

THE INTRACELLULAR SITE OF SYNTHESIS OF MITOCHONDRIAL RIBOSOMAL PROTEINS IN *NEUROSPORA CRASSA*

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ABSTRACT

The intracellular site of synthesis of mitochondrial ribosomal proteins (MRP) in *Neurospora crassa* has been investigated using three complementary approaches. (a) Mitochondrial protein synthesis in vitro: Tritium-labeled proteins made by isolated mitochondria were compared to ^{14}C -labeled marker MRP by cofractionation in a two-step procedure involving isoelectric focusing and polyacrylamide gel electrophoresis. Examination of the electrophoretic profiles showed that essentially none of the peaks of in vitro product corresponded exactly to any of the MRP marker peaks. (b) Sensitivity of in vivo MRP synthesis to chloramphenicol: Cells were labeled with leucine- ^3H in the presence of chloramphenicol, mitochondrial ribosomal subunits were subsequently isolated, and their proteins fractionated by isoelectric focusing followed by gel electrophoresis. The labeling of every single MRP was found to be insensitive to chloramphenicol, a selective inhibitor of mitochondrial protein synthesis. (c) Sensitivity of in vivo MRP synthesis to anisomycin: We have found this antibiotic to be a good selective inhibitor of cytoplasmic protein synthesis in *Neurospora*. In the presence of anisomycin the labeling of virtually all MRP is inhibited to the same extent as the labeling of cytoplasmic ribosomal proteins. On the basis of these three types of studies we conclude that most if not all 53 structural proteins of mitochondrial ribosomal subunits in *Neurospora* are synthesized by cytoplasmic ribosomes.

INTRODUCTION

Mitochondria from *Neurospora* (Küntzel and Noll, 1967; Rifkin et al., 1967) and from many other organisms (Borst and Grivell, 1971, review) are known to contain ribosomes and ribosomal RNA different from those of the cytoplasm. The available evidence, especially that obtained from nucleic acid hybridization studies, indicates that mitochondrial ribosomal RNA of *Neurospora* (Wood and Luck, 1969) and all other organisms so far studied (Borst and Grivell, 1971) are transcription products of the mitochondrial genome.

In contrast, studies on the intracellular site of biosynthesis of mitochondrial ribosomal proteins (MRP) have been less definitive.

Results obtained by Neupert et al. (1969 *a, b*), Küntzel (1969 *a*), and Davey et al (1969) suggest that most of the proteins of the mitochondrial ribosome are synthesized by the ribosomes of the cytoplasm. However, these studies do not rule out the possibility that a few MRP are products of the organelle. This possibility had to be considered seriously in view of other experimental

findings. Linnane et al. (1968 *a, b*) studied a mutation of yeast which renders mitochondrial protein synthesis insensitive to erythromycin and which is transmitted by nonchromosomal inheritance. These authors suggest that erythromycin resistance may result from a modification of the ribosome itself, possibly a ribosomal protein whose synthesis would be coded for by mitochondrial DNA. In *Neurospora*, Rifkin and Luck (1971) have shown that the cytoplasmically inherited "poky" mutation is characterized by a defect in the assembly of the small subunit of mitochondrial ribosomes. The fact that the ribosomal RNA cistrons do not seem to be effected suggested the possibility of a ribosomal protein defect.

The possibility that mitochondria may have retained control for the synthesis of only a few specific MRP was intriguing, and the problem deserved further study. In this paper we shall describe experiments in which this question has been investigated in detail using three complementary experimental approaches: (*a*) in vitro protein synthesis by isolated mitochondria, (*b*) in vivo protein synthesis in the presence of chloramphenicol, a specific inhibitor of mitochondrial protein synthesis, (*c*) in vivo protein synthesis in the presence of anisomycin, a specific inhibitor of cytoplasmic (extramitochondrial) protein synthesis. The results of these studies suggest that all MRP are synthesized outside of the organelle, in the cytoplasm of the cell.

MATERIALS AND METHODS

Materials

Anisomycin (2-*p*-Methoxyphenylmethyl-3-acetoxy-4-hydroxypyrrolidine) was a gift from Chas. Pfizer and Co., Inc., New York. Chloramphenicol and cycloheximide were of commercial grade. Ludox (type HS) Colloidal Silica was a gift from E. I. du Pont de Nemours & Co., Inc., Wilmington, Del. Nonidet P-40 was a gift from the Shell Chemical Corp., New York.

Growth of Cells

All experiments were done with *Neurospora crassa* EM 5256, a wild-type No. 424 from the Fungal Genetics Stock Center, Humboldt State College, Arcata, Calif. Methods for the growth of conidia and for the growth of *Neurospora* in liquid culture have been described previously (Luck, 1963). In general, cells approaching late logarithmic growth were used (15 hr at 25°C).

Preparation of Cell Fractions

Unless otherwise specified, all cell fractionation procedures were carried out at 3°–4°C.

Mitochondria were prepared by two different methods. The method used to obtain mitochondrial fractions free of contamination by cytoplasmic ribosomes was isopycnic flotation in sucrose gradients as previously described (Lizardi and Luck, 1971). The second method, which was used to prepare mitochondria for in vitro incubation, consisted of isopycnic flotation in gradients of colloidal silica (Ludox-HS, du Pont). This method is a modified version of the procedures described by Pertoft et al. (1967, 1968). A detailed account of the method has been given elsewhere (Lizardi, 1972).

Mitochondrial ribosomes were prepared as follows: A pellet of mitochondria prepared by the sucrose flotation method was suspended in 20 mM Tris-Cl pH 7.6, 0.5 M KCl, 12 mM MgCl₂. Nonidet P-40 was added to 2%, and the lysate was clarified at 41,000 g_{av} for 10 min in the Spinco 40 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) The clear supernatant was layered on top of a step sucrose gradient consisting of 2.7 ml of 2.0 M sucrose, 20 mM Tris-Cl pH 7.6, 0.2 M KCl, 12 mM MgCl₂, and 2.0 ml of 1.5 M sucrose, 20 mM Tris-Cl pH 7.6, 0.5 M KCl, 12 mM MgCl₂. Centrifugation was carried out at 300,000 g_{max} for 27 hr (3°C) in the IEC-A321 rotor (International Equipment Co., Needham Heights, Mass.). A colorless, transparent ribosome pellet was obtained and was sometimes used directly as a source of total ribosomal protein from mitochondria.

To obtain purified mitochondrial ribosomal subunits, a modification of the puromycin method of Blobel and Sabatini (1971) was used. A ribosomal pellet prepared in the aforementioned manner was suspended in 0.6 ml of 25 mM Tris-Cl pH 7.6, 0.05 M KCl, 5 mM MgCl₂. Then 1.2 ml of buffer containing 0.75 M KCl and 20 mM MgCl₂ were added to give a final ion concentration of 0.5 M KCl and 15 mM MgCl₂. Puromycin-HCl was added to 1 mM, and the ribosomes were incubated for 10 min at 37°C. The material was then cooled to 4°C and layered on a 10% to 29.6% isokinetic sucrose gradient made up in the same buffer. After centrifugation at 27,000 rpm for 8.5 hr in the Spinco SW27 rotor (Beckman), the 37S and 51S subunit peaks were localized and recovered. Each subunit fraction was diluted with ½ vol of buffer containing 0.05 M KCl and 5 mM MgCl₂, and the subunits were pelleted for 7 hr at 310,000 g_{max} (3°C) in the IEC-A321 rotor. The resulting pellets were used directly for extraction of ribosomal protein.

In some experiments it was necessary to study the total mitochondrial protein remaining after isolation of the ribosome pellet. In this case, the supernatant

fraction remaining above the ribosome pellet (after the 1.5 M–2.0 M sucrose step gradient) was collected *in toto*, β -mercaptoethanol added to 20 mM, gassed with N_2 , and stored at $-85^\circ C$. This material, which is comprised of several colored layers from the centrifuge tube, is referred to as the “mitochondrial postribosomal fraction.”

The “cytosol” fraction was prepared from a crude postmitochondrial supernatant. The material was centrifuged at 41,000 g_{av} for 15 min in the Spinco 40 rotor to remove any remaining mitochondrial fragments. Cytoplasmic ribosomal subunits were prepared from the cytosol fraction, using the same puromycin-dissociation method previously described for mitochondrial ribosomes.

In Vitro Incubation of Mitochondria

Mitochondria prepared by the colloidal silica method were suspended in the following buffer: 30 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid (HEPES)-KOH pH 7.7, 0.1 M sucrose, 0.4% polyvinylpyrrolidone, 0.1% bovine serum albumin, 45 mM KCl, 15 mM $MgCl_2$, 0.5 mM $MnSO_4$, 0.5 mM $CaCl_2$, 2 mM NH_4Cl , 10 mM potassium phosphate, 1.5 mM spermidine, 20 mM α -ketoglutarate, 75 $\mu g/ml$ synthetic amino acid mixture (minus leucine), 140 mM adenosine diphosphate, 40 mM cytidine diphosphate, 40 mM uridine diphosphate, 80 mM guanosine diphosphate 100 $\mu g/ml$ cycloheximide. The concentration of mitochondria was about 2.5 mg of protein/ml. Bacterial counts at the end of a 40 min incubation at $27^\circ C$ ranged from 100 to 500 bacteria/ml.

When protein was to be extracted after incubation, a further purification step was used in order to select intact mitochondria and eliminate most contaminating bacteria: After incubation, the mitochondria were quickly chilled and pelleted by centrifugation at 14,800 g_{av} for 10 min in the Spinco 40 rotor. They were resuspended in a small volume of 15 mM HEPES-KOH pH 7.7, 0.44 M sucrose, 0.5 mM ethylenediaminetetraacetic acid (EDTA), and layered over a linear sucrose gradient (0.67 M–1.90 M) made up in 15 mM HEPES pH 7.7, 0.1 mM EDTA. After centrifugation at 40,000 rpm for 1 hr in the Spinco SW41 rotor the mitochondrial band was collected, diluted with 2 vol of buffer containing 0.15 M sucrose, and pelleted by centrifugation at 17,000 g_{av} for 15 min. The mitochondrial pellet was used immediately for extraction of protein.

Isolation of Ribosomal Proteins

Ribosomal proteins to be used for isoelectric focusing fractionation were prepared using lithium chloride-urea. This procedure is based on the method of Spitnik-Elson (1965) for the extraction of ribo-

somal proteins from bacterial ribosomes. Ribosome pellets containing about 300 μg of RNA plus protein were dissolved in 0.3 ml of 10 mM Tris-Cl pH 7.0, 4 mM EDTA, 60 mM β -mercaptoethanol. Then 0.9 ml of 8 M urea, 3.33 M LiCl was added. The suspension was allowed to stand at $4^\circ C$ for 20 hr, then at $0^\circ C$ for 12 hr. The material was then centrifuged at 40,000 g_{av} for 15 min at $2^\circ C$ in the Spinco 40 rotor. The protein-containing supernatant was recovered and LiCl was removed by diafiltration in an Amicon 8M-C dialysis-concentration apparatus (UM-2 membrane) (Amicon Corp., Lexington, Mass.) using as dialysis solution 8.2 M urea, 27 mM dithiothreitol, pH 8.6. The dialyzed material was saturated with N_2 and incubated at $24^\circ C$ for $2\frac{1}{2}$ hr in a sealed container to obtain reduction of disulfides.

A related method was used to prepare a ribosomal protein-enriched fraction from whole mitochondria which has been previously incubated *in vitro* in the presence of labeled amino acids. The mitochondrial pellet (about 10 mg protein) was suspended in 1.4 ml of 10 mM Tris-Cl pH 7.0, 4 mM EDTA, 60 mM β -mercaptoethanol. Then 4.5 ml of 8 M urea and 3.33 M LiCl were added. The suspension was dispersed by mild sonication (3×6 sec, at $0^\circ C$), and allowed to stand at $4^\circ C$ for 20 hr, then at $0^\circ C$ for 12 hr. The material was then centrifuged at 40,000 g_{av} for 15 min at $2^\circ C$ in the Spinco 40 rotor, the supernatant was recovered, and marker ribosomal proteins (labeled with another isotope, and suspended in LiCl-urea) were mixed with it at this point. The mixture was dialyzed against distilled water for 4 hr, and then precipitated with an equal volume of 20% trichloroacetic acid (TCA). The precipitate was collected and extracted twice with ether, once with ethanol-ether (1:1) at $37^\circ C$ for 60 min, and twice more with ether to remove lipid contaminants. It was dried with a stream of nitrogen, suspended in 5 ml of 8.2 M urea (adjusted to pH 3.3 with HCl), and dialyzed against the same solution for 4 hr at $4^\circ C$. The pH was then adjusted to 8.7, dithiothreitol crystals were added to a final concentration of 50 mM, and the pH was readjusted to 8.7. After gassing with N_2 the solution was allowed to stand for 3 hr at $24^\circ C$ in a sealed beaker. It was then dialyzed overnight ($4^\circ C$, with one change) against 6.25 M urea, 21.5% sucrose, 2 mM dithioerythritol. Finally, it was clarified by centrifugation at 60,000 g_{av} for 30 min ($2^\circ C$) in the Spinco 40 rotor. The supernatant containing the proteins soluble in 6.25 M urea, 2 mM dithioerythritol was used immediately for fractionation by isoelectric focusing.

Isolation of Total Mitochondrial Protein

The mitochondrial postribosomal fraction, prepared as described previously, was dialyzed for 36

hr at 4°C against three changes of 10 mM Tris-HCl pH 7.0, 6 M urea, 2.5 M LiCl, 13 mM β -mercaptoethanol, 2 mM EDTA, and 0.1% Nonidet P-40. This treatment brings about almost total disruption of mitochondrial membranes, since no pellet was obtained after a clarifying spin at 40,000 g_{av} for 20 min in the Spinco 40 rotor. The material was subsequently dialyzed for 4 hr against the same buffer minus LiCl-urea. The protein was then precipitated with an equal volume of 20% TCA. The pellet was subjected to lipid extraction and disulfide reduction as described earlier, except that 0.5% Nonidet P-40 was added in the reduction step. The material was then clarified by centrifugation at 80,000 g_{av} for 35 min in the Spinco 40 rotor. The supernatant, which contained about 95% of the original protein, was used for analysis in isoelectric focusing columns or gels.

Column Isoelectric Focusing

Isoelectric focusing in sucrose gradients was done as described in the LKB manual 1-8100-E01 (Laboratorie och Kemikaliska Produkter [LKB], Stockholm). The gradient was made up in the presence of 6.25 M urea, 2 mM dithioerythritol (and also, sometimes, Nonidet P-40). The ampholyte concentration was 2%. Isoelectric focusing was carried out for 44–48 hr at 12°C, at a maximum voltage of about 550 v. 1 ml fractions were collected (110 ml), the pH was measured, and fractions were counted. The fractions of interest were pooled, 150 μ g of carrier commercial protein mixture were added (cytochrome *c*, myoglobin, and bovine serum albumin), and each pooling was precipitated with 4 vol of 13% TCA. After standing for 1 hr in ice, the precipitate was collected in 13 mm Whatman GFC filters, washed with ether, and dried. The filters were placed in conical bottom tubes, 0.3 ml of electrophoresis loading buffer was added, and the protein was allowed to dissolve for 4 hr at 4°C. The samples were then immediately used for gel electrophoresis in urea gels.

Gel Isoelectric Focusing

Gels 28 cm in length were cast inside 0.5 cm (inside diameter) tubes. The gel composition was 5% acrylamide, 0.25% bisacrylamide, 8 M urea, 0.5% Nonidet P-40, 2% ampholyte. Catalysts were 0.022% ammonium persulfate and 0.05% TEMED. Cathode buffer was 4% ethylenediamine and anode buffer was 0.8% citric acid. For anodic loading of sample the gels were pre-run for 10 hr to remove residual catalyst. The sample was then loaded in 8 M urea, 10% glycerol, 0.5% Nonidet P-40, 25 mM dithioerythritol. A maximum voltage of 1200 v was reached after 24 hr. The run was continued (always at 4°C)

for a total of 26 hr, at which time the gels were immediately removed for staining and counting. Staining took place in 0.01% bromophenol blue, 10% acetic acid, 5% mercuric chloride (4 hr). Destaining was accomplished by dipping the gel for 12 hr in 2% acetic acid and then for 24 hr in "stain" solution diluted with a 1000-fold excess of water. To slice the gels they were placed for 5 hr in 20% glycerol containing traces of stain and then frozen with dry ice, at which point they could be sliced with an equidistant wire apparatus. Counting of radioactivity in slices was done as described below for standard polyacrylamide gels

Electrophoresis in Urea Gels

Electrophoresis of proteins in 8 M urea at pH 4.3 was done according to the method of Leboy et al. (1964), as described by Hallberg and Brown (1969). The gel concentration was 11% acrylamide and 0.55% bis-acrylamide. After electrophoresis, the gels were sliced with an equidistant wire apparatus and processed as follows: slices were incubated in 0.4 ml of 10% hydrogen peroxide for 24 hr at 47°C. Each vial then received 10 ml of the following cocktail: 100 g naphthalene, 8 g butyl-P.B.D. (Ciba Pharmaceutical Co., Summit, N. J.), and *p*-dioxane to 1 liter. Scintillation counting was done in a Beckman LS-250 room temperature counter (Beckman Instruments, Inc., Fullerton, Calif.).

Electrophoresis in Sodium Dodecyl Sulfate-Urea Gels

This procedure is a slight modification of the method of Maizel (1969) for discontinuous gel electrophoresis in the presence of sodium dodecyl sulfate (SDS). The separating gel was 17 cm long and consisted of 11% acrylamide and 0.55% bis-acrylamide. The spacer gel was 1.5 cm long and consisted of 3.2% acrylamide, 0.16% bis-acrylamide. Polymerization of the spacer gel was catalyzed by light. In addition to Tris-HCl, the gel buffers contained 8 M urea and 0.1% SDS. The electrode buffer did not contain urea.

The protein load was prepared as follows; Ribosomal subunit pellets were given a quick rinse with H₂O, and then suspended in a buffer consisting of 20 mM Tris-HCl pH 6.8, 8 M urea, 1.5% SDS, 1% β -mercaptoethanol. The subunits were dissolved at room temperature (20–30 min) and then heated at 98°C for 1–2 min in order to dissolve any remaining metastable aggregates of RNA and protein. The clear solution was loaded on the gels (40–80 μ g protein/gel) and electrophoresis carried out towards the anode at 10 v/cm. It was continued until the cytochrome *c* marker had penetrated 16 cm into the gel. The gels were either sliced immediately or

stained in 0.1% Coomassie brilliant blue (ICI America, Inc., Stamford, Conn.), 7.5% acetic acid, 45% methanol. Destaining was done in 7% acetic acid-5% methanol and finally in 7% acetic acid. Processing of slices for scintillation counting was carried out as described above for urea gels.

Analytical Methods

Protein was determined by the method of Lowry et al. (1951), using crystallized bovine plasma albumin as standard.

RESULTS

Amino Acid Incorporation by Isolated Mitochondria

Mitochondrial fractions prepared by the colloidal silica method are highly suitable for the study of amino acid incorporation. Systematic electron microscopic examination of such fractions indicates that structurally intact mitochondria are the major component, with minor contributions from mitochondrial fragments, nuclear fragments, and very rare whole cell fragments. On the basis of electrophoresis of RNA from these preparations in a system which clearly resolves mitochondrial

and cytoplasmic ribosomal species (Lizardi and Luck, 1971), it could be determined that RNA from cytoplasmic ribosomes constitutes only 5–8% of the total ribosomal RNA. In the presence of α -ketoglutarate these preparations show a respiratory control ratio of 2.7, and incorporate labeled amino acids and adenosine diphosphate (ADP)- ^3H (or uracil- ^3H) into TCA-precipitable material for a period of 40–50 min without additional energy sources.

Preliminary studies of the incorporation of ADP- ^3H (30 min) by these mitochondrial fractions (Lizardi, 1972) indicated that the major product was a high molecular weight RNA, larger than the RNA of the large ribosomal subunit. Furthermore, there was very little newly synthesized RNA associated with ribosomes isolated at the end of the incubation. Accumulation of a probable precursor ribosomal RNA species, very limited labeling of m-ribosomal RNA, and failure to find newly synthesized RNA with ribosomes suggested that ribosome assembly does not take place at an appreciable rate under in vitro conditions. This being the case the biosynthesis of MRP could not be studied properly by looking for newly synthesized proteins in ribosomes re

TABLE I
Percentage Recovery of Protein Mass and Radioactivity from Mitochondria Incubated In Vitro and from the Marker Ribosomal Proteins (^{14}C) Added Subsequently

| Fraction | Protein (Lowry) | ^3H (TCA ppt) | ^{14}C (TCA ppt) |
|----------------------------------------------------------------------------------------------|-----------------|------------------------|---------------------------|
| | <i>cpm</i> | <i>cpm</i> | <i>cpm</i> |
| (a) Final purified mitochondrial pellet | (100) | (100) | — |
| Added marker ribosomal protein | — | — | (100-ribosomes) |
| (b) Recovery after LiCl-urea solubilization | 74 | 78 | 98.6 |
| (c) Recovery after TCA precipitation, lipid extraction, and dialysis against 8 M urea pH 3.3 | 73 | 67 | 96.1 |
| (d) Recovery after final clarifying spin. (This is the isoelectric focusing column load) | 55 | 42 | 95.7 |

Mitochondria were prepared with colloidal silica and incubated in vitro as described in Materials and Methods. The label was 25 mCi of L-leucine- ^3H , 15 Ci/mmol. After 40 min of incubation, the mitochondria were repurified as described in Materials and Methods and immediately used for LiCl-urea extraction.

Marker MRP labeled with leucine- ^{14}C was obtained from isopycnic flotation mitochondria. The mitochondria were obtained from cells labeled with L-leucine- ^{14}C , 41.6 mCi/mmol (100 μg /150 ml culture flask). Label was added 30 min after conidial inoculation. The ribosomes were obtained by pelleting through 2 M sucrose, as described in Materials and Methods. The entire pellet was used for LiCl-urea extraction.

The ^{14}C - and ^3H -labeled extracts (LiCl-urea soluble) were mixed and processed together for isoelectric focusing. Samples were taken at each step, precipitated with TCA, and used for protein (Lowry) and cpm (filter) recovery determinations.

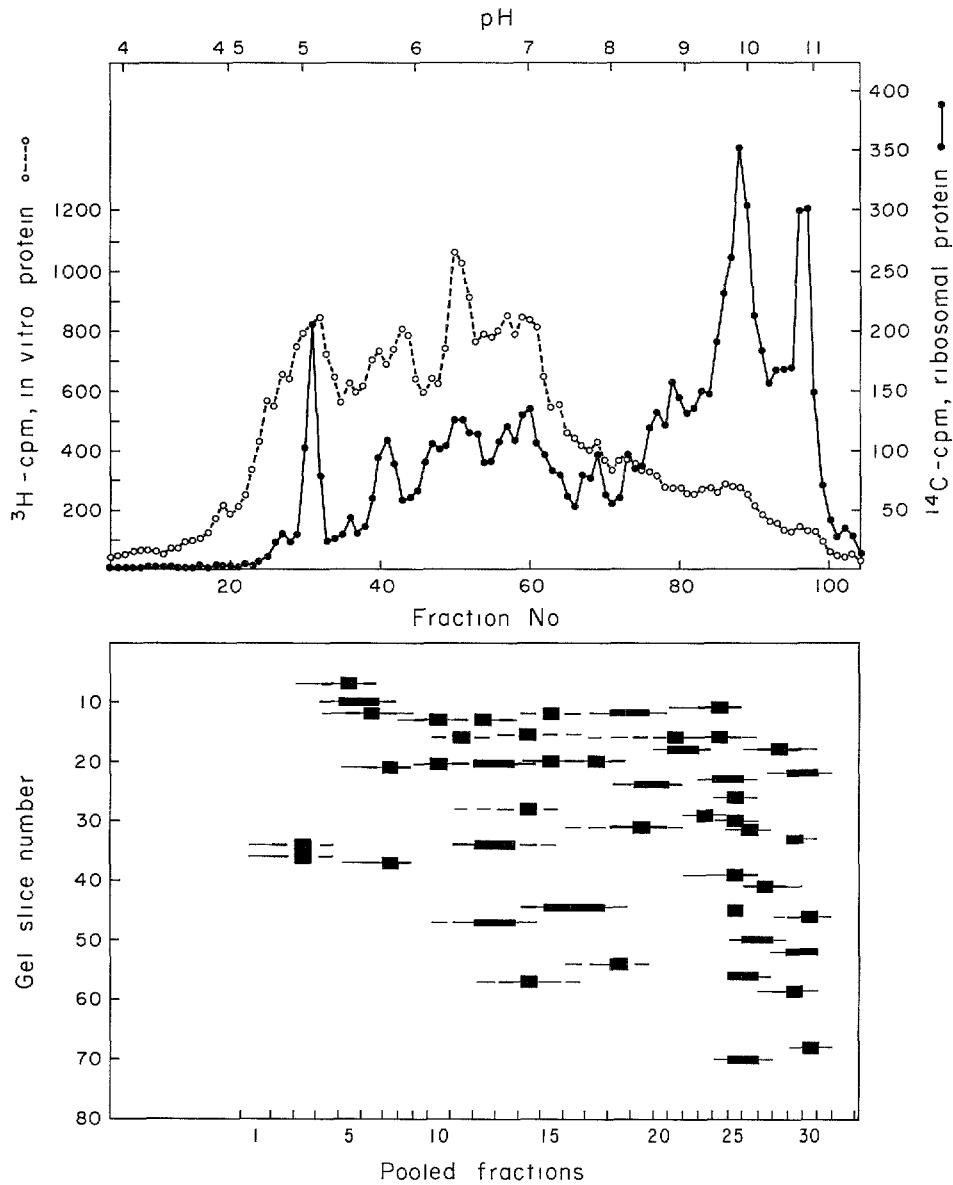


FIGURE 1 Isoelectric focusing and gel electrophoretic fractionation of ³H in vitro product and ¹⁴C marker MRP. The labeled material prepared as described in the legend to Table I was fractionated by isoelectric focusing using pH 3-10 ampholytes. After pH determination and counting of fractions, the fractions were pooled in 31 groups and subjected to polyacrylamide gel electrophoresis in urea gels. (See Materials and Methods for details.) The position of the ¹⁴C-labeled proteins was determined by analysis of relative peak heights, and plotted in the lower diagram. The direction of electrophoresis is from top to bottom, toward the cathode. The major blocks denote the gel position in which a given peak is highest relative to its peak height in adjacent gels. The lateral spread was usually three or four gels. The expected spread for a single protein was verified by examination of the staining patterns of commercial protein standards (myoglobin; cytochrome *c*) in the same type of fractionation.

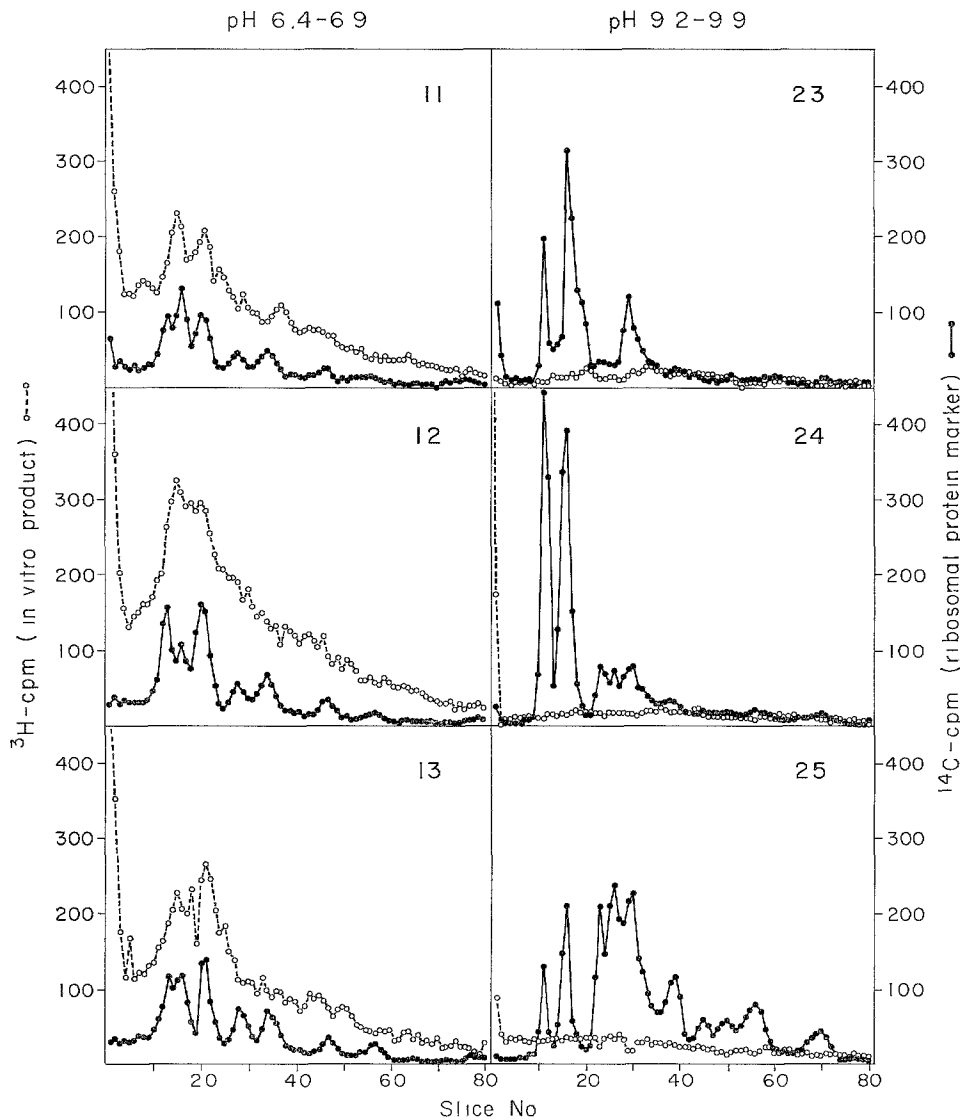


FIGURE 2 Polyacrylamide gel electrophoresis of pooled fractions from isoelectric focusing. These are the ^3H and ^{14}C radioactivity profiles of 6 of the 31 gels included in the lower diagram in Fig. 1. The broken line (open circles) shows the distribution of ^3H -labeled in vitro product, which was not depicted in Fig. 1. In these gels the cytochrome *c* used as visible marker was located in slices 95-97.

covered after incubation. This approach had already been used, with negative results, by Neupert et al. (1969 *a*). In our system any ribosomal proteins synthesized by isolated mitochondria could be expected to be present in detectable amounts only in the "soluble" phase, or in ribosome precursor particles. We, therefore, elected to solubilize whole mitochondria at the end of incubation,

using LiCl-urea. This procedure, which effectively solubilizes bacterial and eukaryotic ribosomal proteins, has recently been used for the extraction of ribosomal proteins from whole amphibian embryos (Hallberg and Brown, 1969).

An in vitro experiment was carried out as follows: Mitochondria isolated by the colloidal silica method were incubated for 40 min in the pres-

ence of leucine- ^3H , and subsequently extracted *in toto* with LiCl-urea. The LiCl-urea-soluble material was mixed with ^{14}C -labeled marker ribosomal proteins, and fractionated by isoelectric focusing followed by gel electrophoresis to obtain almost complete resolution of the individual ^{14}C -marker proteins. The ^3H and ^{14}C profiles were then compared to see if any peaks of marker MRP (^{14}C) coincided with peaks of *in vitro* synthesized protein. A more detailed account of methodology is given in the legends of Table I and Fig. 1.

Table I shows the recovery of protein mass and radioactivity during extraction and processing of the ^3H -labeled material and the ^{14}C -labeled marker MRP, which are mixed after step *b*. There is essentially no loss of ^{14}C radioactivity (95.7% yield) during extraction and processing; in contrast, the yield of ^3H radioactivity is only 42%. The yield of total mitochondrial protein mass is also low (55%). This could be expected because a large percentage of the mitochondrial protein mass consists of exceedingly insoluble membrane proteins.

The top part of Fig. 1 shows the fractionation obtained by isoelectric focusing. As expected, the bulk of the MRP- ^{14}C consists of basic proteins with isoelectric points as high as pH 11. In separate experiments using isoelectric focusing in polyacrylamide gels, it was determined that there are no MRP with isoelectric points higher than 11. There is virtually no ^{14}C -labeled material below pH 4.5. In contrast, the bulk of the ^3H -labeled *in vitro* product consists of acidic proteins, and a small amount of material between pH 7 and 10. Resolution of individual proteins, however, is not obtained in this first dimension of fractionation.

The bottom part of Fig. 1 shows the second dimension of fractionation, which consists of polyacrylamide gel electrophoresis of 31 pooled fractions from the isoelectric focusing column. Only the MRP- ^{14}C marker is shown in this diagram. The dark blocks indicate the positions of major ^{14}C peaks; the lines indicate the minor peaks resulting from the spread of major peaks in the first dimension (*isoelectric focusing*). In total, 47 major ^{14}C peaks were detected. We know that this is an underestimate of the total number of MRP. Later we will show that with more complete fractionation 53 peaks can be detected. Thus, there must be a few overlaps of MRP in this diagram, but probably no more than six. The block diagram shows clearly that the ^{14}C peaks in the original

isoelectric focusing fractionation result from the overlap of a large number of MRP.

Having shown that the ^{14}C marker proteins can be resolved by this two-dimensional technique, let us consider the ^3H -labeled proteins. The number of ^3H peaks was large, so large in some gels that resolution was not complete. The unexpected complexity of the *in vitro* product led us to make an estimate of the peak size which would be expected for a presumptive MRP synthesized by isolated mitochondria. Considering the fraction of total mitochondrial protein represented by MRP (1.5%), and by making an estimate of the fraction of mitochondrial protein synthesized by the organelle itself (<16%), it was possible to use the data of Table I and the total tritium counts recovered in the 31 gels (280,000 cpm) to determine that a ribosomal protein of average molecular weight (circa 25,000) could be expected to have a peak height of 200–250 cpm in a single gel of lower Fig. 1.

Fig. 2 shows the actual radioactivity profiles of gels number 11–13 and 23–25 from the same experiment. The pH range encompassed by each group of gels is indicated at the top of the figure. Gels 23–25 are typical of the range above pH 8.5.

TABLE II
 $^3\text{H}/^{14}\text{C}$ Ratio of Cell Fractions in Presence and Absence of Chloramphenicol (CAP)

| Fraction | $^3\text{H}/^{14}\text{C}$ (+ CAP) | $^3\text{H}/^{14}\text{C}$ (- CAP) |
|--------------------------------|---------------------------------------|---------------------------------------|
| Cytosol | 13.77 | 18.49 |
| Mitochondria | 13.68 | 19.73 |
| Cytoplasmic ribosomal pellet | 9.80 | 15.26 |
| Mitochondrial ribosomal pellet | 11.22 | 17.51 |

Mass label was L-leucine- ^{14}C 41.6 mCi/mmol (83 $\mu\text{g}/100$ ml culture flask). Pulse label was L-leucine- ^3H 9 Ci/mmol (2.5 mCi/100 ml culture flask). Chloramphenicol was added from a slurry (100 mg/ml) to give a final concentration of 4 mg/ml. As chase, 37.5 mg of cold leucine were added to each flask. The recovery culture flasks contained 10 mg of cold leucine in 150 ml of growth medium. Preparation of cell fractions was as described in Materials and Methods (mitochondria prepared by isopycnic flotation, and ribosomal subunits prepared with puromycin). Mitochondrial and cytosol radioactivity was measured from TCA precipitates. Samples of ribosomes and ribosomal subunits were counted directly.

There are a considerable number of ^{14}C -labeled marker MRP peaks, and no distinguishable tritium peaks corresponding to any of them. As a matter of fact, the ^3H profile never rises above 85 cpm in the range of gels 19–31. Gels 11–13 on the other hand, are representative of the range of pH 5 to 8.5. The number of ^3H peaks above 100 cpm is very large and they are not always well resolved. For this reason it was not possible to map the ^3H peaks in a diagram of the type shown in Fig. 1 (bottom). In spite of this problem, it could be determined that among all gels there were no more than a few ^3H - ^{14}C superimpositions.

Of the 47 marker MRP peaks only 3 showed some degree of "coincidence" with tritium peaks. None of these three coincidences was perfect, as we will illustrate for one of them: The ^{14}C -labeled marker MRP present in gels 12–13, slice 20, coincides with a tritium peak in both gels. However, the ^{14}C -labeled marker MRP present in slice 20 of gel 11, which is apparently the same protein, does not exactly coincide with a tritium peak (they differ by one gel slice). A similar situation was found with the other two marker MRP showing some degree of coincidence (gels 10 and 15).

In trying to interpret these three cases of coin-

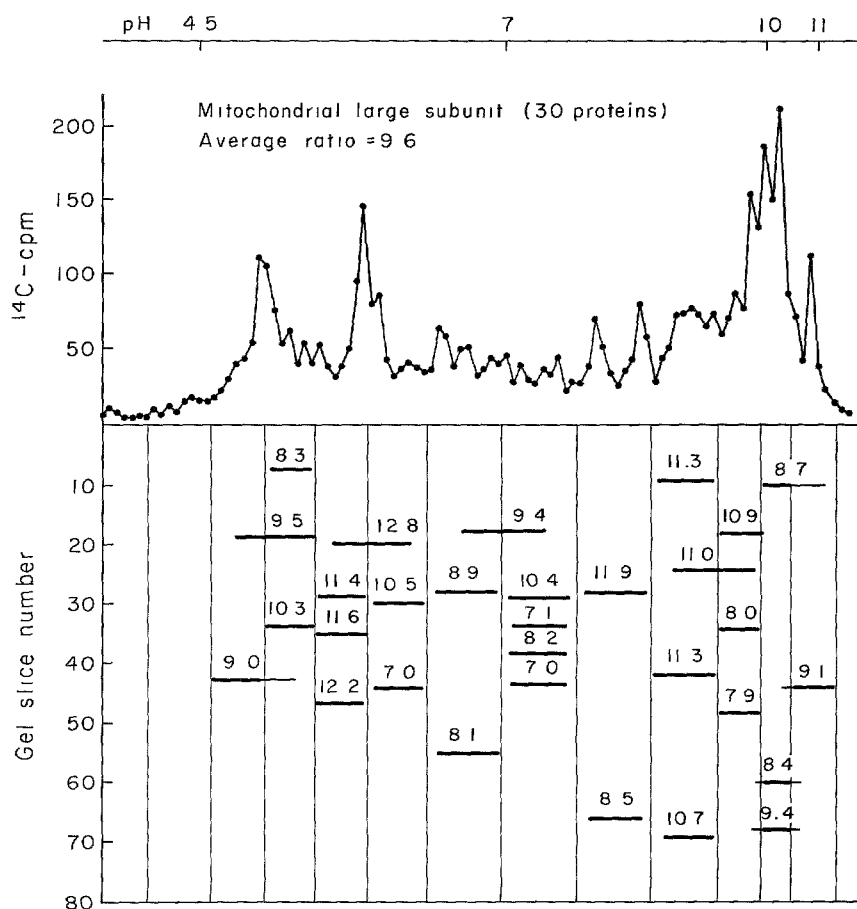


FIGURE 3 Isoelectric focusing-gel electrophoresis fractionation of proteins from the mitochondrial large ribosomal subunit: ^3H : ^{14}C ratios of ^{14}C peaks. Mitochondria were obtained from chloramphenicol-treated cultures labeled as described in Table II. Proteins from the mitochondrial large ribosomal subunits were extracted with LiCl-urea and processed for isoelectric focusing as described in Materials and Methods. Isoelectric focusing fractionation and subsequent gel electrophoresis of pooled fractions was done as described in Fig. 1 (this time 12 gels were used). Each gel was sliced and counted, the ^{14}C mass profile was plotted, and the ^3H : ^{14}C ratio of each ^{14}C peak determined. The peaks are plotted diagrammatically as horizontal lines in each gel; the number above each line is the ^3H : ^{14}C ratio.

cidence we made an estimate of the possibility of a random superimposition of marker bands (47) and significant product peaks (51) in the circa 2250 possible gel positions represented by lower Fig 1. We determined that at least one superimposition could be expected on the basis of chance. While it is clear that 44 marker MRP do not correspond to products of in vitro protein synthesis, the significance of the three coincidences remains uncertain. The unexpected heterogeneity of in vitro products, and the clustering of peaks in the gels showing coincidences makes it infeasible to test the relationship by further procedures such as peptide comparisons.

In Vivo Studies Using Chloramphenicol

Chloramphenicol is known to be a selective inhibitor of mitochondrial protein synthesis (Wheeldon and Lehninger, 1966; Linnane et al, 1967; Kuntzel, 1969 *b*) We have studied the effect of the drug on MRP biosynthesis in exponentially growing cultures. Leucine-¹⁴C was added to cultures and after 12 hr of growth (circa 5 cell mass doublings) chloramphenicol was added at 4 mg/ml. After 15 min, leucine-³H was added for a 30 min labeling period which was terminated by the addition of excess unlabeled leucine. After 20 min, cultures were filtered and transferred to

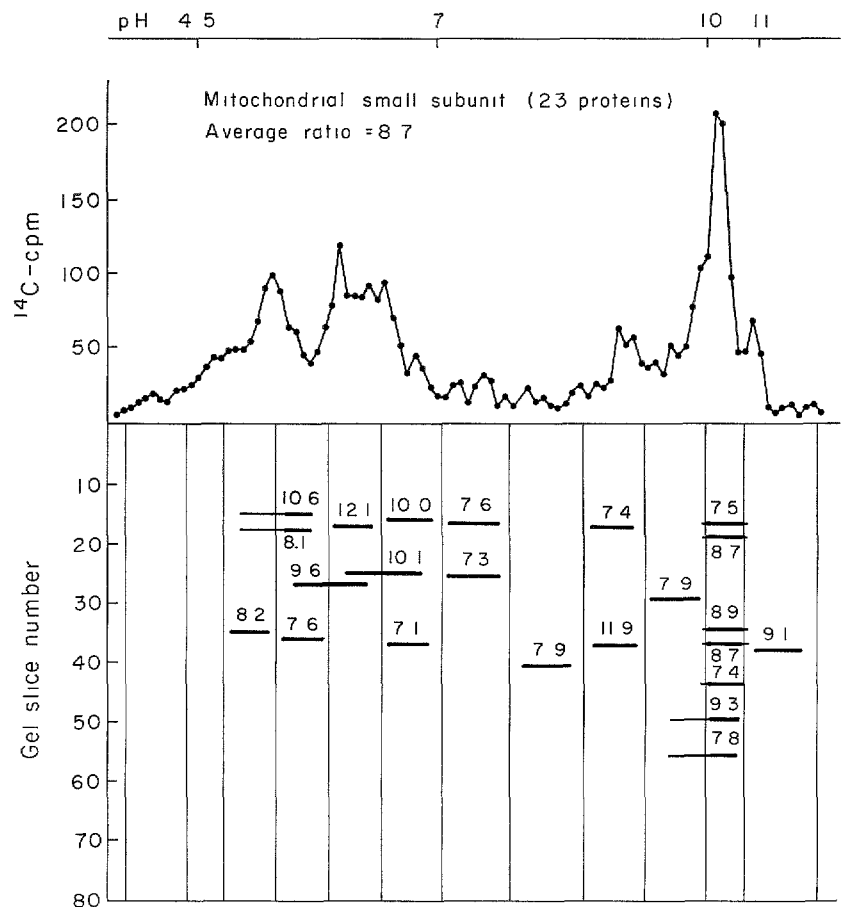


FIGURE 4. Isoelectric focusing-gel electrophoresis fractionation of proteins from the mitochondrial small ribosomal subunit: ³H:¹⁴C ratios of ¹⁴C peaks. Mitochondria were obtained from chloramphenicol-treated cultures as described in Table II. Proteins from the mitochondrial small ribosomal subunit were extracted with LiCl-urea and processed for isoelectric focusing as described in Materials and Methods. Isoelectric focusing fractionation and subsequent gel electrophoresis of pooled fractions was done as described in Fig. 1 (in this experiment 12 gels were used). See legend to Fig. 3 for additional details.

fresh medium where they were allowed to grow for 2 hr. In this protocol leucine- ^{14}C serves as a protein mass label and leucine- ^3H as label for proteins synthesized during chloramphenicol treatment. Products of mitochondrial protein synthesis would be expected to have a low ratio of $^3\text{H}:^{14}\text{C}$ radioactivity. The inclusion of a 2 hr recovery period in the absence of chloramphenicol was to provide an opportunity for MRP to be assembled into ribosomes and, therefore, to minimize the effect of MRP pools and any specific sequence of MRP assembly into subunits. It can be estimated that during the recovery period the mass of newly assembled mitochondrial ribosomes corresponds to circa 57% of the initial population. Therefore, we considered it reliable to analyze the results of this experiment by studying MRP present in subunits obtained from mitochondrial ribosomes (see Materials and Methods) at the end of the experiment.

Table II shows the ratios of $^3\text{H}:^{14}\text{C}$ radioactivity obtained in the preparative steps of such an experiment. The ratio is lower for all cell fractions of drug-treated cells compared with controls. This is to be expected since the cell mass doubling time is prolonged in chloramphenicol treated cells (Rifkin, 1969). The fact that the ratio for cytosol and that for total mitochondria are not significantly different confirms earlier results (Rifkin, 1969), and is consistent with the conclusion that mitochondria synthesize a minority of their constituent proteins.

It was important to obtain an estimate of the degree of inhibition obtained with the drug. Protein was obtained from the mitochondrial post-ribosomal fraction as described in Materials and Methods, and fractionated by isoelectric focusing followed by gel electrophoresis, slicing, and counting of the gels. The analysis revealed an exceedingly large number of peaks. Most had

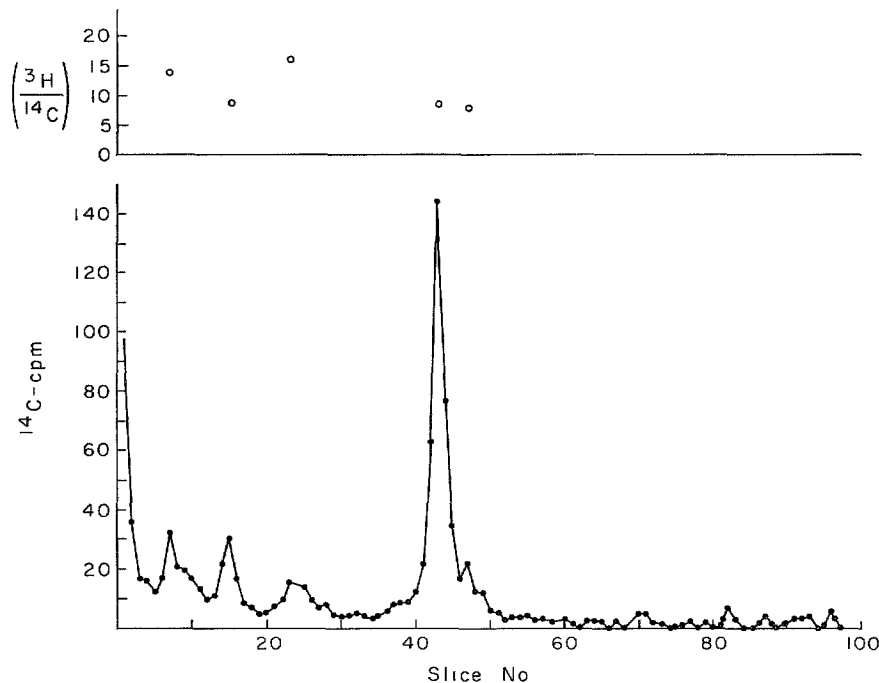


FIGURE 5 Polyacrylamide gel electrophoresis of material released by puromycin dissociation. $^3\text{H}:^{14}\text{C}$ ratio of individual peaks. Mitochondria were obtained from chloramphenicol-treated cultures labeled as described in Table II. The load zone (0-6S) of the sucrose gradient used for separation of puromycin-dissociated ribosomal subunits from mitochondria was collected. Commercial carrier proteins (bovine serum albumin, 50 μg ; ovalbumin, 50 μg ; and cytochrome *c*, 50 μg) were added, followed by $\frac{1}{2}$ vol of 30% TCA. The TCA precipitate was collected in a small glass-fiber filter, rinsed with ether, and dried. 0.3 ml of SDS-urea buffer was added to dissolve the precipitate. The solution was used for electrophoresis in SDS-urea gels. The gels were sliced, counted, and the $^3\text{H}:^{14}\text{C}$ ratios of peaks calculated.

$^3\text{H}/^{14}\text{C}$ radioactivity ratios of about 14, but a few were as low as 4.8. Since resolution was incomplete it is possible that the lowest ratios were below 4.8. However, using the value 4.8 and the average ratio 13.7, it can be estimated that protein synthesis of chloramphenicol-sensitive components was inhibited by 65% or more.

As shown in Table II, the radioactivity ratios for mitochondrial ribosomes are slightly higher than the ratios for cytoplasmic ribosomes. Based on this comparison, chloramphenicol treatment does not appear to inhibit selectively synthesis of MRP as a group. Polyacrylamide gel electrophoresis (urea gels) of the proteins of cytoplasmic ribosomal subunits revealed components with ratios in the range 6.5–9.5. It seems that the synthesis of ribosomal proteins, in general, was depressed relative to other cytosol proteins. The decreased cell growth rate in the presence of chloramphenicol may have caused a slight shift-down in ribosome biosynthesis, affecting both cytoplasmic and mitochondrial ribosomes.

Figs. 3 and 4 show the analysis of MRP from chloramphenicol-treated cells. In this case, the

isoelectric focusing-gel electrophoresis-fractionation scheme was applied separately to the proteins of each subunit. In this way, it was possible to obtain what appears to be complete resolution of all the proteins in each subunit. The small subunit was found to contain 23 different proteins, the large subunit 30. As far as we know, this is the first report on the actual number of proteins present in mitochondrial ribosomes, and it shows that these ribosomes are as complex as bacterial ribosomes in terms of protein content. It should be emphasized that the ribosomal pellet from which these subunits were obtained contained no ribosomal RNA with the electrophoretic mobility of cytoplasmic species (for electrophoretic method see Lizardi and Luck, 1971), and, therefore, the results are not complicated by contamination from cytoplasmic ribosomes.

The numbers in Figs. 3 and 4 are the $^3\text{H}:^{14}\text{C}$ ratios of the individual MRP. In general, they are similar to the ratios of cytoplasmic ribosomal proteins, although somewhat more variable. In spite of this variability, the lowest ratios are only 26–28% below the average, and could be ex-

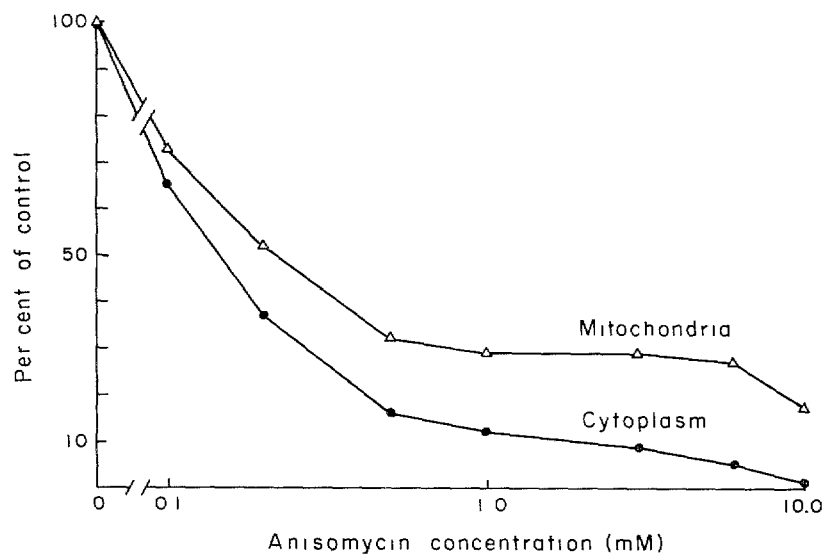


FIGURE 6 Incorporation of leucine- ^3H into cytoplasmic and mitochondrial fractions (in vivo) in the presence of different concentrations of anisomycin. Anisomycin was added to 14.5-hr *Neurospora* cultures to give the final concentrations indicated in the plot. 7 min later each culture flask received 0.2 mCi of leucine- ^3H , 1 Ci/mole. Growth was continued for another 20 min, at which point the cultures were chilled and used for preparation of cytoplasmic supernatant and mitochondria (isopycnic flotation). The fractions were precipitated with TCA, rinsed, and dissolved in 1 M NaOH. Fractions were used for scintillation counting (toluene-NCS) and for protein determination (Lowry). Results are plotted as per cent specific radioactivity of controls (no antibiotic). The specific radioactivities of control cytoplasm and control mitochondria were not significantly different.

plained on the basis of pool size differences. The very lowest MRP ratios are no lower than the typical ratios of cytoplasmic ribosomal proteins. Clearly, none of the ratios is as low as would be expected of a typical chloramphenicol-sensitive protein (65% or more below the average). On the basis of these observations we conclude that MRP cannot be distinguished from cytoplasmic proteins in terms of their sensitivity to chloramphenicol.

The mitochondrial ribosomal subunits used in this experiment were prepared by puromycin dissociation of ribosomes. We have found that in addition to releasing nascent peptide chains, puromycin induces the release from ribosomes of a few discrete polypeptides of relatively high molecular weight. The most reproducible of these is a protein of mol wt $\sim 34,000$ (determined in SDS gels). Fig. 5 shows the polyacrylamide gel profile of the proteins recovered from the soluble phase after puromycin dissociation of mitochondrial ribosomal subunits. The protein of mol wt 34,000 is present in slice 43. The scale above the gel shows that the $^3\text{H}/^{14}\text{C}$ ratios of these proteins range from 8 to 16. Therefore, all of these proteins, including the peak at slice 43, seem to be insensitive to chloramphenicol.

In Vivo Studies Using Anisomycin

It seemed desirable to carry out an *in vivo* experiment, complementary to the chloramphenicol treatment, in which cytoplasmic protein synthesis was selectively inhibited. Cycloheximide has been used for this purpose by Küntzel (1969 *a*) and Neupert et al. (1969 *b*), who have studied MRP synthesis in *Neurospora*. Anisomycin (2-*p*-Methoxyphenylmethyl-3-acetoxy-4-hydroxy-pyrrolidine) has been shown to be a powerful inhibitor of the peptidyl transferase reaction in eukaryotic ribosomes, yet has no inhibitory effect on bacterial protein synthesis. Preliminary experiments (Fig. 6) showed that anisomycin could be used as a selective inhibitor of cytoplasmic protein synthesis in *Neurospora*. As seen in the fig., amino acid incorporation in the cytoplasm can be inhibited to about 15% of control values, while about 16% of the incorporation into mitochondria remains resistant. The selectivity of the drug in this system appears to be comparable to that reported with cycloheximide under optimal conditions (Sebald et al., 1969; Hawley and Greenawalt, 1970), although in our experience, anisomycin

selectivity is more reproducible. The concentration of anisomycin necessary to achieve optimal inhibition (10 mM) is about 1000 times higher than the concentration reported to inhibit protein synthesis by isolated eukaryotic ribosomes (Grollman, 1967; Neth et al., 1970).

The time-course of leucine- ^3H incorporation into cytosol and mitochondrial fractions in the presence of 10 mM anisomycin (*in vivo*) was investigated. Label was given 7 min after addition of drug, and incorporation was measured at 10, 20, and 30 min thereafter. It was found that the rate of incorporation slowed down significantly with time. We believe that the slowdown is a secondary effect resulting from the absence of cytoplasmic protein synthesis. In order to minimize such secondary effects it was desirable to make the period of anisomycin pretreatment as short as possible. It was found that label could be added as early as 30 sec after addition of the drug without danger of excessive residual incorporation by the cytoplasm. In view of this, a preincubation time

TABLE III
 $^3\text{H}/^{14}\text{C}$ Ratios of Cell Fractions in the Presence and Absence of Anisomycin

| Fraction | $^3\text{H}/^{14}\text{C}$ (- aniso) | $^3\text{H}/^{14}\text{C}$ (+ aniso) | Percent- age of control ratio |
|----------------------------------|-----------------------------------------|-----------------------------------------|----------------------------------------|
| Cytosol | 59.1 | 0.79 | 1.3 |
| Mitochondria | 61.3 | 10.4 | 16.9 |
| Cyto. large ribosomal subunit | 36.7 | 0.25 | 0.7 |
| Cyto. small ribosomal subunit | 42.2 | 0.31 | 0.7 |
| Mito. large ribosomal subunit | 47.2 | 0.19 | 0.4 |
| Mito. small ribosomal subunit | 52.9 | 0.51 | 1.0 |

Neurospora cultures (12 flasks) were grown for 14.5 hr in the presence of amino acids- ^{14}C (20 $\mu\text{g}/\text{flask}$ of an equimolar mixture of L-leucine, lysine, arginine, and valine, 10 mCi/mMole). At this time, anisomycin crystals were added to a final concentration of 10 mM. 50 sec later, a mixture of amino acids- ^3H was added (2.0 mCi/flask of an equimolar mixture of L-leucine, lysine, arginine, and valine, 16 Ci/mMole). After 30 min, a "cold" amino acid mixture was added as chase (40 mg of mix/flask). The cultures were harvested after 12 min of chase. Cell fractionation and counting of fractions were as in Table II.

of only 50 sec was used in all subsequent experiments

Our attempts to use anisomycin for a labeling with recovery experiment similar in design to the chloramphenicol experiment of the previous section were not successful. In contrast to chloramphenicol treatment, anisomycin treatment reduces the total amount of protein synthesis to such low levels that only a small percentage of the added leucine- ^3H is utilized during the labeling period. Even after chase and culture transfer, some leucine- ^3H remains and is actively incorporated by cytoplasmic ribosomes during the recovery period in the absence of the drug; this being the case, the selective inhibition of the cytoplasm becomes blurred.

We chose to study MRP synthesis using the following experimental design. Amino acids- ^{14}C were added early in growth as protein mass label. Anisomycin (10 mM) was added after $14\frac{1}{2}$ hr of growth. ^3H -labeled amino acids were added 50 sec after the drug. After a 30 min labeling period, excess nonradioactive amino acids were

added for 12 min in order to reduce the amount of radioactivity present in nascent peptide chains at the time of harvesting the cells. Longer "chase" periods were not used because after 45 min of drug treatment the inhibition is irreversible, and there was danger of increased proteolytic activity. After harvesting, ribosomal subunits were prepared using puromycin and their proteins were fractionated to determine individual $^3\text{H}:^{14}\text{C}$ ratios. A low $^3\text{H}:^{14}\text{C}$ ratio is interpreted as evidence of anisomycin inhibition. The main problem expected with this kind of experiment is that the $^3\text{H}:^{14}\text{C}$ ratio of ribosomal proteins obtained from ribosomes is dependent on individual protein pool sizes and on ribosome assembly. If mitochondrial ribosome assembly were to stop soon after addition of the drug, the ratios of all MRP would be very low regardless of whether their synthesis was inhibited or not. To determine whether mitochondrial ribosomes continue to be assembled after addition of anisomycin, we followed the fate of the mitochondrial ribosomal RNA synthesized in the presence of the drug. It was found that

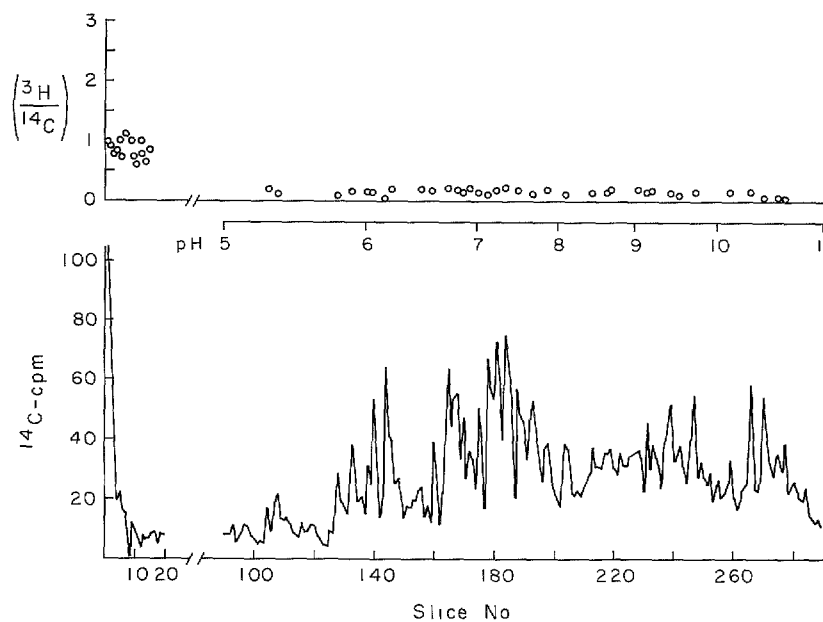


FIGURE 7 Polyacrylamide gel isoelectric focusing of MRP from the large subunit: $^3\text{H}:^{14}\text{C}$ ratios of individual peaks. Mitochondria were obtained from anisomycin-treated cultures labeled as described in Table III. MRP was extracted from the mitochondrial large subunit using LiCl-urea, and used for isoelectric focusing in polyacrylamide gels (see Materials and Methods). In this case the protein was loaded at the anode (slice 1). Gel slicing was slightly uneven due to the softness of the 5% acrylamide gel matrix. An approximate calibration of the pH gradient was obtained as follows: A duplicate (empty) gel was sliced in several small segments, and each segment placed in a small test tube containing 0.6 ml of distilled water. The segment was crushed with a glass rod and its pH was measured.

after labeling for 30 min with uracil-5'- ^3H in the presence of anisomycin (2 min preincubation), the mitochondrial ribosomal monomers contain labeled 25S and 19S ribosomal RNA. In the same experiment newly synthesized ribosomal RNA was also found in ribosomal subunits and in polysomes. Comparison of the total amount of ribosomal RNA radioactivity incorporated into ribosomes in anisomycin-treated vs. control cultures showed that during the 30 min labeling period the drug-treated cells assembled approximately one-third as many mitochondrial ribosomes as the controls. This finding will be useful for the interpretation of ^3H : ^{14}C ratios of mitochondrial ribosomal proteins.

Table III shows the results of the previously described double-labeling experiment. The ^3H : ^{14}C ratios for total proteins of cytosol and mitochondria show the typical anisomycin inhibition effect: the ratio for cytosol is 1.3% of control, that for mitochondria 16.9% of control. Cytosol proteins from anisomycin-treated cells were fractionated in SDS-urea polyacrylamide gels to determine the ratios of individual protein peaks. It was found that the ratios ranged from 0.40 to 1.44, clustering around 0.7–0.8. Therefore, no anisomycin-re-

sistant proteins were detectable in cytosol. Proteins from whole mitochondria of the same cells were also fractionated in SDS-urea gels. This analysis revealed ratios ranging from 0.98 to 61.2 (average ratio equals 10.4). The fractions with very high ratios represent the anisomycin-resistant proteins synthesized inside the mitochondria, and remain uncharacterized. Their ratios were over 50 times higher than the typical ratios of cytosol proteins. This is the kind of difference expected when cytoplasmic protein synthesis has been selectively inhibited by 98.5%.

The ratios for cytoplasmic ribosomal subunits from drug-treated cells (Table III) are about 0.7% of control ratios. This apparently reflects the combined effects of inhibited protein synthesis and reduced ribosome assembly. The ratios for mitochondrial ribosomal subunits are about as low as those of cytoplasmic ribosomal subunits. As mentioned earlier, mitochondrial ribosome assembly in the presence of anisomycin is reduced to about one-third of control values. On the basis of a reduction in ribosome assembly alone the ^3H : ^{14}C ratio of mitochondrial ribosomal subunits from anisomycin-treated cultures would be reduced to 14–33% of control values. The exact

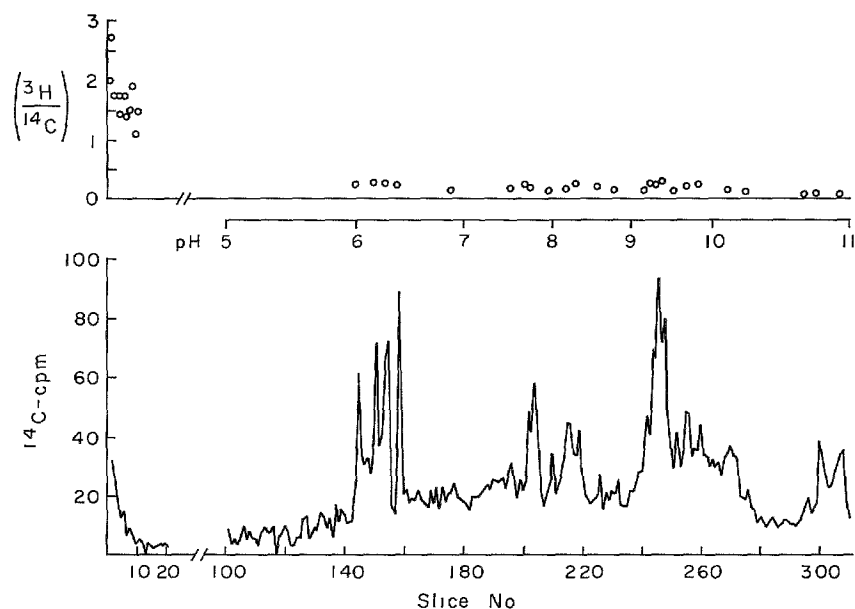


FIGURE 8 Polyacrylamide gel isoelectric focusing of MRP from the small subunit: ^3H : ^{14}C ratio of individual peaks. Mitochondria were obtained from anisomycin-treated cultures labeled as described in Table III. MRP was extracted from the mitochondrial small subunit using LiCl-urea and used for isoelectric focusing in polyacrylamide gels (See Materials and Methods).

value would depend on the detailed time course of failure of ribosome assembly. The observed ratios are in the range of 0.4–1.0% of controls, definitely too low to be explained merely on the basis of reduced ribosome assembly. We conclude that the synthesis of MRP, examined as a group, has been strongly inhibited by anisomycin.

To examine the ratios of individual proteins, LiCl-urea-extracted ribosomal proteins were fractionated by isoelectric focusing in polyacrylamide gels. We found that these 28-cm-long gels allowed almost complete resolution of all the proteins of each subunit. Fractionation of the proteins of the cytoplasmic large ribosomal subunit showed that the $^3\text{H}/^{14}\text{C}$ ratios of individual proteins ranged from 0.14 to 0.38. The results of the fractionation of proteins from the large and small mitochondrial ribosomal subunits are shown in Figs. 7 and 8. All MRP peaks were in the range of pH 5–11,

except for a small amount of material which stayed at the gel origin (anode). All the proteins which penetrated the gels showed $^3\text{H}/^{14}\text{C}$ ratios within a range of uniformly low values (0.07–0.23 for the large subunit, 0.1–0.3 for the small subunit). All these ratios are as low or lower than those of cytoplasmic ribosomal proteins. However, the small amount of nonpenetrating material which smeared from gel slice 1 to 15 showed a somewhat higher $^3\text{H}/^{14}\text{C}$ ratio: about 0.9 in the case of the large subunit, about 1.5–2.5 in the small subunit (Figs. 7 and 8, respectively).

To study further the nonpenetrating material the experiment was repeated, this time fractionating the mitochondrial ribosomal proteins in SDS-urea polyacrylamide gels, in which there is almost 100% penetration. Again, almost all MRP show very low ratios (~ 0.25). However, there was some material of strikingly high ratio

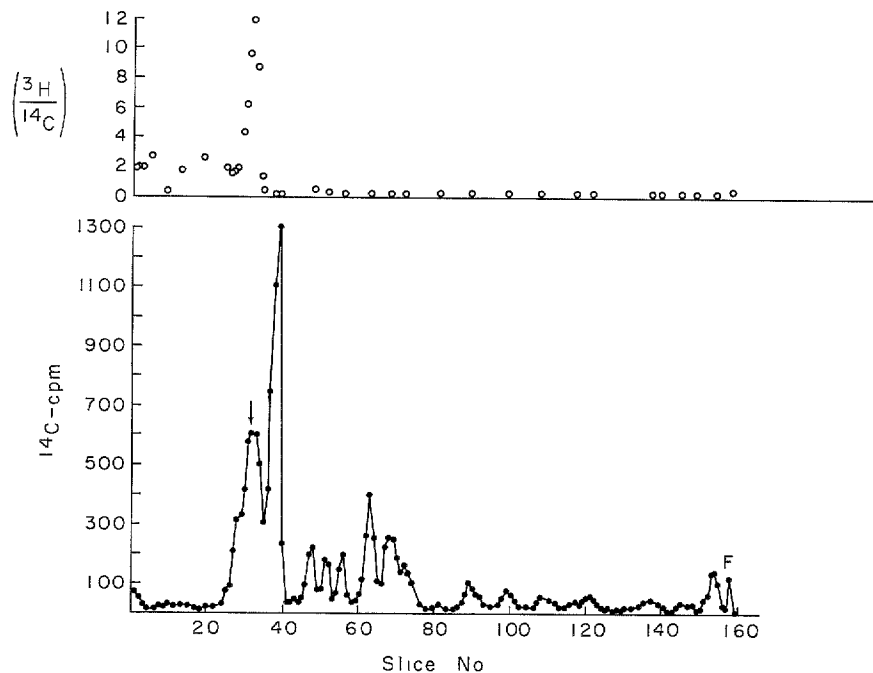


FIGURE 9 Polyacrylamide gel electrophoresis (SDS-urea) of MRP from the small subunit: $^3\text{H}:^{14}\text{C}$ ratios of individual peaks. Mitochondria were obtained from anisomycin-treated cultures labeled as described in Table III. Mitochondrial small ribosomal subunits, prepared with puromycin, were dissolved in SDS-urea- β -mercaptoethanol buffer and used directly for electrophoresis in SDS-urea polyacrylamide gels (see Materials and Methods). The gels were sliced and counted using standard methods. A calibration of molecular weight vs. R_f was obtained by electrophoresis of commercial protein standards (bovine serum albumin, ovalbumin, α -chymotrypsinogen, myoglobin, and cytochrome *c*). On the basis of this calibration, MRP range in mol wt from 12,000 to 63,000 daltons. Examination of stained gels showed that many of the peaks in the radioactivity profile actually represent two adjacent bands.

among the proteins of the small subunit (Fig. 9, gel slice 32). In other gels, which were run for a much longer time, this material was resolved into two peaks, both of which were tritium-rich. The $^3\text{H}:^{14}\text{C}$ ratio of the radioactive peaks was 40–100 times higher than the ratios of the other MRP in the gel. This is the ratio expected for an “anisomycin-resistant” protein.

When we attempted to reproduce this finding in other experiments, the anisomycin-resistant material was only occasionally found in association with the small subunit. It was sometimes present in protein obtained from other zones of the sucrose gradients used to prepare ribosomal subunits, namely the 58S–70S region. These zones did not contain appreciable amounts of ribosomal material.

Our failure to recover consistently the anisomycin-resistant material in association with the main peak of mitochondrial small ribosomal subunit has led us to conclude that it is not a true “structural” protein of mitochondrial ribosomal subunits. Two possibilities occur to us regarding the nature of the anisomycin-resistant protein(s): (a) it is a random contaminant of preparations of mitochondrial ribosomes, (b) it is an unusual ribosomal protein which, since it is recovered in variable and less than stoichiometric amounts, is perhaps associated with a specific subpopulation of ribosomes (for example, membrane-bound ribosomes). We should mention that the solubility properties of the anisomycin-resistant protein are rather different from those of typical MRP. The protein is only partially soluble in LiCl-urea, and requires SDS for complete solubilization. Further studies are in progress to elucidate the nature of this material.

We have shown in Figs 7 and 8 that all reproducible MRP have very low $^3\text{H}:^{14}\text{C}$ ratios. In other analyses (not shown) it was found that the ratios of puromycin-released proteins were also very low (~ 0.25). There was a threefold range of variation among the ratios of MRP in each subunit. This variation, which was also observed in cytoplasmic ribosomal proteins, is probably due mainly to the following: (a) differences in the pool size of each protein; (b) differences in the time of binding of the proteins to nascent ribosomal RNA during the course of ribosome assembly. In spite of this variation, the highest ratio of a reproducible MRP (0.30) was no higher than the typical ratios of cytoplasmic ribosomal proteins (0.14–0.38). The great similarity between

the ratios of MRP and cytoplasmic ribosomal proteins is strong evidence in favor of a common, anisomycin-sensitive, site of synthesis.

DISCUSSION

The intracellular site of synthesis of MRP has been investigated by three complementary approaches. Although there is inherent uncertainty in each procedure, taken together the results suggest strongly that all MRP are products of cytoplasmic protein synthesis.

Amino acid incorporation experiments with mitochondrial fractions indicated that for at least 44 of the 47 marker MRP resolved, no comparable mitochondrial products were made. It could be argued that in this case isolated mitochondria fail to synthesize proteins which might normally be synthesized *in vivo*. In view of what must be a complex interrelationship between mitochondria and the cell, the argument is plausible but must be balanced against our finding that isolated mitochondria did synthesize a large variety of polypeptides of relatively large molecular weight.

In vivo experiments with chloramphenicol showed that not one of the 53 MRP which were resolved showed significant inhibition of synthesis by the drug. The major uncertainty in the interpretation of this result is the question of whether chloramphenicol, even at the highest concentration it was possible to use, uniformly inhibited the synthesis of all proteins made by mitochondria. Although it has been reported that chloramphenicol causes 87–95% inhibition of amino acid incorporation by isolated mitochondria from *Neurospora* (Lamb et al., 1968; Hawley and Greenawalt, 1970), the actual level of inhibition attainable *in vivo* has not been accurately determined. Under our *in vivo* experimental conditions chloramphenicol appeared to inhibit the synthesis of some mitochondrial proteins by at least 65%. We also know that under similar conditions the appearance in mitochondria of spectrophotometrically detectable cytochromes *b* and *a-a₃* appears to be completely inhibited (Rifkin, 1969). However, the possibility that some specific mitochondrion-synthesized proteins were spared inhibition by chloramphenicol cannot be ruled out.

The *in vivo* studies using anisomycin showed that this drug inhibited the labeling of essentially all the proteins of mitochondrial ribosomal subunits. The inhibition of labeling of MRP was

rapid, and in each case as complete as that of cytoplasmic ribosomal proteins. In view of the fact that the drug preincubation time was only 50 sec, it seems unlikely that the inhibition of MRP synthesis was a secondary effect resulting from inhibition of cytoplasmic protein synthesis.

The possible contribution of pools of MRP to the results of the anisomycin experiments merits consideration. We know that the pool sizes for most MRP are not very large because mitochondrial ribosomal subunits reach a specific activity almost as high as that of whole cell protein after only 30 min of labeling with ^3H -labeled amino acids (in the absence of anisomycin). However, we have not investigated the pool sizes for individual MRP. One could conceive of an anisomycin-resistant MRP with a very large pool size, which would exhibit a very low specific activity when labeled during anisomycin treatment. In this case a large pool size and limited ribosome assembly would combine so that the MRP could have a specific activity as low as that of truly anisomycin-sensitive MRP. Although we cannot rule out this possibility with the data of the anisomycin experiment, we can show that it is incompatible with the results of the chloramphenicol experiment. If such an anisomycin-resistant MRP existed, it would be expected to be at least partially sensitive to chloramphenicol. Therefore, in the chloramphenicol experiment the protein would have shown a very low specific activity relative to other MRP, due to the combined effects of chloramphenicol inhibition and pool dilution. No low-specific-activity protein was observed in the chloramphenicol experiment. We conclude that the results of the anisomycin experiments reflect true inhibition in synthesis of all MRP.

Considering all the evidence obtained by means of these three different experimental approaches, we conclude that all proteins of mitochondrial ribosomes are synthesized outside of the mitochondria, presumably by cytoplasmic ribosomes. It should be stressed that these conclusions apply only to those proteins which are tightly associated with ribosomes and are consistently recovered under our high-salt isolation conditions. Whether mitochondria play a role in the synthesis of other ribosome-associated components, such as factors of protein synthesis, membrane-binding proteins, etc., remains to be seen. The anisomycin-resistant protein sometimes found in association with the small subunit could

represent such a component. Our conclusions for individual MRP are in agreement with those reached by Neupert et al (1969 *a, b*) and Kuntzel (1969 *b*) who studied the labeling of whole mitochondrial ribosomes.

Our findings do not help to explain the observations of Linnane et al (1968 *a, b*) on erythromycin-resistant mitochondrial ribosomes, or those of Rifkin and Luck (1971) on the ribosome assembly defect of the poky mutant. Results obtained recently with *Staphylococcus aureus* (Lai and Weisblum, 1971) suggest that resistance to erythromycin could depend on modification of large subunit ribosomal RNA (methylation of adenine). Thus, one mechanism by which mitochondrial genes could determine functional properties of mitochondrial ribosomes, is by directing the synthesis of enzymes which modify the ribosomal RNA. Of course, it is possible that structural genes for MRP are present in mitochondrial DNA but are translated in the cytoplasm. Finally, enzymes coded by mitochondrial DNA could bring about specific chemical modification of some MRP once they have entered the mitochondrion.

The last possibility raises additional questions. Could some MRP be identical or chemically modified variants of cytoplasmic ribosomal proteins? For *Neurospora*, based on results with carboxy methyl cellulose chromatography (Kuntzel, 1969 *a, b*), gel electrophoresis (Rifkin, 1969), and our own experience with isoelectric focusing gels and SDS-urea gel electrophoresis, it appears that most MRP are significantly different from their cytoplasmic counterparts. A definitive answer will require more stringent structural comparisons, e.g., peptide mapping or antibody cross-reactivity studies.

Whether or not MRP are unique, their penetration into mitochondria from a cytoplasmic site of synthesis poses a problem. The inner mitochondrial membrane is known to be impermeable to small molecules such as NADH, and to proteins such as ribonuclease and DNase (Luck and Reich, 1964). On the other hand, isolated amphibian mitochondria are able to internalize high molecular weight polynucleotides (Swanson, 1971) with some discrimination of base composition and secondary structure. Whether a similar discriminative mechanism for transport of MRP exists remains a subject for further experimentation.

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