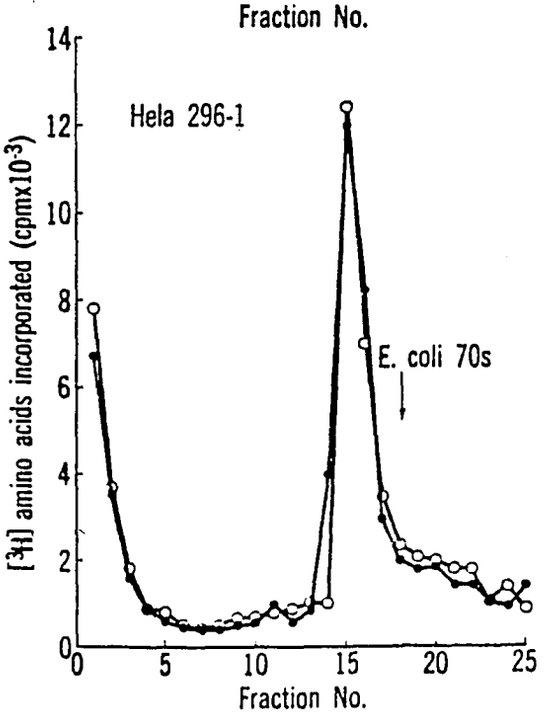
Figure 14. Sucrose gradient centrifugation of nascent polypeptides synthesized by mitochondrial ribosomes from HeLa S_3 and HeLa 296-1 cells in presence and absence of chloramphenicol

Mitochondrial ribosomes from HeLa S_3(A) and HeLa 296-1 (B) cells were incubated in a poly(C,U)-directed polypeptide synthesis mixture (containing cycloheximide) in the presence (●-●) or absence (o-o) of 100 µg/mL chloramphenicol. After incubation, the newly synthesized nascent polypeptides were visualized by centrifugation through a 10 to 30% sucrose gradient as described in Figure 5. The gradient fractions were prepared for liquid scintillation counting as described in the Experimental Procedures Section.

14A


Hela S3
E. coli 70s
[3H] amino acids incorporated (cpmx10-3)
Fraction No.
0
1
2
3
4
5
6
7
0
5
10
15
20
25

14B


Hela 296-1
E. coli 70s
[3H] amino acids incorporated (cpmx10-3)
Fraction No.
0
2
4
6
8
10
12
14
0
5
10
15
20
25

chondrial ribosomes from HeLa 296-1 (Figure 14B). On the other hand, chloramphenicol decreased by 50% the nascent polypeptide associated with mitochondrial ribosomes from HeLa S_3 (Figure 14A). The mitochondrial ribosome preparation from HeLa S_3 used in this experiment was heavily contaminated with cytoplasmic (74S) ribosomes. As expected, chloramphenicol had only a small inhibitory effect (ca. 20%) on the amount of nascent polypeptide associated with these cytoplasmic ribosomes (Figure 14A).

There have been several reports in the literature of cases where selection for resistance to a drug resulted in selection for cross-resistance to other drugs as well (Bunn et al., 1970). That is, a single mutational event which conferred resistance to one antibiotic could sometimes, simultaneously, confer resistance to a seemingly unrelated antibiotic. In order to check for this possibility in HeLa 296-1, resistance to a variety of protein synthesis inhibitors was investigated. Table 7 shows the effects of these inhibitors, each at a concentration of 2 mM, on poly(C,U)-directed polypeptide synthesis by mitochondrial and cytoplasmic ribosomes.

Carbomycin and spiramycin, both macrolide antibiotics, and mikamycin (streptogramin A), all of which inhibit bacterial peptide bond formation, seemed to have little effect on any of the ribosomes assayed. Lincomycin and chloramphenicol, on the other hand, both inhibited polypeptide synthesis by mitochondrial ribosomes from HeLa S_3, while cytoplasmic ribosomes and mitochondrial ribosomes from HeLa 296-1 were virtually unaffected.

Table 7. The effect of various antibiotics on poly(C,U)-directed polypeptide synthesis on mitochondrial and cytoplasmic ribosomes[a]

	Cytoplasmic Ribosomes		Mitochondrial Ribosomes HeLa S_3		Mitochondrial Ribosomes HeLa 296-1	
Antibiotic (2 mM)	CPM[b]	%	CPM[b]	%	CPM[b]	%
None	72,900	100	10,400	100	20,500	100
Carbomycin	64,800	89	10,300	100	14,700	72
Spiramycin	76,100	104	9,900	95	23,900	116
Mikamycin	46,800	64	11,100	106	15,000	73
Chloramphenicol	63,900	88	1,800	17	18,800	92
Lincomycin	61,000	84	4,800	46	17,000	83
Erythromycin	37,500	51	8,200	79	30,000	146
Anisomycin	10,400	14	6,300	69	18,300	89

[a]Assays were conducted as described on Table 3.

[b]The values reported were corrected for incorporation observed in an assay mixture lacking ribosomes (16 X 10^3 cpm).

The effect of erythromycin on this system is quite enigmatic. While mitochondrial ribosomes from HeLa S_3 seem to be relatively unaffected by the drug and cytoplasmic ribosomes are sensitive, mitochondrial ribosomes from HeLa 296-1 seem to be stimulated. Erythromycin is known to be an inhibitor of mitochondrial ribosomes in vitro (Ibrahim et al., 1974) but not cytoplasmic ribosomes, so an interpretation of this result is not possible at this time and must await further investigation. Anisomycin, a potent inhibitor of cytoplasmic ribosomes, had little effect on polypeptide synthesis by mitochondrial ribosomes.

Attempts to Prepare Mitochondrial Ribosomal Subunits Active in Polypeptide Synthesis

The results presented thus far clearly show that the mutation to chloramphenicol resistance directly affects the susceptibility of mitochondrial ribosomes when tested in vitro. In order to identify the mitochondrial ribosome subunit modified by this mutation, mitochondrial ribosomes were dissociated and attempts were made to test their activity in vitro. Dissociation was accomplished by dialysis of the mitochondrial ribosomes against 1 L of low Mg^{2+} KMEDT buffer (1 mM Mg^{2+}) overnight at $4^{o}C$. Because the usual preparation of ribosomes contained large amounts of subunits (see Figure 1), 55S monomers isolated from a previous sucrose gradient were used. The subunits were separated by centrifugation on a 10-30% sucrose gradient in low Mg^{2+} KMEDT buffer for 4 hours

at 40,000 rpm in a Beckman type SW 40Ti rotor (Figure 15). After dialysis the monomers were completely dissociated into subunits of 40S and 30S (Figure 15). The accumulation of labeled material at the top of the gradient suggests that despite purification on a sucrose gradient, the preparation still contains nuclease activity.

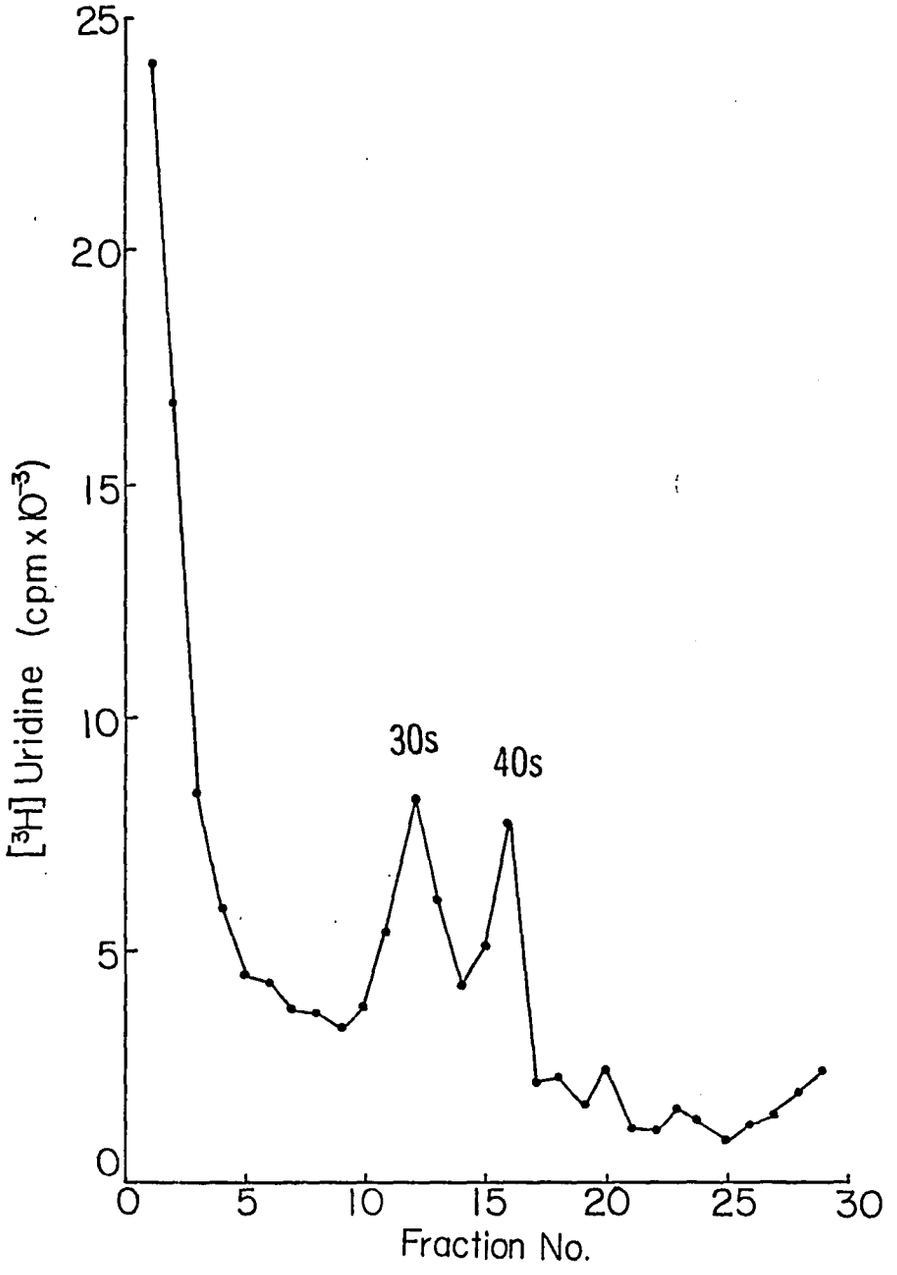
When isolated mitochondrial ribosomal subunits were pooled and tested for activity, they were found to be completely inactive in poly(U)-directed polypeptide synthesis. Subsequent attempts by others in this laboratory have produced significantly improved conditions for subunit preparation; however, they have not been able to demonstrate poly(U)-directed polypeptide synthesis with these preparations (D. Hacker, personal communication).

From preliminary experiments (data not shown) it was known that neither sucrose gradient centrifugation nor incubation in low Mg^{2+} buffers was sufficient to inactivate the mitochondrial ribosomes. 55S monosomes active in poly(U)-directed polypeptide synthesis could be isolated from sucrose gradients containing 10 mM Mg^{2+} and ribosomes preparations incubated in 1 mM Mg^{2+} but not placed on sucrose gradients still retained full activity. These results suggested that some factor normally present on the ribosome was lost during separation of the subunits.

Denslow and O'Brien (1979) and Ulbrich *et al.* (1980) had shown that elongation factor G, which contaminates mitochondrial ribosomal preparations under normal isolation conditions, is lost when ribosomes are

Figure 15. Dissociation of mitochondrial ribosomes by dialysis against low magnesium buffer

HeLa S_3 mitochondrial ribosomes labeled *in vivo* with [^{3}H]-uridine were dialysed overnight (4°C) against low magnesium (1 mM) KMEDT buffer. They were then separated by centrifugation through a 10 to 30% sucrose gradient as described in Figure 5 and 1/10th volume of each fraction was prepared for liquid scintillation counting as outlined in the Experimental Procedures Section.


25
20
15
10
5
0
[³H] Uridine (cpm x 10⁻³)
30s
40s
0
5
10
15
20
25
30
Fraction No.

washed with high salt-containing buffers and that this factor could not be replaced by the analogous E. coli factor. It could, however, be replaced by the homologous factor present in mitochondrial S-100. With this in mind, supernatant factors were prepared from the mitochondria of rat liver, rabbit liver and HeLa cells (See Experimental Procedures Section). None of these preparations stimulated poly(U)-dependent polypeptide synthesis by mitochondrial ribosomes or their subunits. In fact, each contained a potent inhibitor of poly(U)-directed polypeptide synthesis on E. coli ribosomes (Table 8).

Several attempts were made to separate the inhibitor from the components in S-100 required for protein synthesis. Overnight dialysis of rat liver mitochondrial S-100 at $4^{o}C$ against KMEDT buffer (1 L) failed to remove the inhibitor (Table 8).

When an aliquot of rat liver mitochondrial S-100 was chromatographed on Sephadex G-75, two peaks were clearly resolved (Figure 16). The first peak (excluded volume) contained mostly protein; the second (included volume) contained mostly RNA, as judged by the A_{280}/A_{260} ratio of the fractions. When these fractions were tested, all were found to inhibit polypeptide synthesis by E. coli ribosomes (Table 9).

Chromotography of rat liver motochondrial S-100 on DEAE cellulose separated three peaks (Figure 17). The first peak (mostly protein) was eluted with starting buffer (0.02 M phosphate buffer); the second peak eluted with 0.25 M phosphate buffer; the third (mostly RNA) eluted with 0.25 M phosphate buffer containing 1.0 M NaCl. All three fractions contained significant amounts of inhibitory activity (Table 9).

Table 8. Effect of mitochondrial S-100 preparations on poly(U)-directed polypeptide synthesis with *E. coli* ribosomes[a]

Mitochondrial S-100 added	% Control
None	100
Rat liver	5
Rat liver (dialyzed)	7
Rabbit liver	6
HeLa	5

[a]Assays were conducted as described in Table 3, except that they contained 0.5 A_{260} of *E. coli* ribosomes. Except for the control, each mixture contained approximately 30 μg of the indicated mitochondrial S-100 in addition to 300 μg of *E. coli* S-100.

Figure 16. Chromatography of mitochondrial S-100 on Sephadex G-75

One mL of rat liver mitochondrial S-100 (ca. 4mg) was applied to an 11 mL Sephadex G-75 column equilibrated with KMEDT buffer. The sample was eluted with the same buffer. The first mL off the column was discarded and 1 mL fractions were collected for the first 4 mL after which 2 mL fractions were collected. The absorbance at 280 nm (■-■) and at 260 (o-o) was determined for each fraction. The width of the bars in the figure (□-□) indicates the fraction volume collected.

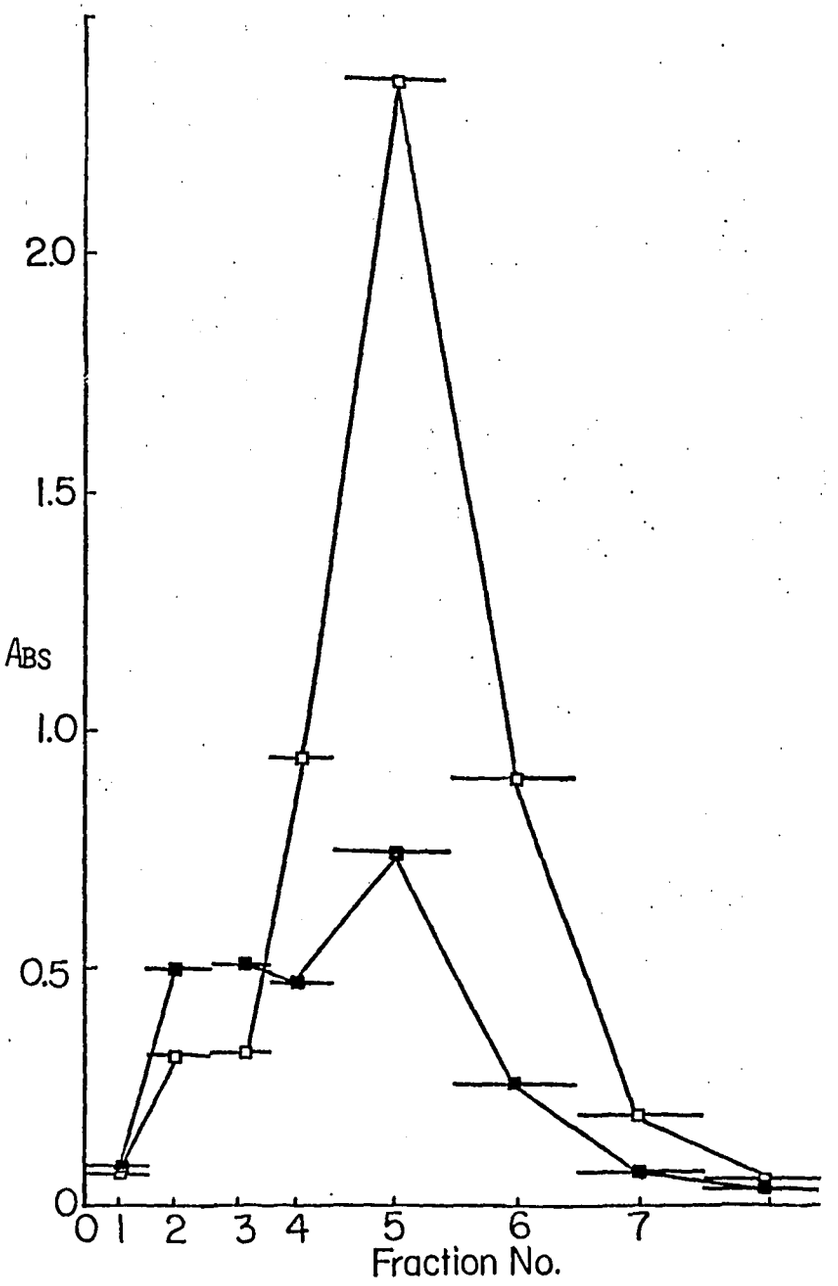

2.0
1.5
ABS
1.0
0.5
0
0 1 2 3 4 5 6 7
Fraction No.

Table 9. Inhibition of poly(U)-directed polypeptide synthesis on *E. coli* ribosomes by rat liver mitochondrial S-100 fractions separated on Sephadex G-75 and DEAE-cellulose (DE-32)[a]

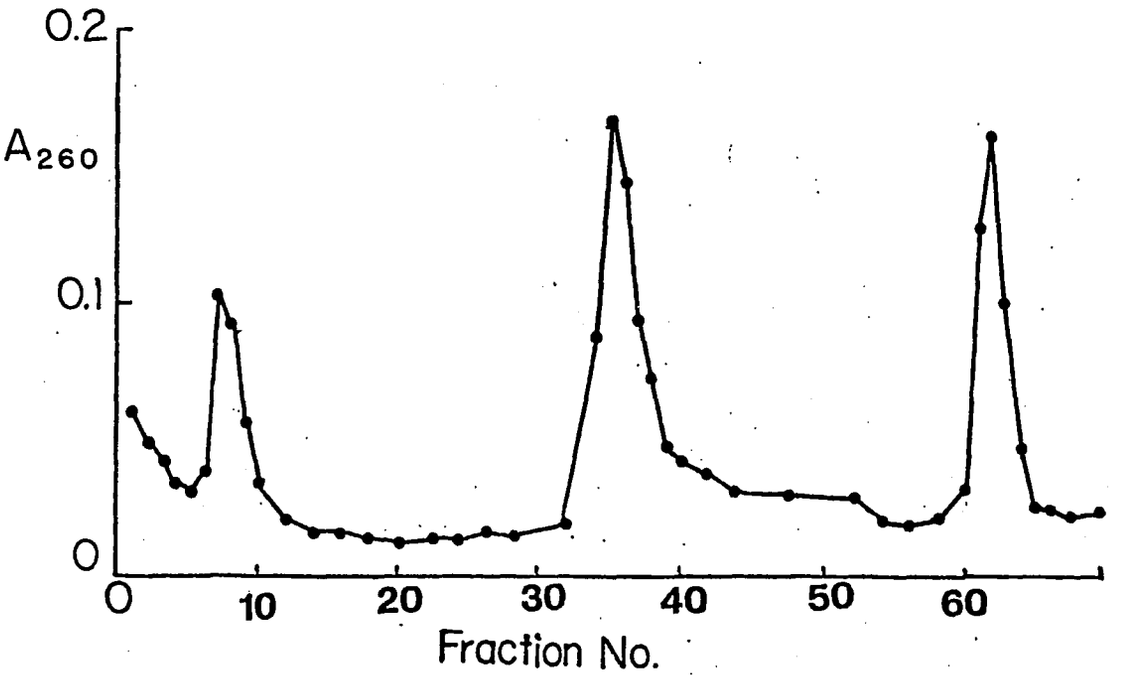
Mitochondrial S-100 fraction[b]		
Sephadex G-75	CPM	% Control
None	123,600	100
Fraction 2 + 3	20,300	16
Fraction 4	16,200	13
Fraction 5	23,600	19
Fraction 6	68,600	57
DEAE-cellulose		
None	81,900	100
Fraction 6-9	29,800	36
Fraction 32-38	17,600	22
Fraction 61-64	53,900	66

[a]Assays were conducted as described in Table 3, except each tube contained 0.5 A_{260} of *E. coli* ribosomes. After incubation, samples were processed for liquid scintillation counting as described in the Experimental Procedures Section.

[b]Approximately 15 µg of protein was added.

Figure 17. Chromatography of mitochondrial S-100 on DEAE-cellulose (DE-32)

2 mL of mitochondrial S-100, dialyzed against 0.02 M phosphate buffer (pH 7.4), were applied to a DEAE-cellulose column equilibrated with the same buffer. 1 mL fractions were collected. Fractions 1 to 27 were eluted with starting buffer, fractions 28 to 53 were eluted with 0.25 M phosphate (pH 7.4) and fractions above 53 were eluted with the same buffer containing 1 M NaCl. The absorbance at 260 nM was determined for each fraction.


0.2
A_{260}
0.1
0
0
10
20
30
40
50
60
Fraction No.

The inhibitory activity found in mitochondrial S-100 could be due to a RNase of lysosomal origin. To test for this possibility, a rat liver mitochondrial S-100 was assayed for RNase activity (Table 10). Significant RNase activity was detected in the mitochondrial S-100 preparation, equivalent to 0.5 μg of commercial pancreatic RNase per 100 μg S-100 protein.

Table 10. RNase activity of a mitochondrial S-100 preparation[a]

Enzyme source	A_{260}	% of Total RNA in supernatant
None	0.002	0
RNase (5 µg)	0.917	9
Rat Liver Mitochondrial (100 µg) S-100	0.093	0.9

[a]The assay was conducted as described in the Experimental Procedures Section. In a final volume of 0.6 mL, 0.1 mL of the solution to be assayed was incubated for 30 minutes at 37°C with 62 A_{260} of the RNA. The RNA was then precipitated with an equal volume of 1N HCl, 76% ETOH and pelleted by centrifugation. A 0.5 mL aliquot of the supernatant was diluted to 4 mL with H_2O and the absorbance at 260 nm was determined.

DISCUSSION

Isolation and Purification of Mitochondrial Ribosomes Active in Polypeptide Synthesis

Two major impediments to purification of mitochondrial ribosomes active in *in vitro* polypeptide synthesis were encountered in these studies. They were the persistence of cytoplasmic ribosomes in the preparations and the variability of different preparations in their competence for polypeptide synthesis. The two problems were related in that attempts to separate mitochondrial ribosomes from cytoplasmic ribosomes after lysis of the mitochondria, e.g., by sucrose gradient centrifugation, resulted in preparations with reduced synthetic activity. It was, therefore, necessary to find conditions which allowed the preparation of intact mitochondria free of contaminating cytoplasmic ribosomes and to use these "clean" mitochondria in the preparation of mitochondrial ribosomes. In a first attempt, mitochondria were washed with digitonin, a detergent known to remove the outer membrane of human liver mitochondria (Benga *et al.*, 1979) and, presumably, the cytoplasmic ribosomes adhering to it as well. Although this procedure significantly reduced the activity of contaminating cytoplasmic ribosomes, it failed to eliminate their presence altogether.

In 1973, Greco *et al.* had shown that washing rat liver mitochondria with EDTA reduced contamination by cytoplasmic ribosomes. We concluded that a combination of the two procedures (digitonin plus EDTA washing) might yield mitochondria free of cytoplasmic ribosomes. Although Avadhani and Rutman (1974) had successfully used EDTA and digitonin to

prepare Ehrlich acites cell mitochondrial ribosomes, EDTA washing was nonetheless employed with some trepidation. There has been considerable controversy over the sedimentation behavior of the Neurospora mitochondrial ribosome. Datema et al. (1974) claimed a value of 80S for the monomer while Michel et al. (1977) suggested that the actual sedimentation value of the ribosome was 73S. According to Datema et al., the slower sedimenting species resulted from damage incurred by washing the mitochondria with EDTA. Because no assay for function was available for the Neurospora mitochondrial ribosome, the question could not be resolved in a straightforward manner. As we had previously established that we could assay HeLa cell mitochondrial ribosomes for poly(U)-directed polypeptide synthesis in vitro, we decided that any significant damage incurred by the ribosomes due to washing the mitochondria with EDTA prior to lysis, could be detected as a loss of activity in this assay.

Human mitochondrial ribosomes prepared from EDTA and digitonin washed mitochondria were free of contaminating cytoplasmic ribosomes as judged by sucrose gradient centrifugation (Figure 6) and by extraction and analysis of the ribosomal RNA in polyacrylamide gels (Figure 8). In consideration of the somewhat limited sensitivity of these assay techniques, a complete absence of cytoplasmic ribosomes cannot be ensured. However, if present, contaminating cytoplasmic ribosomes must represent less than 0.1% of the total ribosome population (calculated from the relative amounts of each rRNA apecies seen on polyacrylamide gels) (Figure 3). The mitochondrial ribosome preparations were active in both poly(U) and poly(C,U)-directed polypeptide synthesis when supplemented

with tRNA and supernatant factors from E. coli. Polypeptide synthesis by mitochondrial ribosomes had a magnesium optimum distinctly different (12 mM) from that of cytoplasmic ribosomes (7 mM) when assayed under the same conditions (Figure 10). The mitochondrial ribosomes were also resistant to inhibition by drugs known to act on cytoplasmic ribosomes, e.g., cycloheximide and anisomycin (Table 7). Taken together these findings clearly demonstrate that the procedure described above for the purification of mitochondrial ribosomes results in a preparation which is essentially free of contaminating cytoplasmic ribosomes, is active in polypeptide synthesis in vitro when supplied with exogenous factors and is therefore, entirely suitable for use in the studies of differential antibiotic sensitivity discussed below.

The Effect of Chloramphenicol and Other Antibiotics on In Vitro Polypeptide Synthesis by Hela S_3 and 296-1 Mitochondrial Ribosomes

Poly(U)-directed polypeptide synthesis by E. coli ribosomes is known to be less sensitive to inhibition by chloramphenicol than is polypeptide synthesis directed by either natural messengers or other synthetic messengers not specifying polyphenylalanine exclusively, e.g., poly(C,U) (Pestka, 1977; Vazquez, 1979). The exact molecular mechanism of this resistance is not known but it has been suggested that either some aspect of the secondary structure of the messenger (poly(U)) or the hydrophobicity or aromaticity of the amino acid (phenylalanine) allows this aminoacyl-tRNA to compete successfully with chloramphenicol for occupation of the A site on the ribosome (Pestka, 1977). Nonetheless, Ibrahim et al. (1974) and Greco et al. (1973) both

reported that chloramphenicol inhibited poly(U)-directed polypeptide synthesis by mitochondrial ribosomes in vitro. Avadhani and Rutman (1974) reported that in an S-25 prepared from Ehrlich acites cell mitochondria, synthesis of polyphenylalanine was inhibited by chloramphenicol.

In more recent reports, Denslow and O'Brien (1978, 1979) compared the effect of chloramphenicol on the peptidyltransferase of bovine liver mitochondrial ribosomes and E. coli ribosomes. When assayed using [^{3}H]-N-acetyl-leucyl-tRNA and puromycin, twice the concentration of chloramphenicol was required for 50% inhibition of mitochondrial ribosomes than E. coli ribosomes. More to the point, Ulbrich et al. (1980), using rat liver mitochondrial ribosomes and homologous supernatant factors, reported that even at levels 10 times those used by previous investigators (500 μg/mL) chloramphenicol inhibited poly(U)-directed polypeptide synthesis by only 15%. In the experiments reported in this thesis, no inhibition of poly(U)-directed polypeptide synthesis on human mitochondrial ribosomes could be detected even at chloramphenicol concentrations of 400 μg/mL. The reason for the different sensitivities reported by these various groups is not clear, but as a result of our inability to show inhibition of polyphenylalanine synthesis by chloramphenicol in the sensitive (S_3) cell line, another messenger had to be found.

Poly(C,U)-directed polypeptide synthesis had previously been shown to be as sensitive to inhibition by chloramphenicol as polypeptide synthesis directed by natural messenger, when assayed in a system containing E. coli ribosomes (Kucan and Lipmann, 1964; Vazquez, 1966). With poly(C,U) as message, chloramphenicol was an effective inhibitor

of polypeptide synthesis on HeLa S_3 mitochondrial ribosomes as well (Table 5). On at least one occasion, however, a level of inhibition intermediate between that of the sensitive line and the resistant line was seen. It seems clear from these results that the mitochondrial ribosome is the component affected by the cytoplasmically inherited mutation to chloramphenicol resistance in the HeLa cell line 296-1.

When the effect of other antibiotics on poly(C,U)-directed polypeptide synthesis was assayed using cytoplasmic ribosomes and mitochondrial ribosomes from each cell line, several unexpected results were obtained. First, polypeptide synthesis was, regardless of the source, resistant to carbomycin, spiramycin and mikamycin, drugs known to be effective against prokaryotic ribosomes (Table 7). Concentrations of these drugs higher than those used (2 mM) may be required to show an effect on mitochondrial polypeptide synthesis. It is known that E. coli ribosomes have a 250-fold higher affinity for carbomycin than do bovine liver mitochondrial ribosomes (Denslow and O'Brien, 1978). Because of the limited solubility of carbomycin, it might be difficult to demonstrate inhibition of mitochondrial ribosome function by this drug. These results are at odds with the report of Ibrahim and Beattie (1974) that carbomycin was more effective than chloramphenicol (on a molar basis) at inhibiting poly(U)-directed polypeptide synthesis by rat liver and yeast mitochondrial ribosomes. Ibrahim and Beattie also found that only carbomycin was able to block the binding of [^{14}C]-chloramphenicol to rat liver mitochondrial ribosomes, erythromycin and lincomycin being comparatively ineffective at equimolar concentrations. This

suggests that carbomycin has a greater affinity for mitochondrial ribosomes than does lincomycin or chloramphenicol. The results reported here and those of Denslow and O'Brien suggest that the opposite may be true. Whether this difference reflects an authentic species difference between the mitochondrial ribosomes of rats on the one hand and cows and humans on the other remains to be seen.

In a peptidyl transferase assay, Denslow and O'Brien (1978) observed that mitochondrial ribosomes were more sensitive to chloramphenicol than to lincomycin (on a molar basis). This observation is supported by the data presented here. At the same concentration (2 mM) lincomycin inhibited poly(C,U)-directed polypeptide synthesis by 54% whereas chloramphenicol inhibited the reaction by 83%. In contrast, E. coli ribosomes are more sensitive to lincomycin than chloramphenicol (Denslow and O'Brien, 1978).

The observation that mitochondrial ribosomes from HeLa 296-1 are relatively unaffected by either chloramphenicol or lincomycin suggests that induction of chloramphenicol resistance has led to an alteration of the binding site for lincomycin as well. Both drugs are known to bind to the large subunit of the bovine liver mitochondrial ribosome (Denslow and O'Brien, 1978) and to have overlapping binding sites on the E. coli ribosome (Fernandez-Munoz et al., 1971; Vazquez, 1966). It is not difficult to imagine that a mutation to chloramphenicol resistance might lead to a reduced affinity of the ribosome for lincomycin as well. It must be noted, however, that this is the first and thus far only report of cross resistance between these two drugs.

Attempts to Prepare Mitochondrial Ribosomal Subunits Active in In Vitro Polypeptide Synthesis

From the results noted above, it was clear that some macromolecular constituent of the mitochondrial ribosome itself had been altered by the mutation to a chloramphenicol resistant phenotype. The next logical step was identification of the affected subunit. Two different approaches were taken in these attempts. The most straight-forward involved dissection of the elongation reaction into its constituent parts. It has been established for both E. coli and bovine liver mitochondrial ribosomes that the major binding site for chloramphenicol is on the large subunit (Denslow and O'Brien, 1978) where the drug inhibits the peptidyl-transferase center of both species of ribosome.

Despite many attempts, we have never successfully applied the peptidyl transferase assay to human mitochondrial ribosomes. The reasons for this failure are not clear. It is possible that the ribosome preparations were contaminated with lysosomal hydrolases (DeVries and Van der Koogh-Schuuring, 1973). These would cause backgrounds too high to permit meaningful results.

The second and more promising approach required separation of the ribosomes from both sensitive and resistant strains into subunits and then recombining the subunits into heterologous ribosomes. Assaying these recombinant ribosomes for in vitro sensitivity to chloramphenicol would then reveal the mutated subunit. As shown in the Results Section, subunits could be separated without difficulty but upon recombining, even

homologous ribosome preparations were without activity. The most likely explanation for this observation is the loss of contaminating elongation factors required for polypeptide synthesis on mitochondrial ribosomes. Denslow and O'Brien (1979) and, more recently, Ulbrich et al. (1980) have reported an absolute requirement for homologous elongation factor G by mitochondrial ribosomes. It is possible that during the process of isolating subunits, which requires centrifugation in high-salt buffers, this factor, normally remaining with the ribosome during preliminary purification, is lost or damaged. The recombined subunits, now devoid of elongation factor G and unable to use the bacterial analogue, would be incompetent for in vitro polypeptide synthesis. It has since been found that high salt washed HeLa mitochondrial ribosomes have an absolute requirement for mitochondrial S-100 (T. Girard, Iowa State University, personal communication).

Because of the above mentioned considerations, we decided to prepare mitochondrial S-100 supernatant factors (see Experimental Procedures Section). In every attempt and with mitochondria from all sources tried, the S-100 preparations were uniformly contaminated with a high molecular weight inhibitor of polypeptide synthesis which co-purified with the mitochondria (Table 8). At least one of the preparations was found to contain RNase activity. As digitonin had been used to preferentially eliminate contaminating lysosomes from the mitochondrial preparations (Loewenstein et al., 1970), hydrolases should have been a minimal problem. It has been noted that the method chosen to evaluate mitochondrial protein (O.D. at A550) gave varying results depending on the exact buffer

composition and method used to resuspend the mitochondria. This could have caused the digitonin treatment to be less effective than it might have been (J. Horowitz, Iowa State University, personal communication).

Recent findings suggest that the mutation is not likely to have affected a ribosomal protein. The only ribosomal proteins known to be encoded in mitochondrial DNA are found on the small subunit (Lambowitz et al., 1979; Terpstra et al., 1979) which is not believed to be the major binding site for chloramphenicol (see Introduction). Additionally, Dujon (1980) has reported that the large subunit mitochondrial rRNAs from two different chloramphenicol resistant strains of yeast each contain a single base change from wild type. In more recent reports, Kearsey and Craig (1981) and Blanc et al. (1981) have shown that similar single base changes also occur in the large subunit mitochondrial rRNAs from chloramphenicol resistant mouse (3T3 and LA9) and human (HeLa B) cell lines. They note that all 5 point mutations occur in one or the other of two distinctive sequences (10 and 13 bases long) which occur toward the 3' end of these molecules. In wild type cells from yeast, mouse and human these sequences are completely conserved. The 23S rRNA of E. coli also contains a region in which these sequences occur (Brosius et al., 1980) suggesting they may play some fundamental role in protein synthesis, perhaps involving peptidyl transferase center (Blanc et al., 1981).

It has been noted (C. D. Wallace, Stanford University, personal communication) that a similar base change occurs in the 16S mitochondrial ribosomal RNA of HeLa 296-1. This observation combined with the data

presented in this thesis provide strong evidence that the chloramphenicol resistant phenotype of HeLa 296-1 is the direct result of a single base change in large subunit rRNA of the mitochondrial ribosome.

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