Messenger Ribonucleic Acid Metabolism in Mammalian Mitochondria. Isolation and Characterization of Polyribosomes from Ehrlich Ascites Mitochondria†

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ABSTRACT: The Mg^{2+} precipitation procedure of R. D. Palmiter ((1974) *Biochemistry 13,* 3606) has been used for preparative scale isolation of polysomes from Ehrlich ascites mitochondria. Digitonin-washed mitochondria used for isolating the polysomes contain no detectable reduced nicotinamide adenine dinucleotide phosphate–cytochrome *c* reductase and over 200-fold reduced hexokinase activity. The mitochondrial polysomes exhibit a heterogeneous sedimentation and appear to contain highly aggregated particles ranging over hexamers. These polysomes are sensitive to RNase, (ethylenedinitrilo)tetraacetic acid and puromycin. Mitochondrial polysomes are active in protein synthesis when supplied with supernatant enzymes from the homologous mitochondrial source or from *Escherichia coli.* Cytoplasmic enzymes, however, appear to be completely inactive. Protein synthesis by mitochrondrial polysomes is sensitive to chloramphenicol and resistant to cycloheximide and emetine. The procedure yields particles containing intact rRNAs. The extent of cytoplasmic RNA contaminating the total mitochondrial RNA or mitochondrial polysomal RNA has been estimated to be negligible.

Several reports have appeared on the isolation of mt[1] (mitochondria) specific polyribosomes from unicellular eukaryotes, as well as from animal cells (for reviews, see Dawid, 1972; Avadhani et al., 1975). Polymeric particles isolated from mitochondria of *E. gracilis* (Avadhani and Buetow, 1972a,b), *Neurospora* (Agsteribbe et al., 1974), *S. cerevisiae* (Mahler and Dawidowicz, 1973; Cooper and Avers, 1974; Ibrahim and Beattie, 1975), and *Tetrahymena* (Allen and Suyama, 1972) have been shown to exhibit the physical, chemical, and biological properties expected of polyribosomes. Previous attempts at isolation of mt-specific polyribosomes from animal cells have, however, encountered difficulties (Perlman and Penman, 1970; Ojala and Attardi, 1972). Recently, we have reported the isolation of membrane associated polysomes from Ehrlich ascites mitochondria (Avadhani et al., 1974). These polysomes are sensitive to EDTA and RNase, as well as puromycin. However, the membrane-associated particles might represent only a fraction of the mt polyribosome pool. In this paper we have adopted the Mg^{2+} precipitation technique of Palmiter (1974) to isolate mt polyribosomes on a preparative scale. The procedure described yields mt polyribosomes relatively free of cytoplasmic contamination. Some of the biochemical and biological properties of these mt polyribosomes have been studied. A portion of the results included in this paper were reported elsewhere (Lewis et al., 1975) as an abstract.

Experimental Procedures

General. All the glassware was acid cleaned and heat-sterilized. As far as possible, sterile reagents and conditions were used.

Materials. Ehrlich ascites hypotetraploid carcinoma cells were grown in the peritoneal cavity of Swiss colony mice for 7 days. The cells were harvested as described before (Chun et al., 1969).

Isolation of Mitochondria. Unless otherwise stated, all the following steps were carried out at 0–4 °C. The procedure for mt isolation was modified from Avadhani et al. (1974). Freshly harvested cells were washed twice with 0.15 M NaCl and 10 mM Tris-HCl (pH 7.5). Packed cells were resuspended in 4–5 volumes of a hypotonic buffer (10 mM Tris-HCl (pH 7.5), 60 mM KCl, 2 mM $Mg(CH_3COO)_2$) and incubated at 0–4 °C for 10 min to lyse the red blood cells. This treatment also causes the softening of the cell membranes facilitating the cell lysis. Cells were homogenized in a medium containing 10 mM Tris-HCl (pH 7.5), 100 mM KCl, 2 mM EDTA, and 0.3 M sucrose (buffer A) and mitochondria were prepared by differential centrifugation as described earlier (Avadhani et al., 1974). The crude mt pellet was resuspended in 3 volumes of buffer A containing 0.1% DEP (v/v) (diethyl pyrocarbonate) and purified by treatment with digitonin. A 1% solution of digitonin in buffer A was added to the mt suspension to obtain a ratio of 1 mg of digitonin:50 mg of mt protein. After repeated mixing (~5 times) on a Vortex Genie, mitochondria were pelleted at 8000*g* for 10 min. The resultant pellet was treated once more with digitonin as described above except that a concentration of 1 mg of digitonin/100 mg of mt protein was used.

Preparation of mt Polyribosomes. Purified mitochondria were washed once with buffer A containing 0.1% DEP and the resultant pellet was suspended in approximately three volumes of buffer B (25 mM Tris-HCl (pH 7.8), 20 mM $Mg(CH_3COO)_2$, 120 mM KCl, 5 mM 2-mercaptoethanol) with 200 μg/ml of heparin and 1% Nonidet NP40 (American Shell Co.). The suspension was gently homogenized with three strokes of a loose-fitted glass homogenizer. The lysate was

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[1] Abbreviations used are: mt, mitochondria or mitochondrial; DEP, diethyl pyrocarbonate; poly(A), poly(adenylic acid); lr, large ribosomal; sr, small ribosomal; ip, intraperitoneal; EDTA, (ethylenedinitrilo)tetraacetic acid; OD, optical density; DEAE, diethylaminoethyl; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

TABLE I: Purity of mt Preparations.[a]

mt Preparation	NADPH Cytochrome *c* Reductase Content (μM Cytochrome Reduced/mg of Protein)	Hexokinase Act. (μM NADP Reduced mg^{-1} Protein min^{-1})
Control	0.0094	0.028
Purified	0.0	0.00013

[a] Mitochondria were prepared as described under the Experimental Procedures. Control mt refers to the preparation washed twice with buffer A and purified mt refers to the preparation washed twice with digitonin. Assays for NADPH cytochrome *c* reductase and hexokinase were carried out as described under the Experimental Procedures.

centrifuged at 25 000*g* for 20 min. The supernatant fraction was aspirated and diluted with buffer B containing 200 μg/ml of heparin and 1% NP40 to obtain a concentration of 10–12 mg of protein/ml. The diluted supernatant fraction was adjusted to 130 mM with $Mg(CH_3COO)_2$ by adding an appropriate volume of a 2 M stock solution and incubated on ice for 1 h. The incubation mixture was then layered over a 10-ml cushion of 0.5 M sucrose in buffer B (containing 200 μg/ml of heparin) and centrifuged at 70 000*g* for 30 min in a SW 25.1 rotor of Beckman L-2 ultracentrifuge at 0–4 °C. The supernatant and the sucrose layers were decanted and discarded. The polysome pellet was rinsed twice and suspended in buffer B containing 200 μg/ml of heparin by gentle stirring with a glass rod and stored in liquid nitrogen.

For the in vitro protein synthesis experiments, polysomes were prepared without use of DEP or heparin. The amount of polysomes was estimated by OD 260-nm readings using a conversion factor of 11.5 OD = 1 mg.

Isolation of RNA. RNA was extracted from various cellular fractions such as microsomes, mitochondria, or polysomes by the sodium dodecyl sulfate–phenol–chloroform method of LaTorre and Perry (1973).

Preparation of RNA-Free Supernatant Enzyme. A strain of *E. coli* A19 was obtained from Dr. J. M. Clark, Jr., University of Illinois, Urbana. A highly active 30 000*g* supernatant fraction (S-30) from *E. coli* was prepared by the method of Clark et al. (1965). Ehrlich ascites cytoplasmic 30 000*g* supernatant (S-30) and mitochondrial 25 000*g* supernatant (S-25) fractions were prepared as described before (Avadhani and Rutman, 1974, 1975). Various S-30 or S-25 fractions were layered over a cushion of 1 M sucrose in buffer B (1.5 ml of sucrose:3.5 ml of S-30 or S-25) and centrifuged in a SW-50 rotor of Beckman L-2 ultracentrifuge at 165 000*g* for 8 h at 0–4 °C. The supernatant fraction above the sucrose layer was carefully aspirated out and treated three times with prewashed DEAE-cellulose (Avadhani and Buetow, 1974) to prepare RNA-free enzyme fractions. The RNA-free enzyme fractions were dialyzed and stored in liquid nitrogen until used.

In Vitro Protein Synthesis. In vitro protein synthesis assays were carried out in 0.2-ml final volume. The reaction mixture contained 30 mM Tris-HCl (pH 7.8), 100 mM KCl, 5 mM $Mg(CH_3COO)_2$, 5 mM 2-mercaptoethanol, 1 mM ATP, 0.3 mM GTP, 3 mM phosphoenolpyruvate, 40 μM each of unlabeled L-amino acids (methionine, glutamine, tryptophan, cysteine, and asparagine), 100 μg of RNA-free supernatant enzyme, 50 μg of polysomes (0.6 OD at 260 nm), 40 μg (1.0 OD at 260 nm) of *E. coli* tRNA (Sigma), 0.5 μCi of ^{3}H-labeled 15 amino acid mixture and 0.32 μg of pyruvate kinase. The reaction was carried out at 35 °C for 45 min. The reaction was stopped by adding 7 ml of 5% Cl_3CCOOH to each tube. Tubes were heated at 90 °C for 15 min, chilled in ice, and filtered through Millipore filter disks (HAWP). The filters were washed at least four times with 5% Cl_3CCOOH, dried at 80 °C, and counted with 10 ml of Cab-O-Sil in a Packard Tricarb spectrometer at 35% efficiency.

Electrophoresis of RNA. Procedure for the electrophoresis of RNA on polyacrylamide gels was essentially as described by Bishop et al. (1967). Gels were scanned at 260 nm using a Gilford 2400A spectrophotometer.

Analytical Procedures. NADPH–cytochrome *c* reductase and hexokinase activity were assayed using the method of Masters et al. (1967) and Sauer (1964, 1968), respectively.

Protein was estimated by the method of Lowry et al. (1951) and RNA was estimated according to Ciriotti (1955).

Results

Purity of Mitochondrial Preparations. The purity of the mitochondrial preparation was determined by analyzing the marker enzymes. NADPH–cytochrome *c* reductase, a microsome-specific enzyme, can be conveniently used to determine the extent of contaminating microsomes in the mitochondrial isolates (Masters et al., 1967). Further, the enzyme hexokinase is known to be associated with the outer membrane of tumor cell mitochondria (Sauer, 1964, 1968) and this enzyme can be used as the marker enzyme for mitochondrial outer membrane in Ehrlich ascites system (Dr. C. Ritter, personal communication). As shown in Table I, the crude mitochondrial pellet contains significant amounts of contaminating microsomes, as revealed by the extent of enzyme detected. Mitochondria purified with digitonin, on the other hand, contain no detectable NADPH–cytochrome *c* reductase. Additionally, the purified mitochondrial preparation contains roughly a 200-fold reduced amount of hexokinase activity as compared with the crude mitochondria.

Isolation of Mitochondrial Polyribosomes. Isolation of mt polyribosomes was carried out using large amounts of cells. For each experiment, 600 and 800 ml of packed cells withdrawn from 500 to 800 tumor bearing animals were used. The sedimentation profile of mt polysome has been presented in Figure 1A. The preparation is sensitive to EDTA, RNase, as well as to puromycin. Treatment with RNase results in the degradation of polyribosomes into monomeric particles (Figure 1B). Treatment with puromycin and EDTA, on the other hand, results in the dissociation of polyribosomes into 29S and 40S subunits (Figure 1C,D), with little absorbance at 260 nm in the polyribosome region.

In order to have a rough approximation of the cellular content of mitochondrial specific polyribosomes, cells prelabeled with ^{32}P for 24 h were fractionated and the distribution of cold Cl_3CCOOH insoluble radioactivity in various fractions was determined. As shown in Table II, under the experimental conditions described, the cytoplasmic fraction contains about 60% of the total radioactivity, while the mt fraction contains only about 6.5% of the radioactivity. Further, almost 25% of the total cellular radioactivity can be recovered in the cytoplasmic polyribosome fraction, while only 0.6% of the radioactivity is associated with mitochondrial polyribosomes isolatable by the present technique.

Composition of Mitochondrial Polyribosomes. The RNA and protein contents of the cytoplasmic and mt polyribosomes are presented in Table III. The mitochondrial polyribosomes have an absorbance ratio at 260:280 nm of about 1.6 and contain 38–39% RNA and about 61% protein, as against 48% RNA and 52% protein for the cytoplasmic polyribosomes.

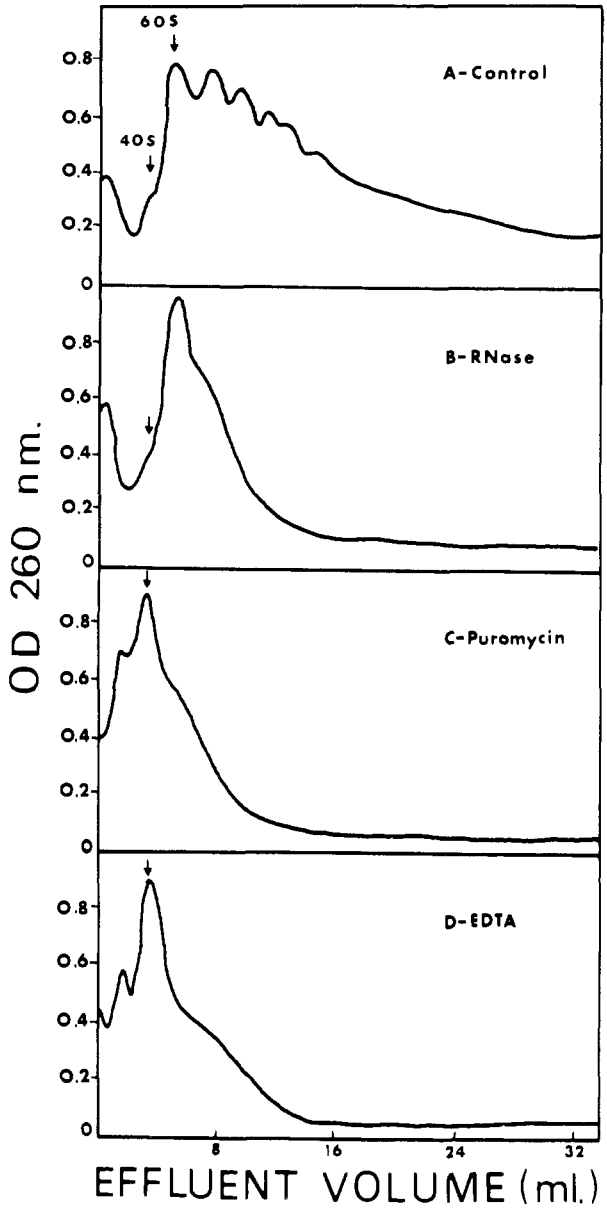

FIGURE 1: Sedimentation pattern of mt polysomes. mt polysomes were prepared from unlabeled cells as described under the Experimental Procedures. About 12 OD units (at 260 nm) of control polysomes and 5–7 OD units (at 260 nm) of treated polysomes were layered on 35 ml of linear sucrose gradients (10–36%) in a buffer containing 10 mM Tris-HCl (pH 7.5), 100 mM KCl, and 10 mM $Mg(CH_3COO)_2$ and centrifuged in an SW 27 rotor of Beckman L2 Ultracentrifuge at 121 000*g* for 2 h. For various treatments, polysomes were taken in 1 ml of 10 mM Tris-HCl (pH 7.5), 100 mM KCl, and 20 mM $Mg(CH_3COO)_2$ and incubated on ice for 15 min either with 200 μg of puromycin, or 5 μg of pancreatic RNase, or 5 μmol of EDTA. The control sample without any addition was also incubated similarly before layering on the gradient.

TABLE II: Relative Proportions of mt and Cytoplasmic Polyribosomes.[a]

Cell Fractions	% of Cold Cl_3CCOOH Insoluble Radioactivity[a,b]
Total cell homogenate	100
Post mitochondrial supernatant	60.7
Crude mt fraction	13.4
Cytoplasmic polysomes	6.2
mt polysomes	24.7
	0.6

[a] Cells were labeled with ^{32}P for 24 h by injecting (ip) 200 μCi of [^{32}P]orthophosphate (New England Nuclear) into mice bearing 6-day old tumors. Details of cell lysis and fractionation were as described under the Experimental Procedures. Post mitochondrial supernatant (25 000*g* supernatant) was used to prepare cytoplasmic polysomes. Radioactivity in various samples was determined by mixing an aliquot with 7.0 ml of 10% Cl_3CCOOH at 0–4 °C. The resultant precipitate was collected on Millipore filters, washed three times with cold Cl_3CCOOH (7.0 ml each) and counted with 10 ml of Cab-o-Sil scintillation gels. [b] Mean of two independent estimates.

TABLE III: RNA and Protein Composition of mt Polysomes.[a]

Polysomes	A_{260nm}/A_{280nm}	Composition	
		RNA (%)	Protein (%)
Mitochondrial			
Expt 1	1.61	39	61
Expt 2	1.63	38	62
Expt 3	1.63	38.5	61.5
Cytoplasmic	1.73	48.3	51.7

[a] RNA and protein contents were determined as described under the Experimental Section.

Such low-RNA and high-protein contents have been reported for other mammalian mitochondrial ribosomes (Hamilton and O'Brien, 1974; O'Brien et al., 1974; de Vries and Kroon, 1974; Leister and Dawid, 1974).

In order to be useful for studies on mRNAs, etc., it is necessary that the isolation procedure should result in minimal degradation of the RNA. The electrophoretic pattern of RNA extracted from mitochondrial polysomes (see Figure 2) shows two components designated as L (larger rRNA) and S (smaller rRNA). Both these RNA components migrate faster than the respective rRNAs from the cytoplasmic ribosomes (Figure 2). Further, the electropherogram shows little or no 28S and 18S RNAs in the mitochondrial polyribosomal RNA.

Biological Activity of Mitochondrial Polyribosomes. The protein synthetic activity of mt polyribosomes is presented in Table IV. Mitochondrial polyribosomes prepared by the Mg^{2+} precipitation technique can participate in protein synthesis when supplied with supernatant enzymes, tRNA, and other components. The activity is dependent on the presence of ATP, GTP, and a nucleotide triphosphate generating system. The system is sensitive to EDTA, and chloramphenicol, an inhibitor known for its specific effect on bacterial and mitochondrial ribosomes (Beattie, 1971; Küntzel, 1971; Pestka, 1971). At the highest level of chloramphenicol used, i.e., 80 μg, the protein synthesis is inhibited by over 90%. Inhibitors of cytoplasmic protein synthesis, such as cycloheximide (Siegel and Sisler, 1965) and emetine (Pestka, 1971), on the other hand, exhibit little effect on protein synthesis by mitochondrial polyribosomes.

The specificities for various soluble enzymes for protein synthesis by mt and cytoplasmic polyribosomes are presented in Table V. Mitochondrial polyribosomes exhibit maximum activity with soluble enzymes from homologous sources or with similar fractions from *E. coli*. The soluble enzyme fraction from the cytoplasmic compartment shows less than 5% activity with mt polyribosomes. Cytoplasmic polyribosomes, on the other hand, are fully active only with soluble enzymes from the homologous source. Bacterial and mitochondrial enzymes show

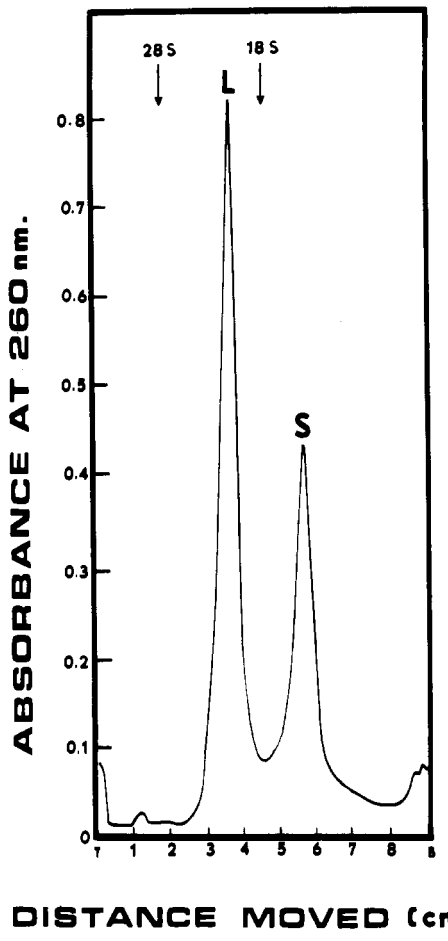

FIGURE 2: Electrophoretic analysis of RNA extracted from mt polysomes. About 40 μg of RNA was electrophoresed on 2.5% polyacrylamide gels as described under Experimental Procedures.

negligible activity with these cytoplasmic polyribosomes. These results are in agreement with previous reports on factor specificity of mitochondrial polyribosomes from unicellular eukaryotes (Avadhani and Buetow, 1972a,b; Allen and Suyama, 1972; deVries and Kroon, 1974) and mitochondrial ribosomes from lower, as well as higher eukaryotes (Küntzel, 1971; Denslow and O'Brien, 1974; Avadhani and Rutman, 1974; Agsteribbe et al., 1974; Swanson, 1973; Greco et al., 1973).

Discussion

Recently several laboratories have focused attention on the characterizaton of mt mRNAs and the information content of the mt genome (Avadhani et al., 1974; Hirsch and Penman, 1974; Ojala and Attardi, 1974; Cooper and Avers, 1974; Agsteribbe et al., 1974). Although affinity chromatography methods using poly(A) as marker can be useful to isolate some mitochondrial mRNAs (Hirsch and Penman, 1974; Ojala and Attardi, 1974), it has been shown that such poly(A)-containing RNAs might not account for all of the mRNA species being translated within mitochondria (see Avadhani et al., 1974; Lewis et al., 1975). It was, therefore, thought necessary to standardize a method to isolate mitochondrial polyribosomes on a preparative scale that could serve as the source of mRNAs for further studies.

The Mg^{2+} precipitation procedure of Palmiter (1974) was chosen because of several reasons. Besides being simple, this method has been shown to permit quantitative isolation of polyribosomes from several cytoplasmic and bacterial systems

TABLE IV: Protein Synthetic Activity of mt Polysomes.[a]

Additions or Omissions	^3H cpm Incorporated[b]	% Act.
Complete system	11 210	100
−Polysomes	395	3
−Supernatant enzyme	819	7
−ATP, GTP, and CTP	1 918	17
−PEP and pyruvate kinase	2 782	24
+EDTA (1 μmol)	800	6
+Chloramphenicol (40 μg)	4 395	39
+Chloramphenicol (80 μg)	691	6
+Emetine (120 μg)	10 942	97
+Cycloheximide (150 μg)	11 170	99

[a] Protein synthesis assays were run using *E. coli* supernatant enzymes as described under the Experimental Procedures. [b] Mean of three estimates.

TABLE V: Enzyme Specificity of Mitochondrial and Cytoplasmic Polysomes.[a]

Source of Polysomes	Source of Supernatant Enzymes	^3H cpm Incorporated[b]
None	Cytoplasm	380
None	Mitochondria	293
None	*E. coli*	324
Cytoplasm	Cytoplasm	14 346
Cytoplasm	*E. coli*	1 210
Cytoplasm	Mitochondria	985
Mitochondria	Mitochondria	11 621
Mitochondria	*E. coli*	12 905
Mitochondria	Cytoplasm	552

[a] Protein synthesis assays were run as described under Experimental Procedures. Both mitochondrial and cytoplasmic polysomes when added were at 50 μg (0.6 OD at 260 nm) level. [b] Values derived from three estimates.

(Palmiter, 1974). Mitochondrial polysomes isolated by this method exhibit heterogeneous sedimentation in sucrose gradients with optical density (at 260 nm) ranging over the hexameric region. These polyribosomes are sensitive to RNase, EDTA, and puromycin, and, therefore, exhibit properties different from polysome-like structures reported for HeLa cell mitochondria (Ojala and Attardi, 1972). Further, the problem of aggregation generally experienced with varied mitochondrial preparations (Avadhani and Buetow, 1972b; Avadhani et al., 1974; Ojala and Attardi, 1972; Michel and Neupert, 1973) is greatly reduced in mitochondrial polyribosomes prepared by the present Mg^{2+} precipitation method. This improved solubility property could be due to the high-salt concentrations used during the isolation.

Results presented in Table II indicate that only 0.6% of ^{32}P counts from steady state labeled cells are recovered in the mt polysome fraction. After making appropriate corrections, these results suggest that at the most 3–4% of total cellular polyribosomes are intramitochondrially located. Further, it is well known that mt preparations from animal sources are heavily contaminated with microsomes and associated cytoplasmic particles. It was, therefore, decided to use biochemical analysis of marker enzymes (see Table I), double-labeling experiments and electron microscopic visualization (results not included) to ensure the purity of mitochondria and mt polysomes.

Additional evidence for the purity of mitochondrial polysomes can be derived from protein synthesis experiments presented in Tables IV and V. Protein synthesis by mitochondrial polysomes is highly sensitive (up to 90%) to chloramphenicol and is almost totally insensitive to cycloheximide and emetine, known to be the specific inhibitors of cytoplasmic protein synthesis (Beattie, 1971; Küntzel, 1971; Siegel and Sisler, 1965). Also, mitochondrial polyribosomes require soluble enzymes from homologous source or from *E. coli* for complete activity. As expected, the cytoplasmic enzymes are completely inactive with mitochondrial polysomes. Analytical results presented in Table III point out a major distinction between mitochondrial and cytoplasmic polyribosomes. Mitochondrial polysomes have the low-RNA and high-protein contents, characteristic of mitochondrial ribosomes from animal sources (Hamilton and O'Brien, 1974; deVries and Kroon, 1974; Leister and Dawid, 1974). On the basis of our current knowledge on mitochondrial ribosomes and mitochondrial protein synthesis (Beattie, 1971, Küntzel, 1971; Borst, 1972; Mahler, 1973; Schatz and Mason, 1974; Kroon and Saccone, 1974; Avadhani et al., 1974) it appears that the present polysomes isolated from Ehrlich ascites mitochondria are indeed organelle specific and, as estimated in several control experiments described above, these mitochondrial polyribosomes appear to contain negligible amounts of cytoplasmic contamination. The electrophoretic pattern of polyribosomal RNA shows (Figure 3) that, as in bacterial and cytoplasmic systems (Palmiter, 1974), the Mg^{2+} precipitation procedure yields mitochondrial particles containing intact rRNAs. Since the present mitochondrial preparations contain highly aggregated particles, and also, since the mRNA fraction released by puromycin treatment has a heterogeneous size distribution of 8S to 16S (Lewis et al., 1976) it appears that there is no extensive degradation of polysomes and ribosomes during isolation. The degree of intactness of mRNAs released from these mitochondrial polysomes, however, remains to be established.

In conclusion, the work reported in this paper provides the first rigorous procedure for the isolation and characterization of biologically active polyribosomes from mammalian mitochondria.

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