bination of many functions and end up as a general sort of pattern more or less similar in all organ systems. It will be interesting to test this hypothesis by comparing the tRNA patterns of clones of lymphoid cells making a single antibody or endocrine organs which export a limited number of protein hormones.

However, even if this hypothesis were established it would be difficult to decide whether the tRNA variations were due to the variable requirements of the different cells (different messengers to be translated) or whether the synthesis of each of the tRNAs was independently regulated at the transcription level and the different tRNA species then regulated which messengers could be translated or controlled other cellular functions. In a previous study (Mushinski *et al.*, 1970) we could not show any particular effect on the *in vitro* translation of rabbit hemoglobin messenger using tRNA from one or another mouse plasmacytoma. Clearly further biological and biochemical studies are needed to better understand the meaning of these different distributions of isoaccepting tRNAs.

Acknowledgments

I thank Drs. Michael Potter and K. Robert McIntire for supplying the tumors used in this study, and Dr. Potter for his encouragement and helpful advice. I am grateful to Dr. I. Bernard Weinstein for his critical reading of the manuscript and to Dr. F. K. Millar for her assistance with the computerization of the calculations. The technical assistance of Miss Vivienne Nye and Miss M. Joy Martin is gratefully acknowledged.

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In Vivo Synthesis, Molecular Weights, and Proportions of Mitochondrial Proteins in *Neurospora crassa**

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ABSTRACT: Mitochondria of wild-type *Neurospora crassa* synthesized three major proteins, comprising 10% of their total protein, with molecular weights of 33,500, 27,700, and 17,500 in the relative proportions 64:20:16. Minor proteins, about 1% of the total, revealed weights of 11,000, 21,000, and 25,000. A mitochondrial mutant *mi*-1 also synthesized three proteins. For these, the overall specific activities were identical with those of the three proteins from wild type, but their proportion and electrophoretic mobility differed. Mitochondrial proteins in wild type of molecular weights between 2500 and 10,000 were synthesized by cytoribosomes but did not appear in the cytosol itself. They comprised 12% by weight of the total mitochondrial protein, and they apparently were not products of either turnover or incomplete synthesis.

Although it is now well established that mitochondria synthesize some of their own proteins, that these proteins are localized in the inner membrane and are insoluble in water, and that they comprise between 5 and 15% by weight of the total mitochondrial protein (Ashwell and Work, 1970), the physicochemical, genetical, and functional properties of these proteins are not presently well defined. Definition of these properties should contribute to an understanding of mitochondrial biogenesis (Roodyn and Wilkie, 1968) and differentiation (Flavell, 1971).

* Contribution No. 1372 of the Laboratories of Molecular Biology and Genetics, The University of Wisconsin, Madison, Wisconsin 53706. *Received March 25, 1971.* Research supported by the College of Agriculture and Life Sciences and the Graduate School of The University of Wisconsin, by the National Institutes of Health (GM-15751), and by The American Cancer Society (E-560). We thank Dr. E. S. Thomas for her assistance in the preparation of this manuscript.

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In this paper we correlate the intracellular sites of synthesis (either cyto- or mitoribosomes) of mitochondrial proteins in *Neurospora crassa* with their molecular weights and proportions.

Materials and Methods

Cell Culture and Radioisotope Procedures. Stock cultures of wild-type *N. crassa* 74A (strain 5.5A Yale, FGSC No. 936)[1] are maintained on Fries' minimal medium (Beadle and Tatum, 1945) supplemented with 2% sucrose and 1.5% agar at 30° for 3–4 days in dim light and then are transferred to continuous light at room temperature. Conidia from 1- to 2-week-old cultures are transferred to 100 ml of a medium enriched with yeast extract, Casamino Acids, and 1% glucose (Howell *et al.*, 1971) in a 1-l. florence flask to a final concentration of 1×10^6 per ml. The flasks are shaken with an eberbach reciprocal shaker at 150 excursions/min for 16 hr at 30°. Mycelia are collected by filtration, washed with 100 ml of Fries' medium, and transferred to a 500-ml florence flask with 50 ml of Fries' medium supplemented with 2% sucrose. The mutant *mi*-1 (FGSC No. 343, Isolate No. 3627-1A) is similarly cultured except that stocks are grown in flasks of the enriched medium on agar to enhance conidiation and mycelia are harvested at 24 hr before transfer to Fries' medium. The mutant was genetically pure as evidenced by its growth rate and its segregation in backcrosses to wild-type 74A (N. Howell, unpublished data).

Mycelia are incubated 1 hr at 30° on the reciprocal shaker in Fries' minimal sucrose medium. Then, if the inhibitors cycloheximide or chloramphenicol are used before the injection of radioactive leucine, they are put into the flasks. Cycloheximide is put into the medium to a final concentration of 100 μg/ml from an aqueous solution of 5 mg/ml, and the mycelia are incubated for 5–15 min before the injection of radioactive leucine into the medium. The variation in the time of incubation with inhibitor, before the incubation with radioactive leucine, did not affect the extent of inhibition of protein synthesis. Alternatively, powdered chloramphenicol is put into the medium to a final concentration of 2 mg/ml and the flask is shaken for 20 min before the injection of the radioactive leucine.

Radioactive leucine, either L-[U-^{14}C]leucine (50 μCi) or L-[4,5-^{3}H]leucine (100 μCi), is introduced into a 50-ml culture. The flask is shaken at 30° for 30 min. Then a solution of L-[^{12}C]leucine is pipetted into the culture to secure a final concentration of 2 mM. Mycelia are incubated in the shake culture for 30 min at 30°, collected by filtration on a Büchner funnel with Whatman No. 1 paper, washed rapidly with 200 ml of distilled water, and blotted dry with paper towels. The wet weight yields of mycelia are: 74A, 3.0–3.5 g; *mi*-1, 2.0–2.2 g.

Experiments with Two Inhibitors Together. When chloramphenicol and cycloheximide are used together, powdered chloramphenicol is dissolved to a concentration of 3 mg/ml in 50 ml of Fries' minimal medium with 2% sucrose in a 500-ml florence flask. Exponential-phase, growing mycelia from the enriched medium are incubated in Fries' medium, aseptically transferred into Fries' medium with chloramphenicol, and incubated in the shake flask for 15 min at 30°. Then cycloheximide is introduced into the flask; it is shaken for 7 min, labeling is as described, and mycelia are harvested.

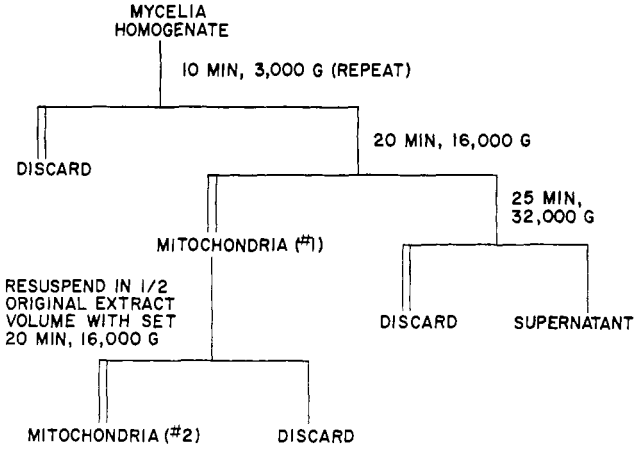

FIGURE 1: Flow diagram of differential centrifugation of mycelia homogenate.

Disruption of Mycelia and Differential Centrifugation. Wet mycelia (1 g) are mixed with 2 g of acid-washed, quartz sea sand and 10 ml of SET and ground 45 sec with mortar and pestle at 0°. Fractions of the homogenate are collected by differential centrifugation at 0–5° as in Figure 1.

Yields of mitochondrial pellet No. 2 (Figure 1) after differential centrifugation are (in milligrams of protein/g of mycelial wet weight): 0.2–0.3 for 74A and 0.5–0.6 for *mi*-1. Protein concentrations are determined by the procedure of Lowry *et al.* (1951).

Method of Dissolving Protein for SPAGE. Mitochondria or 32,000*g* supernatant are isolated and immediately heated in a buffered solution of the detergent sodium dodecyl sulfate to dissolve the protein and to inactivate proteolytic enzymes as follows. Mitochondria are dispersed in 0.3 ml of water. The solution is diluted with 0.075 ml of 10% SDS and 0.05 M H_3PO_4 (previously adjusted to pH 6.8 with Tris) to make a final solution of 0.7–1.5 mg of protein/ml in 2% SDS and 0.01 M H_3PO_4 at pH 6.8. The 32,000*g* supernatant is similarly diluted with detergent solution. The solutions are immediately heated to 100° for 3 min, cooled, and adjusted to contain 8 M urea and 1% mercaptoethanol.

Sucrose Density Gradient Centrifugation. The crude mitochondrial pellet obtained after one 16,000*g* centrifugation (no. 1, Figure 1) is suspended in SET and layered on a 5-ml 30–60% (w/v) sucrose gradient containing 1 mM EDTA and 50 mM Tris-Cl (pH 7.4, 25°). The gradient is centrifuged at 4° for 2 hr at 50,000 rpm in a SW 65 Ti rotor of an L2-65B Spinco centrifuge. Samples, each containing 8 drops, are collected from a hole in the bottom of the centrifuge tube. One part of each sample is saved at 4° to be assayed the following day for radioactivity and cytochrome oxidase; the other part is immediately dissolved with SDS, urea, and mercaptoethanol as just described.

SDS–Polyacrylamide Gel Electrophoresis (SPAGE). Two systems of SPAGE are employed (Swank and Munkres, 1971). In each, the gels contain 0.1% SDS and 8 M urea. In the first (SPAGE-1), the concentrations of acrylamide and bisacrylamide are 8 and 0.27%, respectively. In the second (SPAGE-2) these concentrations are 12.5 and 1.25%. Under our conditions, the first resolves proteins in the molecular

[1] Abbreviations used are: FGSC, from Fungal Genetics Stock Center, Humboldt College, Arcata, Calif. 04421. SET, solution containing 0.5 M sucrose, 1 mM EDTA, and 0.05 M Tris-Cl (pH 7.4); SDS, sodium dodecyl sulfate; BPB, bromophenol blue; SPAGE, SDS-polyacrylamide gel electrophoresis.

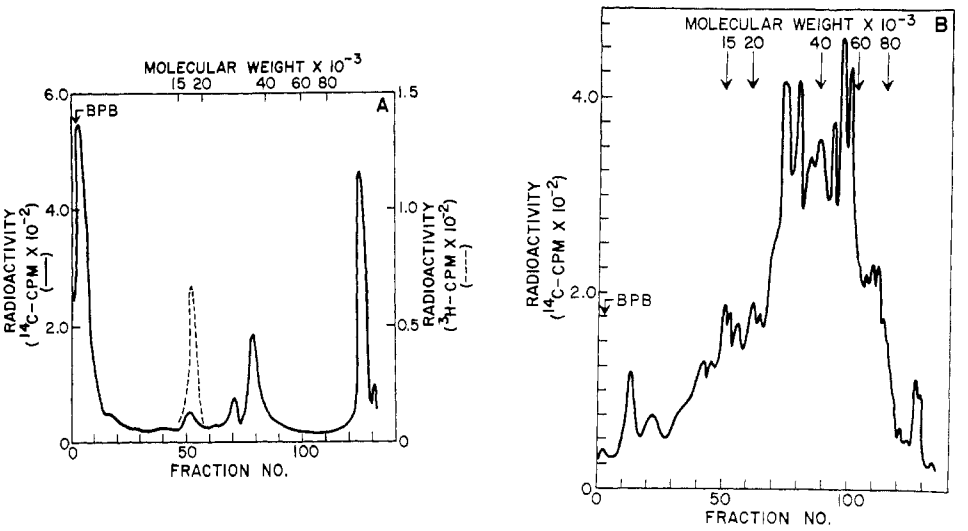

FIGURE 2: Inhibition of the *in vivo* incorporation of [^{14}C]leucine into mitochondrial proteins by cycloheximide. (A) Mitochondria (180 μg of protein, no. 2, Figure 1) from mycelia of wild-type 74A labeled with [^{14}C]leucine in the *presence of cycloheximide* were dissolved in SDS, urea, and mercaptoethanol. Their proteins were separated in SPAGE-1 together with bromophenol blue and [^{3}H]myoglobin. Fractions (1 mm) of the gel were assayed for radioactivity: ^{14}C (—) and ^{3}H (--) (expt 293-1). The anode is on the left in this and in all subsequent figures. The position of bromophenol blue (BPB) is indicated by the arrow. (B) Mitochondria (80 μg of protein, no. 2) labeled in the absence of cycloheximide were analyzed as in part A (expt 281-1). The molecular weight scale indicated on this and subsequent figures was calculated from separate experiments with reference to myoglobin (Swank and Munkres, 1971).

weight ranges of 100,000–10,000; the second, 40,000–2000. Gels are 13 cm in length and 0.6 cm in diameter. A potential of 7.5 V/cm is applied for 17–18 hr at 25°. To mark the electrophoretic front, 0.005 ml of a bromophenol blue solution (0.02% BPB in 40% sucrose and 0.01 M H_3PO_4 adjusted to pH 6.8 with Tris) is layered beneath the sample solution. In some experiments, 0.020 ml of a solution of sperm-whale myoglobin (which was methylated with [^{3}H]dimethyl sulfate) is also added to the sample solution as an internal standard (Kiehn and Holland, 1970). Molecular weights are determined as previously described (Dunker and Rueckert, 1969; Swank and Munkres, 1971). Proteins are stained in gels with coomassie brilliant blue with the solvent previously described for electrophoretic destaining, but the washing rather than the electrophoretic destaining procedure is used because the latter tends to remove oligopeptides from the gel.

Gels are divided into 1-mm (±1.3%) segments with a Gilson automatic fractionator. The crushed segments are washed directly into scintillation vials with 0.36 ml of 0.1% SDS and 0.1 N NaOH. After the addition of 1.5 ml of 0.2 N NaOH to each vial, they are incubated for 24 hr at 37°. A solution (5 ml) containing toluene, Packard Permafluor (25×), and Triton X-100 in the proportions 1.92:0.08:1.00 (v/v) is then placed into each vial. The vials are shaken on a reciprocal shaker for 1 hr at 5° and their radioactivity is determined with a Packard scintillation counter set at 5° and a 5- or 10-min counting interval.

Chemicals. Chloramphenicol was obtained from Sigma Chemical Co.; cycloheximide from the Upjohn Co.; uniformly labeled L-[^{14}C]leucine (specific activity 216 mCi/mmole) from Calbiochem; and L-[4,5-^{3}H]leucine (specific activity 53.9 Ci/mmole) from Schwarz BioResearch Co.

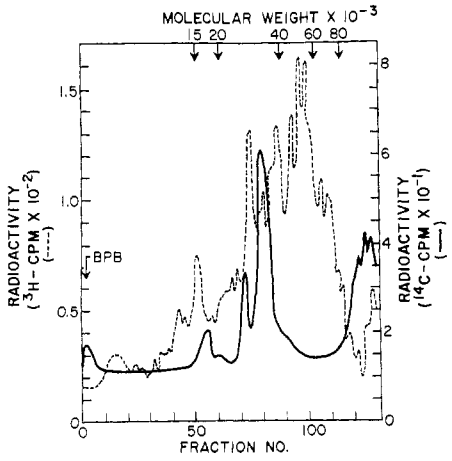

FIGURE 3: Comparative inhibition *in vivo* of the incorporation of radioactive leucine into mitochondrial proteins by cycloheximide or chloramphenicol. Incorporation of [^{14}C]leucine into 53 μg of mitochondrial protein in the presence of cycloheximide (—). Incorporation of ^{3}[H]leucine into 97 μg of mitochondrial protein in the presence of chloramphenicol (--) (expt 288-2). Other conditions as in Figure 2.

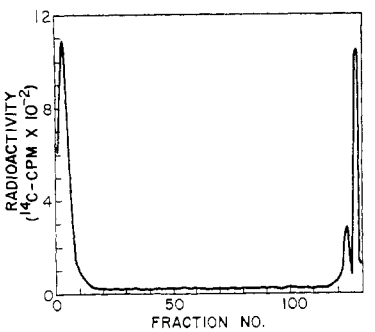

FIGURE 4: Inhibition *in vivo* of the incorporation of radioactive leucine into mitochondrial proteins by the combined action of cycloheximide and chloramphenicol. Mitochondria (90 μg of protein) from mycelia labeled with [^{14}C]leucine in the presence of both cycloheximide (100 μg/ml) and chloramphenicol (3 mg/ml) were analyzed as in Figure 2A (without myoglobin).

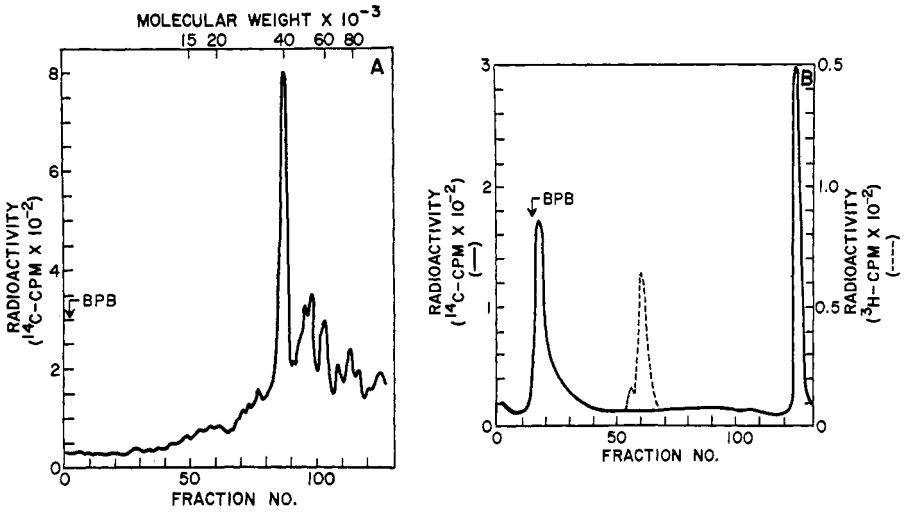

FIGURE 5: Inhibition of the *in vivo* incorporation of radioactive leucine into extramitochondrial proteins by cyclohexmide. (A) The 32,000*g* supernatant (Figure 1, 58 μg of protein) from mycelia labeled with [^{14}C]leucine in the *absence of cycloheximide* was analyzed as in Figure 2 (expt 281-3). (B) The 32,000*g* supernatant (180 μg) of protein) from mycelia labeled with [^{14}C]leucine in the *presence of cycloheximide* was analyzed as in Figure 2 (expt 293-3). [^{14}C]- (—) and [^{3}H]myoglobin (--).

Results

Products of Intrinsic Synthesis. Electropheroradiograms of mitochondrial proteins indicate that only three major proteins were synthesized with cycloheximide inhibition, but at least 25 were synthesized without that inhibition (some of the radioactivity at the top of the gel is free leucine) (Figure 2A,B). Figure 3 indicates that the synthesis was inhibited more by cycloheximide than by chloramphenicol. Because the chloramphenicol-resistant proteins were so numerous, it is not obvious whether the synthesis of the three cycloheximide-resistant ones was *completely* sensitive to chloramphenicol, but at least that synthesis was *partially* sensitive to it (compare Figures 2B and 3). Furthermore, when both inhibitors were used together, none of the three proteins was labeled (Figure 4). To determine if the three arose by resistance of cytoribosomes to cycloheximide, the effect of that drug upon the synthesis of cytosol proteins was tested. Because the synthesis of the cytosol proteins was completely inhibited by cycloheximide (Figure 5A,B), they were not the source of the three mitochondrial proteins. Rather, we conclude that these three major proteins were synthesized by mitoribosomes.

We observed material which was at the top of the gels, which was from either mitochondria or cytosol, and whose synthesis was resistant to both cycloheximide and chloramphenicol (Figures 2A,B, 4, etc.). In gels with bigger pores (5% acrylamide) it still remained at the top, indicating that it was either heavy (>200,000), or neutral in charge or both. Dialysis of mitochondria against water did not remove it, indicating that it was either large or in firm association with them. The following experiments indicated that such material was scarcely found in mitochondria of greater purity.

Crude mitochondria that were labeled *in vivo* with radioactive leucine in the presence of cycloheximide and subjected to isopycnic centrifugation revealed the distribution of cytochrome oxidase and radioactivity in Figure 6. Relative to cytochrome oxidase as a marker of either mitochondria or

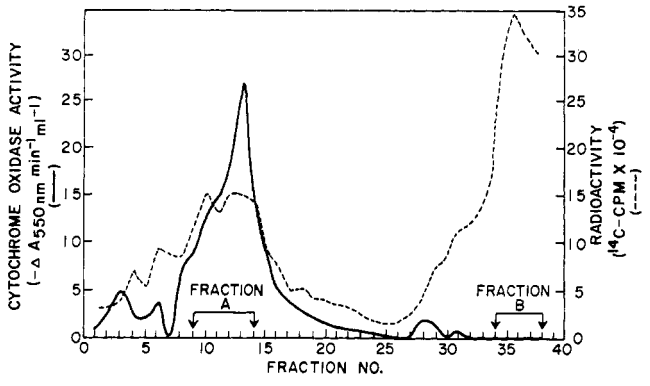

FIGURE 6: Sucrose density gradient profile of cytochrome oxidase activity and radioactivity of crude mitochondrial fraction labeled *in vivo* in the presence of cycloheximide. Crude mitochondria (1.2 mg, Figure 1, no. 1) from mycelia labeled with [^{14}C]leucine in the presence of cycloheximide were centrifuged in a sucrose density gradient as described in Methods and assayed for cytochrome oxidase (—) and radioactivity (--).

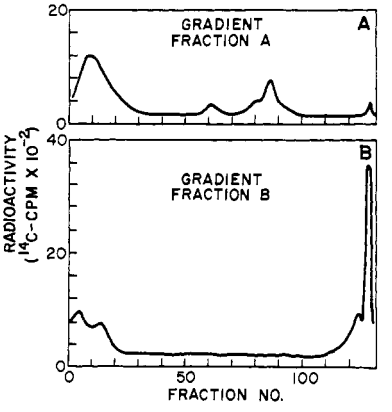

FIGURE 7: SPAGE analysis of fractions from sucrose density gradient centrifugation of crude mitochondria. (A) fraction A (90 μg of protein) from the experiment of Figure 6 was assayed for radioactivity after electrophoresis as described in Figure 2 (expt R-2-2). (B) Fraction B (25 μg) from the experiment of Figure 6 was assayed as in Figure 2 (expt R-2-3).

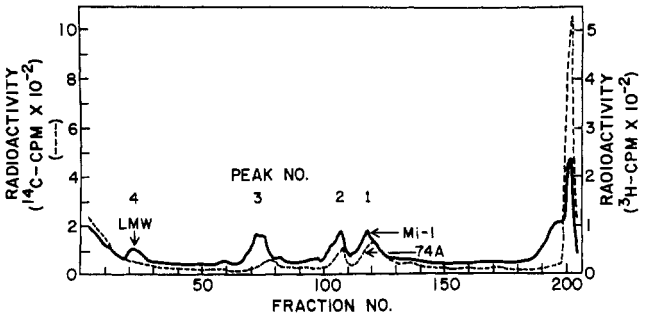

FIGURE 8: SPAGE coelectropheroradiogram of cycloheximide-resistant mitochondrial proteins of *N. crassa* wild-type 74A and mutant *mi*-1. Mitochondria (127 μg) from mycelia of 74A labeled with [^{14}C]leucine in the presence of cycloheximide were coelectrophoresed (SPAGE-1) with mitochondria (200 μg) of *mi*-1 mycelia labeled with [^{3}H]leucine in the presence of cycloheximide. The combined mitochrondrial proteins were dissolved, electrophoresed in 200 $\times$ 0.6 mm polyacrylamide gels, and analyzed as in Figure 2 (expt 295).

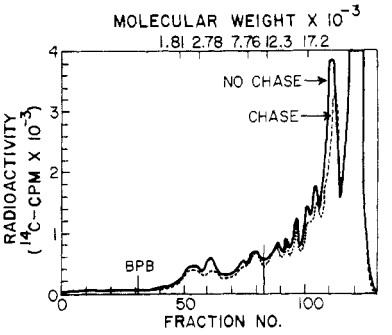

FIGURE 9: SPAGE analysis of mitochondrial proteins after the *in vivo* incorporation of [^{14}C]leucine with or without a postincubation with [^{12}C]leucine. Whole mitochondria (before and after pulse chase *in vivo*) were dissolved, the proteins were electrophoresed, and analyzed in separate gels (SPAGE-2 system). No chase: mitochondrial proteins (180 μg) from mycelia that were incubated for 30 min with [^{14}C]leucine (expt 275-1). Chase: mitochondrial protein (180 μg) was labeled as above followed by a 30-min chase with 500-fold molar excess of [^{12}C]leucine (expt 275-2).

their inner membrane (Cassady and Wagner, 1971), 56% of the radioactivity was not mitochondrial (fractions 26–38, fraction B). (The occurrence of several bands of cytochrome oxidase probably indicates damaged mitochondria because extraction by hypotonic lysis of protoplasts yielded only one band at the density of the main one in Figure 6 (Scott *et al.*, 1971).) Proteins of fractions A, B, and the original mitochondria were compared (Figure 7). The gradient purification led to a 9-fold reduction of the concentration of the unknown material in mitochondria, with no change in the relative proportions and the molecular weights of the other three proteins. None of these three was in fraction B. Therefore, the three proteins synthesized by mitochondria, unlike the unknown material, were firmly associated with mitochondria and were not displaced by the initial differential or the subsequent isopycnic centrifugation.

The mutant *mi*-1 exhibits non-Mendelian inheritance (Mitchell and Mitchell, 1952), defects in electron transport (Eakin and Mitchell, 1970), structurally abnormal mitochondria (K. D. Munkres, unpublished data), and transmission of its hereditary factor by way of its mitochondria (J. F. Wilson, personal communication). The proteins synthesized by its mitochondria also differed from those of wild type in proportion and electrophoretic mobility. The greater mobility of the three mutant proteins relative to tritiated myoglobin indicated, by the simplest interpretation, that their molecular weights were about 4% less than those of wild type (Table I). That difference was also observed when the wild type and mutant proteins were electrophoresed together (Figure 8), and was consistently observed in three or four preparations of each (Table I).

TABLE I: Molecular Weights and Relative Proportions of the Polypeptides Synthesized by Mitochondria of *N. crassa*.[a]

	Strain			
	Wild-Type 74A		Mutant *mi*-1	
Peak No.	Mol Wt	Rel Prop.[b]	Mol Wt	Rel Prop.[b]
1	33,500 ± 1800	64 ± 10	32,000 ± 1500	43 ± 8
2	27,700 ± 1400	20 ± 6	26,600 ± 1100	24 ± 6
3	17,700 ± 500	16 ± 4	17,000 ± 400	24 ± 6
4	>10,000	*c*	>10,000	9 ± 3

[a] Mean and standard deviations of three or four experiments. [b] Relative proportion: the area under each of the three peaks on electropheroradiograms was integrated, corrected for background radioactivity, and expressed as percentage of the sum of the areas. [c] Trace amount, less than 1%. Peaks 4 in mutant and wild type may not be equivalent in molecular weight.

Products of Extrinsic Synthesis (Oligopeptides). Because the SPAGE-1 system in the experiments just described did not resolve the proteins whose weights are below 10,000 (Figure 2B), we used the SPAGE-2 system to do so. The experiments just described indicated that the synthesis of these oligopeptides, although they were unresolved, was inhibited by cycloheximide but not by chloramphenicol (Figures 2A,B, 3, and 4). The results of subsequent experiments, which were identical with these previous ones except with SPAGE-2 rather than SPAGE-1, confirmed the differential inhibition by the two antibiotics. Therefore, these little proteins were synthesized by way of cytoribosomes, but were not retained in the cytosol itself (Figure 5A; Swank *et al.*, 1971).

The synthesis and the molecular weight of a component (peak 4, Figure 8), prominent in the mitochondria and the cytosol of mutant and barely detectable only in mitochondria of wild type was, unlike the oligopeptides just described, resistant to cycloheximide and below 2000; it is not clear whether its synthesis is sensitive to chloramphenicol. More refined analysis of its molecular weight and that of the material at the top of the gel was not possible with the electrophoretic methods.

Figure 9 illustrates the effect of a "pulse chase" upon the proportions of mitochondrial proteins. At least four radioactive oligopeptide classes were observed, constituting 11–13% of the radioactivity of all of the protein. Since the decrease in the proportion of the oligopeptides was 8%, equivalent to the decrease that was calculated on the basis of only a dilution by the chase (where 30 min is equivalent to one-fifth of the generation time), apparently no oligo-

peptide class consisted of either intermediates of rapid turnover or products of incomplete synthesis. Additional studies of the biogenesis and other properties of these oligopeptides are reported in the accompanying paper (Swank *et al.*, 1971).

Validity of the Molecular Weight Determinations. Mitochondria were routinely heated at 100° for 3 min in buffered SDS solution which was then adjusted to 8 M urea and 1% mercaptoethanol. These conditions dissolved all mitochondrial protein: 98% of the radioactivity of fully labeled mitochondria migrated into the gels after electrophoresis. Heating of proteins in SDS solution at 100° prevents proteolysis that may occur at room temperature (Pringle, 1970). Other experiments indicated that enzymatic cleavage of peptide bonds was not an artifact in these preparations (Swank *et al.*, 1971). Heating at 100° probably did not cleave labile peptide bonds because no cleavage of 17 standard proteins was observed under these conditions (Swank and Munkres, 1971). Moreover, the patterns of stained mitochondrial proteins after SPAGE were identified in samples treated at either 60 or 100°. That the products of mitochondrial protein synthesis were stable in the SDS–urea solvent was also indicated by the observation of quantitatively identical electropheroradiograms from proteins that were stored either 1 day at 25° or 6 days at 5°.

Since electropherograms were the same (either by absorbancy or radioactivity measurements) regardless of whether the electrophoresis was performed with or without mercaptopropionic acid in the cathodic buffer, apparently very few of the mitochondrial proteins have rapidly oxidizable disulfides, such as was observed with some proteins from other sources (Swank and Munkres, 1971).

The coefficient of variation of the means of the molecular weights of the proteins synthesized by mitochondria in these experiments was about 5% (Table I). Since this degree of accuracy was obtained for a large number of standard proteins in the molecular weight range of interest here (Dunker and Rueckert, 1969; Swank and Munkres, 1971), these estimates were probably a close approximation to the actual weights. A few proteins such as lysozyme and ribonuclease (Dunker and Rueckert, 1969), however, in this type of analysis may deviate from the actual weight by as much as 15%.

Discussion

Mitochondria of *N. crassa* synthesized six classes of their proteins whose molecular weights were *33,500*, *27,700*, 25,000, 21,000, *17,500*, and 11,000. (The weights of the three predominant ones, together comprising 10% of the total mitochondrial protein, are italicized.) The radioactivity of the protein weighing 21,000 was about 1% of the radioactivity of all protein (Figure 3). Two other proteins, similar in magnitude of radioactivity to the 21,000 one and weighing 25,000 and 11,000, were observed with the SPAGE-2 system. In addition, experiments with SPAGE-1 with gels that were 200 rather than 130 mm in length, and in which the time of electrophoresis was 24 rather than 17 hr, indicated that the 33,500 component may be separated into two components of closely related weights and proportions (G. I. Sheir, unpublished). Thus, clearly six and probably seven classes were synthesized by these mitochondria.

At least two additional classes whose molecular weights were above 200,000 and below 2000 also may be synthesized by mitochondria. But because of the limitations of the electrophoretic analysis and the interference by a high molecular weight component whose synthesis was resistant to both cycloheximide and chloramphenicol, a detailed analysis of these classes was not possible.

This number of molecular weight classes of proteins was similar to that reported by Sebald *et al.* (1968, 1969). They separated the proteins synthesized by *N. crassa* mitochondria by electrophoresis on the basis of both charge and size and observed four major and six minor ones. Because a similar number was observed with either *in vitro* or *in vivo* synthesis, they concluded that the same proteins were synthesized in the two conditions. Unfortunately, however, neither the comparative electrophoretic mobilities nor the individual proportions of the proteins synthesized in the two conditions were reported. We may conclude, however, from these observations that since about the same number of proteins was observed by either method of electrophoretic analysis, there was little or no charge heterogeneity within each weight class, a conclusion that was confirmed by analysis of the isoelectric points of the proteins (G. I. Sheir and K. D. Munkres, in preparation).

The molecular weights observed here are similar to those reported by Yang and Criddle (1970) in *Saccharomyces carlsbergensis:* 15,000, 20,000, 26,500, and 40,000. Although these workers observed fewer classes, it is likely that their purification procedure eliminated some of the minor ones.

With the assumption that the leucine composition of the proteins synthesized by mitochondria is the same as that of proteins synthesized for mitochondria by cytoribosomes, we calculate (after correction for the radioactive material which contaminated mitochondria and which appeared not to be synthesized by either cyto- or mitoribosomes) that 10.7% of the total mitochondrial protein was synthesized by mitoribosomes. This number was similar to that obtained by others, not only in *N. crassa* but also in *S. cerevisiae* and rats. With *N. crassa*, Sebald *et al.* (1971) estimated, after correcting for leucine pool size, a value of 8% and Hawley and Greenawalt (1970) estimated, without that correction, 14%. In *S. cerevisiae*, Kellerman *et al.* (1971) reported 12% in derepressed and 5% in glucose-repressed cells and Schweyen and Kaudewitz (1970) reported 9%. In agreement with these estimates of proportion of mitochondrial proteins synthesized by mitochondria *in vivo*, Beattie *et al.* (1970) estimated 10% with rat liver mitochondria *in vitro*.

Although there is evidence in the case of a mutant (*mi*-1) in *N. crassa* to indicate that this mutation, presumably of a structural gene in mitochondria, leads to structural and functional alteration of mitochondrial membrane protein (Woodward and Munkres, 1966; Munkres and Woodward, 1966; G. I. Sheir and K. D. Munkres, unpublished data), Sebald *et al.* (1968) report that mutant's mitochondria fail to synthesize *in vitro* a protein synthesized by wild type mitochondria. Thus, they infer that the mutant mitochondria are defective in the *synthesis* of mitochondrial protein rather than being defective in the *structure* of that protein. However, it is not clear whether the latter experiments were adequate to detect structural alterations.

Our experiments failed to substantiate either the claim of Sebald *et al.* (1968) of a missing cycloheximide-resistant protein from mutant *mi*-1 or the conclusion of Neupert *et al.* (1971) that the mutant is defective in mitochondrial protein synthesis. However, there are a number of differences between their procedures and ours. First, the effect of culture age may be an important variable. Sebald and Neupert grew wild type

for 2 days and mutant for four into stationary phase; we examined, by the use of enriched medium, young exponentially growing cells between 16 and 24 hr old. Thus, their observations may be attributed to autolytic or regulatory differences unrelated to the mutation. The nature of the culture medium is a second important variable. Sebald and Neupert used a minimal salts–glucose medium, which appears to repress mitochondrial biogenesis as evidenced by differences in cytochrome oxidase activity, the yields of mitochondria in isolation, and the appearance of mitochondria *in situ* (Howell *et al.*, 1971; K. D. Munkres and C. A. Zuiches, unpublished data); we used an enriched medium containing yeast extract, Casamino Acids, and glucose. Third, Sebald used an electrophoretic system which does not allow analysis of either molecular weight or charge of proteins and may not completely dissociate protein aggregates. Fourth, Sebald used isolate no. 3637-1A; we, 3627-1A. Finally, he analyzed an insoluble subfraction of mitochondrial protein after *in vitro* synthesis: the *mi*-1 mitochondria were 10-fold less active than wild type, and in his autoradiographic comparison of electrophoretic patterns, there was 5-fold less mutant protein radioactivity than in wild type.

These considerations emphasize important physiological and biochemical factors in the analysis of such mitochondrial mutants. However, because the greatest uncertainty in studies of this and similar mutants stems from the lack of detailed genetic analyses, one can readily conceive of genetic events which might lead to quantitative and qualitative alterations in *both* the mitochondrial translational system and the products of that translation.

Whether the apparent differences observed here between wild type and mutant protein mobilities represent differences in molecular weight or charge or both remains to be determined. Although a general opinion is that SPAGE separates proteins *only* on the basis of their molecular weight, that generality is only true within the routine error in reproducibility of about 5%; however, SPAGE coelectrophoresis of differentially labeled proteins as applied here is more sensitive and can reveal a single charge difference and difference in mobilities as low as 1% (Strauss and Kaesberg, 1970).

At present, we shall not speculate about possible genetic or other mechanisms by which the apparent differences between the mutant and wild-type mitochondrial proteins could arise. Since the mutant is not isochromosomal to the wild-type 74A, the differences may be unrelated to the extrachromosomal mutation.[2] Experiments about the genetics of this mutant and similar newly isolated ones will be reported (Howell and Munkres, 1971).

Unknown Functions of Proteins Synthesized by Mitochondria. The frequency distribution of numbers of mitochondrial polypeptides in *N. crassa* as a function of their molecular weight in the range 2000–100,000 is approximately normal (K. D. Munkres, unpublished data), and the weights of those synthesized by mitochondria are closely distributed about the mean of that distribution; hence the latter are not unusual in their molecular weights. They are unusual, however, in their insolubility and location in the inner mitochondrial membrane (Ashwell and Work, 1970). Because of the molecular weights, the insolubility, the location, and in some cases genetic and synthetic observations by others too lengthy to review here, we suggest that some of the following proteins conceivably could be the functional equivalents of the ones synthesized by mitochondria: oligomycin-sensitivity-conferring protein, 18,000 (MacLennan and Tzagoloff, 1968); adenosine triphosphatase subunit, 25,000 (Senior and Brooks, 1970); nonheme iron protein, 25,000 (Rieske *et al.*, 1964); succinate dehydrogenase subunit, 30,000 (Righetti and Cerletti, 1971); cytochrome *b*, 28,000 (Goldberger *et al.*, 1961); and cytochrome oxidase subunit, 26,500 (Chuang and Crane, 1971).

[2] Preliminary studies of an isolate of *mi*-1, derived from seven backcrosses to 74A, indicate that only the difference in peak 3 (mol wt 17,500) persists upon backcrossing (G. I. Sheir, unpublished data). That result could be interpreted as an indication that the differences in the other proteins reflect nuclear gene differences between these strains that clearly are not isogenic with respect to nuclear genes. On the other hand, it is not known and cannot be determined with existing methods whether the original mutant differs from the wild type in one or several extrachromosomal genes. Furthermore, since the genetic fate of extrachromosomal (mitochondrial) genes in backcrosses is not known, very little can be deduced about the genetic basis of the differences in the proteins. That there are indeed differences in the properties of these proteins, whether in the original or in the backcrossed mutant, is further substantiated in isoelectric focusing experiments (G. I. Sheir, unpublished data).

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Subcellular and Generic Distribution, Molecular Weights, and Proportions of Oligopeptides*

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ABSTRACT: *Neurospora crassa* mitochondria exhibited six proteins of molecular weight in the range of 2600 to 8800, together comprising 10–13% by weight and 35–45% by mole proportion of mitochondrial protein. A submitochondrial fraction, designated P_0, yielded the same molecular weights in two methods of molecular sieving. An insoluble membrane fraction and fraction P_0 were enriched in the concentration of these oligopeptides whose molecular weights were the same as those in whole mitochondria of *N. crassa*. In mitochondria of *Saccharomyces cerevisiae* and bovine heart, oligopeptides were similar to those of *N. crassa* in number of classes, molecular weights, and proportion. We observed the same classes and an enrichment of oligopeptides in both the electron transport particles and fraction P_0 of bovine mitochondria, but only a small quantity of one peptide of 3500 in bovine mitochondrial adenosine triphosphatase. Only small amounts and numbers of classes of oligopeptides appeared in the cytosol or microsomes of *N. crassa* or in purified rat liver nuclear membrane, and none appeared in synaptic complex of porcine brain. Nuclei of *N. crassa* and both neurofibrils and myelin of porcine brain, all of which were probably contaminated by mitochondria, yielded fewer classes and lower proportion of oligopeptides than did pure mitochondria. No experiment thus far has yielded any evidence indicating that the oligopeptides are products of proteolysis either *in vivo* or *in vitro;* rather, they appear to be synthesized directly by way of cytoribosomes.

Electrophoresis in polyacrylamide gels which contain the detergent sodium dodecyl sulfate (Shapiro and Maizel, 1969; Dunker and Rueckert, 1969; Weber and Osborn, 1969) (SPAGE[1]) permits analysis of monomeric molecular weights and proportions of polypeptides[2] in complex mixtures such as viruses (Dunker and Rueckert, 1969), ribosomes (Dzionara *et al.*, 1970), membranes (Schnaitman, 1969; Kiehn and Holland, 1970a), and even whole cells (Kiehn and Holland, 1970b). Studies of the molecular weights of mitochondrial proteins of *Neurospora crassa* and bovine heart by these SPAGE methods, and by the method of Hedrick and Smith (1968) reveal, however, that a substantial proportion of the total protein is less than 10,000 (Munkres *et al.*, 1971; Swank *et al.*, 1971), and that the methods are inadequate for high resolution of these small proteins. This paper describes the application of two improved methods of electrophoresis in studies of the subcellular and generic distribution, molecular weights, and proportions of oligopeptides in the range of 2000–10,000 in eucaryotic cells, with emphasis upon *N. crassa*.

* Contribution No. 1372 of the Laboratories of Molecular Biology and Genetics, The University of Wisconsin, Madison, Wisconsin 53706. *Received March 25, 1971.* Research supported by the College of Agricultural and Life Sciences and the Graduate School of The University of Wisconsin, by the National Institutes of Health (GM-15751), and by The American Cancer Society (E-560). We thank Dr. E. S. Thomas for her assistance in the preparation of this manuscript.

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[1] Abbreviations used are: SDS, sodium dodecyl sulfate; BPB, bromophenol blue; ETP, electron transport particles; P_0, mitochondrial oligopeptide fraction; SPAGE, SDS–polyacrylamide gel electrophoresis; Temed, *N,N,N′,N′*-tetramethylethylenediamine.

[2] Here oligopeptides refers to polypeptides whose molecular weights are below 10,000, and polypeptides to all monomeric proteins.

Materials and Methods

Methods of stock maintenance and culture, and *in vivo* labeling with radioactive leucine were described by Swank *et al.* (1971).