7. The exposure is developed with Kodak liquid x-ray developer for 5 min (a type 6B safe light can be used). Fixed x-ray films are washed 20 min in running water and air dried. Where a finer grain is desired, Kodak SO-146, x-ray film is used. This film requires about four times the exposure of the RP/R2 film. For a comparison of the autoradiograph with the stained gel, the paper is marked with ^{35}S-labeled ink to allow proper alignment when the autoradiograph is superimposed over the gel.

Figure 2 shows typical results obtained when cells are labeled with $^{35}SO_4^{2-}$ in the presence and absence of cycloheximide and chloramphenicol. In the absence of antibiotics, a large number of bands are observed; cycloheximide inhibits the incorporation of label into most of these and the remaining bands (more than 20 can be visualized) correspond to intrinsic mitochondrial translation products.

[7] Biogenesis of Mitochondrial Proteins in HeLa Cells[1]

By GIUSEPPE ATTARDI and EDWIN CHING

A considerable amount of evidence supports the view that biogenesis of mitochondria, both in lower and higher eukaryotic cells, consists of two processes: the gross formation of the mitochondrial membranes and the differentiation of the mitochondrial *anlagen* into organelles active in oxidative phosphorylation.[2] While the gross formation of mitochondria appears to be solely controlled by the nuclear genome through the participation of the cytoplasmic protein synthesizing apparatus, the differentiation of "inactive" mitochondria into energy-producing organelles requires both components coded for by nuclear genes and synthesized on cytoplasmic ribosomes and components specified by mitochondrial genes and synthesized on mitochondrial ribosomes.[2-4] Therefore, the assembly of functional mitochondria involves the cooperation of the extramitochondrial translation machinery and of the organelle-specific counterpart. In particular, the extramitochondrial protein-synthesizing apparatus provides the protein components of the outer membrane and of the mitochondrial matrix, all or nearly all the proteins involved in replication

[1] The research on which this article is based has been supported by Grant GM-11726 from the National Institute of Health.

[2] G. Attardi, P. Costantino, J. England, D. Lynch, W. Murphy, D. Ojala, J. Posakony, and B. Storrie, *in* "Genetics and Biogenesis of Mitochondria and Chloroplasts" (W. Birky, P. Perlman and T. Byers, eds.), p. 3. Ohio State Univ. Press, Columbus, 1975.

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and transcription of mitochondrial DNA and in mitochondrial translation, and most of the proteins of the inner mitochondrial membrane[2,3]. By contrast, the mitochondrial machinery produces a relatively small number of polypeptides, which are mostly, if not exclusively, polypeptide constituents of the respiratory complexes and of the oligomycin-sensitive ATPase.[3,5-13]

In HeLa cells, relatively little is known about the mitochondrial proteins synthesized in the extramitochondrial compartment, apart from the evidence suggesting that their nature is the same as in other eukaryotic cells.[14,15] The large number of these proteins, the contamination of the mitochondrial fraction by other membrane components,[16] and the limitation of the fractionation techniques have so far hampered the study of the biogenesis of these proteins. By contrast, the polypeptides synthesized in the organelles have been more easily accessible to analysis due to the *in vivo* use of selective inhibitors of cytoplasmic protein synthesis, like cycloheximide[17-20] and emetine,[21-23] and to the development of mitochondrial protein-synthesizing systems utilizing isolated organelles.[17,24] In this chapter, we describe the procedures which have been successfully employed in this laboratory in the study of mitochondrial protein synthesis in HeLa cells and the main results thus obtained.

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[24] G. Attardi, P. Costantino, J. England, M. Lederman, D. Ojala, and B. Storrie, *Acta Endocrinol., Suppl.* **180**, 263 (1973).

Experimental Procedures

Cell Growth and Labeling. HeLa cells of the S3 clonal strain are generally used for these studies. These are grown in suspension in modified Eagle's medium[25] with 5% calf serum (generation time about 22 hr).

Long-term labeling of total mitochondrial proteins is carried out by exposing exponentially growing cells for two or three generations to one or more labeled amino acids, the type and amount of isotope depending upon the requirements of the experiment. For pulse labeling of the products of mitochondrial protein synthesis, ^{3}H-isoleucine, ^{3}H-leucine, or ^{35}S-methionine are the radioactive amino acids of choice, because of the comparatively higher levels of incorporation they give.[26] In these experiments, exponentially growing cells are centrifuged down, resuspended at a concentration of 1×10^6 to 3×10^6 cells/ml in warm medium (lacking the particular amino acid used for labeling and supplemented with 5% dialyzed calf serum), and incubated for 30 min at 37° prior to the pulse. Either cycloheximide or emetine (100 μg/ml) is used to inhibit cytoplasmic protein synthesis. Cycloheximide is utilized in the experiments where a reversibility of the block of cytoplasmic protein synthesis after the pulse is required,[18,27]; in all other cases, emetine is preferred because it produces a more complete inhibition of cytoplasmic protein synthesis.[22,23,28] In all cases, the inhibitor is added to the culture 5 min prior to the labeling. As a control, a parallel culture is labeled under identical conditions in the presence of both the chosen inhibitor of cytoplasmic protein synthesis and chloramphenicol (100 μg/ml), an effective inhibitor of mitochondrial protein synthesis.[17,19,29] Pulse labeling is ended by pouring the cell suspension over frozen and crushed NKM (0.13 *M* NaCl, 0.005 *M* KCl, 0.001 *M* $MgCl_2$) in a flask immersed in a salt-ice bath.

Subcellular Fraction.[16,17,28] All operations described below are carried out at 2°–4°. The labeled cells are washed three times with NKM and then resuspended in 6 volumes of 0.01 *M* Tris buffer (pH 6.7 at 25°), 0.01 *M* KCl, 0.00015 *M* $MgCl_2$. After 2 min of swelling, the suspension is homogenized with an A. H. Thomas Teflon pestle homogenizer (motor-driven pestle, ~1500 rpm), using a sufficient number of strokes to break 60–70% of the cells. After addition of sucrose to 0.25 *M*, the homogenate is centrifuged at about 1160 g_{av} for 3 min to sediment nuclei, unbroken cells and large cytoplasmic debris. The supernatant is spun at 5000 g_{av} for 10 min; the pellet thus obtained is resuspended in 0.25 *M* sucrose in 0.01 *M*

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[26] E. Ching, P. Costantino, and G. Attardi, *Biochem. Biophys. Res. Commun.* **79**, 451 (1977).
[27] B. Colombo, L. Felicetti, and C. Baglioni, *Biochim. Biophys. Acta* **119**, 109 (1966).
[28] P. Costantino and G. Attardi, *J. Mol. Biol.* **96**, 291 (1975).
[29] A. M. Kroon, *Biochim. Biophys. Acta* **108**, 275 (1965).

Tris buffer (pH 6.7), 0.00015 *M* $MgCl_2$ (one-half of the volume of the homogenate). After a spin at 1100 g_{av} for 2 min to sediment any residual nuclei the suspension is recentrifuged at 5000 g_{av} for 10 min. The pellet is resuspended in 0.25 *M* sucrose in 0.01 *M* Tris acetate buffer (pH 7.0) at a concentration of 2–4 mg protein per milliliter, as determined by the Lowry method. To prepare the sample for electrophoretic analysis, the suspension, in a volume of 1–3 ml (in a tube immersed in iced water), is sonicated twice for 10 sec, with a 1-min interval [we use a Branson sonifier (Model S-125, 20 kHz) with a microprobe], and immediately utilized or stored frozen at −60°.

Analysis of the products of mitochondrial protein synthesis can also be carried out on lysates of cells pulse labeled with an amino acid in the presence of emetine. For this purpose, the labeled cells, washed three times with NKM, are lysed with sodium dodecyl sulfate (SDS) buffer [0.01 *M* Tris buffer (pH 7.0), 0.1 *M* NaCl, 0.001 *M* EDTA, 0.5% SDS; 3.0 ml per 0.1 ml packed cell volume], sonicated for 10 sec (twice for 5 sec at room temperature) with the Branson sonifier, and dialyzed for 18 hr against 0.5% SDS (150 volumes, 3 changes) at room temperature. In whole cell lysates, the proportion of emetine resistant incorporated radioactivity which is not sensitive to chloramphenicol is higher than in mitochondrial lysates; however, the possibility of enzymatic degradation during extraction is greatly reduced by this method.

Chloroform–Methanol Extraction.[28] A sample of the sonically disrupted 5000 g_{av} mitochondrial fraction (0.2–0.7 mg protein in 0.05–0.15 ml) is mixed with 2 ml of a 2 : 1 (v/v) mixture of chloroform and methanol. The suspension is incubated at 50° for 30 min, and the insoluble residue pelleted by centrifugation at 1500 g_{av} for 5 min. The extract and the residue are dried under nitrogen and dissolved in 0.2 ml 2.5% SDS.

Polyacrylamide Gel Electrophoresis. CYLINDRICAL GELS. A modification of the system described by Weber and Osborn[30] is used. The samples to be applied to the gels, in 0.2 ml of 2.5% SDS, are brought to 5% mercaptoethanol and, in some cases, heated at 70° for 30 min to inactivate any protease present. In some experiments, the SDS lysates are treated for 30 min at pH 10, 37°, to break any peptidyl-tRNA complexes, and then neutralized. After addition of glycerol to 10% and bromophenol blue to ~0.001%, the samples are applied to 0.6 × 23 cm 12.5% polyacrylamide gel (1 : 40 *N,N'*-methylenebisacrylamide) in 0.1 *M* $NaPO_4$ buffer (pH 7.0), 0.1% SDS in the presence of ammonium persulfate and *N,N,N',N'*-tetramethylethylenediamine (TEMED). Electrophoresis is carried out at room temperature in 0.1 *M* $NaPO_4$ buffer (pH 7.0), 0.1% SDS, for 13–43 hr at 7.5 mA/gel in a Canalco model 1400 apparatus. The gels are frac-

[30] K. Weber and M. Osborn, *J. Biol. Chem.* **244**, 4406 (1969).

tionated in an extrusion apparatus similar to that described by Maizel,[31] using 0.05% SDS as eluant. Parallel gels of marker proteins, treated as the radioactive samples, are run routinely. These are stained overnight with 25% isopropyl alcohol, 10% acetic acid, 0.025–0.05% Coomassie Brilliant Blue (Schwarz/Mann), then for 6–9 hr in 10% isopropyl alcohol, 10% acetic acid, 0.0025–0.005% Coomassie brilliant blue; destaining is carried out in 5% isopropyl alcohol, 10% acetic acid, until the background clears (procedure modified from Fairbanks *et al.*[32]). The positions of the protein bands are correlated with the fraction number of the parallel fractionated gels on the basis of the relative migration of the tracking dye, as measured before staining or fractionation.

SLAB GELS. The design of the slab gels stand is as described by Studier.[33] Gels 15 to 30 cm long and 1 to 3 mm thick are normally used, depending on the particular requirements of the experiment.

The gel solutions are poured in a stand consisting essentially of a base with a trough and a vertical rod holding a clamp to keep the plates in place. The glass-spacer-glass sandwich is placed into the stand, the trough is filled with the acrylamide "shelf" solution and the solution is allowed to polymerize to seal the bottom of the sandwich.

a. *SDS-polyacrylamide gradient gels.* The acrylamide-bisacrylamide gradient solution is prepared with a standard sucrose gradient apparatus and pumped into the bottom of the glass-spacer-glass sandwich by means of two metal tubes connected with the mixing chamber (starting from the solution of lowest concentration). After the solution is dispensed, the tubes are carefully pulled out. The gradient solution can also be dispensed into the sandwich from the top by letting it run down the wall, starting with the solution of highest concentration. However, with this method, considerable turbulence may occur during layering, especially with long gels.

The shelf consists of 12% polyacrylamide (1 : 75 bisacrylamide) in 0.19 *M* Tris-HCl, pH 8.8, 0.1% SDS, and 0.002 *M* EDTA (polymerized with TEMED and ammonium persulfate). The separating gel consists of a linear 15–25% polyacrylamide gradient (constant 1 : 75 bisacrylamide) in 0.19 *M* Tris-HCl, pH 8.8, 0.1% SDS, and 0.002 *M* EDTA. The 1-cm stacking gel consists of 5% polyacrylamide (1 : 37.5 bisacrylamide) in 0.0625 *M* Tris-HCl, pH 6.8, 0.1% SDS, and 0.002 *M* EDTA. The reservoir buffer consists of 0.05 *M* Tris-HCl, pH 8.3, 0.38 *M* glycine, 0.1% SDS, and 0.002 *M* EDTA. The samples are prepared in 0.0125 *M* Tris-HCl, pH 6.8,

[31] J. V. Maizel, *in* "Methods in Virology" Vol. V (K. Maramorosch and H. Koprowski, eds.), p. 179. Academic Press, New York, 1971.

[32] G. Fairbanks, T. L. Steck, and D. F. H. Wallach, *Biochemistry* **10**, 2606 (1971).

[33] F. W. Studier, *J. Mol. Biol.* **79**, 237 (1973).

1% SDS, 0.002 EDTA, 1% 2-mercaptoethanol, 10% glycerol, and 0.0025% bromophenol blue. The electrophoresis is carried out at about 10 V/cm until the dye reaches the bottom (about 12 hr); fans are used to prevent overheating of the gel during electrophoresis.

b. *SDS-polyacrylamide/8 M urea gels.* These gels are a modification of the urea gels described by Downer *et al.*[34] The shelf consists of 6% polyacrylamide (1 : 75 bisacrylamide), 8 *M* urea in 0.1 *M* H_3PO_4-Tris, pH 6.8, and 0.1% SDS (polymerized with TEMED and ammonium persulfate). The separating gel consists of 15% polyacrylamide (1 : 50 bisacrylamide), 8 *M* urea in 0.1 *M* H_3PO_4-Tris, pH 6.8, and 0.1% SDS. A 1-cm stacking gel is utilized consisting of 5% polyacrylamide (1 : 37.5 bisacrylamide), 8 *M* urea in 0.033 *M* H_3PO_4-Tris, pH 6.3, and 0.1% SDS. The reservoir buffer is 0.1 *M* H_3PO_4-Tris, pH 6.8, and 0.1% SDS. The samples are prepared in a near-to-saturated solution of urea in 0.1 *M* H_3PO_4-Tris, pH 8.3, 50 m*M* dithiothreitol, 4% SDS, 10% glycerol and 0.0025% bromophenol blue. The gels are run until the dye reaches the bottom (about 12 hr for a 20-cm gel, when run at a constant current of 180 mA/cm^2). The gels are cooled by fans during electrophoresis.

c. *Visualization of proteins on gels.* Upon completion of the electrophoresis, the sandwich of glass and gel is removed from the stand and the glass plates pried apart. The gels can be stained with Coomassie brilliant blue, if so desired, following the procedure described above for cylindrical gels. They are then photographed over a light box on Kodak Contrast Process Ortho film using a Kodak No. 53 Wratten filter.

For visualization of ^{35}S-methionine-labeled material, direct autoradiography can be used. For this purpose, the stained or unstained gel is placed onto a piece of filter paper and dried down using an apparatus similar to that described by Maizel[31] (Hoefer Sci. Inst., San Francisco, California). In the case of urea gels, urea must be removed by elution in water for 15–30 min. Gels 2 mm or thicker require equilibration in 2% glycerol to prevent cracking during the drying process. The filter paper is taped onto a Kodak RP-5 x-ray film (with the gel facing the film), and placed in an exposure folder. After appropriate exposure, the film is developed in Kodak KLX developer for 5–7 min, put in Kodak Indicator stop bath for 1 min, and fixed for 2–4 min in Kodak Rapid Fixer. The x-ray film is washed in running water for 1 hr and dried.

For visualization of ^{3}H-labeled material or for enhancement of ^{35}S-labeled bands, the indirect fluorographic method[35] is used. For this purpose, the gel is immersed in 20 volumes of dimethyl sulfoxide (DMSO, practical grade; 3 changes, 20 min each), and then in 4 volumes of 22%

[34] N. W. Downer, N. C. Robinson, and R. A. Capaldi, *Biochemistry* **15**, 2930 (1976).
[35] W. M. Bonner and R. A. Laskey, *Eur. J. Biochem.* **46**, 83 (1974).

(w/v) 2,5-diphenyloxazole (PPO) in DMSO for 3 hr under rocking. The gel is placed in running water for 30 min and dried, as indicated above. Exposure is done at −60°.

Two-Dimensional Isoelectric Focusing-SDS Polyacrylamide Gel Electrophoresis. The method used is essentially that described by O'Farrell.[36] Three millimeters × 130 mm gels [4% polyacrylamide (1 : 17.5 bisacrylamide) in 9.15 *M* urea, 2% Triton X-100, and 2% LKB 3–10 ampholines] are prepared in glass tubes and overlaid with 8 *M* urea. The gels are inserted into a standard cylindrical gel electrophoresis apparatus and left to stand at least 3 hr. The 8 *M* urea is replaced with sample lysis medium (9.5 *M* urea, 2% Triton X-100, 2% LKB 3–10 ampholines, and 5% mercaptoethanol), the lower reservoir is filled with 0.01 *N* H_3PO_4 and the upper one with degassed 0.02 *N* NaOH, and the system is prerun at a constant 200 V for 30 min. After removing the sodium hydroxide and sample lysis medium, the samples (50–300 μg in sample lysis medium, with or without 1% SDS) are applied onto the gels, and overlaid with 1% LKB 3–10 ampholines in 9 *M* urea, and the sodium hydroxide is placed back in the upper reservoir. The gels are run at a constant 400 V for 12 hr and then an additional hour at 800 V to sharpen the bands.

After the run, the gels are removed from the columns and immediately processed; if necessary, they can be stored at −60°. To check the pH gradient, one gel is sliced, the focused ampholines are eluted in 0.1 *M* KCl for at least 2 hr, and the pH of each eluate is measured. To determine the fractionation of polypeptides by isoelectric focusing, one gel is stained in 20% 5-sulfosalicylic acid, 25% isopropyl alcohol, and 0.0025% Coomassie brilliant blue for 36 hr and destained in 20% acetic acid.[37] To determine the separation of radioactive components, another gel is sliced, the slices are dissolved in a gel solubilizer (NCS, Amersham/Searle or its equivalent), and counted.

Gels to be run in the second dimension are equilibrated for 2 hr in equilibration buffer [0.0625 *M* Tris-HCl (pH 6.8), 5% mercaptoethanol, 2.3% SDS, and 10% glycerol]. An SDS-polyacrylamide gradient or SDS-polyacrylamide/8 *M* urea slab gel is prepared as previously described, except that no sample wells are formed in the stacking gel. About 50 μl of 0.05% bromophenol blue are added across the top of the stacking gel and 100–200 μl of hot (~80°) 1% agarose in equilibration buffer are layered above the stacking gel. The equilibrated cylindrical gel is immediately laid into the hot agarose layer and sealed into position above the stacking gel with a minimal amount of additional agarose solution. Using the same

[36] P. H. O'Farrell, *J. Biol. Chem.* **250**, 4007 (1975).
[37] H. V. Huang, R. S. Molday, and W. J. Dreyer, *FEBS Lett.* **37**, 285 (1973).

electrophoresis buffer as in single-dimension slab gel runs, electrophoresis is carried out and the gels processed as described above.

In Vitro Mitochondrial Protein Synthesis. Incubation of a 5000 g_{av} crude mitochondrial fraction from HeLa cells with a respiratory substrate, ADP, and inorganic phosphate results in the incorporation of a radioactive amino acid into protein, which can be characterized as mitochondrial by its sensitivity to chloramphenicol and its insensitivity to cycloheximide and RNase.[17] Below are the detailed conditions of incubation.

For analytical purposes, the individual incubation mixtures (set up under sterile conditions) contain, in 0.5 ml, 150–500 μg protein of the 5000 g_{av} mitochondrial fraction, 350 m*M* sucrose, 50 m*M* Tris buffer (pH 7.4 at 25°), 60 m*M* KCl, 7 m*M* $MgCl_2$, 1 m*M* EDTA, 30 m*M* sodium succinate, 2 m*M* ADP, 20 m*M* potassium phosphate, 0.1 m*M* of each amino acid (except the labeled one), and 2.5 μCi (or more) of [4,5-^{3}H]isoleucine or [4,5-^{3}H]leucine or ^{35}S-methionine. Incubation is carried out in 2-ml stoppered tubes at 37° with shaking, and terminated by addition of trichloroacetic acid to 5% and 500 μg of bovine serum albumin. After pelleting, the precipitates are washed twice, by centrifugation, with cold 5% trichloroacetic acid containing 10 m*M* of the amino acid used for labeling, and then dissolved in 1 *M* NaOH at 37° for 1 hr; after reprecipitation with 10% trichloroacetic acid in the presence of 10 m*M* of the amino acid used for labeling and heating at 90° for 15 min in 5% trichloroacetic acid, the precipitates are successively washed with cold absolute ethanol and 3 : 1 ethanol/ether at 60°, and finally dissolved in 1 *N* NaOH. Protein concentration and acid precipitable radioactivity are determined on portions of the final samples.

For polyacrylamide gel electrophoresis analysis of the products of *in vitro* protein synthesis, larger scale incubation mixtures are used; after incubation, the mitochondrial fraction is washed twice [with 0.25 *M* sucrose, 0.01 *M* Tris buffer (pH 6.7), 0.025 KCl, and 0.005 *M* $MgCl_2$] by spinning at 12,000 g_{av} for 10 min and resuspension, and finally lysed as described above.

Results

Figure 1 shows the electrophoretic fractionation in a cylindrical gel of the proteins of a mitochondrial fraction from HeLa cells long-term labeled with both ^{14}C-lysine and ^{14}C-arginine, and then pulse labeled for 1 hr with ^{3}H-isoleucine in the presence of emetine. The lack of correspondence between the ^{3}H radioactivity peaks (representing the products of mitochondrial protein synthesis) and the profile of ^{14}C-labeled total proteins of the mitochondrial fraction indicates that most of these proteins

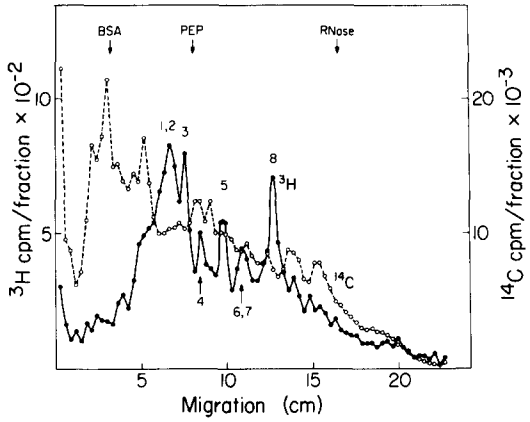

FIG. 1. Electrophoretic profile (26-hr run) of the total proteins of the mitochondrial fraction from HeLa cells labeled for 72 hr with ^{14}C-arginine and ^{14}C-lysine, and then for 1 hr with ^{3}H-isoleucine in the presence of 100 μg/ml emetine (P. Costantino and G. Attardi, unpublished observations).

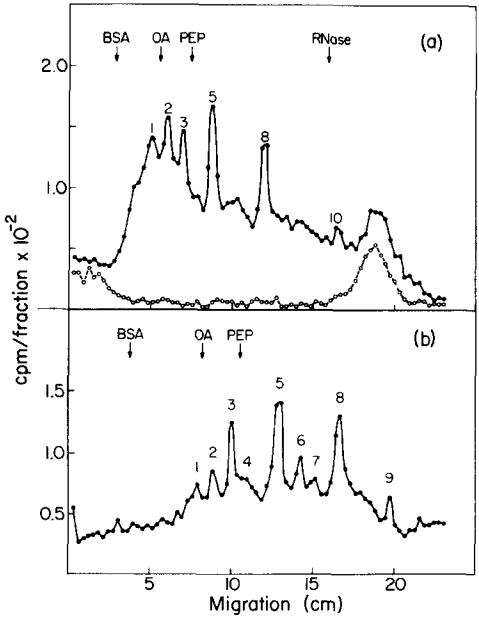

FIG. 2. Electrophoretic profiles of the products of mitochondrial protein synthesis from HeLa cells labeled for 10 min with ^{3}H-isoleucine in the presence of 100 μg/ml emetine, and in the absence (●) or presence (○) of 100 μg/ml chloramphenicol. (a) 26-hr run; (b) 40-hr run.[28]

TABLE I
MOLECULAR WEIGHTS OF THE PRODUCTS OF HeLa CELL MITOCHONDRIAL PROTEIN SYNTHESIS SEPARATED IN A CYLINDRICAL GEL[a]

Electrophoretic component	MW
1	42,000
2	39,000
3	35,000
4	31,500
5	27,500
6	24,000
7	22,500
8	19,500
9	15,000
10	11,500

[a] Costantino and Attardi.[28]

are synthesized on cytoplasmic ribosomes. By this type of electrophoretic fractionation, 10 discrete components have been reproducibly observed, over a heterogeneous background, among the products of HeLa cell mitochondrial protein synthesis[28] (Figs. 1 and 2). The synthesis of these components is completely sensitive to chloramphenicol at 100 μg/ml. (The chloramphenicol-resistant broad peak near the bottom of the gel in Fig. 2, on the basis of the available evidence, does not appear to be due to protein.) It is evident from recent data, described below, that some of these electrophoretic components represent multiple polypeptide species. Control experiments, involving either a direct SDS lysis of whole pulse-labeled cells or the use of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) during cell homogenization and fractionation, tend to exclude the possibility that the discrete products of mitochondrial protein synthesis observed in HeLa cells result from enzymic degradation during extraction. Table I shows the molecular weights of the discrete electrophoretic components identified among the mitochondrial protein products in HeLa cells, as estimated in many experiments from their electrophoretic mobilities, relative to those of standard proteins run in parallel gels. Recent studies[26] have shown that all of the 20 common amino acids are incorporated into these products. [The apparent exceptions of aspartic acid, glutamic acid and glycine are presumably due to pool phenomena.] An investigation of the metabolic properties of the polypeptides synthesized in HeLa cell mitochondria[38] has revealed a crucial role of

[38] P. Costantino and G. Attardi, *J. Biol. Chem.* **252**, 1702 (1977).

cytoplasmically-made proteins in controlling the rate of mitochondrial protein synthesis and in stabilizing the mitochondrial products.

The high degree of hydrophobicity of the products of mitochondrial protein synthesis, which results, at least in part, from the high proportion of hydrophobic amino acids they contain, accounts for their unusual solubility in organic solvents, in particular in chloroform-methanol mixtures. This solubility behavior, previously observed in material from rat liver and yeast, has been confirmed in the HeLa cell system. Here, most of the mitochondrial protein products have been selectively solubilized by a neutral chloroform-methanol mixture, giving a 20- to 30-fold purification with respect to the cytoplasmically synthesized proteins[28] (Fig. 3). At least six of the discrete products larger than 20,000 daltons have been

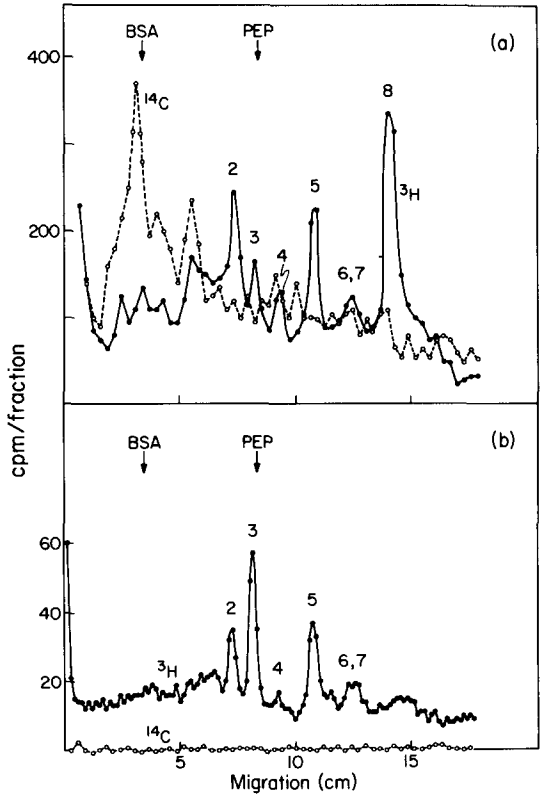

FIG. 3. Electrophoretic profiles of the proteins of the mitochondrial fraction extracted with neutral 2:1 chloroform-methanol mixture (b) and of the insoluble residue (a) from HeLa cells labeled for 48 hr with ^{14}C-arginine and ^{14}C-lysine and then for 1 hr with ^{3}H-isoleucine in the presence of 100 μg/ml emetine.[28]

found to be extracted to an appreciable extent by neutral chloroform-methanol. These solubility properties will undoubtedly facilitate the isolation in pure form of individual polypeptides synthesized in the organelles.

A greater degree of resolution of the polypeptides synthesized in HeLa cell mitochondria can be obtained by slab gel electrophoresis followed by autoradiography or fluorography. As shown in Fig. 4A, at least 19 discrete electrophoretic components (in the molecular weight range between 3500 and 51,000 daltons) can be recognized after fractionation on an SDS-polyacrylamide gradient slab gel (I) of the mitochondrial products labeled for 2 hr with ^{35}S-methionine in the presence of emetine. Similarly, at least 18 components are resolved by electrophoresis through an SDS-

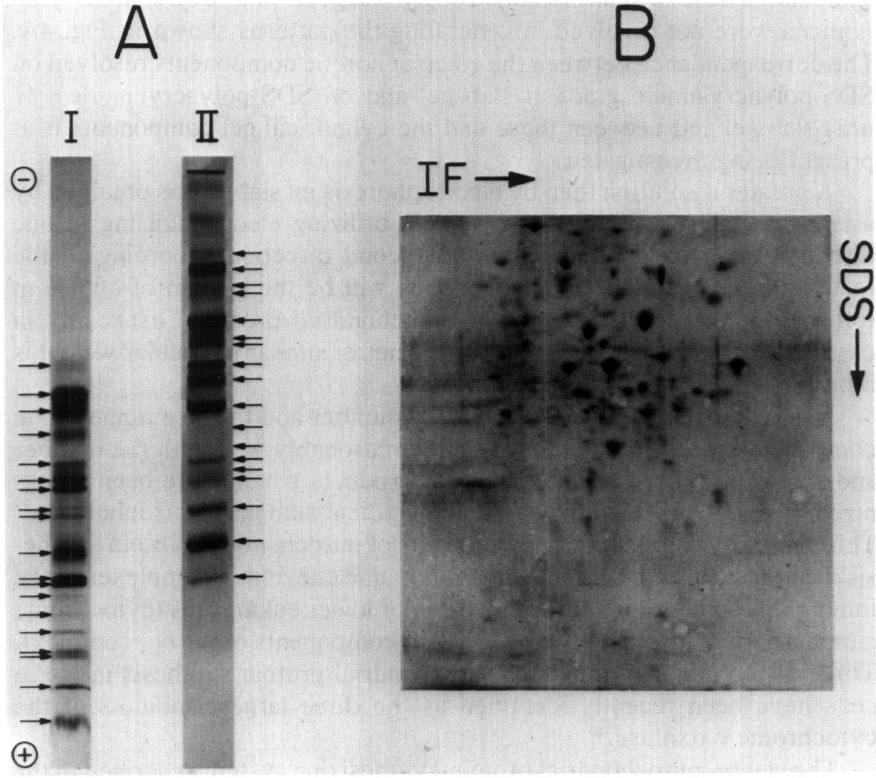

FIG. 4. (A) Fluorographs, after electrophoresis through SDS-polyacrylamide 15 to 25% gradient slab gel (I) or SDS-polyacrylamide/8 *M* urea slab gel (II), of the proteins from the mitochondrial fraction of HeLa cells labeled for 2 hr with ^{35}S-methionine in the presence of 100 μg/ml emetine. 16,000 cpm were run in slot I (5.5-day exposure), 13,000 cpm in slot II (10-day exposure). (B) Stained pattern of the proteins from the mitochondrial fraction of HeLa cells separated by two-dimensional electrofocusing-electrophoresis.

polyacrylamide/8 *M* urea slab gel (II). The actual number of polypeptides synthesized in HeLa cell mitochondria is probably larger than indicated by the discrete electrophoretic components recognized on either of the two slab gel systems shown here. Preliminary experiments of fractionation by bidimensional polyacrylamide/8 *M* urea and polyacrylamide gradient slab gel electrophoresis have indicated that some of the discrete bands shown in Fig. 4A consist of more than one component. Control experiments utilizing chloramphenicol have shown that all the above mentioned discrete components are synthesized in mitochondria. The same components were observed if PMSF was present throughout cell homogenization and preparation of the mitochondrial proteins, or if a total HeLa cell lysate was directly analyzed on either of the two slab gel systems. These observations strongly suggest that degradation phenomena were not involved in generating the patterns shown in Fig. 4A. The correspondence between the electrophoretic components resolved on SDS-polyacrylamide gradient slab gel and on SDS-polyacrylamide/8 *M* urea slab gel and between these and the cylindrical gel components is at present being investigated.

A greater resolution than by electrophoresis on slab gels is obtained by a two-dimensional fractionation system utilizing electrofocusing in one direction and electrophoresis in the second direction according to the technique of O'Farrell[36] (Fig. 4B). This will be the system of choice in future work on the biogenesis of mitochondrial proteins, especially in cases where resolution of molecular species of similar molecular weight is a problem.

It is interesting to note that both the number and the size range of the components resolved on slab gels agree reasonably well with the number and size of the discrete mitochondrial products which have been recognized in yeast by SDS-polyacrylamide gradient slab gel electrophoresis.[39] This similarity suggests that the products of mitochondrial protein synthesis in animal cells are components of the same enzymatic complexes of the inner mitochondrial membrane as those of lower eukaryotes (cytochrome *c* oxidase, oligomycin-sensitive ATPase, components of the bc_1 complex). Three of the major products of mitochondrial protein synthesis in HeLa cells have been recently identified as the three largest subunits of the cytochrome *c* oxidase.[40]

The *in vitro* mitochondrial protein-synthesizing system described in the previous section has been shown to exhibit characteristics of energy requirements and of response to inhibitors of protein synthesis similar to those which have been reported for mitochondria from other organisms.[17]

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Furthermore, the electrophoretic profile of the proteins synthesized *in vitro* is very similar to that of the mitochondrial proteins labeled *in vivo* in the presence of an inhibitor of cytoplasmic protein synthesis, although the rates of synthesis differ in the two systems.[41] This indicates that, qualitatively, the protein-synthesizing activity of the isolated mitochondria reproduces fairly closely the *in vivo* activity. This *in vitro* system may prove to be very valuable in future studies on the synthesis, processing and integration into the inner membrane of the mitochondrial polypeptide products.

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[8] Mitochondrial Ribosomes of *Neurospora crassa*: Isolation, Analysis, and Use

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Introduction

Mitochondrial ribosomes are central constituents of the mitochondrial transcription-translation system which is linked to the mitochondrial DNA.[1–5] The functional and structural characteristics of *Neurospora* mitochondrial ribosomes have been intensively investigated. They were the first mitochondrial ribosomes to be isolated and positively characterized as mitochondrial.[6,7] They represent a good system for the study of mitochondrial ribosomes and their reactions from a variety of reasons: (a) they can be isolated in high quantities from fast-growing *Neurospora* cells; (b) they can be obtained in pure condition, since it is possible to remove cytoplasmic ribosomes and membraneous material; (c) mutations in *Neurospora* affecting ribosome structure and functions are available. On the other hand, there are a number of allied problems in the biogenesis of mitochondria in which a knowledge of mitochondrial ribosomes is a prerequisite to a meaningful experimental approach.

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