# BIOCHEMICAL AND FUNCTIONAL CHARACTERIZATION OF THE PRODUCTS OF MITOCHONDRIAL PROTEIN SYNTHESIS IN HeLa CELLS

Thesis by

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To my parents

who made it all possible

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**Abstract**

Various aspects of the biology of mitochondria have been studied by many investigators. Recently, much of this research has concentrated on the molecular biology and biogenesis of the organelle. Investigations presented here more carefully resolve and characterize the products of mitochondrial protein synthesis.

The in vivo incorporation of each of the 20 common amino acids, under conditions allowing translation only on mitochondrial ribosomes, is investigated. Nearly all of the common amino acids are incorporated into products displaying the electrophoretic pattern characteristic of polypeptides synthesized by HeLa cell mitochondria.

A number of improvements are devised to increase the resolution of these mitochondrially synthesized polypeptides. A highly resolving gradient polyacrylamide SDS gel system is described which resolves these proteins into 19 discrete species. A second SDS-urea gel system is described which resolves 17 species. The correspondence of species between these two high resolution and the earlier electrophoretic systems is determined.

Experiments show these species to reflect in vivo polypeptides of mitochondrial origin and are probably not related as precursor to product. By combination of the SDS-urea and gradient gel systems, a bidimensional gel reproducibly resolves 25 discrete polypeptide components which satisfy the criterion for mitochondrial synthesis.

Estimation of the molecular weights of these species, when summed up and compared to the size of the human mitochondrial genome, can fully deplete the non-overlapping single strand coding capacity.

As a model for mitochondrial membrane enzyme biogenesis, the cytochrome c oxidase enzymatic complex is chosen for study. This enzyme is purified from human placental mitochondria and characterized to contain seven discrete subunits when analyzed on an SDS-urea gel. By electrophoretic analysis, the human enzyme is quite similar to that purified by any of a number of different methods from beef heart mitochondria.

When HeLa cell mitochondria, labeled under conditions allowing translation only on mitochondrial ribosomes, are added to human placental mitochondria and the cytochrome c oxidase complex purified, the three largest of the seven purified subunits contain labeled components. Consequently, the three large subunits of the enzyme complex are synthesized on mitochondrial ribosomes; conversely the four small subunits must be synthesized on cytoplasmic ribosomes.

## Table of Contents


| | | Page |
|---|---|---|
| **Chapter 1** | An Introduction to the Biology of the Mitochondrial System | 1 |
| | Introduction | 2 |
| | Morphology | 3 |
| | Molecular biology | 6 |
| | Organelle function and biogenesis | 10 |
| | Mitochondrial protein synthesis | 11 |
| | Cytochrome c oxidase | 16 |
| | References | 20 |
| **Chapter 2** | In vivo Incorporation of Different Amino Acids into Electrophoretically Characteristic Polypeptides Synthesized by HeLa Cell Mitochondria | 32 |
| | Summary | 33 |
| | Introduction | 33 |
| | Materials and Methods | 35 |
| | Results | 36 |
| | Discussion | 38 |
| | References | 42 |
| **Chapter 3** | Biogenesis of Mitochondrial Proteins in HeLa Cells | 43 |
| | Introduction | 44 |
| | Experimental Procedures | 46 |
| | Results | 51 |
| **Chapter 4** | High Resolution Analysis and Characterization of the Mitochondrial Translation Products in HeLa Cells | 58 |
| | Summary | 59 |
| | Introduction | 60 |
| | Experimental Procedures | 61 |
| | Results | 64 |
| | Discussion | 69 |
| | References | 77 |
| **Chapter 5** | Isolation, Subunit Composition and Site of Synthesis of Human Cytochrome c Oxidase | 96 |
| | Abstract | 98 |
| | Introduction | 99 |
| | Experimental Procedure | 101 |
| | Results | 108 |
| | Discussion | 117 |
| | References | 127 |
| **Chapter 6** | Update and Outlook for the Future | 150 |
| | Mitochondrial 4S RNA species | 151 |
| | A quick comment of evolutionary relationships | 154 |
| | Recent work on the cytochrome c oxidase and the the other respiratory chain complexes | 156 |
| | References | 162 |

# Chapter 1

## An Introduction to the Biology of the Mitochondrial System

## Introduction

All eukaryotic cells, by definition, contain mitochondrial organelles, structures which display a limited degree of autonomy from the nuclear genome. Nevertheless, mitochondrial organelle biology is usually associated with two particular concepts, its function as the energy source for cellular metabolism and its proposed origin from symbiotic evolution. The first is certainly true since the mitochondrial inner membrane contains the oxidative phosphorylation chain, which converts 95% of the recovered metabolic energy from glucose into the usable form stored in ATP (Hogeboom et al., 1943; Kennedy and Lehninger, 1948). The second is still a matter of reasonable dispute based on some peculiar properties of the organelle which are similar to prokaryotic organisms (Margulis, 1970; Cohen, 1970, 1973; Raff and Mahler, 1972; Mahler and Raff, 1975).

In reality the mitochondrion is an organelle with extensive interactions with the rest of the cell. Due to this complexity the research on mitochondrial biology has utilized many different approaches. The organelle contains a separate amplified DNA genome which is transcribed and translated to produce multiple polypeptide species, all using biosynthetic machinery localized within the organelle. The mechanisms of DNA replication, transcription, and translation, along with the structural and functional characteristics of the products, are incompletely understood (Borst and Grivell, 1978). The large number of metabolic pathways localized within its membranes make it a biochemical system interesting beyond its importance in the study of the mechanism of respiration and energy transduction The mitochondrial DNA gives it genetic behavior which is distinct from the Mendelian behavior displayed by nuclear genes (Gillham, 1974; Wilkie, 1975; Tzagoloff, 1977). The cell biology of the organelle population is studied because of its position in much of cellular metabolism and its relationship to other cellular components,

but its biogenesis is particularly intriguing because cooperation and interaction of nuclear and mitochondrial components are necessary in biosynthesis of specific respiratory enzyme complexes (Tzagoloff et al., 1973; Schatz and Mason, 1974).

To develop a background useful to appreciate the work presented here, a brief introduction to the system is included below. Though there are differences between mitochondria from various organisms, the mitochondrial DNA informational content and most of the biological principles appear to be evolutionarily conserved. I have worked on human mitochondrial proteins, mostly in the S3 strain of human HeLa cells, but have also used both human placentas and beef heart as sources for purification of the cytochrome c oxidase enzymatic complex. The descriptions below apply to mitochondria in general with most of the information extrapolated from studies on yeast, Neurospora, beef heart and many other sources, but with specific details from the human system pointed out where appropriate. Many reviews on particular aspects of the mitochondrion and at various levels of detail have been published (Lehninger, 1964; Wainio, 1970; Mahler, 1973a,b; Schatz and Mason, 1974; Milner, 1976; O'Brien, 1976; Tzagoloff, 1977; Borst and Grivell, 1978).

**Morphology**

Mitochondria are a dynamic population of similar entities which are constantly in motion and with the capacity to split off portions and to fuse back together (Frederic, 1958). The mechanism of splitting is analogous to budding in contrast to processes leading to even division. The organelles can take a wide range of forms depending on the particular cell type and metabolic state (Wainio, 1970; Ernster and Kuylenstierna, 1970). There is some evidence for structural and functional heterogeneity in the organelle population, which suggests that there may also be intracellular heterogeneity and functional specialization of different organelles (Swick et al., 1967; Bahr, 1971; Storrie and Attardi, 1973b).

The numbers may range from a single "megamitochondrion" in some yeast cells to hundreds in animal cells to thousands in large protozoa. Upon electron microscopic observation of single tissue sections, it may appear that there are multiple separate organelles which are actually joined together at a different level, thus requiring serial sectioning and morphometric reconstruction to determine the true mitochondrial number (Brandt et al., 1974; Berger, 1973; Stevens, 1977). Furthermore, during biochemical purification and cell disruption the organelles may be split and broken to reform into smaller but more numerous bodies.

The more familiar shape of the organelle is similar to a potato, sometimes round but often longer in one dimension and somewhat ovoid and irregular. Although the forms do vary dramatically depending on the source, in rat liver common dimensions are about 0.6 μ in diameter and 0.8 μ long, approximately on the order of the size of a bacterium (Berger, 1973).

The organelle has a double membranous structure composed of two distinct and separate lipid-bilayers. The outer membrane gives the external shape described above while the inner membrane has a further variation in form depending again on cell type and metabolic state. The total inner membrane is much larger in surface area than the cell plasma membrane, crudely and conservatively estimated to be as much as 50X; this is accommodated by extensive pleating and invaginations into the contained "matrix" volume. This large surface area provides for a larger amount of membrane-dependent enzyme function in a small volume, relevant to the mechanism of oxidative phosphorylation.

The outer and inner membranes differ in many properties, as summarized in Table I. Structurally, the outer membrane is relatively rigid and inflexible while the inner membrane is flexible and changes shape with metabolic state (LeMasters, 1978). Under normally active metabolic conditions the inner membrane

**Table I**

Comparison of properties of outer and inner mitochondrial membranes

| Property | Outer Membrane | Inner Membrane |
|---|---|---|
| flexibility | rigid | flexible |
| form | smooth | folded; invaginated |
| metabolite permeability | permeable | impermeable |
| large molecule permeability | < ∿10K MW | $H_2O$ impermeable |
| % protein composition | ∿40% | ∿70% |
| lipids: | | |
| cholesterol:phospholipid* | ∿1:10 | ∿1:50 |
| cardiolipin | virtually absent | present |
| phosphatidyl-inositol | much more abundant | present |

*High cholesterol leads to digitonin sensitivity

is moderately swelled while in the inactive (condensed) metabolic state the membrane (and matrix) are condensed to a small volume leaving a large intermembranous space. The inner membrane is also subject, in purified organelle preparations, to osmotic effects.

As in virtually all other biological membranes, both of these show fluidity and asymmetry in both lipid and protein components and in function (Höchli and Hackenbrock, 1976, 1977; Clarke, 1976). The inner membrane clearly displays morphological asymmetry having knob-like structural elements on the matrix face. These elements composed of a head and stalk are portions of the oligomycin-sensitive ATPase enzymatic complex.

An obvious morphological consequence of the double membrane is the presence of four distinct compartments besides the outside (cytoplasmic) environment. These are the outer membrane itself, the intermembranous space (called the "external" compartment--presumably relative to the inner matrix), the inner membrane, and the inner matrix.

Each compartment contains its own characteristic profile of structural and functional components, many of which serve as specific markers for that compartment (Schnaitman and Greenawalt, 1968; Ernster and Kuylenstierna, 1970). The elucidation of mechanisms to generate these specific distributions of protein components among the different compartments remains as an important biological puzzle.

**Molecular Biology**

The mitochondrial genome codes for the structural RNA species used in the organelle's translation apparatus and for the mitochondrial-ribosome synthesized polypeptides involved in the energy transduction function of the mitochondrion (O'Brien, 1976). Although expression is not necessary for growth of the organelle's

membrane structure, blockage or absence of gene expression prevents further formation of the functional units of the oxidative phosphorylation chain (Clark-Walker and Linnane, 1967; Linnane and Haslam, 1970; Nagley and Linnane, 1970; Lenk and Penman, 1971; Storrie and Attardi, 1973a).

The mitochondrial genome in vivo is predominantly a covalently closed circular double-stranded molecule in HeLa cells and is likewise in most other systems, though some protozoa appear to contain true linear DNAs (Borst and Flavell, 1976; Borst, 1977). Significant purification of mitochondrial DNA can be achieved because of a different GC content from the nuclear DNA composition and thus a modified density or by taking advantage of differential dye intercalation into closed circular molecules causing density shifts (Radloff et al., 1967). Contour lengths seem to be longer in lower organisms than in higher animals ranging from about 25 μ in yeast to about 5 μ in human and other mammalian cells, although the angiosperms have large mitochondrial genomes of about 30 μ (Borst and Flavell, 1976). Under normal conditions the multiply-copied genome, ranging from about five to several hundred separate copies per organelle, appears to be identical within each of the individual cells of an organism.

The replication of mitochondrial DNA has been extensively studied, but the details of the enzymology remain unresolved (Robberson et al., 1972; Kasamatsu et al., 1974; Gillum and Clayton, 1978; Brown et al., 1978). Though it is unclear whether the organelle utilizes a unique organelle-specific polymerase, it and the other associated proteins are coded for in the nucleus and translated on cytoplasmic ribosomes (Bolden et al., 1977). Thereafter they are imported into the organelle.

The mitochondrial DNA molecules are reportedly located in "chondriolites" which are interpreted as clusters of a few molecules attached to the inner membrane

(Williamson, 1976). There is evidence for a membrane attachment of mitochondrial DNA which may reflect a membrane-bound replication complex as found in the bacteria (Albring et al., 1977).

The potential informational content of the genome is much larger than that necessary to code for the known functional polypeptides. In yeast, only about half of the polypeptides have been functionally identified which, when combined with structural RNA genes corresponds to about 16% of the available "classical" coding capacity (Borst, 1977). Genetic studies indicate that there should be no more unidentified polypeptides associated with the oxidative phosphorylation chain (Tzagoloff et al., 1976). The transcription products in HeLa cells include genes for two ribosomal RNA species, multiple 4S (tRNA) species and 18 poly(A)-containing species which include putative mitochondrial messenger RNAs (Attardi et al., 1976). The human mitochondrial genome has a 5 μ contour length corresponding to about 16,500 nucleotides. A map of the structural RNA species of HeLa cell mitochondria is shown in Figure 1; the poly(A)-containing species have not been included.

The RNA polymerase presumably also comes from the nuclear genome and is synthesized on cytoplasmic ribosomes (Barath and Kuntzel, 1972; Scragg, 1976). Although the possibility does still exist, there is good evidence for the lack of transport of large RNA species into or out of the organelle (Amalric et al., 1978; Borst and Grivell, 1978). Consequently, all of the RNA species required for translation would come from the mitochondrial DNA, including the 12S and 16S rRNAs, the necessary tRNAs and the messenger species. However, there is good evidence that most of the proteins involved in translation are of nuclear origin (Davey et al., 1969).

The actual number of tRNA species coded on the genome is somewhat unclear. Experiments indicate that the mitochondrial translation system contains

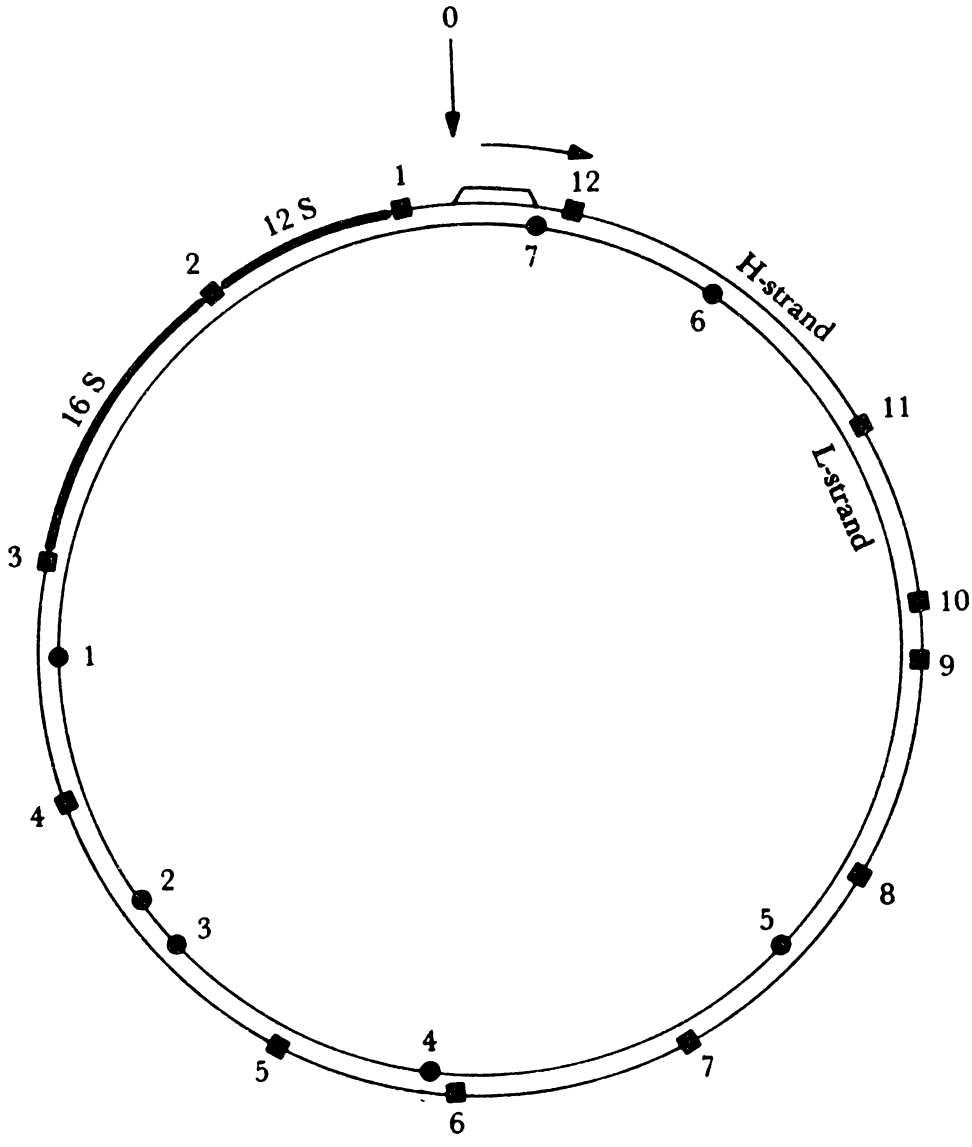


**Figure 1.** Map of 19 4S and the two ribosomal RNA species on HeLa cell mitochondrial DNA. The origin and direction of H-strand DNA replication are indicated by the arrows.

a sufficient number of species to utilize all of the 20 common amino acids but other experiments had failed to identify that many tRNAs on the genome (see Chapter 2).

The 18 poly(A)-containing transcripts present a minor problem. Though at least some are presumptive messenger species and all can be isolated from "polysomal" structures, they have not yet been translated by in vitro systems (Amalric et al., 1978). Mitochondrial transcription maps and understanding of the mechanisms involved are incomplete, along with uncertainty of the true relationship of the RNA species to the mitochondrially synthesized polypeptides (Van Ommen and Groot, 1977; Battey and Clayton, 1978; Moorman et al., 1977, 1978). However, most of the RNA transcripts contain sufficient coding capacity for the largest of the mitochondrially synthesized polypeptides.

**Organelle Function and Biogenesis**

As pointed out above, the mitochondrion is not simply a double membrane containing the oxidative phosphorylation chain. The organelle is actually an important structure which contains a wealth of necessary and important enzymes. Among the biochemical pathways contained within the mitochondrion are fatty acid oxidation, heme biosynthesis, many of the amino acid biosynthetic pathways and the Krebs cycle (Mahler and Cordes, 1971; Lehninger, 1975). There are, in addition to minor enzymatic activities, membrane transport systems, the apparatus necessary for the maintenance and expression of the mitochondrial genome and the possibility of significant structural elements, the overwhelming majority of which are nuclear coded and cytoplasmically synthesized (Borst and Grivell, 1978). Consequently, when mitochondrial protein synthesis is inhibited or absent, the organelle still continues structural growth (Linnane and Haslam, 1970; Storrie and Attardi, 1973).

The biogenesis of the organelle is much like that of other cellular membranous systems. Growth presumably does not occur de novo, but by addition of both lipid and protein components to preexisting organelles or at least to a primer membranous structure (Luck, 1963, 1965; Linnane and Haslam, 1970).

The double membrane structure gives rise to the four compartments described above; with the large number of enzymes contained within, localization of particular enzymes must be coordinated to generate a complex functional system (Schnaitman and Greenawalt, 1968). The topology, local environment and membrane permeability to substrates may be very important in the arrangement of particular enzyme pathways within the organelle compartments (Ernster and Kuylenstierna, 1970). And since a great number of these biochemical enzymes are translated in the cytoplasm, they must be carefully directed to the correct location in the final organelle (Godinot and Lardy, 1973; Waksman et al., 1977; Shore et al., 1979; Chua and Schmidt, 1979).

An equally fascinating problem involves the enzymes in the oxidative phosphorylation pathway, particularly the cytochrome c oxidase considered here, along with the cytochrome $bc_1$ and the oligomycin-sensitive ATPase enzymatic complexes. These multisubunit complexes contain polypeptides coded for by the nucleus and cytoplasmically synthesized, and other polypeptides coded for and synthesized within the mitochondrion (Tzagoloff et al., 1973; Schatz and Mason, 1974; see Chapter 5). The interrelationship between the genomes and the regulation and coordination of subunit synthesis in assembly of the complex comprise an important problem worthy of further research (Poyton and Kavanaugh, 1976; Poyton and McKemmie, 1976; Maccecchini et al., 1979; Coté et al., 1979).

**Mitochondrial Protein Synthesis**

In spite of the general conception of the similarities in the mitochondrial

and prokaryote translation machinery, their similarities are not overwhelming, particularly in view of the large variation in structure of mitochondrial ribosomes from various organisms (O'Brien, 1976). Table II compares the properties of the three protein synthesizing apparatuses.

The ribosomes are structurally different from the three different sources, both in the sizes of the rRNA species and in the ribonuclear-protein subunit sizes. Functionally, the translation mechanism in mitochondria appear to be similar to prokaryotes in many respects, particularly antibiotic sensitivity. However, many of these properties may well result from the mitochondrial ribosomal proteins, which are coded (with one exception) by the nuclear genome. Consequently, although the mitochondrial ribosomes may be functionally homologous to the bacterial ribosomes, the properties arise from expression of nuclear genes.

A complementary inhibitor sensitivity of mitochondrial ribosomes compared with cytoplasmic ribosomes leads to a labeling scheme for mitochondrially synthesized polypeptides. Labeling in the presence of cycloheximide or emetine allows only mitochondrial ribosomes to function making the mitochondrially synthesized polypeptides labeled (Ennis and Lubin, 1964; Colombo et al., 1966; Perlman and Penman, 1970). Parallel labeling with either cytoplasmic ribosome inhibitor plus chloramphenicol, a mitochondrial ribosome inhibitor, serves as a control for background nonribosomal incorporation (Kroon, 1965; Lederman and Attardi, 1970; Galper and Darnell, 1971; Costantino and Attardi, 1975; Jeffreys and Craig, 1976).

Eukaryotic ribosomes initiate translation with methionine at appropriate AUG codons, while prokaryotic ribosomes use a formyl-methionine residue. The mitochondrial ribosome also initiates with the formyl-methionine moiety presumably resulting in blocked methionine amino termini on primary mitochondrially synthesized protein components (Galper and Darnell, 1969; Mahler et al., 1972; Buse

**Table II**

Comparisons of protein synthesizing apparatuses

| | Eukaryote cytoplasm | Animal mitochondria | Prokaryote |
|---|---|---|---|
| large rRNA size | 28S | 16S | 23S |
| small rRNA size | 18S | 12S | 16S |
| 5S RNA | 5S | 4S(?) | 5S |
| whole ribosome | 80S | 60S | 70S |
| large subunit | 60S | 45S | 50S |
| small subunit | 40S | 35S | 30S |
| initiator tRNA | met | formyl-met | formyl-met |
| poly-A tail | 150-200 | 60-80 | none |
| 5' mG cap | present | absent | absent |
| antibiotics: | | | |
| chloramphenicol | insensitive | sensitive | sensitive |
| emetine | sensitive | insensitive | insensitive |
| cycloheximide | sensitive | insensitive | insensitive |

and Steffens, 1976). This property provides an additional method to specifically label the proteins synthesized on mitochondrial ribosomes. This has the advantage of avoiding antibiotics but requires that no processing of the amino terminus of the polypeptides occurs and gives only one label per polypeptide making detection quite difficult (Feldman and Mahler, 1974).

Another approach has been to isolate temperature-sensitive cytoplasmic protein synthesis mutants. Labeling at the nonpermissive temperature allows specific incorporation into the polypeptides synthesized on mitochondrial ribosomes (Wallace et al., 1975). Others have prepared purified mitochondria to synthesize mitochondrial components in vitro (Lederman and Attardi, 1970; Poyton and Groot, 1975).

There are a limited number of functionally identified polypeptide products of mitochondrial protein synthesis (Tzagoloff et al., 1973; Schatz and Mason, 1974; Borst and Grivell, 1978; see Chapter 5). Although the mitochondria contain hundreds of polypeptides, including structural components of mitochondrial ribosomes, polymerases, aminoacyl transferases and other protein factors besides the metabolic enzymes described above, only a small percentage are mitochondrially coded and synthesized. Many of these are contained in those important multisubunit enzymatic complexes involved in oxidative phosphorylation. Those complexes containing identified mitochondrially synthesized subunits are the cytochrome $bc_1$ (complex III), the cytochrome c oxidase (complex IV) and the oligomycin-sensitive ATPase (complex V) (Katan et al., 1976; Weiss, 1976; Weiss and Ziganke, 1977; Rubin and Tzagoloff, 1973b; Mason and Schatz, 1973; Koch, 1976; Sebald, 1977; Rascati and Parsons, 1979b; see Chapter 5; Tzagoloff and Meagher, 1972; Jackl and Sebald, 1975). There is also some evidence for a mitochondrially synthesized ribosomal protein (Lambowitz et al., 1976).

Mitochondrial DNA sequence information is beginning to appear from yeast and human sources. One of the mitochondrially coded ATPase subunits has been sequenced and aligned with a portion of the DNA sequence in yeast (Macino and Tzagoloff, 1979a,b; Hensgens et al., 1979). Portions of the human mitochondrial genome from HeLa cells have been sequenced, and it may be only a short time before protein sequence information may allow alignment and mapping of some of the species resolved by work presented here (Crews et al., 1979; Crews, personal communication).

Good genetic evidence exists in yeast that there are no further unidentified mitochondrially synthesized species which directly affect the activity of the oxidative phosphorylation chain components (Tzagoloff et al., 1976). However, there also exists evidence that there are many additional polypeptides which are synthesized on mitochondrial ribosomes (see Chapter 4; Douglas and Butow, 1976).

A substantial fraction of the included research here involved the development of techniques and increased separation of the mitochondrially synthesized polypeptide species labeled with the use of antibiotics as described above (see Chapter 3). The mitochondria synthesize about 3% of cellular protein. Our mitochondrial purification procedure at best would give us a 10-fold enrichment in mitochondria due to elimination of cytoplasmic soluble and membrane proteins, which finally means 30% of the remaining protein in our mitochondrial preparation is mitochondrially synthesized. A 3 hr labeling incubation (longer times are toxic in the presence of antibiotics) which makes up about 15% of a 22-24 hr healthy generation in these HeLa cells should allow a corresponding 15% more synthesis to occur. Thus, 15% of the 30% purified mitochondrial products, or 4.5% of the total final sample will contain label which will be spread out over multiple species.

Since about 70% of the proteins contained in the sample of mitochondrial proteins are not even mitochondrially synthesized and consists of the above-mentioned combination of randomly sized cytoplasmically synthesized enzymes, it is not presently possible to obtain mass purified individual species. Virtually all of the work must be done with biosynthetically-labeled proteins using analyses insensitive to enormous mass contamination, and if it were possible to partially mass purify a specific mitochondrially synthesized polypeptide, about 80% of the mass of the mitochondrially synthesized component would be unlabeled.

Technologically, using high specific activity $^{35}$S-methionine (which emits a relatively high energy β particle upon decay) for labeling allows the work described here to be completed. No other radioisotopically-labeled amino acid gives a sufficient combination of detectability, specific activity, and reasonable cost.

With these techniques, 25 discrete products of mitochondrial protein synthesis are resolved. The molecular weights of the species have been estimated and virtually saturate the mitochondrial single-strand nonoverlapping coding capacity which remains after the structural rRNAs and 20 4S RNA lengths are considered (see Chapter 4). Consequently, it appears as if all of the products of mitochondrial protein synthesis have been resolved and await functional assignments.

**Cytochrome c Oxidase**

The oxidative phosphorylation chain is the enzymatic pathway which utilizes the oxidation potential of pyruvate and extracts almost 95% of the biologically recovered energy from a molecule of glucose. This substantial energy yield is converted into the production of ATP.

The respiratory chain is integrally associated with the inner mitochondrial membrane. The electron transport chain, as shown in Figure 2, consists of the enzymatic complexes NADH dehydrogenase (complex I), succinate dehydrogenase

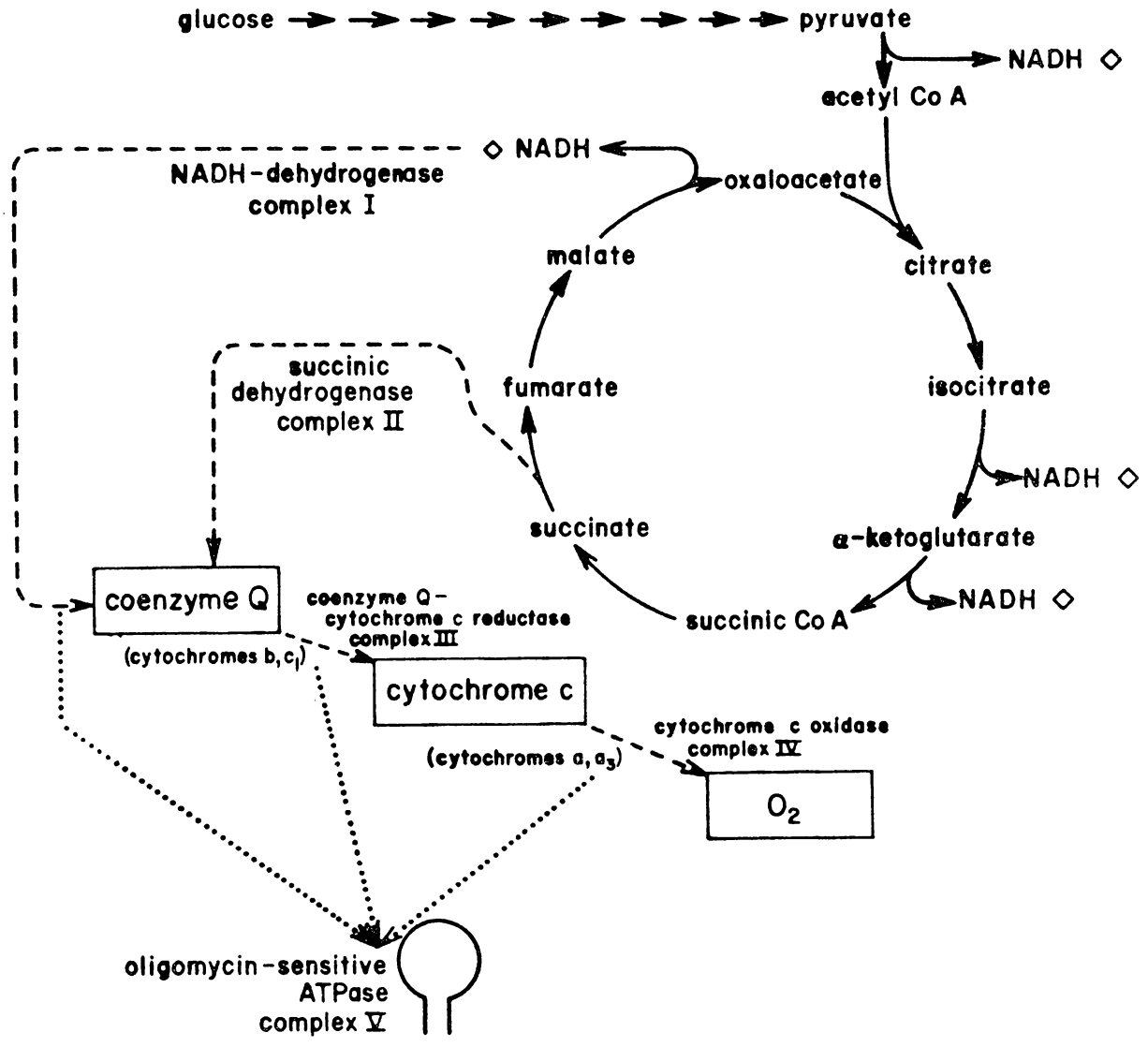


**Figure 2.** The relationship between the Krebs cycle and oxidative phosphorylation in the respiratory chain.

(complex II), the cytochrome $bc_1$ complex (coenzyme Q-cytochrome c reductase or complex III) and the cytochrome c oxidase (complex IV). They are numbered complexes I-IV because each activity can be separated from the others and purifies as an intact multipeptide complex (Hatefi et al., 1961, 1962; Hatefi, 1966). The mitochondrial ATPase also purifies as an intact multipeptide complex (which corresponds structurally to the above-mentioned "knob" structures) and is designated complex V. Although it is closely linked functionally to complexes I-IV, it is not truly considered to be an electron transport chain component, but is included in the larger activity comprising oxidative phosphorylation.

The mechanism of oxidative phosphorylation appears to be essentially as described in the chemiosmotic theory which proposes that electron transport is coupled with production of a proton gradient across the inner mitochondrial membrane. As an electron is passed from NADH (or succinate) to coenzyme Q, to cytochrome c and finally to molecular oxygen, the enzymatic complexes perform work in exporting protons across the membrane. The energy contained in the proton gradient across the inner membrane is then utilized by the oligomycin-sensitive ATPase to generate the production of ATP (Mitchell, 1961, 1975; Racker, 1970; Boyer, 1975). This mechanism would explain why the inner membrane has such a large surface area, thus allowing more of the membrane-dependent proton translocation enzymes to operate within a small volume.

The cytochrome c oxidase, being the terminal oxidase in the respiratory pathway, is one of the most prevalent mitochondrial enzymes. It turns out to be the product of both the mitochondrial and nuclear genomes, and so was chosen as a model for study of the coordination and regulation of expression of the two genomes.

Structurally, the complex has been studied from many sources and found to be resolved into six to eight polypeptide subunits which span the membrane

in an asymmetric conformation (Kuboyama et al., 1972; Mason et al., 1973; Rubin and Tzagoloff, 1973a; Briggs et al., 1975; Phan and Mahler, 1976; Downer et al., 1977; Buse and Steffens, 1977; Höchli and Hackenbrock, 1978; Rascati and Parsons, 1979a). It contains two hemes, a and $a_3$, which are structurally similar (or identical) but display slightly different properties and molecular environments (Gibson et al., 1965). Subunits are presumably present in equimolar amounts, a single copy per complex, with the larger subunits being of mitochondrial origin (Rubin and Tzagoloff, 1973b; Mason and Schatz, 1973; Koch, 1976; Sebald, 1977; Rascati and Parsons, 1979b).

We show that the human form of the enzyme is quite similar to that from other sources in its subunit composition and site of synthesis of the polypeptides (see Chapter 5).

## References

Albring, M., Griffith, J. and Attardi, G. (1977) Association of a protein structure of probable membrane derivation with HeLa cell mitochondrial DNA near its origin of replication. Proc. Nat. Acad. Sci. **74**, 1348-1352.

Amalric, F., Merkel, C., Gelfand, R. and Attardi, G. (1978) Fractionation of mitochondrial RNA from HeLa cells by high-resolution electrophoresis under strongly denaturing conditions. J. Mol. Biol. **118**, 1-25.

Attardi, G., Amalric, F., Ching, E., Costantino, P., Gelfand, R. and Lynch, D. (1976) Informational content and gene mapping of mitochondrial DNA from HeLa cells. In: The Genetic Function of Mitochondrial DNA (eds. C. Saccone and A. M. Kroon), pp. 37-46. Elsevier/North-Holland, Amsterdam.

Bahr, G. F. (1971) A unit mitochondrion: DNA content and response to X-irradiation. Adv. Cell Mol. Biol. **1**, 267-292.

Barath, Z. and Kuntzel, H. (1972) Induction of mitochondrial RNA polymerase in Neurospora crassa. Nature New Biol. **240**, 195-197.

Battey, J. and Clayton, D. A. (1978) The transcription map of mouse mitochondrial DNA. Cell **14**, 143-156.

Berger, E. R. (1973) Two morphologically different mitochondrial populations in the rat hepatocyte as determined by quantitative three-dimensional electron microscopy. J. Ultrastructural Res. **45**, 303-327.

Bolden, A., Noy, G. P. and Weissbach, A. (1977) DNA polymerase of mitochondria is a $\gamma$-polymerase. J. Biol. Chem. **252**, 3351-3356.

Borst, P. (1977) Structure and function of mitochondrial DNA. In: International Cell Biology 1976-1977 (eds. B. R. Brinkley and K. R. Porter), pp. 237-244. Rockefeller University Press.

Borst, P. and Flavell, R. A. (1976) Properties of mitochondrial DNAs. In: Handbook of Biochemistry and Molecular Biology, 3rd Edition, Nucleic Acids, Vol. 2 (ed. G. Fasman), pp. 363-374. CRC Press, Cleveland.

Borst, P. and Grivell, L. A. (1978) The mitochondrial genome of yeast. Cell **15**, 705-723.

Boyer, P. D. (1975) A model for conformational coupling of membrane potential and proton translocation to ATP synthesis and to active transport. FEBS Lett. **58**, 1-6.

Brandt, J. T., Martin, A. P., Lucas, F. V. and Vorbeck, M. L. (1974) The structure of rat liver mitochondria: A reevaluation. Biochem. Biophys. Res. Comm. **59**, 1097-1103.

Briggs, M., Kamp, P. F., Robinson, N. C. and Capaldi, R. A. (1975) The subunit structure of the cytochrome c oxidase complex. Biochemistry **14**, 5123-5128.

Brown, W. M., Shine, J. and Goodman, H. M. (1978) Human mitochondrial DNA: Analysis of 7S DNA from the origin of replication. Proc. Nat. Acad. Sci. **75**, 735-739.

Buse, G. and Steffens, G. J. (1976) Analysis of the polypeptide chains of cytochrome oxidase from beef heart mitochondria. In: Genetics and Biogenesis of Chloroplasts and Mitochondria (eds. Th. Bucher et al.), pp. 189-194. Elsevier/North-Holland, Amsterdam.

Chua, N. H. and Schmidt, G. W. (1979) Transport of proteins into mitochondria and chloroplasts. J. Cell Biol. **81**, 461-483.

Clark-Walker, G. D. and Linnane, A. W. (1967) The biogenesis of mitochondria in Saccharomyces cerevisiae. A comparison between cytoplasmic respiratory-deficient mutant yeast and chloramphenicol-inhibited wild type cells. J. Cell Biol. **34**, 1-14.

Clarke, S. (1976) Protein asymmetry in the inner membrane of rat liver mitochondria. J. Biol. Chem. **251**, 1354-1363.

Cohen, S. S. (1970) Are/were mitochondria and chloroplasts microorganisms? Am. Sci. **58**, 281-289.

Cohen, S. S. (1973) Mitochondria and chloroplasts revisited. Am. Sci. **61**, 437-445.

Colombo, B., Felicetti, L. and Baglioni, C. (1966) Inhibition of protein synthesis in reticulocytes by antibiotics. I. Effects on polysomes. Biochim. Biophys. Acta **119**, 109-119.

Costantino, P. and Attardi, G. (1975) Identification of discrete electrophoretic components among the products of mitochondrial protein synthesis in HeLa cells. J. Mol. Biol. **96**, 291-306.

Coté, C., Solioz, M. and Schatz, G. (1979) Biogenesis of the cytochrome $bc_1$ complex of yeast mitochondria. J. Biol. Chem. **254**, 1437-1439.

Crews, S., Ojala, D., Posakony, J., Nishiguchi, J. and Attardi, G. (1979) Nucleotide sequence of a region of human mitochondrial DNA containing the precisely identified origin of replication. Nature **277**, 192-198.

Davey, P. J., Yu, R. and Linnane, A. W. (1969) The intracellular site of formation of the mitochondrial protein synthetic system. Biochem. Biophys. Res. Comm. **36**, 30-34.

Douglas, M. G. and Butow, R. A. (1976) Variant forms of mitochondrial translation products in yeast: Evidence for location of determinants on mitochondrial DNA. Proc. Nat. Acad. Sci. **73**, 1083-1086.

Downer, N. W., Robinson, N. C. and Capaldi, R. A. (1976) Characterization of a seventh different subunit of beef heart cytochrome c oxidase. Similarities between the beef heart species and that from other species. Biochemistry **15**, 2930-2936.

Ennis, H. L. and Lubin, M. (1964) Cycloheximide: Aspects of inhibition of protein synthesis in mammalian cells. Science **146**, 1474-1476.

Ernster, L. and Kuylenstierna, B. (1970) Outer membrane of mitochondria. In: Membranes of Mitochondria and Chloroplasts (ed. E. Racker). Van Nostrand Reinhold Company, New York.

Feldman, F. and Mahler, H. R. (1974) Mitochondrial biogenesis. Retention of terminal formylmethionine in membrane proteins and regulation of their synthesis. J. Biol. Chem. **249**, 3702 3709.

Frederic, J. (1958) Recherches cytologiques sur le chondriome normal ou soumis a l'expérimentation dans des cellules vivantes cultivées in vitro. Arch. Biol. **69**, 167-349.

Galper, J. B. and Darnell, J. E. (1969) The presence of N-formyl-methionyl-tRNA in HeLa cell. Biochem. Biophys. Res. Comm. **34**, 205-213.

Galper, J. B. and Darnell, J. E. (1971) Mitochondrial protein synthesis in HeLa cells. J. Mol. Biol. **57**, 363-367.

Gibson Q. H., Palmer, G. and Wharton, D. C (1965) The binding of carbon monoxide by cytochrome c oxidase and the ratio of the cytochromes a and $a_3$. J. Biol. Chem. **240**, 915-920.

Gillham, N. W. (1974) Genetic analysis of the chloroplast and mitochondrial genomes. Ann. Rev. Genet. **8**, 347-391.

Gillum, A. M. and Clayton, D. A. (1978) Displacement-loop replication initiation sequence in animal mitochondrial DNA exists as a family of discrete lengths. Proc. Nat. Acad. Sci. **75**, 677-681.

Godinot, C. and Lardy, H. A. (1973) Biosynthesis of glutamate dehydrogenase in rat liver. Demonstration of its microsomal localization and hypothetical mechanism of transfer to mitochondria. Biochemistry **12**, 2051-2059.

Hatefi, Y. (1966) The functional complexes of the mitochondrial electron-transfer system. Comp. Biochem. **14**, 199-231.

Hatefi, Y., Haavik, A. G., Fowler, L. R. and Griffiths, D. E. (1962) Studies on the electron transport system. XLII. Reconstitution of the electron transfer system. J. Biol. Chem. **237**, 2661-2669.

Hatefi, Y., Haavik, A. G. and Griffiths, D. E. (1961) Reconstitution of the electron transfer system. I. Preparation and properties of the interacting enzyme complexes. Biochem. Biophys. Res. Comm. **4**, 441-453.

Hensgens, L. A. M., Grivell, L. A., Borst, P. and Bos, J. (1979) Nucleotide sequence of the mitochondrial structural gene for subunit 9 of yeast ATPase complex. Proc. Nat. Acad. Sci. **76**, 1663-1667.

Höchli, L. and Hackenbrock, C. R. (1978) Cytochrome c oxidase from rat liver mitochondria: Purification and characterization. Biochemistry **17**, 3712-3719.

Höchli, M. and Hackenbrock, C. R. (1976) Fluidity in mitochondrial membranes: Thermotropic lateral translational motion of intramembrane particles. Proc. Nat. Acad. Sci. **73**, 1636-1640.

Höchli, M. and Hackenbrock, C. R. (1977) Thermotropic lateral translational motion of intramembrane particles in the inner mitochondrial membrane and its inhibition by artificial peripheral proteins. J. Cell Biol. **72**, 278-291.

Hogeboom, G. H., Schneider, W. C. and Palade, G. E. (1948) Cytochemical studies of mammalian tissues. I. Isolation of intact mitochondria from rat liver; some biochemical properties of mitochondria and submicroscopic particulate material. J. Biol. Chem. **172**, 619-636.

Jackl, G. and Sebald, W. (1975) Identification of two products of mitochondrial protein synthesis associated with mitochondrial adenosine triphosphatase from Neurospora crassa. Eur. J. Biochem. **54**, 97-106.

Jeffreys, A. J. and Craig, I. W. (1976) Analysis of proteins synthesized in mitochondria of cultured mammalian cells. Eur. J. Biochem. **68**, 301-311.

Kasamatsu, H., Grossman, L. I., Robberson, D. L., Watson, R. and Vinograd, J. (1974) The replication and structure of mitochondrial DNA in animal cells. Cold Spring Harbor Symp. Quant. Biol. **38**, 281-288.

Katan, M. B., Loosbroek, N. V. H. and Groot, G. S. P. (1976) The cytochrome $bc_1$ complex of yeast mitochondria. Eur. J. Biochem. **70**, 409-417.

Kennedy, E. P. and Lehninger, A. L. (1948) Intracellular structures and the fatty acid oxidase system of rat liver. J. Biol. Chem. **172**, 847-848.

Koch, G. (1976) Synthesis of the mitochondrial inner membrane in cultured Xenopus laevis oocytes. J. Biol. Chem. **251**, 6097-6107.

Kroon, A. M. (1965) Protein synthesis in mitochondria. III. On the effects of inhibitors on the incorporation of amino acids into protein by intact mitochondria and digitonin fractions. Biochim. Biophys. Acta **108**, 275-284.

Kuboyama, M., Yong, F. C. and King, T. E. (1972) Studies on cytochrome oxidase. VIII. Preparation and some properties of cardiac cytochrome oxidase. J. Biol. Chem. **247**, 6375-6383.

Lambowitz, A. M., Chua, N. H. and Luck, D. J. L. (1976) Mitochondrial ribosome assembly in Neurospora. Preparation of mitochondrial ribosomal precursor particles, site of synthesis of mitochondrial ribosomal proteins and studies on the poky mutant. J. Mol. Biol. **107**, 223-253.

Lederman, M. and Attardi, G. (1970) In vitro protein synthesis in a mitochondrial fraction from HeLa cells: Sensitivity to antibiotics and ethidium bromide. Biochem. Biophys. Res. Comm. **40**, 1492-1500.

Lehninger, A. L. (1964) The Mitochondrion, Molecular Basis of Structure and Function. W. A. Benjamin, Inc., New York.

Lehninger, A. L. (1975) Biochemistry. Worth Publishers, Inc., New York.

LeMasters, J. J. (1978) Possible role of the mitochondrial outer membrane as an oncotic regulator of mitochondrial volume. FEBS Lett. **88**, 10-14.

Lenk, R. and Penman, S. (1971) Morphological studies of cells grown in the absence of mitochondrial-specific protein synthesis. J. Cell Biol. **49**, 541 546.

Linnane, A. W. and Haslam, J. M. (1970) The biogenesis of yeast mitochondria. Curr. Topics Cell Reg. **2**, 101-172.

Luck, D. J. L. (1963) Formation of mitochondria in Neurospora crassa. A quantitative autoradiographic study. J. Cell Biol. **16**, 483-499.

Luck, D. J. L. (1965) Formation of mitochondria in Neurospora crassa. A study based on mitochondrial density changes. J. Cell Biol. **24**, 461-487.

Maccecchini, M. L., Rudin, Y., Blobel, G. and Schatz, G. (1979) Import of proteins into mitochondria: Precursor forms of the extramitochondrially made $F_1$-ATPase subunits in yeast. Proc. Nat. Acad. Sci. **76**, 343-347.

Macino G. and Tzagoloff, A. (1979a) Assembly of the mitochondrial membrane system: Partial sequence of a mitochondrial ATPase gene in Saccharomyces cerevisiae. Proc. Nat. Acad. Sci. **76**, 131-135.

Macino, G. and Tzagoloff, A. (1979b) Assembly of the mitochondrial membrane system. The DNA sequence of a mitochondrial ATPase gene in Saccharomyces cerevisiae. J. Biol. Chem. **254**, 4617-4623.

Mahler, H. R. (1973a) Mitochondria: Molecular Biology, Genetics, and Development. Addison-Wesley, Reading, Massachusetts.

Mahler, H. R. (1973b) Biogenetic autonomy of mitochondria. CRC Crit. Rev. Biochem. **1**, 381-460.

Mahler, H. R. and Cordes, E. H. (1971) Biological Chemistry. Harper and Row, New York.

Mahler, H. R. Dawidowicz, K. and Feldman, F. (1972) Formate as a specific label for mitochondrial translation products. J. Biol. Chem. **247**, 7439-7442.

Mahler, H. R. and Raff, R. A. (1975) The evolutionary origin of the mitochondrion: A non-symbiotic model. Int. Rev. Cytol. **43**, 1-124.

Margulis, L. (1970) Origin of Eucaryotic Cells. Yale University Press, New Haven, Connecticut.

Mason, T L., Poyton, R. O., Wharton, D. C. and Schatz, G. (1973) Cytochrome c oxidase from bakers' yeast. I. Isolation and properties. J. Biol. Chem. **248** 1346-1354.

Mason, T. L. and Schatz, G. (1973) Cytochrome c oxidase from bakers' yeast. II. Site of translation of protein components. J. Biol. Chem. **248**, 1355-1360.

Milner, J. (1976) The functional development of mammalian mitochondria. Biol. Rev. **51**, 181-209.

Mitchell, P. (1961) Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. Nature **191**, 144-148.

Mitchell, P. (1975) The protonmotive Q cycle: A general formulation. FEBS Lett. **59**, 137-139.

Moorman, A. F. M., Van Ommen, G. J. B. and Grivell, L. A. (1978) Transcription in yeast mitochondria: Isolation and physical mapping of messenger RNAs for subunits of cytochrome c oxidase and ATPase. Mol. Gen. Genet. **160**, 13-24.

Moorman, A. F. M., Verkley, F. N., Asselbergs, F. A. M. and Grivell, L. A. (1977) Yeast mitochondrial messenger RNA is not correctly translated in heterologous cell-free systems. In: Mitochondria 1977. Genetics and Biogenesis of Mitochondria (eds. W. Bandlow et al.), pp. 385-399. Walter de Gruyter, New York.

Nagley, P. and Linnane, A. W. (1970) Mitochondrial DNA deficient petite mutants of yeast. Biochem. Biophys. Res. Comm. **39**, 989-996.

O'Brien, T. (1976) Mitochondrial protein synthesis. In: Protein Synthesis (ed. E. H. McConkey), pp. 249-307. Marcel Dekker, Inc., New York.

Perlman, S. and Penman, S. (1970) Mitochondrial protein synthesis: Resistance to emetine and response to RNA synthesis inhibitors. Biochem. Biophys. Res. Comm. **40**, 941-948.

Phan, S. H. and Mahler, H. R. (1976) Studies on cytochrome oxidase, partial resolution of enzymes containing seven or six subunits from yeast and beef heart, respectively. J. Biol. Chem. **251**, 257-269.

Poyton, R. O. and Groot, G. S. P. (1975) Biosynthesis of polypeptides of cytochrome c oxidase by isolated mitochondria. Proc. Nat. Acad. Sci. **72**, 172-176.

Poyton, R. O. and Kavanaugh, J. (1976) Regulation of mitochondrial protein synthesis by cytoplasmic proteins. Proc. Nat. Acad. Sci. **73**, 3947-3951.

Poyton, R. O. and McKemmie, E. (1976) The assembly of cytochrome c oxidase from Saccharomyces cerevisiae. In: Genetics and Biogenesis of Chloroplasts and Mitochondria (eds. Th. Bücher et al.), pp. 207-214. Elsevier/North-Holland, Amsterdam.

Racker, E. (1970) Function and structure of the inner membrane of mitochondria and chloroplasts. In: Membranes of Mitochondria and Chloroplasts (ed. E. Racker), pp. 127-171. Van Nostrand Reinhold Company, New York.

Radloff, R., Bauer, W. and Vinograd, J. (1967) A dye-buoyant density method for the detection and isolation of closed circular duplex DNA in HeLa cells. Proc. Nat. Acad. Sci. **57**, 1514-1521.

Raff, R. A. and Mahler, H. R. (1972) The non-symbiotic origin of mitochondria. Science **177**, 575-582.

Rascati, R. J. and Parsons, P. (1979a) Purification and characterization of cytochrome c oxidase from rat liver mitochondria. J. Biol. Chem. **254**, 1586-1593.

Rascati, R. J. and Parsons, P. (1979b) Biosynthesis of cytochrome c oxidase by isolated rat liver mitochondria. J. Biol. Chem. **254**, 1594-1599.

Robberson, D. L., Kasamatsu, H. and Vinograd, J. (1972) Replication of mitochondrial DNA. Circular repetitive intermediates in mouse L cells. Proc. Nat. Acad. Sci. **69**, 737-741.

Rubin, M. S. and Tzagoloff, A. (1973a) Assembly of the mitochondrial membrane system. IX. Purification, characterization, and subunit structure of yeast and beef cytochrome oxidase. J. Biol. Chem. **248**, 4269-4274.

Rubin, M. S. and Tzagoloff, A. (1973b) Assembly of the mitochondrial membrane system. X. Mitochondrial synthesis of three of the subunit proteins of yeast cytochrome oxidase. J. Biol. Chem. **248**, 4275-4279.

Schatz, G. and Mason, T. L. (1974) The biosynthesis of mitochondrial proteins. Ann. Rev. Biochem. **43**, 51-87.

Schnaitman, C. and Greenawalt, J. W. (1968) Enzymatic properties of the inner and outer membranes of rat liver mitochondria. J. Cell Biol. **38**, 158-175.

Scragg, A. H. (1976) Origin of the mitochondrial RNA polymerase of yeast. FEBS Lett. **65**, 148-151.

Sebald, W. (1977) Biogenesis of mitochondrial ATPase. Biochim. Biophys. Acta **463**, 1-27.

Shore, G. C., Carignan, P. and Raymond, V. (1979) In vitro synthesis of a putative precursor to the mitochondrial enzyme, carbamyl phosphate synthetase. J. Biol. Chem. **254**, 3141-3144.

Stevens, B. J. (1977) Variation in number and volume of the mitochondria in yeast according to growth conditions. A study based on serial sectioning and computer graphics reconstitution. Biol. Cellulaire **28**, 37-56.

Storrie, B. and Attardi, G. (1973a) Expression of the mitochondrial genome in HeLa cells. XV. Effect of inhibition of mitochondrial protein synthesis on mitochondrial formation. J. Cell Biol. **56**, 819-831.

Storrie, B. and Attardi, G. (1973b) Expression of the mitochondrial genome in HeLa cells. XVII. Heterogeneity of isolated HeLa cell mitochondria as assayed for their enzymatic and in vivo biosynthetic activities. J. Biol. Chem. **248**, 5826-5834.

Swick, R. W., Stange, J. L., Nance, S. L. and Thomson, J. F. (1967) The heterogeneous distribution of mitochondrial enzymes in normal rat liver. Biochemistry **6**, 737-744.

Tzagoloff, A. (1977) Genetic and translational capabilities of the mitochondrion. BioScience **27**, 18-23.

Tzagoloff, A., Foury, F. and Akai, A. (1976) Resolution of the mitochondrial genome. In: The Genetic Function of Mitochondrial DNA (eds. C. Saccone and A. M. Kroon), pp. 155-161. Elsevier/North-Holland, Amsterdam.

Tzagoloff, A. and Meagher, P. (1972) Assembly of the mitochondrial membrane system. VI. Mitochondrial synthesis of subunit proteins of the rutamycin-sensitive adenosine triphosphatase. J. Biol. Chem. **247**, 594-603.

Tzagoloff, A., Rubin, M. S. and Sierra, M. F. (1973) Biosynthesis of mitochondrial enzymes. Biochim. Biophys. Acta **301**, 71-104.

Van Ommen, G. J. B. and Groot, G. S. P. (1977) Transcription map of yeast mitochondrial DNA. In: Mitochondria 1977. Genetics and Biogenesis of Mitochondria (eds. W. Bandlow et al.), pp. 415-424. Walter de Gruyter, New York.

Wainio, W. W. (1970) The Mammalian Respiratory Chain. Academic Press, New York.

Waksman, A., Rendon, A., Cremel, G., Pellicone, C., de Brugiere, J. F. G. (1977) Intramitochondrial intermembranal reversible translocation of aspartate amino transferase and malate dehydrogenase through the inner mitochondrial membrane. Biochemistry **16**, 4703-4707.

Wallace, R. B., Williams, T. M. and Freeman, K. B. (1975) Mitochondrial protein synthesis in a mammalian cell-line with a temperature-sensitive leucyl-tRNA synthetase. Eur. J. Biochem. **59**, 167-173.

Weiss, H. (1976) Subunit composition and biogenesis of mitochondrial cytochrome b. Biochim. Biophys. Acta **456**, 291-313.

Weiss, H. and Ziganke, B. (1977) Partial identification, stoichiometry and site of translation of the subunits of ubiquinone:cytochrome c oxidoreductase. In: Mitochondria 1977, Genetics and Biogenesis of Mitochondria (eds. W. Bandlow, et al.), pp. 463-472. Walter de Gruyter, New York.

Wilkie, D. (1975) Cytoplasmic inheritance and mitochondrial genetics in yeasts. Methods Cell Biol. **12**, 353-372.

Williamson, D. H. (1976) Packaging and recombination of mitochondrial DNA in vegetatively growing yeast cells. In: Genetics, Biogenesis and Bioenergetics of Mitochondria (eds. W. Bandlow et al.), pp. 117-123. Walter de Gruyter, New York.

# Chapter 2

## *In vivo* Incorporation of Different Amino Acids into Electrophoretically Characteristic Polypeptides Synthesized by HeLa Cell Mitochondria

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# IN VIVO INCORPORATION OF DIFFERENT AMINO ACIDS INTO ELECTROPHORETICALLY CHARACTERISTIC POLYPEPTIDES SYNTHESIZED BY HELA CELL MITOCHONDRIA

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SUMMARY - The in vivo incorporation of each of the twenty common amino acids into electrophoretically characteristic polypeptides synthesized by HeLa cell mitochondria has been investigated. Under labeling conditions which allow translation only on mitochondrial ribosomes, incorporation of all the amino acids, except aspartic acid, cysteine, glutamic acid and glycine, has been detected. These exceptions are probably due to problems related to amino acid pool size and/or equilibration.

Several recent investigations have suggested that the tRNA species coded for by HeLa cell mitochondrial DNA are far fewer than those required to read all the 61 amino acid specifying codons, and may not even represent a full complement for the 20 common amino acids (1-4). Furthermore, in a study of the incorporation of different amino acids into acid-precipitable and alkali-resistant material (assumed to represent mitochondrial protein synthesis on the basis of its cycloheximide resistance and chloramphenicol sensitivity), eight amino acids were found not to be incorporated to a significant extent (5). However, these apparently "negative" amino acids did not correspond in any meaningful way to those for which no mitochondrial DNA-coded tRNA had been detected (3). The above mentioned amino acid incorporation work was mostly performed in vitro in order to circumvent problems of amino acid equilibration with the cytoplasmic pools. However, the possible role of the mitochondrial pools in affecting the observed labeling patterns could not be excluded, nor were the mitochondrially synthesized products characterized as polypeptides.



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TABLE I

Amino Acids and Labeling Conditions

| Amino Acid[+] | Labeling Time[‡] (minutes) |
|---|---|
| *[$^{3}$H]alanine (51) | 30 (15) |
| [$^{3}$H]arginine (12,10) | 30 (15), 60 |
| *[$^{3}$H]asparagine (12) | 30 (15), 120 |
| *[$^{3}$H]aspartic acid (13) | 30 (15), 120 |
| [$^{3}$H]cysteine (2.6) | 120, 240 |
| *[$^{3}$H]glutamic acid (13.5) | 30 (15), 120 |
| [$^{3}$H]glutamine (23) | 30 (15), 120 |
| *[$^{3}$H]glycine (33, 43) | 30 (15), 120 |
| [$^{3}$H]histidine (22) | 30 (15), 60 |
| [$^{3}$H]isoleucine (30) | 30 (15), 30 (30), 60 |
| [$^{3}$H]leucine (57, 64) | 30, 30 (15) |
| [$^{3}$H]lysine (50) | 30 (30) |
| [$^{35}$S]methionine (64) | 30 (15) |
| [$^{3}$H]phenylalanine (50) | 30 (15) |
| *[$^{3}$H]proline (17, 46) | 120, 240 |
| [$^{3}$H]serine (1.6, 17, 17) | 60, 120, 240 |
| [$^{3}$H]threonine (1.4) | 120 |
| [$^{3}$H]tryptophan (1.5) | 60 |
| [$^{3}$H]tyrosine (46) | 30 (15), 120 |
| [$^{3}$H]valine (16) | 30 (15) |

All labeling experiments were carried out using medium which lacked the particular amino acid utilized, except in the case of amino acids marked with an asterisk (*), where complete medium was utilized.

[+]In parentheses, specific activity in Ci/mmole of the amino acid in the experiments reported in the table.

[‡]All the amino acids were tested in several experiments; the numbers reported indicate the labeling times utilized (those underlined refer to the experiments illustrated in Figure 1). The numbers in parentheses indicate the length of the chase period, performed by addition of an excess (0.01 M) of unlabeled amino acid.

In the present paper, we have examined the in vivo utilization of different amino acids for mitochondrial protein synthesis in HeLa cells using labeling

conditions designed to minimize possible pool effects. In addition, the radioactive products were analyzed by sodium dodecyl $SO_4$-polyacrylamide gel electrophoresis.

With the exception of aspartic acid, cysteine, glutamic acid and glycine, all of the common amino acids exhibited emetine-resistant and chloramphenicol-sensitive incorporation into polypeptides having the characteristic electrophoretic behavior of the products of mitochondrial protein synthesis.

## MATERIALS AND METHODS

HeLa cells were grown in suspension as previously described (6). Modified Eagle's medium (7) supplemented with 5% dialyzed calf serum was used. Exponentially growing cells were collected by centrifugation, resuspended in the appropriate medium [whenever possible, the particular amino acid being studied was omitted (Table I)] at $5 \times 10^6$ cells/ml and labeled as previously described (6). The isotope concentration used varied between 7.0 and 11.6 μCi/ml. Table I lists the specific activity of the amino acids and the labeling conditions used. For each amino acid two identical cultures were labeled, one in the presence of 100 μg/ml emetine [an effective inhibitor of cytoplasmic protein synthesis (8)], and the other in the presence of both emetine and 100 μg/ml chloramphenicol [the latter, a specific inhibitor of mitochondrial protein synthesis (9-11)]. The preparation of the 5000 $g_{av}$ crude mitochondrial fraction, its disruption by sonication, and the electrophoresis of the sodium dodecyl $SO_4$ solubilized samples through 12.5% polyacrylamide cylindrical gels were carried out as previously described (6). In some experiments, the labeled sample was precipitated with trichloroacetic acid in order to remove labeled non-protein contaminants (see below), dissolved in 100 μl of sample buffer, and neutralized by careful addition of NaOH (using bromphenol blue as an indicator) before being applied to the gel. Peak assignments in the electrophoretic patterns were made by comparison with the typical profile of [$^3$H]isoleucine- or [$^{35}$S]methionine-labeled mitochondrial protein products run

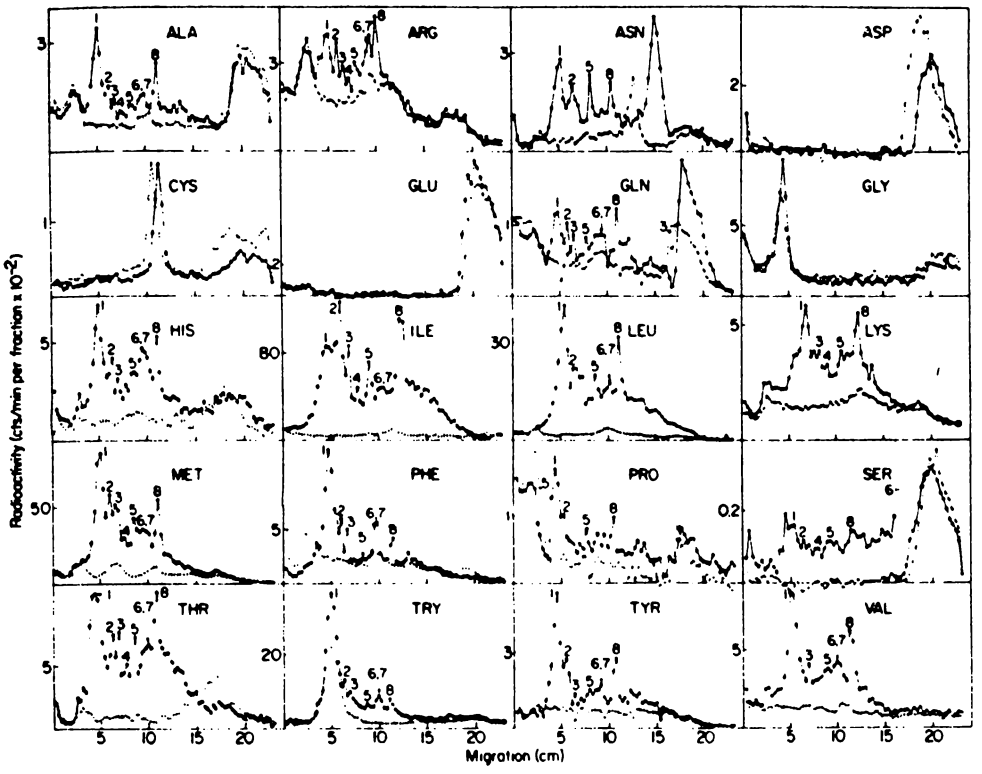


Fig. 1 Electrophoretic distribution (after 24-hr runs) of the products of HeLa cell mitochondrial protein synthesis labeled in vivo with different amino acids. Cell populations were labeled with different amino acids in the presence of 100 µg/ml of emetine and in the absence or presence of 100 µg/ml of chloramphenicol. The counts/min per fraction are normalized to 1 mg protein of the mitochondrial fraction applied to the gel. Peak assignments were made on the basis of the comparison with an internal [$^{35}$S]methionine-labeled standard or with the profile of a [$^{3}$H]isoleucine- or [$^{35}$S]methionine-labeled sample run in a parallel gel. ●----●, radioactivity in the presence of emetine; O----O, radioactivity in the presence of emetine and chloramphenicol.

on the same gel as an internal standard or in a parallel gel. Correction of $^{35}$S spillover into the $^{3}$H channel was made when appropriate.

## RESULTS

The conditions for labeling the products of mitochondrial protein synthesis were chosen so as to minimize problems connected with pool dilution of

the label and to increase the sensitivity of detection of amino acid incorporation. High isotope concentrations and, in some cases, long labeling times were used; furthermore, in some experiments, a chase period was introduced after the pulse, in order to reduce the heterogeneous background in the electrophoretic profiles and to make the polypeptide peaks more evident (12). Figure 1 shows the patterns from 24-hr electrophoretic runs of mitochondrial translation products labeled with different amino acids in the absence or presence of chloramphenicol. The well characterized profile of the mitochondrially synthesized polypeptides labeled with [$^{3}$H]isoleucine (6) or [$^{35}$S]methionine was used as the standard for peak assignment. All the amino acids, with the exception of aspartic acid, cysteine, glutamic acid and glycine, are incorporated into the characteristic electrophoretic components, though to different overall extents. The relative labeling of the individual peaks also varies with different amino acids: for example, phenylalanine gives an unusually high labeling of components 6 and 7.

For some of the amino acids (i.e., alanine, arginine, glutamine, phenylalanine and proline), the electrophoretic distribution of the labeled polypeptides exhibited a relatively high background of chloramphenicol-resistant heterogeneous material (Fig. 1); furthermore, glutamine, proline and serine showed very low absolute levels of incorporation, even under the conditions of most extensive labeling used. However, in all cases, the chloramphenicol-sensitive products showed an electrophoretic profile with the same identifiable peaks as in the profile of the [$^{3}$H]isoleucine labeled proteins.

In some cases, the analysis was complicated by the presence, in the electrophoretic profile, of peaks of emetine- and chloramphenicol-resistant labeled material. Such peaks were found in the low molecular weight region of the gel in the case of the products labeled with [$^{3}$H]aspartic acid, [$^{3}$H]glutamic acid or [$^{3}$H]serine, and in the middle or high molecular weight region, in the case of [$^{3}$H]cysteine or [$^{3}$H]glycine (Fig. 1). A broad peak of emetine- and chloramphenicol-resistant material had been previously observed after [$^{3}$H]isoleu-

cine incorporation (6). Characteristic of this material were its lack of reproducibility in different experiments and its lability, i.e., its tendency to decrease in amount or to change in electrophoretic mobility after storing or freezing and thawing. This lability may explain the lack of correspondence in position and the difference in size of these peaks in the patterns of material labeled in the presence and absence of chloramphenicol (see, for example, the profiles of the products labeled with [$^3$H]asparagine). In several instances, as in the case of [$^3$H]serine or [$^3$H]tyrosine labeled products, it was possible to eliminate this chloramphenicol-resistant material forming discrete peaks by precipitating the labeled sample with trichloroacetic acid and redissolving it in the electrophoresis buffer. The nature of this material is not known; it is possible that it is some kind of amino acid-containing lipid which can be labeled with different amino acids. In any case, the presence of these spurious peaks in the electrophoretic profiles did not affect the recognition of the peaks characteristic of the mitochondrially synthesized polypeptides.

Figure 2 shows the patterns obtained, after 42 hr of electrophoresis, for the mitochondrial translation products labeled with some of the amino acids; the better resolution of the discrete components reveals more clearly the basic similarity among the various electrophoretic profiles.

## DISCUSSION

The present study has extended earlier observations on the utilization of different amino acids for mitochondrial protein synthesis in HeLa cells (5). In particular, all the amino acids previously reported to be incorporated into chloramphenicol-sensitive products were shown here, under more stringent criteria, to be incorporated into mitochondrially synthesized, electrophoretically characteristic polypeptides. More significantly, however, four of the eight amino acids previously reported as apparently non-incorporated, i.e., alanine, arginine, glutamine and lysine, were found in the present work to be actually

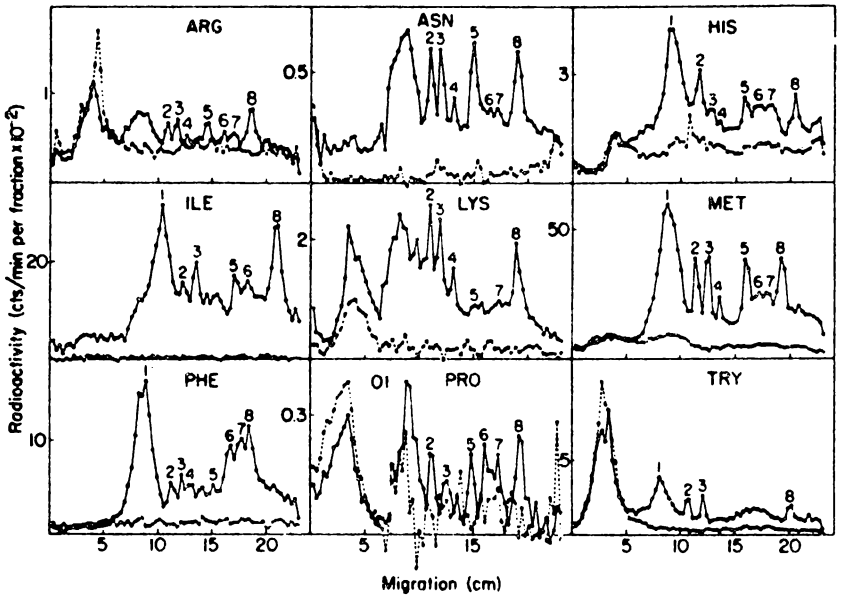


Fig. 2 Electrophoretic profiles, after 42-hr runs, of the products of mitochondrial protein synthesis labeled with selected amino acids. For details see legend for Figure 1.

utilized for protein synthesis. It seems likely that pool phenomena accounted for the very low incorporation obtained with these amino acids in the early experiments. It should be recalled that in vivo labeling of the mitochondrially synthesized polypeptides probably involves equilibration of the radioactive amino acid with both the cytoplasmic and the mitochondrial amino acid pools. The higher isotope concentrations and the longer labeling times used in the present work have reduced these pool effects. This approach, together with the electrophoretic analysis of the products, have increased the sensitivity and reliability of detection of the amino acids utilized by the mitochondrial translation apparatus.

There are reasons to believe that the failure to detect incorporation for aspartic acid, cysteine, glutamic acid and glycine in the present work could still be due to pool phenomena. Aspartic acid and glutamic acid are in fact known to be synthesized within the mitochondria (13), suggesting the

possibility of large mitochondrial pools or slow transport through the mitochondrial membrane via the aspartate-glutamate shuttle. Glycine and cysteine are both components of glutathione, which may enlarge the available pools because of its fairly rapid turnover (14); about six times as much glycine is present in glutathione as in the free pool (15). That glutamic acid and glycine have large cytoplasmic pools in HeLa cells is suggested by the very low levels of incorporation observed for these amino acids, relative to others, in an *in vitro* system measuring protein synthesis by endoplasmic reticulum-bound polysomes in the presence of the 105,000 $g_{av}$ supernatant (5).

The interpretation that large intramitochondrial and/or extramitochondrial pools or uptake restrictions may be responsible for the results obtained with aspartic acid, cysteine, glutamic acid and glycine would also be consistent with other observations. In particular, each of these amino acids has been shown to be present in at least some of the mitochondrially synthesized polypeptide components of the cytochrome c oxidase from yeast (16) and *Neurospora crassa* (17) and of cytochrome b from the latter organism (18,19), and of the oligomycin-sensitive ATPase from yeast (20), and in the presumptive subunits of mitochondrial origin of the cytochrome c oxidase from beef heart (21,22). It should also be recalled that mitochondrial DNA-coded tRNAs specific for these amino acids have been shown to exist in HeLa cell mitochondria (3).

Metabolic conversion from one amino acid to another could have conceivably led to some spurious positive results in the present work. However, the well-known conversions of glutamic or aspartic acid to other amino acids (23) presumably did not play a role in producing spurious labeling data, since, as discussed above, these amino acids were not themselves incorporated to any detectable extent. As concerns other possible relevant interconversions known in animal cells, that of phenylalanine to tyrosine (23) seems to be excluded by the difference in the observed labeling profiles.

The pool phenomena discussed above and the difference in the specific activities of the amino acids and in the labeling conditions make it impossible

to interpret the data of relative incorporation of different amino acids into a given mitochondrially synthesized polypeptide as reflecting directly its amino acid composition. However, the relative incorporation of a given amino acid into different polypeptides is probably a measure of its relative abundance in the various mitochondrial products. The labeling data obtained in the present work will therefore be useful for the selection of the appropriate amino acid likely to give the maximum specific labeling of individual mitochondrial polypeptides in future work.

In a previous study on the specificity of HeLa cell mitochondrial tRNAs, at least 17 species coded for by mitochondrial DNA, which are specific for 16 different amino acids, were detected (3). No tRNAs specific for asparagine, glutamine, histidine or proline were found to hybridize with mitochondrial DNA. These amino acids have been shown in the present work to be incorporated into characteristic mitochondrially synthesized polypeptides. Therefore, if the observations in the tRNA study are not due to experimental difficulties, they would imply an import into the mitochondria of tRNAs coded for by the nuclear genome, which are specific for the four amino acids. Such a possibility is open to direct experimental test. Evidence suggesting a transport of some tRNA species from the nucleo-cytoplasmic compartment has already been presented for Tetrahymena pyriformis mitochondria (24,25).

On the basis of the evidence presented in this work and of the tRNA specificity data discussed above, it seems reasonable to conclude that all amino acids are very probably utilized for mitochondrial protein synthesis in HeLa cells.

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REFERENCES

1. Aloni, Y., and Attardi, G. (1971) J. Mol. Biol. 55, 271-276.
2. Wu, M., Davidson, N., Attardi, G., and Aloni, Y. (1972) J. Mol. Biol. 71, 81-93.
3. Lynch, D. C., and Attardi, G. (1976) J. Mol. Biol. 102, 125-141.
4. Angerer, L., Davidson, N., Murphy, W., Lynch, D., and Attardi, G. (1976) Cell 9, 81-90.
5. Costantino, P., and Attardi, G. (1973) Proc. Nat. Acad. Sci. USA 70, 1490-1494.
6. Costantino, P., and Attardi, G. (1975) J. Mol. Biol. 96, 291-306.
7. Levintow, L., and Darnell, J. E. (1960) J. Biol. Chem. 235, 70-73.
8. Perlman, S., and Penman, S. (1970) Biochem. Biophys. Res. Commun. 40, 941-948.
9. Kroon, A. M. (1965) Biochim. Biophys. Acta 108, 275-284.
10. Linnane, A. (1968) in Symposium on Biochemical Aspects of the Biogenesis of Mitochondria (Slater, E. G., et al., eds.) pp. 333-353, Adriatica Editrice, Bari.
11. Lederman, M., and Attardi, G. (1970) Biochem. Biophys. Res. Commun. 40, 1492-1500.
12. Costantino, P., and Attardi, G. (1977) J. Biol. Chem. 252, 1702-1711.
13. Lehninger, A. L. (1975) in Biochemistry, Worth Publishers, Inc., New York.
14. Henriques, O. B., Henriques, S. B., and Mandelbaum, F. R. (1957) Biochem. J. 66, 222-227.
15. Piez, K. A., and Eagle, H. (1958) J. Biol. Chem. 231, 533-545.
16. Poyton, R. O., and Schatz, G. (1975) J. Biol. Chem. 250, 752-761.
17. Sebald, W., Machleidt, W., and Otto, J. (1973) Eur. J. Biochem. 38, 311-324.
18. Katan, M. B., Pool, L., and Groot, G.S.P. (1976) Eur. J. Biochem. 65, 95-105.
19. Weiss, H., and Ziganke, B. (1976) in Genetics and Biogenesis of Chloroplasts and Mitochondria (Th. Bücher, et al., eds.) 259-266, North Holland, Amsterdam.
20. Sierra, M. F., and Tzagoloff, A. (1973) Proc. Nat. Acad. Sci. USA 70 3155-3159.
21. Downer, N. W., Robinson, N. C., and Capaldi, R. A. (1976) Biochem. 15, 2930-2936.
22. Buse, G., and Steffens, G. J. (1976) in Genetics and Biogenesis of Chloroplasts and Mitochondria (Th. Bücher, et al., eds.) 189-194, North Holland, Amsterdam.
23. Meister, A. (1965) in Biochemistry of the Amino Acids, Vol. II, pp. 593-1020, Academic Press, New York.
24. Chiu, N., Chiu, A.O.S., and Suyama, Y. (1974) 82, 441-457.
25. Chiu, N., Chiu, A., and Suyama, Y. (1975) 99, 37-50.

# Chapter 3

## Biogenesis of Mitochondrial Proteins in HeLa Cells

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.

[3] G. Schatz and T. L. Mason, *Annu. Rev. Biochem.* **43**, 51 (1974).

[4] A. W. Linnane and J. M. Haslam, *Curr. Top. Cell. Regul.* **2**, 101 (1970).



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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.

[5] A. Tzagoloff and P. Meagher, *J. Biol. Chem.* **247**, 594 (1972).
[6] M. F. Sierra and A. Tzagoloff, *Proc. Natl. Acad. Sci. USA* **70**, 3155 (1973).
[7] W. Sebald, W. Machleidt, and J. Otto, *Eur. J. Biochem.* **38**, 311 (1973).
[8] H. Weiss and B. Ziganke, *Eur. Biol. Biochem.* **41**, 63 (1973).
[9] M. S. Rubin and A. Tzagoloff, *J. Biol. Chem.* **248**, 4275 (1975).
[10] G. Jackl and W. Sebald, *Eur. J. Biochem.* **54**, 97 (1975).
[11] M. B. Katan, N. Van Harten-Loosbroek, and G. S. P. Groot, *Eur. J. Biochem.* **70**, 409 (1976).
[12] M. L. Claisse, A. Spyridakis, and P. P. Slonimski, *in* "Mitochondria 1977—Genetics and Biogenesis of Mitochondria" (W. Bandlow, R. J. Schweyen, K. Wolf, and F. Kaudewitz, eds.), p. 337. Walter de Gruyter, Berlin and New York, 1977.
[13] A. Tzagoloff, F. Foury, and A. Akai, *Mol. Gen. Genet.* **149**, 33 (1976).
[14] B. Storrie and G. Attardi, *J. Mol. Biol.* **71**, 177 (1972).
[15] B. Storrie and G. Attardi, *J. Cell. Biol.* **56**, 833 (1973).
[16] B. Attardi, B. Cravioto, and G. Attardi, *J. Mol. Biol.* **44**, 47 (1969).
[17] M. Lederman and G. Attardi, *Biochem. Biophys. Res. Commun.* **40**, 1492 (1970).
[18] H. L. Ennis and M. Lubin, *Science* **146** 1474 (1964).
[19] A. Linnane, *in* "Biochemical Aspects of the Biogenesis of Mitochondria" (E. C. Slater, M. Tager, S. Papa, and E. Quagliariello, eds.), p. 333. Adriatica Editrice, Bari, 1968.
[20] S. Perlman and S. Penman, *Nature (London)* **227**, 133 (1970).
[21] A. P. Grollman, *Proc. Natl. Acad. Sci. USA* **56**, 1867 (1966).
[22] S. Perlman and S. Penman, *Biochem. Biophys. Res. Commun.* **40**, 941 (1970).
[23] D. Ojala and G. Attardi, *J. Mol. Biol.* **65**, 273 (1972).
[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 $1 \times 10^6$ to $3 \times 10^6$ 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 $\mu$g/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 $\mu$g/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*

[25] L. Levintow and J. E. Darnell, *J. Biol. Chem.* **235**, 70 (1960).
[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 $\mu$g 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 $\mu$Ci (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 $\mu$g 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

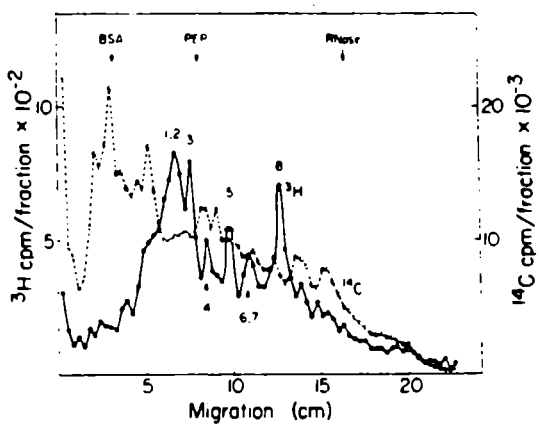


FIG. 1. Electrophoretic profile (26-hr run) of the total proteins of the mitochondrial fraction from HeLa cells labeled for 72 hr with $^{14}$C-arginine and $^{14}$C-lysine, and then for 1 hr with $^3$H-isoleucine in the presence of 100 μg/ml emetine (P. Costantino and G. Attardi, unpublished observations).

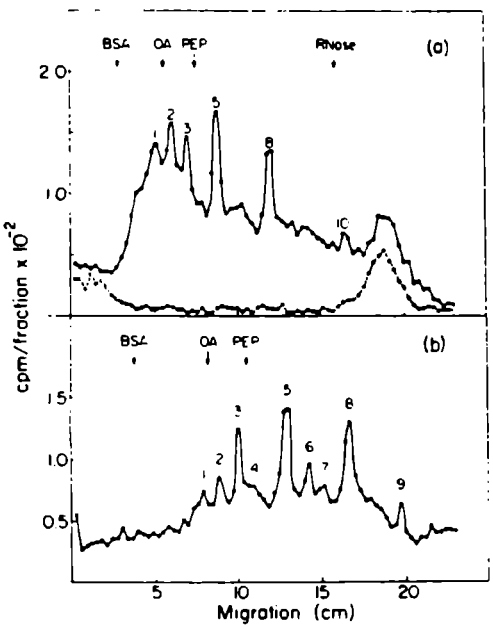


FIG. 2. Electrophoretic profiles of the products of mitochondrial protein synthesis from HeLa cells labeled for 10 min with $^3$H-isoleucine in the presence of 100 μg/ml emetine, and in the absence (●) or presence (○) of 100 μg/ml chloramphenicol. (a) 26-hr run; (b) 40-hr run.[28]

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.[3a]

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[24] (Figs. 1 and 2). The synthesis of these components is completely sensitive to chloramphenicol at 100 $\mu$g/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

[3a] 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

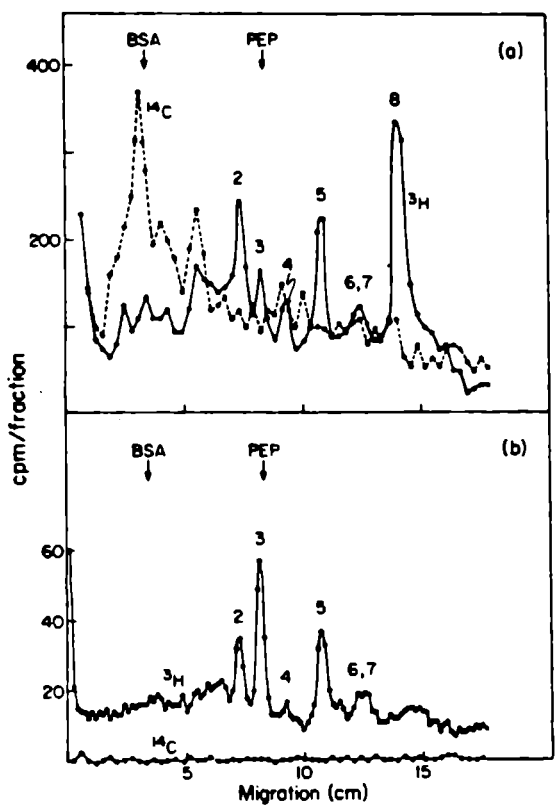


FIG. 3. Electrophoretic profiles of the proteins of the mitochondrial fraction extracted with neutral 2:1 chloroform-methanol mixture (b) and of the insoluble residue (a) from HeLa cells labeled for 48 hr with $^{14}$C-arginine and $^{14}$C-lysine and then for 1 hr with $^{3}$H-isoleucine in the presence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

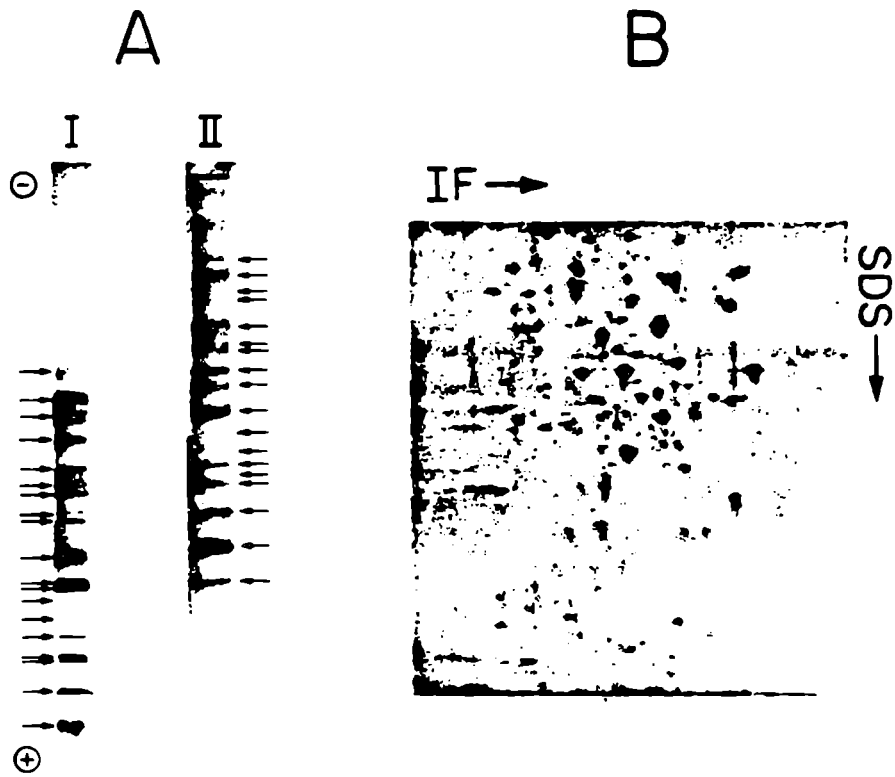


FIG. 4. (A) Fluorographs, after electrophoresis through SDS-polyacrylamide 15 to 25% gradient slab gel (I) or SDS-polyacrylamide/8 *M* urea slab gel (II), of the proteins from the mitochondrial fraction of HeLa cells labeled for 2 hr with $^{35}$S-methionine in the presence of 100 μg/ml emetine. 16,000 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]

[39] M. G. Douglas and R. A. Butow, *Proc. Natl. Acad. Sci. USA* 73, 1083 (1976).
[40] J. Hare, E. Ching and G. Attardi, *J. Biol. Chem.*, submitted (1978).

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.

[41] M. Lederman and G. Attardi, *J. Mol. Biol.* **78**, 275 (1973).

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

*By* W. NEUPERT, F. MILLER, R. MICHEL, and G. HALLERMAYER

### 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.

[1] P. Borst and L. A. Grivell, *FEBS Lett.* **13**, 73 (1971).
[2] P. Borst, *Annu. Rev. Biochem.* **41**, 333 (1972).
[3] T. W. O'Brien and D. E. Matthews, *Handb. Genet.* **5**, 535 (1976).
[4] N. G. Avadhani, F. S. Lewis, and R. J. Rutman, *Sub-Cell. Biochem.* **4**, 93 (1975).
[5] W. Neupert, *in* "Horizons in Biochemistry and Biophysics" (E. Quagliariello, F. Palmieri, and T. P. Singer, eds.), Vol. III, p. 257. Addison-Wesley, Reading, Massachusetts, 1977.
[6] H. Küntzel and H. Noll, *Nature (London)* **215**, 1340 (1967).
[7] M. R. Rifkin, D. D. Wood, and D. J. L. Luck, *Proc. Natl. Acad. Sci. U.S.A.* **58**, 1025 (1967).



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# Chapter 4

## High Resolution Analysis and Characterization of the Mitochondrial Translation Products in HeLa Cells

**Summary**

A highly resolving gradient polyacrylamide SDS gel electrophoresis system is described which resolves the products of HeLa cell mitochondrial protein synthesis into 19 discrete species. A second SDS-urea polyacrylamide gel system is described which resolves these products into 17 discrete species. The correspondence is determined between the species resolved here and those resolved using the phosphate buffered gel system described earlier (Costantino and Attardi, 1975).

Evidence is presented that these species are indeed polypeptides, of mitochondrial origin, present in vivo and probably not related as precursor to product. A bidimensional system resulting from combination of the SDS-urea and gradient gel systems presents evidence for 25 discrete components synthesized on mitochondrial ribosomes. This system has also allowed determination of the correspondence of species run in each of the gel systems.

Molecular weight estimates of these mitochondrial translation products, if summed up and combined with assumptions on the absence of relatedness, number of nucleotides required for coding and estimates of the size of the human mitochondrial genome in HeLa cells, suggest that these products would completely saturate the non-overlapping single strand mitochondrial coding capacity.

## Introduction

The mitochondrion contains its own transcription and translation machinery (Borst and Grivell, 1978). It is generally accepted that the information which comes from the organelle's circular genome is translated on the mitochondrial ribosomes. The different antibiotic sensitivity of the mitochondrial from the cytoplasmic ribosomes allows biosynthetic incorporation of radioactive amino acids specifically into mitochondrially synthesized polypeptides (Schatz and Mason, 1974).

Earlier studies in characterizing these species utilized a phosphate buffered SDS-polyacrylamide column gel system based on that described by Weber and Osborn for analysis of the products (Weber and Osborn, 1969; Costantino and Attardi, 1975). The method of assay was by fractionation of the gel and liquid scintillation counting. These studies utilized the relatively low specific activity and low decay energy $^{3}H$ isotopes but allowed labeling with many different amino acids (Ching et al., 1977). Ten discrete polypeptide species were resolved and extensively studied (Costantino and Attardi, 1975, 1977).

A number of basic improvements have been applied which dramatically increase the power of the resolving system. Use of the higher decay energy $^{35}S$ isotope in methionine as the radioactive label has increased the sensitivity of detection and allowed autoradiography which gives higher resolution. Furthermore the higher specific activity has yielded much greater amounts of radioactivity which has aided further in the sensitivity of detection. Using these improvements increases the resolution to 13 species.

The development of two higher resolution polyacrylamide gel electrophoresis systems has given much greater resolution than the ten previously reported (Costantino and Attardi, 1975). An SDS-urea gel system utilized here resolves

17 discrete species. Another gradient polyacrylamide SDS-gel system can resolve 19 components. These species have been characterized and shown to reflect in vivo polypeptide species of mitochondrial origin. By combining the urea and gradient gel systems into a bidimensional system, 25 discrete polypeptide components have been identified. The correspondences between the three systems have been determined. Finally, the species separable by the urea gel system have been subjected to peptide fragment analysis and at least 7 of the species resolved appear not to contain components structurally related to one another. Molecular weight estimates have been made of these 25 polypeptide species. When comparison is made with the mitochondrial genome's single-stranded coding capacity (after accounting for an assumed 20 tRNA species and the two rRNA species), we find complete saturation.

Functional identification of polypeptides in yeast indicates mitochondrially synthesized polypeptides are present in the oxidative phosphorylation chain, specifically in (and only in) the cytochrome $bc_1$, the cytochrome c oxidase and the oligomycin-sensitive ATPase complexes (Tzagoloff et al., 1975, 1976a,b; Foury and Tzagoloff, 1976). One additional protein has been identified in the yeast and Neurospora mitochondrial ribosomes (Groot, 1974; Lambowitz et al., 1976; LaPolla and Lambowitz, 1977). Though these appear to be the major polypeptide species, many additional minor polypeptides also appear to exist (Douglas and Butow, 1976).

## Experimental Procedures

### Labeling and preparation of HeLa cell mitochondria

HeLa cells of the S3 clonal strain were grown in suspension culture as described (Costantino and Attardi, 1975).

Cells were centrifuged and washed twice with medium lacking methionine but containing 5% dialyzed calf serum. The cells were then resuspended in this medium at 5 x $10^6$ cells/ml. All growth incubations thereafter were performed aerating the flask at 2 liters/min with filtered air containing 5% $CO_2$, a modification which significantly helped to stabilize the pH of the medium at very high cell density.

Five minutes after cell resuspension, antibiotics were added at 100 µg/ml. For inhibition of cytoplasmic ribosomes, emetine was usually the antibiotic of choice unless the reversibility of cycloheximide was important (Perlman and Penman, 1970; Costantino and Attardi, 1975; Ennis and Lubin, 1964; Colombo et al., 1966). For inhibition of mitochondrial ribosomes, chloramphenicol was also added (Kroon, 1965; Lederman and Attardi, 1970). Five minutes after antibiotic addition, [$^{35}$S]methionine (600 Ci/mmole) was added to 8.3 µCi/ml. Cells were incubated for a two-hour labeling period. One hr chases involved either addition of unlabeled methione to 0.01 M if emetine was used or washing twice in complete unlabeled medium plus calf serum and resuspension at 2 x $10^6$ cells/ml to remove cycloheximide.

Upon conclusion of the labeling procedure, the cells were quickly cooled on ice and washed three times with cold 0.13 M NaCl, 0.005 M KCl, 0.001 M $MgCl_2$ (NKM) and then resuspended in 6-cell volumes of 0.01 M Tris Cl (pH 6.7 at 25°), 0.01 M KCl, 0.00015 M $MgCl_2$. After two minutes of swelling, the cell suspension was homogenized in an A.H. Thomas Teflon pestle homogenizer (motor-driven pestle at ~1500 rpm) using enough strokes to break 70-90% of the cells. Sucrose was added to 0.25 M and the homogenate was centrifuged at 1160 $g_{av}$ for 3 min to pellet cell nuclei and other large cellular structures. The supernatant was spun at 5000 $g_{av}$ for 10 min and the pellet carefully resuspended in 8-cell volumes

of 0.25 M sucrose in 0.01 M Tris Cl (pH 6.7 at 25°), 0.00015 M $MgCl_2$. This resuspension was respun at 1160 $g_{av}$ for 3 min and the pellet resuspended in 0.3-cell volumes of 0.25 M sucrose in 0.01 M Tris acetate (pH 7.0). Samples were then sonicated. Final protein concentrations of ~10 mg/ml resulted.

When present, PMSF (to 2 mM) and EGTA (to 5 mM) were added freshly to all preparative solutions starting with cell washings in NKM (James, 1978).

**Whole cell lysates**

Cells were labeled for 2 hours as described above. At the end of the labeling period, the methionine label was chased by addition of unlabeled methionine to 0.01 M. After 1 hr of chase, cells were washed 3X in NKM and resuspended into 0.6-cell volumes of 0.01 M Tris Cl (pH 7.0 at 25°), 0.10 M NaCl, and 0.001 M EDTA, then SDS was added to 0.5%. Samples were then sonicated.

**SDS polyacrylamide gel electrophoresis**

Phosphate buffered SDS-polyacrylamide column gels initially devised by Weber and Osborn were prepared and run as described (Weber and Osborn, 1969; Costantino and Attardi, 1975). Slab gels were run using the same recipe but using 15% acrylamide instead of 12-1/2%. For the slab gels, reservoir buffers were changed about halfway through the run since the pH tended to change.

A 15-25% gradient gel was prepared (1:75 bisacrylamide) and run as described (Studier, 1973).

SDS-urea gels were prepared and run essentially as described (Downer et al., 1976). However, a stacking gel of 1 cm was added, consisting of 5% acrylamide (1:37.5 bisacrylamide), 8 M urea in 0.033 M $H_3PO_4$-Tris (pH 6.3) and 0.1% SDS.

**Two-dimensional gels**

When a track was to be overlaid over another gel, a 12-1/2% polyacrylamide

SDS-urea gel was used instead of 15% and run the appropriate distance. The gel track was cut out, the urea eluted in multiple short changes of water and the track overlaid onto a higher percentage gel. Bubbles at the gel interfaces were carefully removed. The track was overlaid with a solution of 0.1% SDS, 5% 2-mercaptoethanol and dyes and run normally.

### Peptide mapping

Peptide mapping with chymotrypsin was performed with a 12-1/2% polyacrylamide SDS-urea first dimension gel and a straight 15% gel using the gradient gel buffer system essentially as described (Bordier and Crettol-Järvinen, 1979).

### Fluorography and autoradiography

Fluorography and autoradiography were performed as described (Bonner and Laskey, 1974).

## Results

### Resolution

The earlier procedure in this laboratory for gel electrophoresis of the products of mitochondrial protein synthesis was by Weber-Osborn column gels. The radioactivity assay was by scintillation counter and would optimally resolve 10 discrete species which have been extensively studied (Costantino and Attardi, 1975, 1977). The resolution of species labeled by [$^{35}$S]methionine is virtually identical to that by other amino acids (Ching et al., 1977). A profile with the major peaks identified by the earlier designation (using an *) is shown in Figure 1.

Beneath is shown a Weber-Osborn 15% polyacrylamide slab SDS gel of similar material and its chloramphenicol control assayed by fluorography. Thirteen discrete species are indicated with partial identification by the earlier peak

designations. The resolution of the fluorograph shows a number of additional bands which are not apparent in the scintillation counter profile suggesting, perhaps, that fluorography under these conditions is favorable in terms of sensitivity when compared to the scintillation counter. The chloramphenicol control shows in the higher resolution slab system that the presence of all bands is sensitive, thus confirming their dependence on mitochondrial ribosome function.

Subsequent to those studies, higher resolution gel electrophoresis systems have been applied for separation of the products of mitochondrial protein synthesis. A Tris-glycine buffered gradient polyacrylamide slab SDS-gel can resolve 19 discrete bands as shown in Figure 2 (track a), with the reproducible species indicated. Each band is assigned a numerical label (whose significance becomes obvious below) by which it will be referred. Other particular runs may show less resolution or be insufficiently exposed for visualization of the minor bands, but the enaracteristic relative migrations and intensities are very reproducible.

A Tris-phosphate buffered SDS-urea polyacrylamide gel system resolves 17 different bands as shown in Figure 3 (track a). Once again, the reproducible species are indicated and each band is assigned a numerical designation.

**Characterization of labeled species as polypeptides**

The species studied in this work are characterized as polypeptides by the three criteria of the specific labeling by the amino acid methionine, incorporation being dependent on ribosomal function and the pronase sensitivity of these species.

Dependence on ribosomal function is shown in Figure 2 (tracks b and c) by the absence of any species when the labeling is performed in the presence of both of the antibiotics emetine and chloramphenicol, conditions where all ribosomal function should be absent.

Pronase sensitivity of these species is also shown in Figure 2 (tracks d and e). Profiles are shown before and after samples were exposed to 25 μg of pronase at 37° for 50 min. All of the bands have been degraded to very small fragments running very near to the bottom of the gel.

**Correspondence between the Weber-Osborn gel system and the SDS-urea gel system**

The correspondence between the early Weber-Osborn column gel and the urea gel systems was determined by running a two-dimensional gel. The first dimension was run in the urea system and the track was overlaid onto a slab Weber-Osborn gel. Figure 4 shows the result of this experiment. Spot correspondences were made on the combined criteria of relative and absolute migrations and intensities. The results are clear with a few specific points of interest.

Some bands which can be identified in the urea dimension have no corresponding band in the Weber-Osborn gel. On the other hand, some bands of the urea dimension are resolved in the Weber-Osborn dimension, in particular are urea system bands 2,3; 14,15 and 16,17. Also, one component of urea gel band 7,8,9,10 appears to be resolved from the others and comigrates with band 11 on the Weber-Osborn system. This gel also allows an assignment of the previously assigned asterisked numbered bands resolved in Figure 1 with the reference system used in this paper (see Table I). Another interesting observation is the inversion of migrations between the two systems. This has been reported earlier for particular mitochondrial proteins (Capaldi et al., 1977; Groot et al., 1978).

**Correspondence between the SDS-urea and gradient gel systems**

The most highly resolving system utilized a bidimensional combination of the urea and gradient gel systems. The result is shown in Figure 5. The numbering system used above becomes obvious here. From the fluorograph and the drawing,

where there is reproducible evidence for a discrete spot, this is numbered successively from the left (i.e., in increasing electrophoretic mobility in the SDS-urea gel system) and comigrating species in that dimension successively from the top (i.e., increasing electrophoretic mobility in the gradient gel system). Bands in either dimension are numerically designated by the numbered components contained within it.

Components 7,8,9,10 which comigrate in the urea dimension are marginally resolved in the gradient dimension of the bidimensional gel and are clearly separated in the gradient system alone. Spots 15, 17, 18 and 19 are very faint but reproducibly present. Unresolved doublets in the urea dimension of 2,3; 14,15; 16,17 and 22,23 are resolved in the gradient dimension; conversely for the unresolved gradient doublets 4,5; 6,7; and 9,11. Note that component 6 appears to be absent in the urea gel dimension, as is 19 in the gradient dimension. The total resolution comes to 25 discrete components whose estimated molecular weights are presented in Table II.

In the course of other studies on the cytochrome oxidase complex, component 2 or 3 corresponds to cytochrome oxidase subunit I, 13 corresponds to subunit II and 14 corresponds to subunit III (Hare et al., 1979). It can be clearly seen that subunits II and III (i.e., species 13 and 14) are inverted relative to one another in the SDS-urea and gradient gel systems.

**Evidence for in vivo presence of these polypeptides**

The separation of such a large number of polypeptides suggested the possibility of artifactual generation of species by highly site-specific degradative enzymes acting during the purification procedure. Degradation is minimized throughout the preparation procedure by keeping the samples on ice and working as quickly as possible. The entire preparation procedure takes about 2 hours, about 1 hour of which is after cell homogenization.

One experiment was done where PMSF and EGTA were added freshly to all preparation solutions to inhibit protease activity during the mitochondrial purification procedure. Figure 2 (track f) shows that the resulting profile is virtually the same as the normal preparations in the absence of the protease inhibitors.

Finally, to completely eliminate the 2 hours of preparative manipulations, cells were labeled under the appropriate conditions, chased by addition of unlabeled methionine and directly lysed with SDS. This sample, plus a chloramphenicol control sample, are shown in Figure 3 (tracks b and c). It is clear that virtually all the same bands with the possible exceptions of bands 11 and 18 or 19 are present in both samples after either a direct cell lysate or after a mitochondrial purification. Consequently, virtually all the species seen are present in vivo.

**Polypeptide interrelationships**

The question of relationships between these species arises since the number of functionally expected polypeptides is much fewer. It has been shown that the species are not artifactually generated during the mitochondrial preparation, but the possibility of precursor to product relationships does remain.

To address this question, a pulse-chased sample was compared with non-chased material to see if the radioactive label would be chased from precursors in a 1 hour chase. Figure 3 (tracks d and e) shows samples without and with a chase. The two profiles are virtually identical with the exceptions again involving the possible absence of species 11 and 19. Consequently the great majority of the components appear to be reasonably stable.

Peptide fragment analysis is a more direct means to check relationships between different species. Proteolytic digestion was performed by a modification of partial proteolysis within the gel (Cleveland et al., 1977). Although the data do not allow interpretation of the minor species, some information can be derived

for the major species (Figure 6). This analysis has two difficulties, low detectability of the minor species and insensitivity of species to proteolysis (Weiss, 1976). Furthermore, it is limited by the resolution of the first dimension urea gel where many of the species comigrate. However, the major species appear not to be related structurally.

## Discussion

As described above, the biosynthetic incorporation of $^{35}$S-methionine is a good indication that the species labeled here are indeed polypeptides. Furthermore, the ribosomal dependence of incorporation as indicated by the antibiotic emetine (or cycloheximide) coupled with chloramphenicol strongly suggests that these species are polypeptide in nature. In the presence of both types of inhibitors, all ribosome translation should cease and any residual incorporation would result from one of two possible sources. There may conceivably be a subclass of ribosomes which are insensitive to both inhibitors at these levels or there may be non-ribosomal incorporation into components of polypeptide or non-polypeptide nature. However, the absence of any residual incorporation argues strongly that neither of these is occurring. In particular, the absence of the second possibility involving non-ribosomal incorporation implies that all products are synthesized on ribosomes and thus are polypeptides. Finally, the sensitivity of the species to pronase characterizes the final products as protein.

Thus, the possibility is eliminated that one of the bands in these gel patterns results from a methionine charged tRNA.

The criterion used for a polypeptide being synthesized on mitochondrial ribosomes has been the differential antibiotic sensitivities between cytoplasmic and mitochondrial ribosomes. The mitochondrial ribosomes are insensitive to

both antibiotics emetine and cycloheximide but sensitive to chloramphenicol. Consequently, labeling in the presence of appropriate levels of either emetine or cycloheximide alone allows synthesis to continue exclusively on mitochondrial ribosomes. As a further verification of mitochondrial ribosome dependence, the mitochondrial ribosome-specific antibiotic chloramphenicol can also be included in a parallel labeling experiment which inhibits all residual protein synthesis.

It is shown that these numerous species do not arise artifactually by specific protease digestions during the mitochondrial preparation nor do they seem to be particularly unstable species. Mitochondrial purification is done on ice as quickly as possible, taking about 2 hours of which about half is after cell breakage. The presence of the protease inhibitors PMSF (for serine proteases) and EGTA (for $Ca^{++}$ requiring proteases) throughout the mitochondrial purification make degradation during preparation unlikely since the pattern remains the same whether the inhibitors are present or not. Finally, the presence of essentially the same pattern in whole cell lysates labeled under similar conditions eliminates the possibility of dependence on a 2 hr preparation period to generate these species and further indicates that each of these species exists within the cells.

The appearance of this many polypeptide species is a reproducible result under the labeling conditions and gel separation techniques utilized. The size distribution and relative proportions of the species are virtually identical from preparation to preparation, although some runs are slightly higher resolving than others. Consequently, although particular profiles may indicate loss of minor species, these differences may not reflect true differences in the protein profiles. In addition, all of the described experimental samples have actually been run on both of the two high resolution gel systems (SDS-urea and gradient) and confirm the results.

The labeling of these species with $^{35}$S-methionine requires the incorporation of the amino acid into the polypeptide chain. The relative intensity of labeling should depend, besides upon the synthesis rate of the component, on the amino acid composition and polypeptide size. All things equal, a shorter polypeptide should have less label on an equimolar peptide basis than a larger one, and species synthesized in greater amounts will show up more intensely than more scarce components. The composition effect is such that polypeptides completely lacking methionine residues will not be detected, however the mitochondrial ribosomes initiate with formyl-methionine. If there is no post-translational removal of this residue, then the polypeptide should theoretically be detectable.

The overall evidence on amino terminal processing indicates that at least some and probably all species retain their amino terminal methionine. Buse and Steffens have reported that subunits I and II of beef heart cytochrome oxidase have blocked formyl-methionine termini (Buse and Steffens, 1976). The cytochrome b polypeptide is also reported to have a blocked amino terminus (Weiss, 1976). Mahler's use of radioactive formyl groups to label mitochondrially synthesized polypeptides in the absence of antibiotics indicates that at least many of the proteins retain the blocked terminus under the conditions used (Mahler et al., 1972; Feldman and Mahler, 1974).

The number of species resolved depends on the type of gel system utilized. The early Weber-Osborn column gels had resolved about ten species, a larger number of species than were being found by most other systems. Those studies both clearly showed these products to be mitochondrially synthesized and characterized some of their metabolic properties (Costantino and Attardi, 1975, 1977). Since then, conversion of the Weber-Osborn column gel system to a slab gel coupled with autoradiography has resulted in greater resolution and sensitivity, increasing the

number of species to 13. Other groups investigating various mammalian systems have also characterized some 8-13 polypeptides whose electrophoretic profiles appear slightly similar (Jeffreys and Craig, 1976; Yatscoff and Freeman, 1977; Yatscoff et al., 1978). The gel patterns of Jeffreys and Craig look quite similar to the pattern of the major species on the gradient gel system described here. In yeast, another gradient gel system has resolved over 20 discrete polypeptides which satisfy the criteria of mitochondrial protein products (Douglas and Butow, 1976).

The gradient gel system described here gives very good band sharpness and reasonably good separation and can resolve as many as 19 different species. The urea gel system described here gives sufficient resolution to separate 17 discrete polypeptide species. This system gives good separation of the low molecular weight components and a much better molecular weight determination than either of the others. Table II gives the molecular weights of the species determined by this system; sizes range from 51 to 3.5K daltons. However, the mitochondrially synthesized polypeptides are very hydrophobic or may be glycosylated which may cause anomolous migration upon electrophoresis. There are indications that some mitochondrially synthesized proteins may well display anomolous migration behaviors in SDS-polyacrylamide gel electrophoresis and consequently may give erroneous molecular weight estimates (Briggs et al., 1975; Yatscoff and Freeman, 1977; Höchli and Hackenbrock, 1978; Groot et al., 1978). The average size of the mitochondrially synthesized polypeptides is much smaller than the average size of the bulk of the mitochondrial proteins, perhaps a consequence of the informational frugality displayed by the small genome (see Hare et al., 1979).

The numerical assignments in the gel systems are based primarily on the correspondence determined by the bidimensional gels. Where there is any degree of ambiguity, the species are compared making use of relative intensities and relative migrations. Although polypeptides have behaved basically as expected, there are particular phenomena which should be kept in mind. The overlaying of the track from the first dimension between two glass plates can lead to minor contractions and expansions of particular regions of the track. Species run after separation in the first gel system may run slightly differently from the normal single dimension track of the same gel system. In particular, for the urea to Weber-Osborn slab gels, the first dimension is a 12-1/2% polyacrylamide gel laid over a 15% gel. Besides being 15% instead of the normal 12-1/2% polyacrylamide for the Weber-Osborn system, this additional overlaid gel will effectively lengthen the gel but include a slight stacking effect at the 12-1/2 to 15% interface. Furthermore, the effective sample height becomes the height of the overlaid track, a problem since this gel system has little or no inherent stacking capability.

For the urea to gradient bidimensional gels, the normal gradient system contains a low percentage stacking gel. When the 12-1/2% polyacrylamide urea track is overlaid, again the sample height is increased and the stacking effect is less, due residually to the difference in acrylamide concentration and ionic strength. The acrylamide gradient also can be slightly different from gel to gel due to the slight nonreproducibility of gradient gel pouring.

Furthermore, the sample environment is different in the two situations. Normally, when a sample is applied, any particular species is in solution with the entire spectrum of other proteins. After separation in one dimension, each species is flanked only by a relatively small number and a lower overall concentration of neighboring similarly migrating species. Consequently, whatever minor residual

protein interactions might occur in the presence of SDS are different in the two cases and can potentially lead to slightly different migration behavior.

But the overall result combining the urea and gradient gel systems into a higher resolution bidimensional system has allowed the separation of 25 discrete mitochondrially synthesized polypeptides, some of very low labeling intensity. The conditions chosen for labeling and analysis come close to the optimal detection capabilities presently available.

However, the resolution of 25 different polypeptide species raises the question, in view of the limited number of known functional species, of why there are so many. They are indeed real since they do exist in vivo. To the limits of the available technology they appear not to be merely processing intermediates because the patterns appear virtually identical before or after a 1 hr chase with cold methionine in the presence of cytoplasmic protein synthesis. Furthermore, partial digestion peptide analysis shows no obvious structural similarities. Neither of these experiments bear on the possibility that peptides may overlap in different reading frames or that the low intensity bands might share sequences with any of the others, as observed for T antigen of SV40 and polyoma viruses (Paucha et al., 1978; Hutchinson et al., 1978).

The possibility of antibiotic artifacts has not been totally eliminated. Although there is no evidence for such a phenomenon, both antibiotics (which are structurally quite different) might conceivably cause identical premature site-specific termination generating an artifactual homogenous species. On the other hand, the antibiotics may cause repression of synthesis of various species which would increase the true number beyond 25. Two possible approaches which might avoid antibiotic artifacts involve use of formyl label as described above, or the use of temperature-sensitive cytoplasmic protein synthesis mutants (Mahler et al., 1972; Feldman and Mahler, 1974; Wallace et al., 1975; Yatscoff and Freeman, 1977).

Two other sources of artifactual generation of species can also be eliminated. Bacterial or mycoplasma contamination would lead to the same antibiotic sensitivity of products (Tourtellotte et al., 1967). All cell labelings are done both under sterile conditions and in the presence of the antibiotics penicillin and streptomycin. Mycoplasma can also be eliminated since periodic checks of our suspension cell cultures have failed to detect any of these structures.

Although it is not shown directly that each of the 25 components is chloramphenicol sensitive and some may be related to others, the available evidence indicates that they are discrete mitochondrial proteins. In either the urea or gradient dimensions alone, the chloramphenicol tracks are completely devoid of discrete bands, showing no evidence of any insensitive incorporation.

In the event that these 25 species are really independent polypeptides, the mitochondrial genome would thus code for considerably more protein than has been previously identified structurally. Addition of the molecular weights of the products indicates that saturation of the single strand coding capacity has been achieved. As indicated in Table II, these species add up to an estimated 522,000 daltons. Then it might be assumed that each component is completely polypeptide (i.e., no glycosylations) and migrates true to its size. With an average amino acid residue of 120 daltons, this much protein would be composed of 4,350 amino acids. Assuming colinear coding (no overlapping of sequence), 13,000 nucleotides would be required. Adding coding capacity to account for the two ribosomal RNA species (1050 and 1600 nucleotides) plus 20 4S species (1400 nucleotides) sum to near 17,050. The size of the human mitochondrial genome has been estimated to be between 16,300 and 17,200 nucleotides (Ojala and Attardi, 1977; Drouin and Symons, 1979). Thus, the sum of the molecular weights of these 25 species saturates the available non-overlapping single strand coding capacity of

the HeLa mitochondrial genome, making discovery of additional species unlikely. However, it must be once again pointed out that besides these gross assumptions, both polypeptide and nucleotide size estimates may be no better than ±10%.

The search for new functions may be very difficult since there are no real candidates and yeast data indicate that there are no other components in the respiratory chain (Tzagoloff et al., 1976b). Furthermore, the functions already identified are most likely the species which are present in the largest molar amounts. It is quite possible that the remaining species are perhaps not even strictly necessary for enzymatic function. If these other species are normally repressed and become expressed only under appropriate conditions, the search for functionality may indeed be difficult. However, it has been reported that viral infection does not alter the pattern of mitochondrial gene expression (Yatscoff et al., 1978). Consequently, the problem may have reversed itself from a system looking for sufficient polypeptides to account for the known functions to the opposite, or a system with extra polypeptides and not enough known functions.

## References

Bonner, W. M. and Laskey, R. A. (1974) A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**, 83-88.

Bordier, C. and Crettol-Järvinen, A. (1979) Peptide mapping of heterogeneous protein samples. J. Biol. Chem. **254**, 2565-2667.

Borst, P. and Grivell, L. A. (1978) The mitochondrial genome of yeast. Cell **15**, 705-723.

Briggs, M., Kamp, P. F., Robinson, N. C. and Capaldi, R. A. (1975) The subunit structure of the cytochrome c oxidase complex. Biochemistry **14**, 5123-5128.

Buse, G. and Steffens, G. J. (1976) Analysis of the polypeptide chains of cytochrome oxidase from beef heart mitochondria. In: Genetics and Biogenesis of Chloroplasts and Mitochondria (eds. Th. Bucher et al.), pp. 189-194. Elsevier/North Holland, Amsterdam.

Capaldi, R. A., Bell, R. L. and Branchek, T. (1977) Changes in order of migration of polyeptpides in complex III and cytochrome c oxidase under different conditions of SDS polyacrylamide gel electrophoresis. Biochem. Biophys. Res. Commun. **74**, 425-433.

Ching, E., Costantino, P. and Attardi, G. (1977) In vivo incorporation of different amino acids into electrophoretically characteristic polypeptides synthesized by HeLa cell mitochondria. Biochem. Biophys. Res. Commun. **79**, 451-460.

Cleveland, D. W., Fischer, S. G., Kirschner, M. W. and Laemmli, U. K. (1977) Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. **252**, 1102-1106.

Colombo, B., Felicetti, L. and Baglioni, C. (1966) Inhibition of protein synthesis in reticulocytes by antibiotics. I. Effects on polysomes. Biochim. Biophys. Acta **119**, 109-119.

Costantino, P. and Attardi, G. (1975) Identification of discrete electrophoretic components among the products of mitochondrial protein synthesis in HeLa cells. J. Mol. Biol. **96**, 291-306.

Costantino, P. and Attardi, G. (1977) Metabolic properties of the products of mitochondrial protein synthesis in HeLa cells. J. Biol. Chem. **252**, 1702-1711.

Douglas, M. G. and Butow, R. A. (1976) Variant forms of mitochondrial translation products in yeast: Evidence for location of determinants on mitochondrial DNA. Proc. Nat. Acad. Sci. **73**, 1083-1086.

Downer, N. W., Robinson, N. C. and Capaldi, R. A. (1976) Characterization of a seventh different subunit of beef heart cytochrome c oxidase. Similarities between the beef heart species and that from other species. Biochemistry **15**, 2930-2936.

Drouin, J. and Symons, R. H. (1979) Human mitochondrial DNA: Refractoriness of some fragments to cloning in E. coli. In: ICN-UCLA Symposium on Extrachromosomal DNA (eds. C. F. Fox et al.), in press.

Ennis, H. L. and Lubin, M. (1964) Cycloheximide: Aspects of inhibition of protein synthesis in mammalian cells. Science **146**, 1474-1476.

Feldman, F. and Mahler, H. (1974) Mitochondrial biogenesis. Retention of terminal formylmethionine in membrane proteins and regulation of their synthesis. J. Biol. Chem. **249**, 3702-3709.

Foury, F. and Tzagoloff, A. (1976) Assembly of the mitochondrial membrane system. XIX. Genetic characterization of mit$^-$ mutants with deficiencies in cytochrome oxidase and coenzyme $QH_2$-cytochrome c reductase. Mol. Gen. Genetics **149**, 43-50.

Groot, G. S. P. (1974) The biosynthesis of mitochondrial ribosomes in Saccharomyces cerevisiae. In: The Biogenesis of Mitochondria (eds. A. M. Kroon and C. Saccone), pp. 443-452. Academic Press, New York.

Groot, G. S. P., Van Harten-Loosbrock, N. and Kreike, J. (1978) Electrophoretic behavior of yeast mitochondrial translation products. Biochim. Biophys. Acta **517**, 457-463.

Hare, J. F., Ching, E. and Attardi, G. (1979) Isolation, subunit composition and site of synthesis of human cytochrome c oxidase. Biochemistry, submitted for publication.

Höchli, L. and Hackenbrock, C. R. (1978) Cytochrome c oxidase from rat liver mitochondria: Purification and characterization. Biochemistry **17**, 3712-3719.

Hutchinson, M. A., Hunter, T. and Eckhart, W. (1978) Characterization of T antigens in polyoma-infected and transformed cells. Cell **15**, 65-77.

James, G. T. (1978) Inactivation of the protease inhibitor phenylmethylsulfonyl fluoride in buffers. Anal. Biochem. **86**, 574-579.

Jeffreys, A. J. and Craig, I. W. (1976) Analysis of proteins synthesized in mitochondria of cultured mammalian cells. An assessment of current approaches and problems in interpretation. Eur. J. Biochem. **68**, 301-311.

Kroon, A. M. (1965) Protein synthesis in mitochondria. III. On the effects of inhibitors on the incorporation of amino acids into protein by intact mitochondria and digitonin fractions. Biochim. Biophys. Acta **108**, 275-284.

Lambowitz, A. M., Chua, N. H. and Luck, D. J. L. (1976) Mitochondrial ribosome assembly in Neurospora. Preparation of mitochondrial ribosomal precursor particles, site of synthesis of mitochondrial ribosomal proteins and studies on the poky mutant. J. Mol. Biol. **107**, 223-253.

La Polla, R. J. and Lambowitz, A. M. (1977) Mitochondrial ribosome assembly in Neurospora crassa. Chloramphenicol inhibits the maturation of small ribosomal subunits. J. Mol. Biol. **116**, 189-205.

Lederman, M. and Attardi, G. (1970) In vitro protein synthesis in a mitochondrial fraction from HeLa cells: Sensitivity to antibiotics and ethidium bromide. Biochem. Biophys. Res. Commun. **40**, 1492-1500.

Mahler, H., Dawidowicz, K. and Feldman, F. (1972) Formate as a specific label for mitochondrial translation products. J. Biol. Chem. **247**, 7439-7442.

Ojala, D. and Attardi, G. (1977) A detailed physical map of HeLa cell mitochondrial DNA and its alignment with the positions of known genetic markers. Plasmid **1**, 78-105.

Paucha, E., Mellor, A., Harvey, R., Smith, A. E., Hewick, R. M. and Waterfield, M. D. (1978) SV40 large and small T-antigens have identical amino terminal mapping at 0.65 map units. Proc. Nat. Acad. Sci. **75**, 2165-2169.

Perlman, S. and Penman, S. (1970) Mitochondrial protein synthesis: resistance to emetine and response to RNA synthesis inhibitors. Biochem. Biophys. Res. Commun. **40**, 941-948.

Schatz, G. and Mason, T. L. (1974) The biosynthesis of mitochondrial proteins. Ann. Rev. Biochem. **43**, 51-87.

Studier, F. W. (1973) Analysis of bacteriophage T7 early RNAs and proteins on slab gels. J. Mol. Biol. **79**, 237-248.

Tourtellotte, M. E., Pollack, M. E. and Nalewaik, R. P. (1967) Protein synthesis in mycoplasma. Annals N.Y. Acad. Sci. **143**, 130-138.

Tzagoloff, A., Akai, A., Needleman, R. B. and Zulch, G. (1975) Assembly of the mitochondrial membrane system. Cytoplasmic mutants of Saccharomyces cerevisiae with lesions in enzymes of the respiratory chain and in the mitochondrial ATPase. J. Biol. Chem. **250**, 8236-8242.

Tzagoloff, A., Foury, F. and Akai, A. (1976a) Assembly of the mitochondrial membrane system. XVIII. Genetic loci on mitochondrial DNA involved in cytochrome b synthesis. Molec. Gen. Genetics **149**, 33-42.

Tzagoloff, A., Foury, F. and Akai, A. (1976b) Resolution of the mitochondrial genome. In: The Genetic Function of Mitochondrial DNA (eds. C. Saccone and A. M. Kroon), pp. 155-161. Elsevier/North Holland, Amsterdam.

Wallace, R. B., Williams, T. M. and Freeman, K. B. (1975) Mitochondrial protein synthesis in a mammalian cell-line with a temperature-sensitive leucyl-tRNA synthetase. Eur. J. Biochem. **59**, 167-173.

Weber, K. and Osborn, M. (1969) The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**, 4406-4412.

Weiss, H. (1976) Subunit composition and biogenesis of mitochondrial cytochrome b. Biochim. Biophys. Acta **456**, 291-313.

Yatscoff, R. W., Aujume, L., Freeman, K. B. and Goldstein, S. (1978) Interspecific variations in proteins synthesized by mammalian mitochondria. Can. J. Biochem. **56**, 939-942.

Yatscoff, R. W. and Freeman, K. B. (1977) Discrete electrophoretic products of mitochondrial protein synthesis in the Chinese hamster ovary cell line. Can. J. Biochem. **55**, 1064-1074.

**Table I**

Correspondence between the previous (asterisked) numbering system and the present system

| Previous (*) notation | Present notation |
|---|---|
| 1* | 2,3 |
| 2* | 3 of (7,8,9,10) |
| 3* | 11, 1 of (7,8,9,10) |
| 4* | 12 |
| 5* | 16/17 |
| | 14 |
| | 15 |
| | 20 |
| 8* | 13 |
| | 21 |
| | 22, 23 |
| | 24 |

The previous assignment of discrete species in Weber-Osborn column gels (asterisked numbers) is aligned with the more highly resolving slab gel system whose species have been given new numerical assignments (see text).

**Table II**

Molecular weights of the products of mitochondrial protein synthesis estimated on SDS-urea gels

| Species | Molecular weight* | Sum of components* |
|---|---|---|
| 1 | 51 | 51 |
| 2,3 | 45 | 90 |
| 4 | 39 | 39 |
| 5 | 36 | 36 |
| 7,8,9,10 | 29 | 116 |
| 11 | 25 | 25 |
| 12 | 24 | 24 |
| 13 | 20 | 20 |
| 14,15 | 18 | 36 |
| 16,17 | 16 | 32 |
| 18 | 12 | 12 |
| 19 | 10 | 10 |
| 20 | 9 | 9 |
| 21 | 8 | 8 |
| 22,23 | 6 | 6 |
| 24 | 4.5 | 4.5 |
| 25 | 3.5 | 3.5 |
| | Total | 522 |

*in thousands of daltons.

Estimated molecular weights of the species resolved on SDS-urea gels and the total complexity of protein synthesized by mitochondria making appropriate assumptions as described in the text.

**Figure 1.** Resolution of species using the Weber-Osborn gel electrophoresis system. The profile of products of mitochondrial protein synthesis labeled with [$^{35}$S]-methionine either in the presence of emetine alone (●——●) or emetine plus chloramphenicol (O- -O) separated on a 12-1/2% polyacrylamide Weber-Osborn gel and assayed by scintillation counter is shown in the line drawing. The peak assignments are as previously designated (Costantino and Attardi, 1975). The lower profiles EM and EM + CAP are similarly labeled proteins run on a 15% polyacrylamide Weber-Osborn slab gel assayed by fluorography. Bands are labeled corresponding to the column gel with additional unassigned species also indicated.

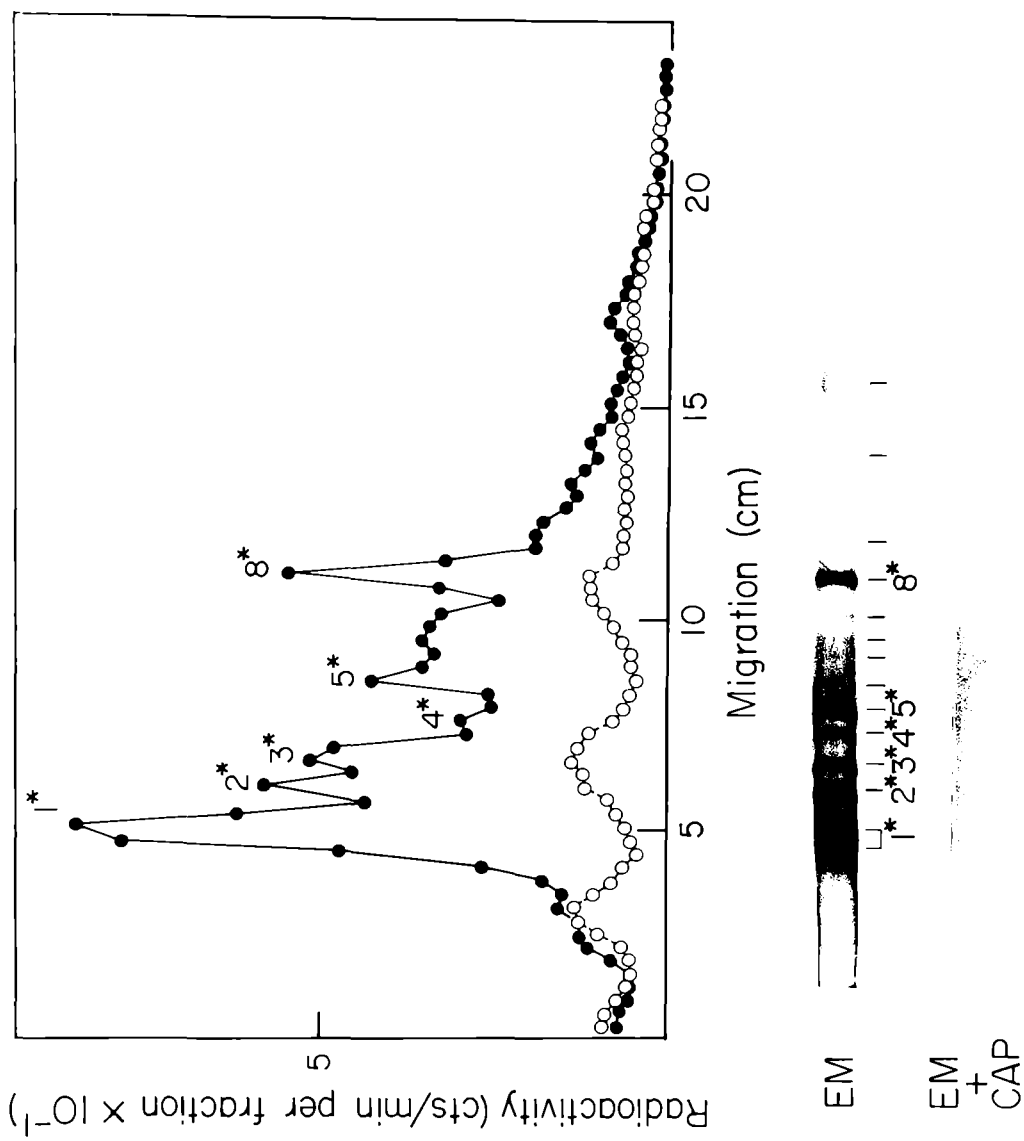
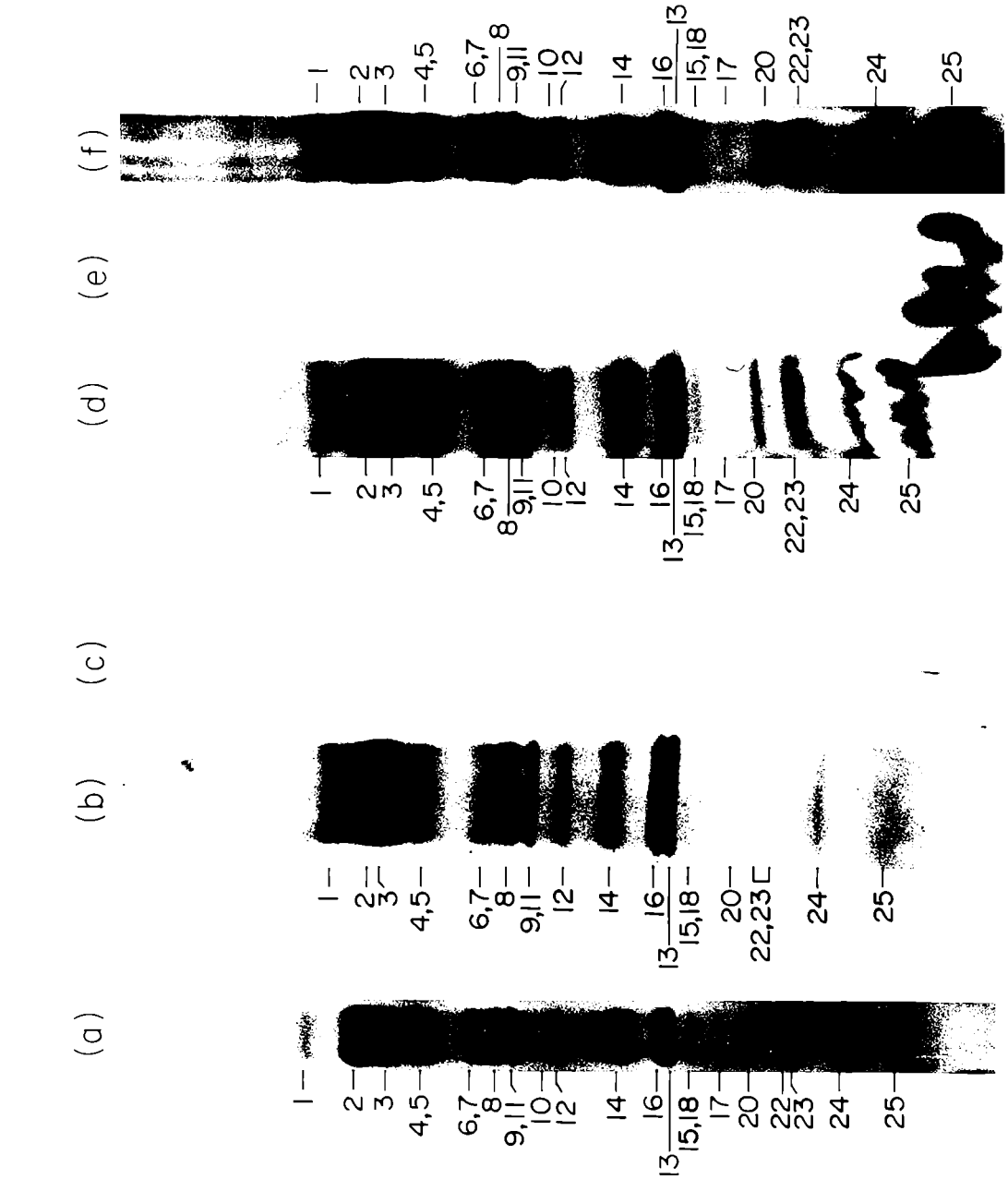

Radioactivity (cts/min per fraction x $10^{-1}$)
5
1*
2*
3*
4*
5*
8*
5
10
15
20
Migration (cm)
EM
EM + CAP
1* 2* 3* 4* 5* 8*

**Figure 2.** Resolution of species using the gradient polyacrylamide SDS gel electrophoresis system. (a) The profile of products of mitochondrial protein synthesis labeled with [$^{35}$S]-methionine in the presence of emetine alone. (b and c) The profiles of the products labeled in the presence of emetine alone, or emetine plus chloramphenicol, respectively. (d and e) The products labeled in the presence of emetine alone, without or with, respectively, incubation with 25 μg of pronase at 37° for 50 min. (f) The products labeled in the presence of emetine with the mitochondrial purification performed in the presence of both fresh PMSF and EGTA in all solutions.


(a)
(b)
(c)
(d)
(e)
(f)
1
2
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4,5
6,7
8
9,11
10
12
14
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15,18
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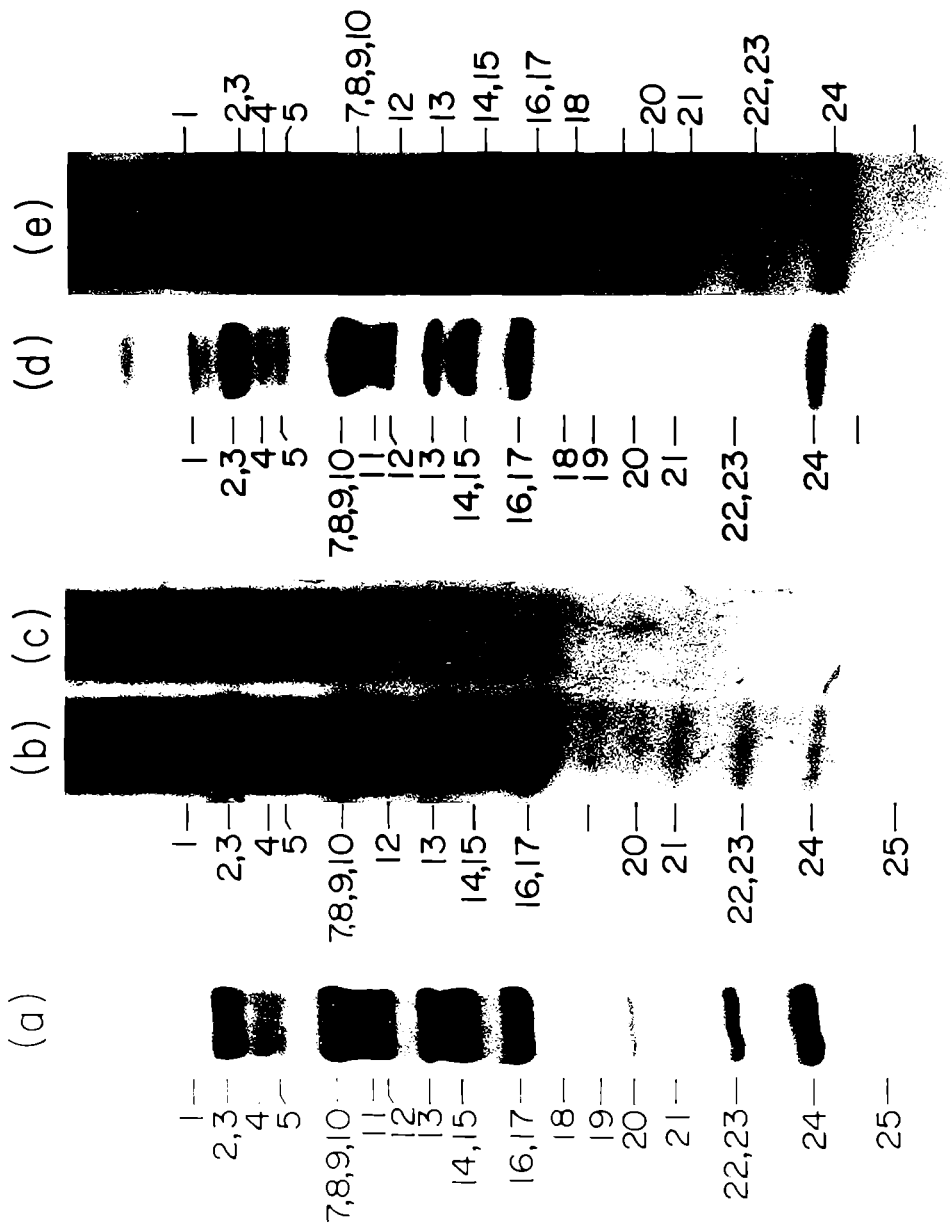
22,23
24
25

**Figure 3.** Resolution of species using the SDS-urea polyacrylamide gel electrophoresis system. (a) The profile of products of mitochondrial protein synthesis labeled with [$^{35}$S]-methionine in the presence of emetine alone. (b and c) The profiles of the products labeled in the presence of emetine alone or emetine plus chloramphenicol, respectively, with a one-hour chase and direct whole cell lysis. (d and e) The products labeled in the presence of cycloheximide alone, without or with, respectively, a one-hour chase. Species of ambiguous identification are indicated but not numbered.


(a)
(b)
(c)
(d)
(e)
1
2,3
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7,8,9,10
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14,15
16,17
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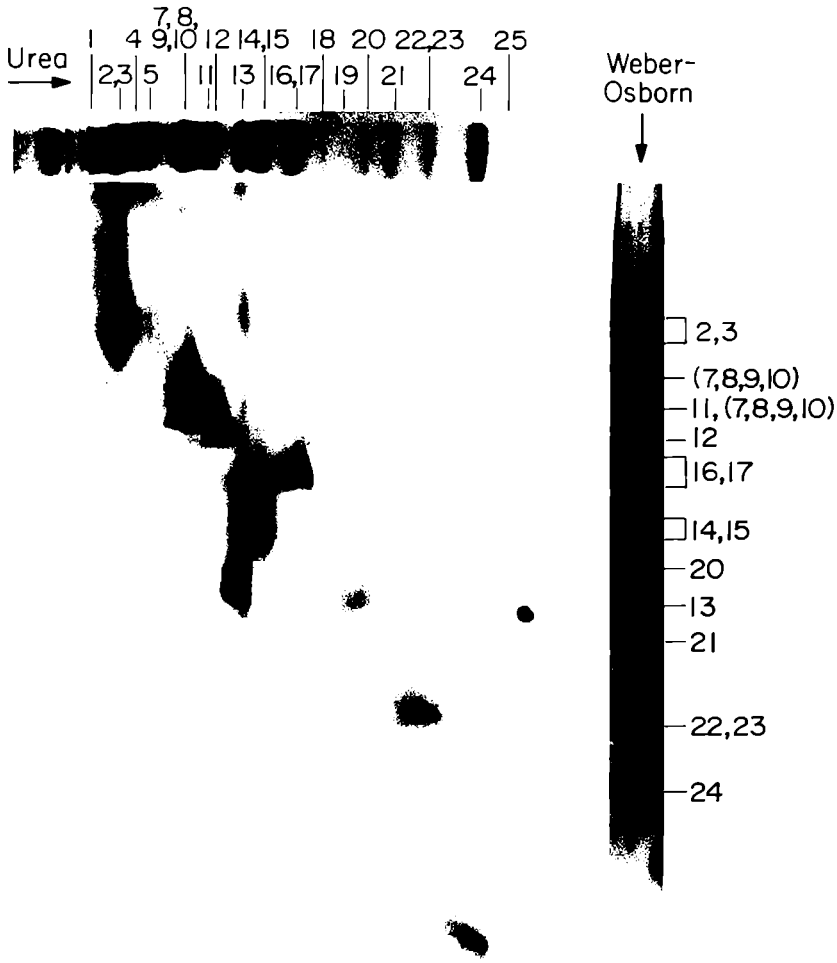
**Figure 4.** Correspondence between species resolved in the SDS-urea polyacrylamide gel system and the Weber-Osborn SDS-polyacrylamide gel system. A track of a 12-1/2% polyacrylamide SDS-urea gel was overlaid onto a 15% Weber-Osborn gel and run. A urea gel pattern and a Weber-Osborn gel pattern are shown alongside to aid in the recognition of species. The interpretation of the gel is shown in the line drawing underneath. The numerical correspondence between the gel systems is indicated by the labels of each of the species in the two dimensions.


Urea
1
4
7,8,
9,10
12
14,15
18
20
22,23
25
2,3
5
11
13
16,17
19
21
24
Weber-
Osborn
2,3
(7,8,9,10)
11, (7,8,9,10)
12
16,17
14,15
20
13
21
22,23
24

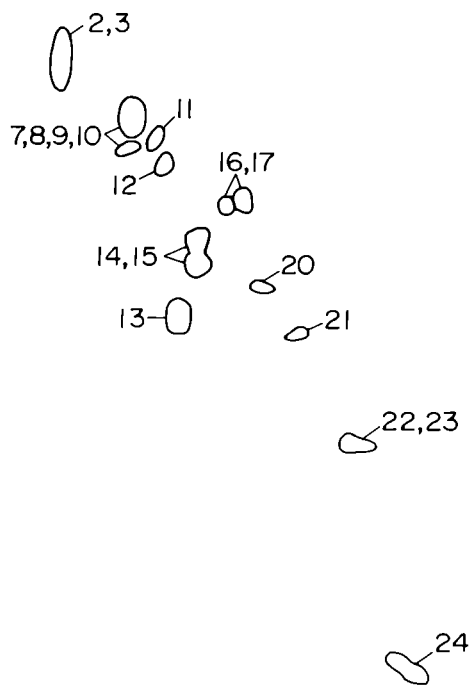

2,3
7,8,9,10
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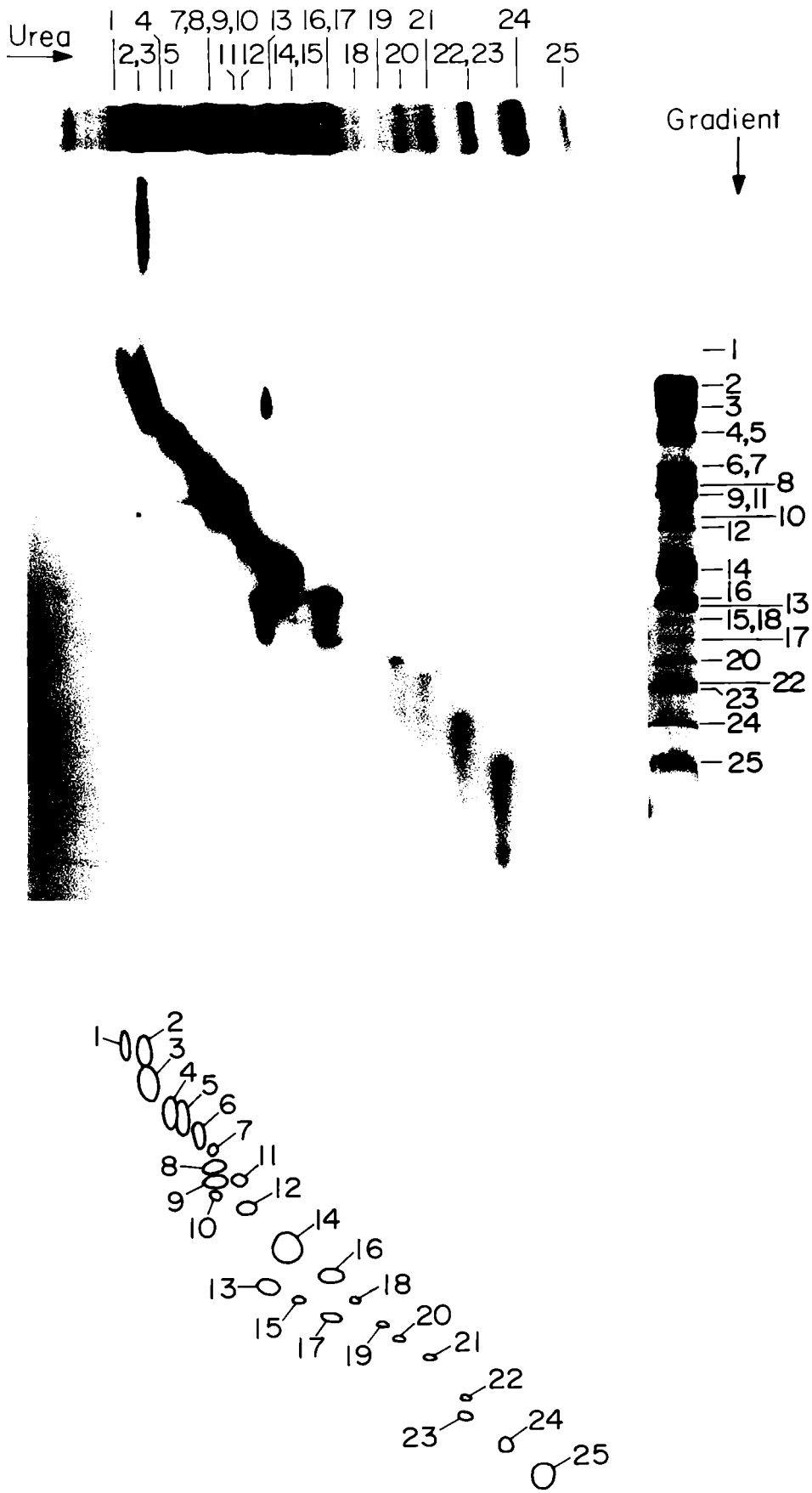
**Figure 5.** Correspondence between species resolved in the SDS-urea polyacrylamide gel system and the gradient polyacrylamide SDS gel system. A track of a 12-1/2% polyacrylamide urea gel was overlaid onto a gradient gel and run. A urea gel pattern and a gradient gel pattern are shown alongside to aid in the recognition of species. The interpretation of the gel is shown in the line drawing underneath. The numerical designations for each species reflect the individual components contained within them.


Urea
1 4 7,8,9,10 13 16,17 19 21 24
2,3 5 11 12 14,15 18 20 22,23 25
Gradient
1
2
3
4,5
6,7
8
9,11
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12
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15,18
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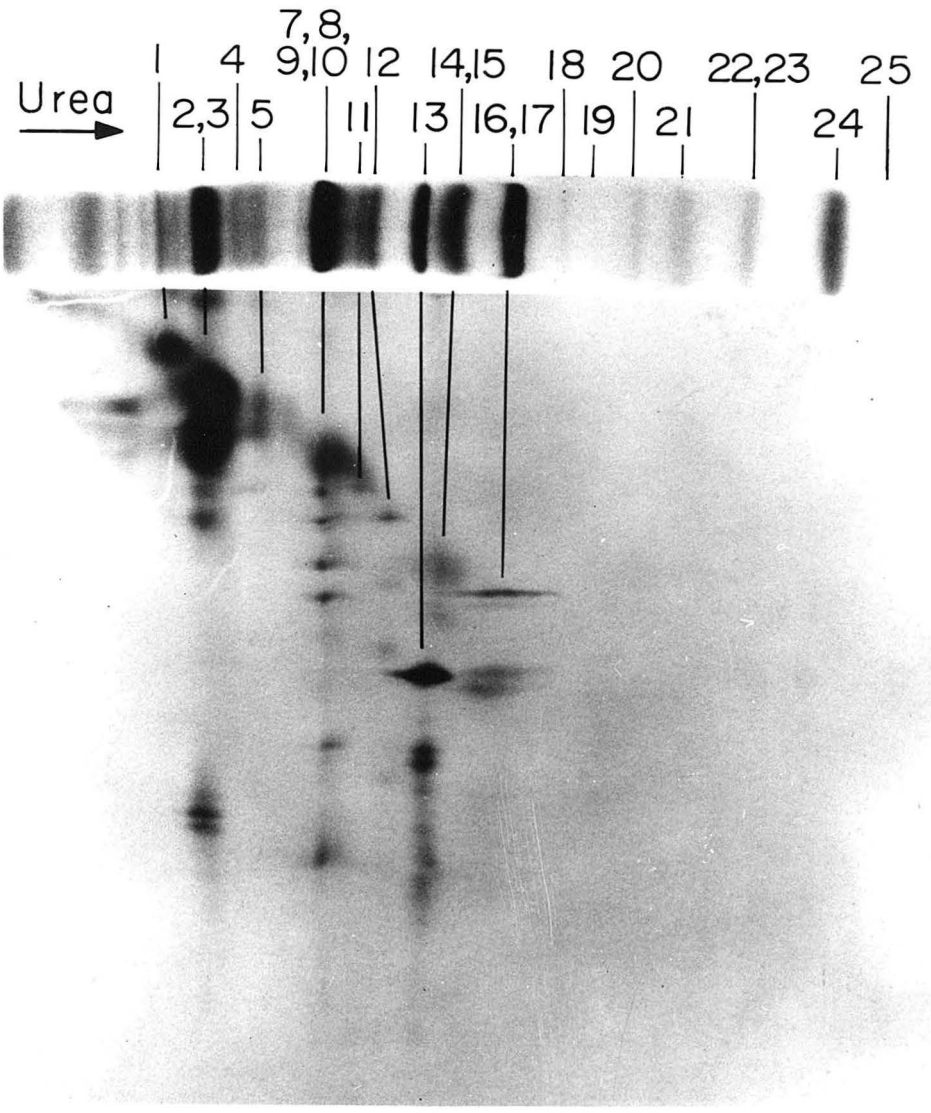
25
1 2 3 4 5 6 7 8 11 9 12 10 14 16 13 18 15 17 20 19 21 22 23 24 25

**Figure 6.** Chymotryptic partial proteolytic analysis of the products resolved by the urea gel system. A track of a 12-1/2% polyacrylamide urea gel was overlaid onto a 15% polyacrylamide gel in the gradient gel buffer system. Chymotrypsin was overlaid, the enzyme was run into the gel and stopped 30 min to allow partial digestion. Electrophoresis was continued to separate the partial digestion products. A urea gel pattern is shown to aid in the recognition of species which is further indicated by the lines.


Urea
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# Chapter 5

## Isolation, Subunit Composition and Site of Synthesis of Human Cytochrome c Oxidase

# Isolation, Subunit Composition and Site of Synthesis of Human Cytochrome c Oxidase †

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ABSTRACT: Cytochrome c oxidase (ferrocytochrome c:oxygen oxidoreductase, EC 1.9.3.1), the terminal oxidase of the respiratory chain in eukaryotic cells, has been purified from human placenta mitochondria. Seven polypeptides have been identified reproducibly by high resolution electrophoresis of the enzyme complex through SDS-urea polyacrylamide gels: these correspond closely in size to the subunits of beef heart cytochrome c oxidase. When HeLa cells, grown in suspension culture, were pulse-labeled with [$^{35}$S]methionine in the presence of cycloheximide to inhibit cytoplasmic protein synthesis, and chased with an excess of unlabeled methionine in the absence of the drug, the mitochondrially synthesized polypeptides were resolved into at least 17 components by SDS-urea polyacrylamide gel electrophoresis. After mixing labeled HeLa mitochondria with human placenta mitochondria and isolating the cytochrome c oxidase, three of the labeled components were found to copurify with the three largest subunits of the complex. We conclude that human cytochrome c oxidase contains seven subunits, the three largest of which are synthesized on mitochondrial ribosomes, while the other four are synthesized in the cytoplasm.

Historically, the stability and high content of respiratory enzymes in beef heart mitochondria have made this the material of choice for probing the physical structure and enzymology of the mitochondrial inner membrane. Recently, however, investigations on the biogenesis and genetics of mitochondria have required the purification and characterization of respiratory enzymes from other sources, in particular, cell systems which are amenable to growth in culture under defined conditions, like yeast, Neurospora and various types of animal cells. Experiments from different laboratories have shown that both cytoplasmic and mitochondrial translation products contribute to the structure of respiratory complexes and of the oligomycin-sensitive ATPase in yeast and Neurospora crassa (see review by Schatz and Mason, 1974). The role of the mitochondrial genome in specifying at least some of the mitochondrially synthesized polypeptide components of these enzymes has been demonstrated recently (Slonimski and Tzagoloff, 1976; Tzagoloff et al., 1976; Borst and Grivell, 1978).

Previous work from this laboratory utilizing electrophoretic fractionation on cylindrical polyacrylamide gels has led to the identification, among the in vivo products of mitochondrial protein synthesis in HeLa cells, of 10 distinct components in the mol. wt. range from about 11,000 to 42,000 (Costantino and Attardi, 1975, 1977). More recently, electrophoresis through slab polyacrylamide gels combined with autoradiography has further resolved the mitochondrially synthesized products into at least 17 discrete [$^{35}$S]methionine-labeled polypeptide bands (Ching and Attardi, manuscript in preparation).

Identification of the functional significance and of the genetic origin of these polypeptides is of primary importance for understanding the informational role of mitochondrial DNA in animal, in particular human, cells. The smaller size of the mitochondrial genome in animal cells as compared to yeast and Neurospora (Borst, 1972) has raised the question of whether there may be a reduced number of structural genes in mitochondrial DNA of higher eukaryotic cells relative to that of lower eukaryotic cells. Although in yeast and mammalian cell mitochondria, the number of polypeptides synthesized on mitochondrial ribosomes appears to be comparable (Beattie, 1971; Borst, 1972; Schatz and Mason, 1974), only a specific identification of these components would allow direct comparisons, and therefore lead to some conclusions about a possible difference in structural gene content between the mitochondrial DNAs of the two sources. A difference in the informational content of mitochondrial DNA of different lower eukaryotic cells has been already well documented in the case of one of the subunits of the ATPase complex, which is coded in mitochondrial DNA in yeast and in the nucleus in Neurospora (Sebald et al., 1977).

In the present work, as a preliminary for an investigation of the biogenesis of cytochrome c oxidase in HeLa cells, we have purified this enzyme from another, more easily available, human material, i.e., placenta mitochondria. We have probed the subunit structure of the human enzyme by direct comparison with that of the beef heart enzyme, and have found that, except for minor variations, the structure of the two enzymes is identical. We have also demonstrated that,

as in yeast and Neurospora crassa (Schatz and Mason, 1974), the three largest among the seven polypeptide components of HeLa cell cytochrome c oxidase are synthesized on mitochondrial ribosomes.

Experimental Procedure

Preparation of Human Placenta Mitochondria. Human placentas were obtained from a local hospital within 1 h of delivery and transported in ice to the laboratory. All manipulations were carried out at 0° to 4°. The tissue was cleaned of connective tissue and diced; about 250 g of tissue was placed in a liter Waring blender (2-speed) with 500 ml of 0.25 M sucrose, 0.001 M EDTA, 0.05 M Tris-$H_2SO_4$ (pH 7.8 at 25°). This suspension was blended at low speed for 1 min and centrifuged at 250 x g for 10 min to separate unbroken cells, nuclei and connective tissue fibers. The supernatant was poured into a flask kept in ice through several layers of cheesecloth (avoiding with care the floating material consisting mostly of fat), and centrifuged at 1300 x g for 5 min to remove any remaining red blood cells and debris. The mitochondria were pelleted by centrifuging the 1300 x g supernatant at 12,000 x g for 10 min in a Sorvall SS-34 rotor. The light brown pellet was resuspended in 0.25 M sucrose, 0.001 M EDTA, 0.01 M Tris-HCl, pH 7.4 (25°) (SET) in one-quarter of the volume of the supernatant and gently homogenized with a Potter-Elvehjem glass-Teflon homogenizer. The mitochondria were reisolated by centrifuging in an SS-34 rotor at 27,000 x g for 15 min, resuspended in one volume of SET and gently homogenized. Four- to five-milliliter portions of this suspension were layered

onto discontinuous gradients consisting of 15 ml of 1.7 M sucrose and 15 ml of 1.0 M sucrose in 0.0001 M EDTA, 0.01 M Tris-HCl (pH 7.0), prepared in Spinco SW27 rotor tubes. The gradients were centrifuged at 70,000 x g for 30 to 40 min. Under these conditions, the mitochondria band at the 1.0 to 1.7 M sucrose interface. The 1.0 M sucrose layer was removed and the interface particulate matter collected with a wide-mouthed pipette. The mitochondria were diluted in 4 to 5 volumes of SET, centrifuged in an SS-34 rotor for 20 min at 27,000 x g and resuspended in one volume of SET. The mitochondria were stored for no more than two weeks at -60° before use.

Labeling and Isolation of HeLa Cell Mitochondria. HeLa cells were grown in suspension as previously described (Amaldi and Attardi, 1968), using modified Eagle's medium (Levintow and Darnell, 1960) supplemented with 5% calf serum. Prior to labeling, the cells were grown for 16 h in the presence of 40 μg/ml chloramphenicol, in order to increase the stability and, presumably, promote the integration into the inner mitochondrial membrane of the mitochondrial protein products synthesized after removal of the drug (Costantino and Attardi, 1977).

The cells were collected by centrifugation, washed twice with medium lacking methionine and finally resuspended in the same medium to 5 x $10^6$ cells/ml. Following 5 min adaptation, cycloheximide was added to 100 μg/ml; 5 min later, [$^{35}$S]methionine (600 mCi/μmole) was added to 8.3 μCi/ml. During the labeling, filtered air containing 5% $CO_2$ was flushed over the medium

at 2 liters/min in order to keep the pH of the medium constant. After 2 h labeling, the cells were collected, washed twice in complete unlabeled medium, resuspended in the same medium to $2 \times 10^6$ cells/ml and incubated for 1 h under aeration conditions. This chase had the purpose of allowing integration of the mitochondrially synthesized polypeptides into the inner mitochondrial membrane under conditions of resumed cytoplasmic protein synthesis (Costantino and Attardi, 1977).

After incubation, the cells were collected, washed with 0.13 M NaCl, 0.005 M KCl, 0.001 M $MgCl_2$, and a 12,000 x g crude mitochondrial fraction was prepared as previously described (Attardi et al., 1969), using SET in the mitochondrial pellet resuspensions.

Preparation of Beef Heat Mitochondria. Beef heat mitochondria were prepared by the method of Löw and Vallin (1963).

Purification of Human Cytochrome c Oxidase. After preliminary tests, the methodology finally chosen for isolating cytochrome c oxidase from human placenta mitochondria, as outlined in Figure 1, follows the rationale developed for yeast cytochrome c oxidase purification (Mason et al., 1973). All manipulations were carried out at 0° to 4°. Mitochondria from about 10 placentas (which had been stored frozen at -60° in one volume of SET) were thawed, diluted with four volumes of SET, resuspended with a Potter-Elvehjem homogenizer, and pelleted in a Sorvall SS-34 rotor at 27,000 x g for 30 min. The washed mitochondria were then resuspended in SET in a final volume of 160 ml, homogenized, and

sonicated in 20 ml aliquots twice for 1 min at 3 A output with a Branson sonifier (model S-125) in 50 ml conical, plastic centrifuge tubes. The sonicated mitochondria were centrifuged in a Spinco type 65 fixed angle rotor at 160,000 x g for 30 min to pellet the membrane fraction.

The membrane fraction was resuspended in 0.05 M $K_2HPO_4$, 0.001 M EDTA, 0.9% KCl to 20 mg protein per ml. Three mg of potassium cholate (from a 20% solution, pH 7.8) were added per mg of protein under stirring, followed by 176 g $(NH_4)_2SO_4$ per liter (30% of saturation). The suspension was stirred gently for 8 h and centrifuged at 20,000 x g for 20 min in a Sorvall SS-34 rotor. After the supernatant was carefully poured off, 62 g of ammonium sulfate per liter (40% of saturation) were slowly added under stirring. The suspension was centrifuged at 57,000 x g in a Spinco 65 rotor for 15 min to remove material forming a brown-green pellet ($P_2$). $P_2$ was resuspended by gentle agitation in 15 ml of 0.5% cholate, 0.25 M sucrose, 0.01 M Tris-HCl, pH 7.4 (CST) per gram of membrane protein processed. Saturated (0°), neutralized ammonium sulfate solution was added dropwise under stirring to the solubilized pellet to 28% saturation. The turbid suspension was centrifuged at 100,000 x g for 10 min to remove material forming a white pellet ($P_3$). To the supernatant, saturated ammonium sulfate was added to 40% saturation; the suspension was centrifuged at 100,000 x g for 10 min to remove material forming a green pellet ($P_4$). $P_4$ was resuspended in 10 ml of 0.02 M $NaPO_4$ (pH 7.0), 1% Triton X-100, and diluted with 20 ml of CST buffer. Saturated ammonium sulfate

was added to 30% saturation and the suspension was centrifuged at 100,000 x g for 10 min after being kept at 0° for 30 min. To the green supernatant (S5), saturated ammonium sulfate was added stepwise with 2% increments starting from 32% up to 40% saturation. Among the pellets obtained by centrifugation (100,000 x g, 10 min) after each ammonium sulfate addition (following a 10-30 min incubation at 0°C), those which contained most of the green material were pooled ($P_6$). Usually, the bulk of the enzyme was precipitated by 34-38% saturated ammonium sulfate.

$P_6$ was resuspended in 1.0 ml of 0.02 M $NaPO_4$ (pH 7.0), 1% Triton X-100, by stirring, and desalted on a Sephadex G-25 (coarse) column (1 x 40 cm), equilibrated with the same buffer. The excluded fractions were combined and applied to a DEAE-cellulose column (Whatman DE-52; 1.2 x 6 cm), equilibrated with 0.02 M $NaPO_4$ (pH 7.0), 1% Triton X-100. The column was washed successively in a stepwise fashion with 20 ml portions of 0.02, 0.05, 0.10 M $NaPO_4$ (pH 7.0), containing 1% Triton X-100. The green absorbance at 423 nm was monitored and of that retained, most ($\sim$60%) was found to be eluted with 0.10 M $NaPO_4$ (Figure 2). Fractions eluting with the 0.1 M $NaPO_4$ front (numbers 53-57 in Figure 2) were combined and stored at -60°, or subjected to further purification on a sucrose density gradient. For this purpose, 0.5 ml samples of DEAE purified cytochrome c oxidase were layered onto 11.5 ml of 5 to 20% linear sucrose gradients in 0.1 M $NaPO_4$ (pH 7.0), 1% Triton X-100, and centrifuged in a Spinco SW41 rotor at 150,000 x g for 24 h at 2°; in other experiments 0.4 ml

samples were centrifuged through 5-25% sucrose gradients in the same buffer in an SW65 rotor at 250,000 x g for 11 h. Fractions of 0.6 ml (SW41 tube) or 0.32 ml (SW65 tube) were collected, and their absorbance at 423 nm and protein concentration determined.

Purification of Beef Heart Cytochrome c Oxidase. The beef heart enzyme was purified as described by Capaldi and Hayashi (1972), or by the method described above for the human enzyme. One preparation of beef enzyme prepared according to the procedure by Kuboyama et al. (1972) was kindly provided by Dr. Sunney Chan.

Polyacrylamide Gel Electrophoresis. Electrophoresis through SDS-urea polyacrylamide slab gels in Tris-phosphate buffer was carried out essentially as described (Downer et al., 1976). A 1 cm stacking gel containing 5% acrylamide (0.133% bis-acrylamide), 8 M urea, 0.03 M Tris-$H_3PO_4$ (pH 6.3) and 0.1% SDS was utilized. The gels were stained with Coomassie Blue, and dried for autoradiography when required.

Molecular weights of the cytochrome c oxidase subunits were determined by comparison with the migration of markers run on the same gel. The following proteins were used as markers in various combinations in different runs: bovine serum albumin (BSA, mol. wt. 66,000); mouse IgA heavy chain ($\gamma$-AH, mol. wt. 58,000); ovalbumin (OA, mol. wt. 46,000); chymotrypsinogen A (CHY A, mol. wt. 26,000); mouse IgA light chain ($\gamma$-AL, mol. wt. 23,000); beef blood globin (GLO, mol. wt. 16,000); cytochrome c (CYT C, mol. wt. 12,400); insulin B (INS B,

mol. wt. 3,400); insulin A chain (INS A, mol. wt. 2,300). A calibration curve was constructed by using the observed migrations of the markers in all the runs, normalized to that of bovine blood globin; from this curve the apparent molecular weights of the markers, under the electrophoretic conditions used, were derived. These apparent molecular weights were used in each electrophoretic run to construct the best fitting line to the observed migrations of the markers in that run (for further details, see legends for Figure 6 and Table III and text). This procedure tended to minimize the imprecision derived from the use of less than the complete set of markers on each gel.

Analytical Procedures. Heme a, b and $c_1$ concentrations were determined from the reduced-minus-oxidized difference spectra at room temperature (Van Gelder, 1963; Vanneste, 1966) and, in the case of heme a, also from the reduced absolute absorption spectrum (Yonetani, 1959) using a Cary 15 spectrophotometer. Heme a concentration of purified cytochrome c oxidase samples was, in addition, determined by their dithionite reduced pyridine hemochromogen (Rieske, 1967), using an $EM_{587} = 24.0\ mM^{-1}\ cm^{-1}$. Oxygen reduction was measured polarographically (Berezney et al., 1972). ATP hydrolysis was measured colorimetrically at 37°, essentially as described (Ernster et al., 1962). Protein concentration was determined as described previously (Lowry et al., 1951), using 1% SDS in the assay buffer (Dulley and Grieve, 1975) and bovine serum albumin as a standard.

Chemicals. Cholic acid (Schwarz/Mann) was recrystallized (Mason et al.,

1973), solubilized with potassium hydroxide, and titrated to pH 8.0. Urea (ultrapure) was obtained from Schwarz/Mann. [$^{35}$S]methionine was obtained from Amersham-Searle Corp.

Results

Heme a Content and Cytochrome c Oxidase Activity of Human Mitochondria. The specific heme a content, as well as the relative content of cytochromes a, b, and $c_1$, in human placenta or HeLa cell mitochondria were found to be strikingly different from those measured in beef heart mitochondria (Table I). The lower specific content of heme a in the mitochondria from the human sources mentioned above was probably not due to the lower degree of purity of the human mitochondrial preparations used here, since oligomycin-sensitive adenosine triphosphatase activity was comparable in placenta and beef heart mitochondria. Human placenta mitochondria reduced molecular oxygen in the presence of reduced cytochrome c (1.18 µatoms 0/min/mg protein at 37°), but the activity was decreased in the presence of 6% cholate (0.66 µatoms 0/min/mg protein), and all activity was lost in a matter of hours in 1% Triton X-100. Since a strong non-ionic detergent was essential in our fractionation procedure, we used specific heme a concentration as a measure of the intact enzyme content; however, we refer to the heme a-protein complex as cytochrome c oxidase.

Purification of Human Placenta Cytochrome c Oxidase. We attempted at first to purify cytochrome c oxidase from cytochromes b, c, and $c_1$ by solubilizing the latter with either Triton X-114 (Jacobs et al., 1966) or deoxycholate (Fowler et al., 1962), but a fairly wide range of concentrations of these detergents did not produce selective solubilization of the b and c cytochromes. On the contrary, we were able to solubilize fairly selectively a heme a-protein complex by the method utilized for the purification of yeast cytochrome c oxidase (Sekuzu et al., 1962; Mason et al., 1972). The purification of the heme a-protein complex is diagrammed in Figure 1. The fractionation scheme approximates that of Mason et al. (1972), except for the following modifications: 1) conditions for salt precipitation of the $P_2$ fraction were changed from 45% to 40% ammonium sulfate saturation, in order to avoid recovering $P_2$ as a "floating pellet"; 2) the fourth ammonium sulfate fractionation step was eliminated. Furthermore, the heme a-protein complex was eluted from the DEAE-cellulose column with 0.10 M $NaPO_4$ (Figure 2) more selectively than with 0.15 M $NaPO_4$. The enzyme so obtained was subjected to several additional purification steps in an attempt to either increase the heme a to protein ratio or to decrease the number of components visualized on SDS-urea polyacrylamide gels. Neither elution from the DEAE-cellulose column with a continuous 0.05 to 0.30 M $NaPO_4$ gradient nor gel filtration through Sepharose 4B in 1% Triton X-100 proved effective in this respect. However, a 60 to 130% enrichment in heme a to protein ratio (as estimated from the $O.D._{423}$/protein ratio), was achieved

in different experiments (the results of two of these are reported in Table II), by centrifuging the DEAE-purified enzyme on a 5 to 20% continuous sucrose gradient in the presence of 1% Triton X-100 (Figure 3).

The purification was monitored at each step by determining the heme a to protein ratio (Table II), by measuring oxygen consumption with ascorbate-cytochrome c as an electron donor (Table II), and by visualizing the polypeptide constitutents on SDS-urea polyacrylamide gels. We obtained at least a 30-fold enrichment in the heme a to protein ratio from the membrane fraction to the final sucrose gradient purified enzyme. The final heme a to protein ratio obtained after the sucrose gradient step, i.e., 4.9 (5.3)[1] and 7.4 (9.4) nmoles heme a/mg protein, in two different experiments, was comparable to that measured in the beef heart cytochrome c oxidase that we purified by the modified Capaldi-Hayashi procedure (see below). As mentioned above, the activity of the enzyme was extremely labile in the presence of Triton X-100 and was irreversibly lost during ammonium sulfate fractionation.

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[1]Here, and below, the first estimate derives from the reduced-minus-oxidized $A_{603}$ (millimolar ext. coefficient = 12), and that in parentheses, if given, from the reduced $A_{603}-A_{630}$ (millimolar ext. coefficient = 16.5). Very similar values were obtained from the dithionite reduced pyridine haemochromogen determinations.

Purification of Beef Heart Cytochrome c Oxidase. Beef heart cytochrome c oxidase was purified by either the method utilized above (cholate-Triton X-100 preparation) or by the procedure of Capaldi and Hayashi (1972). When the former method was utilized, the following modifications were made, since the beef heart enzyme behaved in a strikingly different way from the human placenta enzyme: 1) Mitochondria were sonicated twice for 2 min rather than for 1 min in order to effect maximum breakage; 2) $P_6$ was salted out of solution with stepwise additions of saturated ammonium sulfate from 32 to 42%, instead of 32 to 40%, in order to recover most of the heme a; 3) of the heme a absorbing at 423 nm retained on the DEAE-cellulose column at 0.02 M $NaPO_4$, slightly more than half was eluted with 0.05 M $NaPO_4$, instead of less than 40%, as observed for the human enzyme, and this fraction had the highest heme a/protein ratio. The specific heme a concentration of the final product eluted with 0.05 M $NaPO_4$ was 4.5 nmoles heme a/mg protein.

Beef heart cytochrome c oxidase purified by the Capaldi-Hayashi method had a specific heme a content of 5.6 nmoles heme a/mg protein and was catalytically active. To further purify the enzyme, and to determine if Triton X-100 had any effect on its polypeptide composition or spectral characteristics, the Capaldi-Hayashi enzyme was resuspended in a minimal volume of 1% Triton X-100, 0.02 M $NaPO_4$ (pH 7.0), desalted on a G-25 column in the same buffer, and applied to a DEAE-cellulose column equilibrated with 1% Triton X-100, 0.02 M $NaPO_4$ (pH 7.0). Approximately equal amounts of the absorbance at

423 nm were eluted with 0.05 M $NaPO_4$, this fraction having 10.0 nmoles heme a/mg protein, and with 0.15 M $NaPO_4$, this fraction having 9.6 nmoles heme a/mg protein. Neither fraction retained the ability to reduce molecular oxygen when supplied with reduced cytochrome c. The subunit structure of beef heart cytochrome c oxidase preparations is analyzed below.

Spectral Properties of the Purified Human Cytochrome c Oxidase. Both the reduced-minus-oxidized difference spectrum and the reduced and oxidized absolute absorption spectra for the human enzyme purified up to the DEAE-cellulose stage are shown in Figure 4. At this stage of purification, no evidence of heme b or c or irreducible heme a could be detected. In the reduced form, the enzyme shows absorption maxima at 603, 520, and 444 nm. The characteristic absorbance ratios for the enzyme purified up to either the DEAE-cellulose stage or the sucrose gradient sedimentation stage were identical, and are as follows: $A_{603}$ (reduced):$A_{550}$ (reduced), 2.8; $A_{444}$ (reduced):$A_{603}$ (reduced), 5.1; $A_{423}$ (reduced):$A_{444}$ (reduced), 0.48; $A_{603}$ (reduced):$A_{599}$ (oxidized), 2.2. These values all fall within the acceptable limits, as defined by Lemberg (1969).

Subunit Structure of Beef Heart and Human Cytochrome c Oxidase. The seven subunits of beef heart cytochrome c oxidase were resolved by SDS-urea polyacrylamide gel electrophoresis (Downer et al., 1976), as described in Experimental Procedure (Figure 5). The subunit polypeptides from beef heart preparations were identified by comparison with the published patterns by

Capaldi and collaborators (Downer et al., 1976; Capaldi et al., 1977) on the basis of their electrophoretic mobility. No differences were seen in the mobility of the polypeptide components of the beef heart cytochrome c oxidase as prepared by the procedure of Kuboyama et al. (1972) (Figure 5, slot 2), by the cholate-Triton X-100 procedure (Figure 5, slot 5), by the Capaldi-Hayashi procedure (Capaldi and Hayashi, 1972) (Figure 5, slot 3), or after the enzyme prepared by the latter procedure was treated with Triton X-100 and eluted from a DEAE-cellulose column with 0.05 M or 0.15 M $NaPO_4$ (Figure 5, slot 4). Table III shows the molecular weights of the seven subunits, as estimated in the present work in many experiments from their mobility relative to that of standard proteins; very similar estimates were obtained by using for the marker proteins the apparent molecular weights determined under the electrophoresis conditions used here, as shown in Figure 6, or the real molecular weights. One can see that, apart from subunit I, all the others had apparent molecular weights fairly similar to those reported by Capaldi et al. (1977). The reason for the discrepancy in the apparent mol. wt. of subunit I is unknown. Subunit I aggregated upon disssociating the enzyme in SDS-urea at 100° for 2 min (Downer et al., 1976), and samples treated in this manner lacked the corresponding band. Subunit III was characteristically diffuse (Downer et al., 1976). The beef heart cytochrome c oxidase prepared by the Capaldi-Hayashi procedure showed a relatively small amount of high mol. wt. contaminants, which were in most part eliminated by further purification involving treatment with 1% Triton X-100, adsorption

on DEAE-cellulose and elution with 0.05 M or 0.15 M $NaPO_4$. A characteristic contaminant of about 13,000 daltons mol. wt. was found consistently between subunits IV and V, presumably corresponding to the impurity denoted as "a" by Capaldi et al. (1977). Two other contaminants appeared in variable amounts between subunits V and VI (see also patterns in Figures 7 and 8), and presumably correspond to the impurities with the same mobilities previously described in beef heart preparations (bands "b" and "c" in Capaldi et al., 1977).

Two bands migrating one slightly faster and one slightly slower than subunit VII were seen with variable intensity in different preparations of beef heart cytochrome c oxidase, and often in different runs of the same preparation (compare, for example, patterns in Figures 5, 7 and 8). Identification of the middle band in the triplet as corresponding to subunit VII of Capaldi et al. (1977) was made on the basis of the observations that it was consistently the most intensely stained with Coomassie Blue and that it corresponded in mobility to the only polypeptide present in that region of the gel in the electrophoretic patterns of human cytochrome c oxidase (see below). The enzyme prepared by the cholate-Triton X-100 procedure showed the least degree of contamination by extraneous polypeptides.

The polypeptide composition of human placenta cytochrome c oxidase at various stages of the purification procedure is shown in Figure 7. The effectiveness of the DEAE-cellulose chromatography in removing contaminating polypeptides, especially in the high mol. wt. region of the gel, is apparent (Figure 7, slots 4,5).

A further purification was reproducibly obtained by running the DEAE-cellulose purified (0.1 M $NaPO_4$ eluate) enzyme through a sucrose gradient (see, for example, Figure 7, slot 6). Analysis of the most purified human preparations showed clearly and reproducibly seven polypeptides, which migrated identically or similarly to the seven subunits of beef heart cytochrome c oxidase (Figures 5 and 7), and are presumably homologous to them. In particular, human subunits III, V, VI and VII had identical mobility to the corresponding beef heart subunits. Subunit I migrated consistently somewhat slower and subunit IV somewhat faster than the homologous beef heart components. Subunit II, tentatively identified as the component migrating immediately behind subunit III (Figures 5 and 7), had an electrophoretic mobility distinctly lower than beef heart subunit II; however, the observation that this human polypeptide is synthesized on mitochondrial ribosomes (see below) supports the interpretation that this component is indeed subunit II of cytochrome c oxidase. Sometimes subunit III appeared as a doublet [see for example, Figure 5 (slot 6)]. This possibly reflects the occurrence of heme-free and heme-containing polypeptides with slightly different electrophoretic mobility (Downer et al., 1976); however, the possibility that the slower moving band of the doublet may be a contaminant is suggested by its disappearance after sucrose gradient centrifugation (Figure 7, slot 6). As mentioned above, only one band was observed in the region of beef heart subunit VII and was identified as the homologous human component. Table III shows the molecular weights of the subunits of human cytochrome c oxidase

estimated in many experiments from their mobility relative to that of standard proteins.

Identification of Mitochondrial Translation Products Associated with Human Cytochrome c Oxidase. HeLa cells were exposed to [$^{35}$S]methionine under the conditions described in Experimental Procedure, designed to label specifically the polypeptides synthesized on mitochondrial ribosomes. In particular, exponentially growing cells were labeled with [$^{35}$S]methionine for 2 h in the presence of cycloheximide (to block cytoplasmic protein synthesis), and chased for 1 h with an excess of cold methionine in the absence of the drug. [This chase was designed to allow the assembly of the newly synthesized polypeptides with the cytoplasmically made subunits to form the mature cytochrome c oxidase complex.] Twenty mg of mitochondrial membrane protein derived from such cells were mixed with 580 mg of human placenta mitochondrial membrane protein, and cytochrome c oxidase was purified by the cholate-Triton X-100 procedure. The purification resulted in a selective enrichment of mitochondrial translation products, as judged from the amount of [$^{35}$S]cpm per μg of protein (Table IV). The purified enzyme (34 cpm/μg protein) was electrophoresed, and the slab gel was stained with Coomassie Blue, to visualize the component polypeptides, and subjected to autoradiography to visualize the labeled components (Figure 8). The electrophoretic pattern of the labeled proteins from the total HeLa cell mitochondrial fraction revealed at least 17 bands of varying intensity (Figure 8, autoradiograph). Control experiments

showed that the labeling of all these components is sensitive to 100 μg/ml chloramphenicol, indicating therefore that they are products of mitochondrial protein synthesis (Ching and Attardi, manuscript in preparation). In the gel track containing cytochrome c oxidase from mixed labeled HeLa mitochondria - unlabeled placenta mitochondria, three of the labeled polypeptides (Figure 8, autoradiograph) coincide with the stained cytochrome c oxidase subunits I, II and III (Figure 8, stained pattern), identifying these as mitochondrial translation products. Conversely, the absence of any hint of labeled bands corresponding to subunits IV through VII strongly suggests that these components are synthesized in the cytoplasm.

Discussion

In the present work, the conventional fractionation approach which has been previously used to purify cytochrome c oxidase from beef heart mitochondria was not successful with the mitochondrial membranes of HeLa cell or human placenta origin. This is possibly due to the intrinsically low heme a content of the organelles from these sources and/or to the presence of contaminating membranes which altered the normal lipid environment during extraction. After testing a variety of other procedures, satisfactory results were finally obtained by using a method based on a modification of that devised for the yeast enzyme isolation (Mason et al., 1973). This purification procedure resulted, however, in a final product which had lost its ability to reduce molecular

oxygen. The beef heart enzyme purified in the present work by the cholate-Triton X-100 procedure was also inactive. The factor responsible for this loss of molecular activity of the cytochrome c oxidase appears to be exposure to Triton X-100. In fact, human placenta mitochondria were found to lose their ability to reduce molecular oxygen in the presence of reduced cytochrome c within a few hours after solubilization with this detergent; furthermore, the beef heart enzyme prepared by the Capaldi-Hayashi procedure lost its activity after DEAE-cellulose chromatography in the presence of Triton X-100. Inactivation by Triton X-100 has also been reported for the rat liver enzyme (Rascati and Parsons, 1979a). It seems possible that this loss of activity is due to a perturbation of the membrane lipid environment, which results from exposure of the enzyme complex to this detergent. A previous report had indicated a nearly complete loss of activity of human heart cytochrome c oxidase during purification (Jeffreys and Craig, 1977).

The most extensively purified preparations of cytochrome c oxidase obtained in the present work from human placenta had a specific heme a content [4.9-7.4 nmoles/mg protein, using red-ox $A_{603}$ ($\Delta\epsilon$ = 12 $mM^{-1}$ $cm^{-1}$); 5.3-9.4 nmoles/mg protein, using red $A_{603}$-red $A_{630}$ ($\Delta\epsilon$ = 16.5 $mM^{-1}$ $cm^{-1}$)] comparable to that of the beef heart enzyme [present work: 4.5 nmoles heme a/mg protein, using red-ox $A_{603}$ ($\Delta\epsilon$ = 12 $mM^{-1}$ $cm^{-1}$)], or the yeast enzyme [Mason et al., 1973; 9.4-10.0 nmoles/mg protein, using red $A_{603}$-red $A_{630}$ ($\Delta\epsilon$ = 16.5 $mM^{-1}$ $cm^{-1}$)] purified by the same procedure; it was also similar to that reported for the

human heart enzyme [Jeffreys and Craig, 1977; 7 nmoles heme a/mg protein, using red $A_{603}$-red $A_{630}$ ($\Delta\epsilon$ = 16.5 $mM^{-1}$ $cm^{-1}$)]. The available values for the specific heme a content of beef heart cytochrome c oxidase purified by procedures not involving Triton X-100 cover a fairly wide range: 9.6-10.0 nmoles heme a/mg protein, using red-ox $A_{603}$ ($\Delta\epsilon$ = 12 $mM^{-1}$ $cm^{-1}$) (present work); 7.9 nmoles/mg protein using red $A_{603}$-red $A_{630}$ ($\Delta\epsilon$ = 16.5 $mM^{-1}cm^{-1}$) (Phan and Mahler, 1976a); 9.70 nmoles heme a/mg protein, using dithionite reduced pyridine hemochromogen ($\epsilon_{587}$ = 21.7 $mM^{-1}cm^{-1}$) (Capaldi and Hayashi, 1972); 11 nmoles heme a/mg protein using red-ox $A_{603}$ ($\Delta\epsilon$ = 12 $mM^{-1}cm^{-1}$) (Kuboyama et al., 1972); 12 nmoles heme a/mg protein using red $A_{603}$-red $A_{630}$ ($\Delta\epsilon$ = 16.5 $mM^{-1}cm^{-1}$) (Rubin and Tzagoloff, 1973a). Values of 10.2 and 9.5-13.4 nmoles heme a/mg protein [using red $A_{603}$-red $A_{630}$ ($\Delta\epsilon$ = 16.5 $mM^{-1}cm^{-1}$)] have been reported, respectively, for the Xenopus laevis (Koch, 1976) and the rat liver cytochrome c oxidase (Höchli and Hackenbrock, 1978; Rascati and Parsons, 1979a). A part of the variability in the specific heme a content found by different investigators in the enzyme isolated from different animal sources or also from the same source probably reflect differences in the purification procedure or in the methods used to determine the heme a content, rather than intrinsic differences in the enzyme. In any case, there are reasons to believe that the specific heme content is not necessarily a reliable indicator of the polypeptide purity of the cytochrome c oxidase preparations. Thus, the beef heart enzyme isolated here by the cholate-Triton X-100 method had

an appreciably lower heme a content than the enzyme prepared by other procedures, and still showed a typical pattern on SDS-urea polyacrylamide gel, with the seven subunits of the enzyme in the normal relative amounts; furthermore, and most significantly, this preparation was less contaminated than the others by extraneous polypeptides. Similarly, the most purified preparations of cytochrome c oxidase from human placenta exhibited an electrophoretic pattern as clean, in terms of absence of contaminating polypeptides, and probably cleaner than the best beef heart preparations. A decline in molecular activity of the beef enzyme during purification, in the absence of any change in the essential subunit composition, as judged by polyacrylamide gel electrophoresis in the presence of SDS, has been previously reported and attributed to a reorganization of the multimeric phospholipid-protein complex (Vanneste et al., 1976). Therefore, it seems that heme loss can occur from the enzyme complex during purification in the absence of breakdown of the complex. On the other hand, it has been shown that even preparations of beef heart enzyme with high heme a content and high specific activity, apparently indicative of purity, are contaminated with NADH-acceptor reductases (Penniall et al., 1976) and many extraneous bands on polyacrylamide gels (E. Ching, Y. Hatefi and G. Attardi, unpublished observations). High resolution polyacrylamide gel electrophoresis thus provides an important criterion to evaluate the polypeptide purity of the enzyme. This may be more important than the specific heme content whenever comparisons with a standard pattern and other criteria allow

the identification of the enzyme subunits. Since our purpose in the present work was to be able to identify the mitochondrially and the cytoplasmically synthesized subunits of cytochrome c oxidase, reducing the number of contaminating polypeptides as visualized on high resolution gels was of greater interest to us than obtaining a preparation of high specific activity or heme concentration.

The subunit structure of cytochrome c oxidase has been the object of great controversy. Much of the variability in results has been the consequence of differences in isolation procedures or conditions of gel electrophoresis, of failure to adequately dissociate the enzyme or of selective aggregation of subunits during preparation of the samples for gel electrophoresis. There is general agreement that the yeast and the Neurospora enzymes can be resolved into seven polypeptides, each present in one copy (Rubin and Tzagoloff, 1973a; Sebald et al., 1973; Poyton and Schatz, 1975; Phan and Mahler, 1976a), with similar molecular weights of the individual subunits in the two organisms. As to the animal cell enzymes, Locusta migratoria cytochrome c oxidase has been reported to consist of seven subunits (Weiss et al., 1972) with molecular weights fairly similar to those of the Neurospora enzyme (Sebald et al., 1973), while in the Xenopus enzyme (Koch, 1976), only six subunits have been found. In the rat liver enzyme, six (Rascati and Parsons, 1979a) or seven (Höchli and Hackenbrock, 1978) subunits have been reported. In the beef heart cytochrome c oxidase, either six (Rubin and Tzagoloff, 1973a; Phan and Mahler, 1976a; Yatscoff et al., 1977), seven (Capaldi and Hayashi, 1972; Kuboyama et al.,

1972; Capaldi et al., 1977) or eight subunits (Buse and Steffens, 1976) have been described. There is some evidence to suggest that the failure to detect one of the large subunits (subunit III) in some of the above quoted studies is due to its poor staining with Coomassie Blue and to its not being resolved from subunit II under the conditions of electrophoresis on standard SDS-polyacrylamide gels (Downer et al., 1976).

The thorough investigations by Capaldi and collaborators have clearly shown the effect of different conditions of gel electrophoresis on the order of migration and the apparent molecular weights of the subunits of beef heart cytochrome c oxidase (Capaldi et al., 1977). The molecular weights for the various subunits determined by these authors in SDS-urea gels are 35,300 (I), 25,200 (II), 21,000 (III), 16,200 (IV), 12,100 (V), 6,700 (VI) and 3,400 daltons (VII) (Capaldi et al., 1977). Even the most purified preparations appeared to contain small amounts of other polypeptides in the 8,000-16,000 mol. wt. range, which were interpreted as impurities on the basis of their non-stoichiometric amounts and variability in yield. These impurities have been observed also in the present work; some of the discrepancies in the literature concerning the number and mol. wt. of mammalian cytochrome c oxidase subunits may be due to the presence of these contaminating polypeptides, often in substantial amount. There is also some question about the nature of the smallest polypeptides detected in the beef heart enzyme preparations. While the Capaldi group identified the polypeptide with an apparent mol. wt of 3,400 daltons in SDS-urea

gels and of 4,300 daltons in standard SDS gels as a single, integral component of the enzyme (subunit VII) (Capaldi et al., 1977), in another investigation (Buse and Steffens, 1976), this component was found to be a non-stoichiometric mixture of polypeptides with three N-terminal amino acids.

In the present work, the beef heart enzyme prepared by the Capaldi-Hayashi procedure (Capaldi and Hayashi, 1972), the same further purified on DEAE-cellulose, the enzyme prepared by the method of Kuboyama et al. (1972), and that prepared by the procedure used in this laboratory for the purification of the human enzyme gave identical patterns in SDS-urea gel: this pattern was very similar to that published by Capaldi and collaborators (Downer et al., 1976; Capaldi et al., 1977). The only difference was the presence of three closely migrating bands in the region of the VII subunit, of which the intermediate one was more intensely stained with Coomassie Blue than the other two. It seems possible that these three polypeptides correspond to those described by Buse and Steffens (1976). In the present work, the intermediate, most intensely stained band has been interpreted to represent the VII subunit. The validity of this interpretation is supported by the observation that this intermediate band corresponded in electrophoretic mobility to the only polypeptide of human cytochrome c oxidase in that molecular weight range. The nature of the slower and faster moving, less intensely stained bands is unknown. The molecular weights estimated for the seven subunits of the beef heart enzyme in this work are similar to those published by Capaldi et al. (1977), except that for

subunit I (44,200) which was substantially higher than the one reported by the cited authors (35,300). The reason for this discrepancy is not clear.

The human cytochrome c oxidase purified in this work appears to contain seven major polypeptides. Identification of these as the seven subunits of the enzyme complex has been made on the basis of their size and of the reproducibility of their presence and relative amounts at different stages of the purification procedure. All these subunits, except one, migrate on SDS-urea gels identically or very similarly to the homologous beef heart subunits. The exception is represented by subunit II, which was missing or of low staining intensity in our early preparations, for unexplained reasons, but has consistently appeared as a major component in our more recent preparations. This component was found to have a slightly greater electrophoretic mobility than subunit II of the beef heart cytochrome c oxidase. The significance of this observation is not clear. However, the finding that this polypeptide is synthesized on mitochondrial ribosomes supports our interpretation that it is homologous to subunit II of beef heart cytochrome c oxidase; apparently, this component is more easily removed from the protein complex than the other subunits. We conclude, therefore, that the cytochrome c oxidase protein complex from human mitochondria, as that from beef heart and yeast, contains seven subunits. Fewer components may be required for electron transport function, as shown in yeast (Phan and Mahler, 1976b), but more may be required for other functions (coupling, regulation, integration, etc.). A more definitive evaluation of the number

and nature of the polypeptides which are integral constituents of cytochrome c oxidase must await the results of reconstitution experiments.

The observation reported here of the mitochondrial biosynthetic origin of subunits I, II, and III of the human cytochrome c oxidase extends to the human enzyme the results obtained for the enzyme from yeast (Mason and Schatz, 1973; Rubin and Tzagoloff, 1973b), Neurospora crassa (Sebald et al., 1973), Xenopus laevis (Koch, 1976) and rat liver (Rascati and Parsons, 1979b). Only one mitochondrially synthesized subunit (with electrophoretic mobility corresponding to that of subunit II of the enzyme from human placenta or beef heart) has been detected in one study of the human heart enzyme (Jeffreys and Craig, 1977); in embryonic bovine trachea cells, only two large polypeptides translated in mitochondria have been found to be precipitated by antibody against cytochrome c oxidase (Yatscoff et al., 1977). The reason for these discrepancies is not known, but may be related to the same factors discussed above which affect the apparent subunit composition of the enzyme under different experimental conditions. In any case, it is clear that, in all organisms studied, cytochrome c oxidase is a chimera of mitochondrially translated (and presumably mitochondrial DNA coded) polypeptides and cytoplasmically synthesized (nuclear DNA coded) polypeptides. Although final conclusions must await the complete identification of the mitochondrially synthesized polypeptides in yeast and animal cells, in the particular case of cytochrome c oxidase, there does not appear to be any difference in the structural gene content of mitochondrial

DNA between yeast and animal cells. Important questions which need to be answered in the future concern the mechanism of insertion of the enzyme into the membrane and the mitochondrial and nuclear genetic control of its synthesis.

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References

Amaldi, F., and Attardi, G. (1968), J. Mol. Biol. 33, 737-755.

Attardi, B., Cravioto, B., and Attardi, G. (1969), J. Mol. Biol. 44, 47-70.

Beattie, D. S. (1971), Subcell. Biochem. 1, 1-23.

Berezney, R., Macaulay, L. K., and Crane, F. L. (1972), J. Biol. Chem. 247, 5549-5561.

Borst, P. (1972), Ann. Rev. Biochem. 41, 333-376.

Borst, P., and Grivell, L. A. (1978), Cell 15, 705-723.

Buse, G., and Steffens, G. J. (1976), in Genetics and Biogenesis of Chloroplasts and Mitochondria (Bücher, Th. et al., Eds.), pp. 189-194, North-Holland, Amsterdam.

Capaldi, R. A., Bell, R. L., and Branchek, T. (1977), Biochem. Biophys. Res. Commun. 74, 425-433.

Capaldi, R. A., and Hayashi, H. (1972), FEBS Lett. 26, 261-263.

Costantino, P., and Attardi, G. (1975), J. Mol. Biol. 96, 291-306.

Costantino, P., and Attardi, G. (1977), J. Biol. Chem. 252, 1702-1711.

Downer, N. W., Robinson, N. C., and Capaldi, R. A. (1976), Biochemistry 15, 2930-2936.

Dulley, J. R., and Grieve, P. A. (1975), Anal. Biochem. 64, 136-141.

Ernster, L., Siekevitz, P., and Palade, G. F. (1962), J. Cell Biol. 15, 541-562.

Fowler, L. R., Richardson, S. H., and Hatefi, Y. (1962), Biochim. Biophys. Acta 64, 170-173.

Höchli, L., and Hackenbrock, C. R. (1978), Biochemistry 17, 3712-3719.

Jacobs, E. E., Andrews, E. C., Cunningham, W., and Crane, F. L. (1966), Biochem. Biophys. Res. Commun. 25,87-95.

Jeffreys, A. J., and Craig, I. W. (1977), FEBS Lett. 77, 151-154.

Koch, G. (1976), J. Biol. Chem. 251, 6097-6107.

Kuboyama, M., Yong, F. C., and King, T. E. (1972), J. Biol. Chem. 247, 6375-6383.

Lemberg, M. R. (1969), Physiol. Rev. 49, 48-121.

Levintow, L., and Darnell, J. E. (1960), J. Biol. Chem. 235, 70-73.

Löw, H., and Vallin, I. (1963), Biochim. Biophys. Acta 69, 361-374.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), J. Biol. Chem. 193, 265-275.

Mason, T. L., Poyton, R. O., Wharton, D. C., and Schatz, G. (1973), J. Biol. Chem. 248, 1346-1354.

Mason, T. L., and Schatz, G. (1973), J. Biol. Chem. 248, 1355-1360.

Penniall, R., Elliot, W. B., van Buuren, K. J. H., and van Gelder, B. F. (1976), J. Bioenergetics and Biomembranes 8, 271-292.

Phan, S. E., and Mahler, H. R. (1976a), J. Biol. Chem. 251, 257-269.

Phan, S. E., and Mahler, H. R. (1976b), J. Biol. Chem. 251, 270-276.

Poyton, R. O., and Schatz, G. (1975), J. Biol. Chem. 250, 752-761.

Rascati, R. J., and Parsons, P. (1979a), J. Biol. Chem. 254, 1586-1593.

Rascati, R. J., and Parsons, P. (1979b), J. Biol. Chem. 254, 1594-1599.

Rieske, J. S. (1967), Methods in Enzymol. 10, 488-493.

Rubin, M. S., and Tzagoloff, A. (1973a), J. Biol. Chem. 248, 4269-4274.

Rubin, M. S., and Tzagoloff, A. (1973b), J. Biol. Chem. 248, 4275-4279.

Schatz, G., and Mason, T. L. (1974), Ann. Rev. Biochem. 43, 51-87.

Sebald, W., Machleidt, W., and Otto, J. (1973), Eur. J. Biochem. 38, 311-324.

Sebald, W., Sebald-Althaus, M., and Wachter, E. (1977), in Mitochondria 1977, Genetics and Biogenesis of Mitochondria (Bandlow, W. et al., Eds.), pp. 433-440, Walter de Gruyter, Berlin.

Sekuzu, I., Mizushima, H., and Okunuki, K. (1964), Biochim. Biophys. Acta 85, 516-519.

Slonimski, P. P., and Tzagoloff, A. (1976), Eur. J. Biochem. 61, 27-41.

Tzagoloff, A., Foury, F., and Akai, A. (1976), in The Genetic Function of Mitochondrial DNA (Kroon, A., and Saccone, C., Eds.), pp. 155-161, North-Holland, Amsterdam.

Van Gelder, B. F. (1963), Biochim. Biophys. Acta 118, 36-46.

Vanneste, W. H. (1966), Biochim. Biophys. Acta 113, 175-178.

Vanneste, W. H., Ysebaert-Vanneste, M., and Mason, H. S. (1976), J. Biol. Chem. 249, 7390-7401.

Weiss, H., Lorenz, B., and Kleinow, W. (1972), FEBS Lett. 25, 49-51.

Yatscoff, R. W., Freeman, K. B., and Vail, W. J. (1977), FEBS Lett. 81, 7-9.

Yonetani, T. (1959), J. Biochem. (Tokyo) 46, 917-924.

TABLE I: Concentration of Cytochromes and ATPase Specific Activity in Beef Heart, Human Placenta, and HeLa Cell Mitochondrial Membranes

| | Heme specific concentration[a] (nmoles/mg protein) | | | Specific activity of ATPase (μmoles ATP/min/mg protein) |
|---|---|---|---|---|
| | a, $a_3$ | b | $c_1$ | |
| Beef heart | 1.54 | 0.82 | 0.48 | 2.0 |
| Human placenta | 0.16 | 0.14 | 0.089 | 1.0 |
| HeLa cells | 0.028 | 0.051 | 0.047 | 0.14 |

[a]Heme $a/a_3$, b and $c_1$ concentrations were determined from the reduced-minus-oxidized absorption spectra at room temperature (Vanneste, 1966).

*TABLE II: Summary of Cytochrome c Oxidase Purification*

| | Protein (mg) | Heme a[(1)] (nmoles) | Specific Heme a content (nmoles/mg) | | $OD_{423}$/mg prot. | Specific activity (μatoms 0/min/mg prot. |
|---|---|---|---|---|---|---|
| | | | (1) | (2) | | |
| EXPERIMENT I | | | | | | |
| Total Membrane | 2250 | 360 | 0.16 | 0.092 | - | 0.70 |
| $P_2$ | 512 | 256 | 0.50 | 0.53 | - | 1.06 |
| $P_4$ | 336 | 252 | 0.75 | 0.82 | - | 0 |
| $P_6$ or G-25 Excluded fraction | 56.2 | 61 | 1.09 | 1.20 | - | 0 |
| DEAE 0.10 M $PO_4$ | 10.0 | 35 | 3.50 | 3.80 | 0.28 | 0 |
| SW41 Sucrose gradient peak | 5.8 | 28 | 4.90 | 5.30 | 0.45 | 0 |
| EXPERIMENT II | | | | | | |
| DEAE 0.10 M $PO_4$ | - | - | - | - | .39 | - |
| SW65 Sucrose gradient peak | - | - | 7.4 | 9.4 | .90 | - |

(1) = heme a determined using $\epsilon(603_{reduced} - 603_{oxidized}) = 12.0\ mM^{-1}cm^{-1}$

(2) = heme a determined using $\epsilon(603_{reduced} - 630_{reduced}) = 16.5\ mM^{-1}cm^{-1}$.

TABLE III: Estimates of the Molecular Weight of the Subunits of Cytochrome c Oxidase from Beef Heart and Human Placenta Mitochondria[a]

| Subunits | Beef heart[b] | Beef heart (this work) | | Human placenta (this work) | |
|---|---|---|---|---|---|
| I | 35,300 | 42,300 | (41,300) | 44,600 | (43,200) |
| II | 25,200 | 23,200 | (22,300) | 20,000 | (21,200) |
| III | 21,000 | 18,000 | (17,400) | 18,100 | (18,800) |
| IV | 16,200 | 14,100 | (13,600) | 13,400 | (14,200) |
| V | 12,100 | 9,000 | (8,800) | 8,800 | (9,600) |
| VI | 6,700 | 5,800 | (5,800) | 5,600 | (6,300) |
| VII | 3,400 | 3,600 | (3,600) | 3,600 | (4,200) |

[a]The molecular weights given for the subunits of the beef heart and human placenta enzymes are averages of the estimates made on the basis of standard curves constructed by the least squares method for the individual gel runs, using for the marker proteins the apparent molecular weights derived from Figure 6 (see text for details). In parentheses are the averages of the estimates made by using the real molecular weights for the marker proteins in the construction of the individual standard curves.

[b]Capaldi et al., 1977.

TABLE IV: Purification of Cytochrome c Oxidase from a Mixture of Human Placenta Mitochondria and HeLa Cell Mitochondria Labeled in the Organelle-Specific Translation Products[a]

| | mg protein | Total cpm | cpm/μg protein |
|---|---|---|---|
| Mitochondrial membrane fraction | 600 [580 (PM) 20 (HM)] | 4,710,000 | 7.9 |
| G-25 excluded fraction | 17.5 | 308,000 | 17.6 |
| Cytochrome c oxidase (DEAE-0.10 M $NaPO_4$) | 2.5 | 84,000 | 34.0 |

[a]For details, see text.

PM: human placenta mitochondria; HM: [$^{35}$S]methionine-labeled HeLa cell mitochondria.

FIGURE 1: Flow diagram of cytochrome c oxidase purification from human placenta mitochondria.

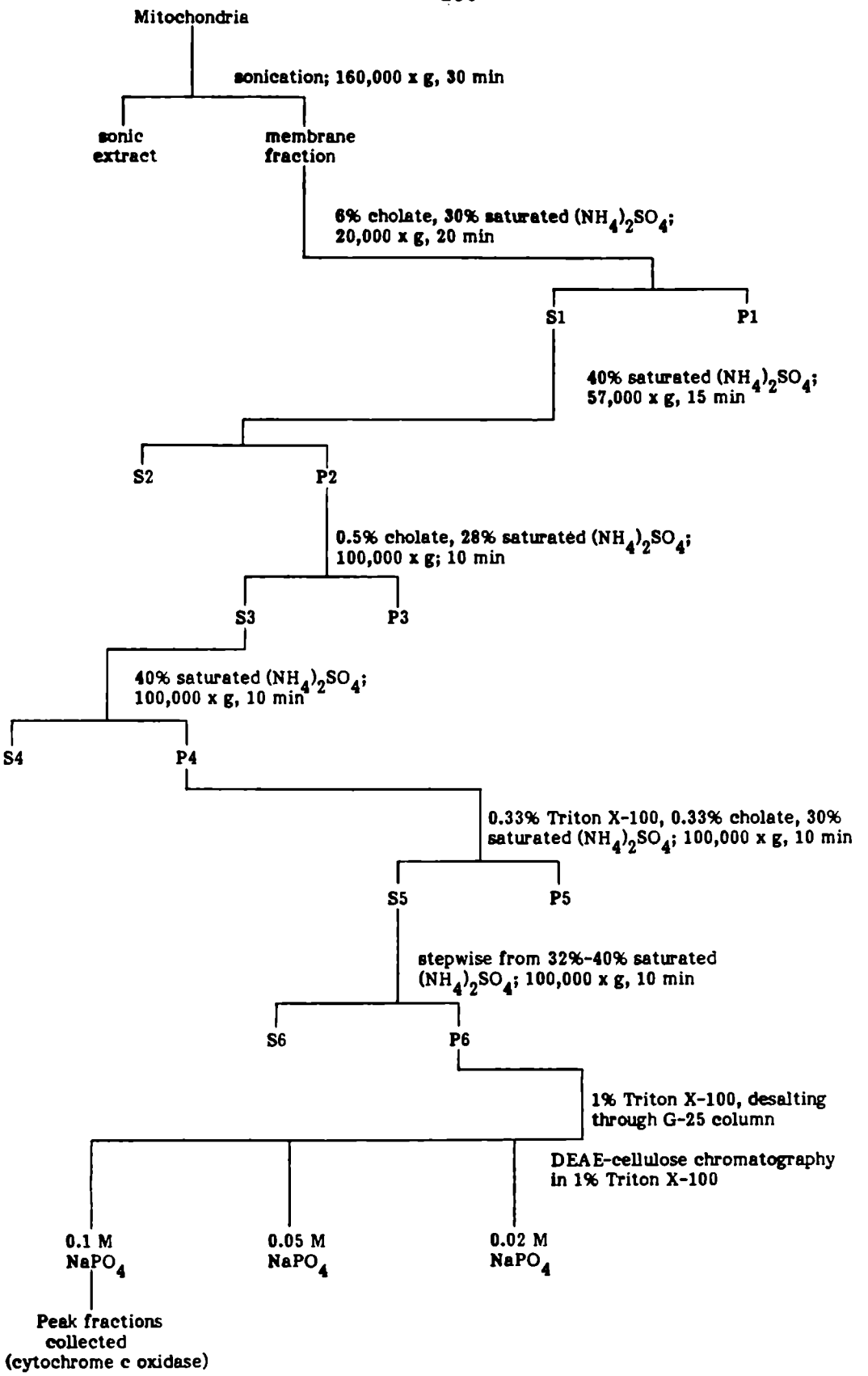

Mitochondria
sonication; 160,000 x g, 30 min
sonic extract
membrane fraction
6% cholate, 30% saturated $(NH_4)_2SO_4$; 20,000 x g, 20 min
S1
P1
40% saturated $(NH_4)_2SO_4$; 57,000 x g, 15 min
S2
P2
0.5% cholate, 28% saturated $(NH_4)_2SO_4$; 100,000 x g; 10 min
S3
P3
40% saturated $(NH_4)_2SO_4$; 100,000 x g, 10 min
S4
P4
0.33% Triton X-100, 0.33% cholate, 30% saturated $(NH_4)_2SO_4$; 100,000 x g, 10 min
S5
P5
stepwise from 32%-40% saturated $(NH_4)_2SO_4$; 100,000 x g, 10 min
S6
P6
1% Triton X-100, desalting through G-25 column
DEAE-cellulose chromatography in 1% Triton X-100
0.1 M $NaPO_4$
0.05 M $NaPO_4$
0.02 M $NaPO_4$
Peak fractions collected (cytochrome c oxidase)

Figure 1

FIGURE 2: Elution profile of human cytochrome c oxidase from the DEAE-cellulose column. The $P_6$ fraction was desalted on Sephadex G-25 and run on the DEAE-cellulose column as described in Experimental Procedure. Fractions of 0.9 ml were collected, and the absorbance at 423 nm and the protein concentration were measured. Fractions 53-57 were pooled, and either frozen or further purified on a sucrose density gradient (see Figure 3).

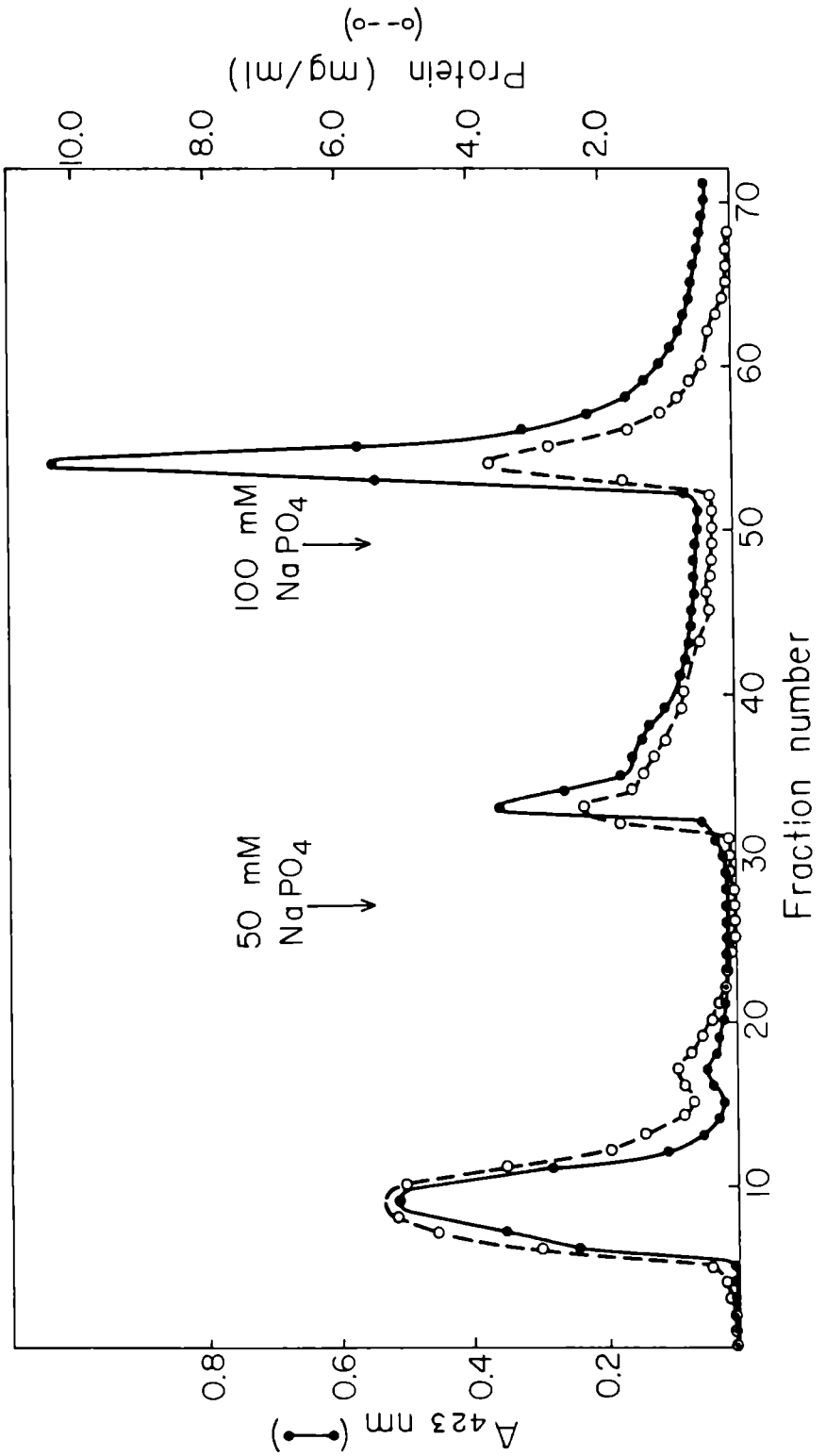

$A_{423\ nm}$ (●—●)
0.2
0.4
0.6
0.8
Protein (mg/ml)
(o--o)
2.0
4.0
6.0
8.0
10.0
10
20
30
40
50
60
70
Fraction number
50 mM
$NaPO_4$
100 mM
$NaPO_4$

FIGURE 3: Sedimentation profile of DEAE-cellulose purified human cytochrome c oxidase after centrifugation in a sucrose density gradient. A 0.5 ml sample of the 0.10 M $NaPO_4$ eluate from the DEAE-cellulose column shown in Figure 2 was run through a 5-20% sucrose gradient, as described in Experimental Procedure.

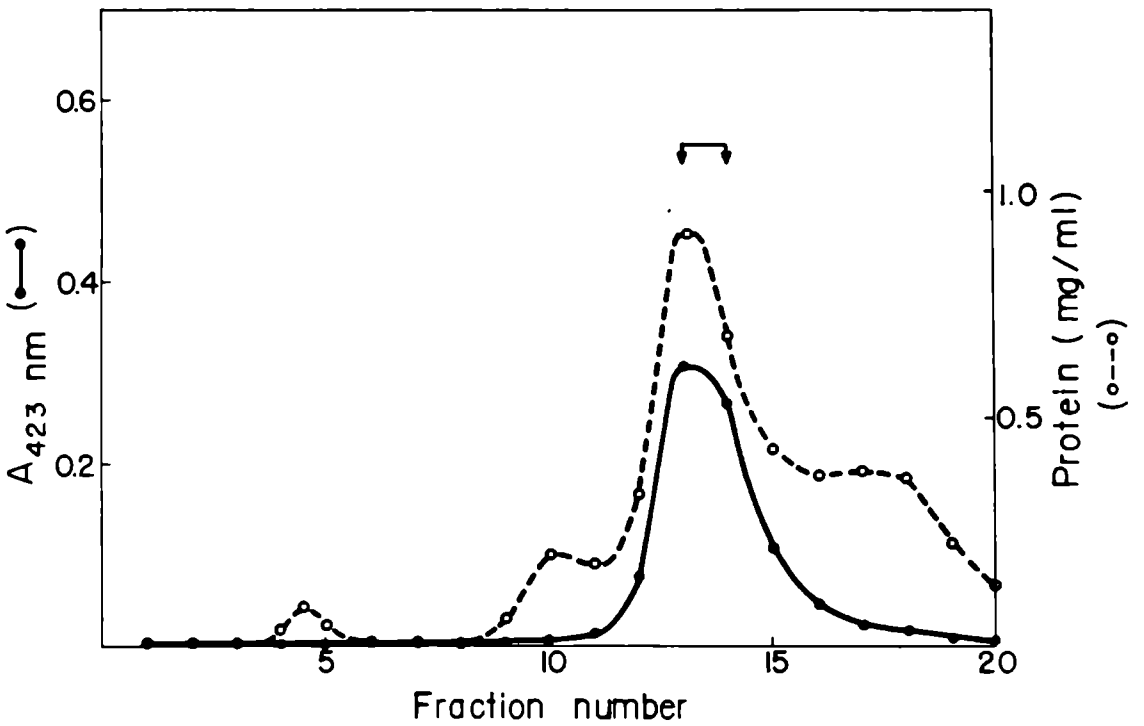

0.6
0.4
0.2
$A_{423}$ nm (—●—)
1.0
0.5
Protein (mg/ml)
(o--o)
5
10
15
20
Fraction number

FIGURE 4: Absolute spectra of human cytochrome c oxidase. Samples were diluted with 0.10 M $NaPO_4$ (pH 7.0) containing 1% Triton X-100 to 0.76 mg of protein per ml, reduced with a few mg of sodium dithionite (solid line) or left oxidized (dashed line), and then analyzed separately in a Cary 15 spectrophotometer against a buffer blank. The insert shows the difference spectrum (reduced-minus-oxidized) obtained by reading samples (0.76 mg of protein per ml), treated as described above, against each other.

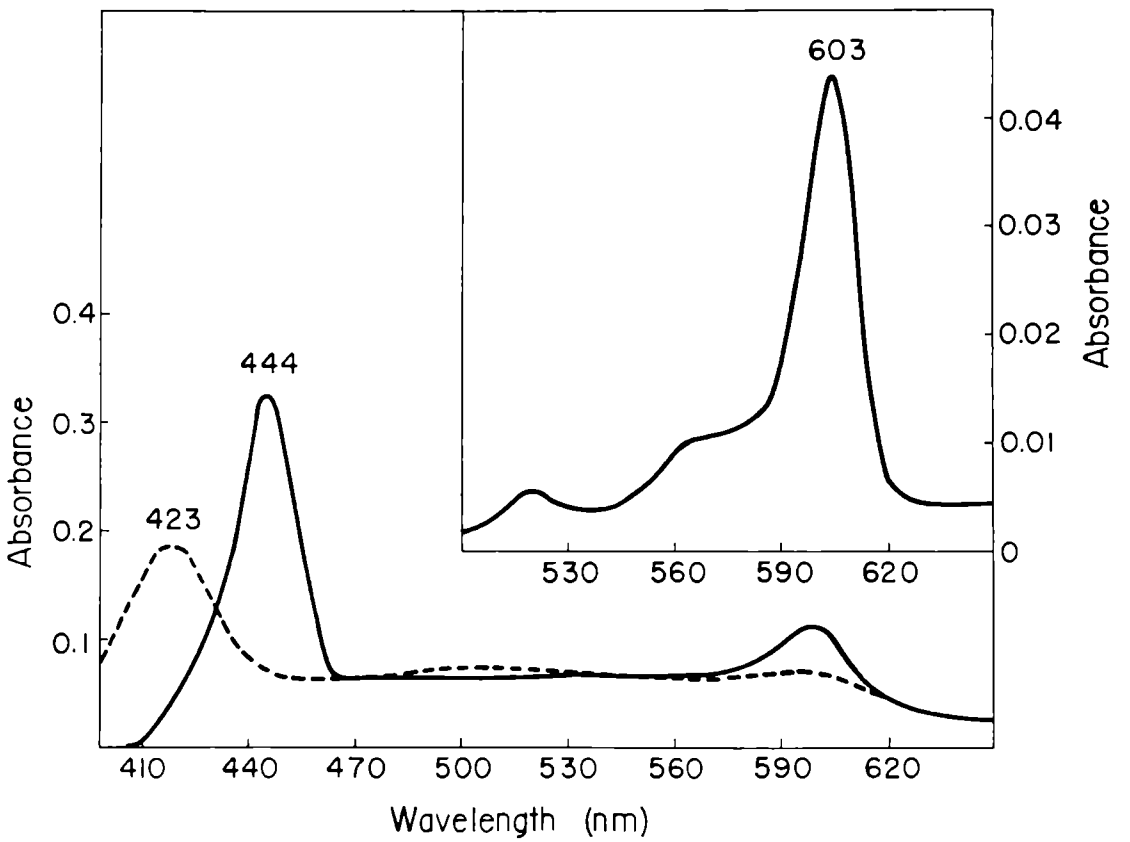

603
444
423
0.4
0.3
0.2
0.1
Absorbance
0.04
0.03
0.02
0.01
0
Absorbance
530
560
590
620
410
440
470
500
530
560
590
620
Wavelength (nm)

FIGURE 5: Electrophoretic patterns in SDS-urea polyacrylamide gels of different preparations of beef heart cytochrome c oxidase and of human placenta cytochrome c oxidase. The beef heart enzyme samples were prepared following the procedure of Kuboyama et al. (1972) (sample 1), that of Capaldi and Hayashi (1972) (sample 2), the latter procedure followed by DEAE-cellulose chromatography (0.15 M $NaPO_4$ eluate following the 0.05 M $NaPO_4$ eluate) (sample 3), and the Mason et al. (1973) procedure (sample 4). The human placenta enzyme had been purified through the DEAE-cellulose chromatography stage (0.10 M $NaPO_4$ eluate).

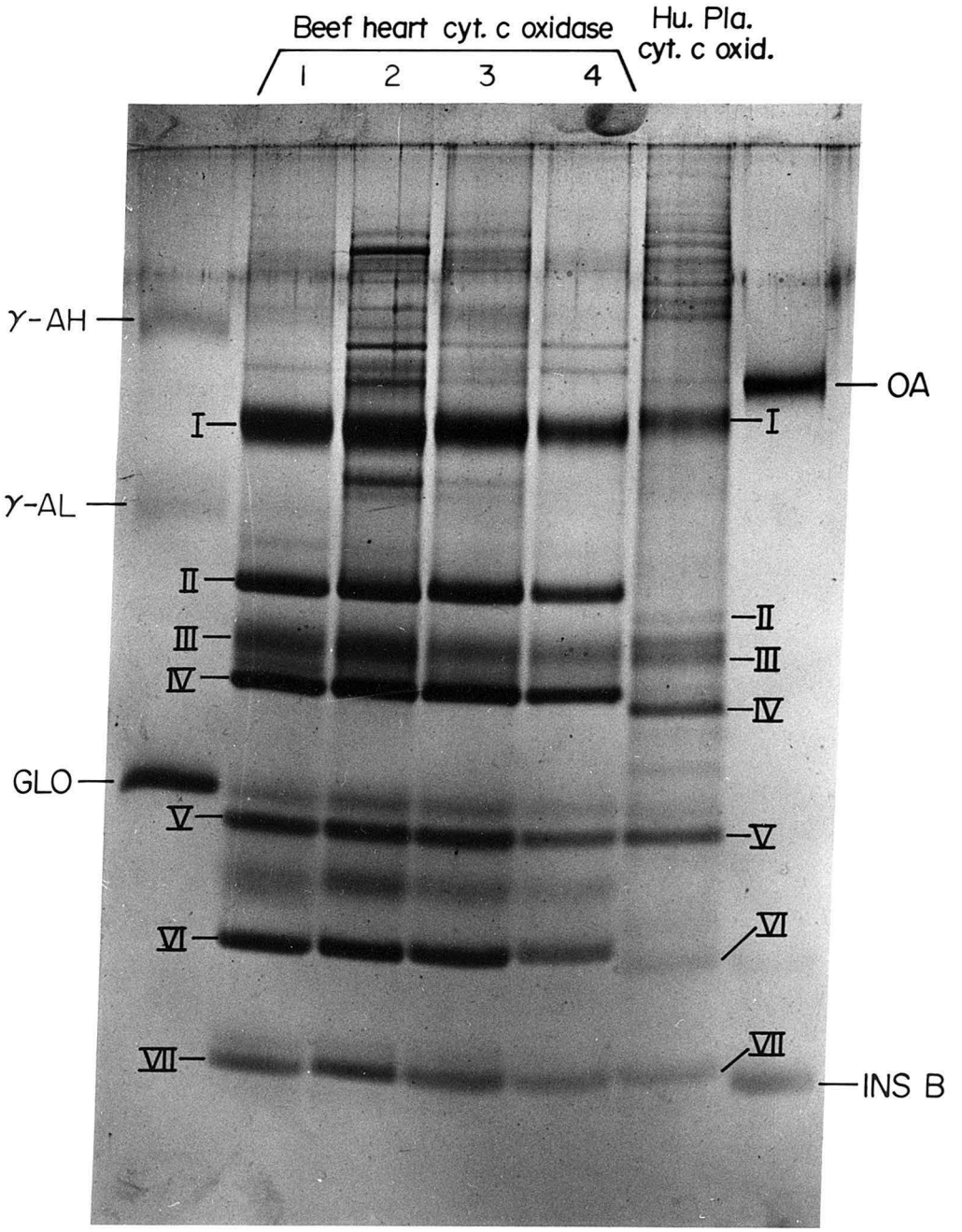

Beef heart cyt. c oxidase
1
2
3
4
Hu. Pla. cyt. c oxid.
γ-AH
γ-AL
GLO
I
II
III
IV
V
VI
VII
OA
INS B

FIGURE 6: Estimation of the apparent molecular weight of standard proteins from their migration in SDS-urea polyacrylamide gels. The standard proteins were run under the same conditions used for the radioactive samples. The migrations observed for the individual markers in different runs were normalized with respect to the migration of globin, which was always present. The open circles represent the average normalized migration values for the individual markers, the best fitting line drawn through them by the least squares method was used to estimate the apparent molecular weight of the standards, as exemplified in the diagram for $\gamma$-AL. See text for details.

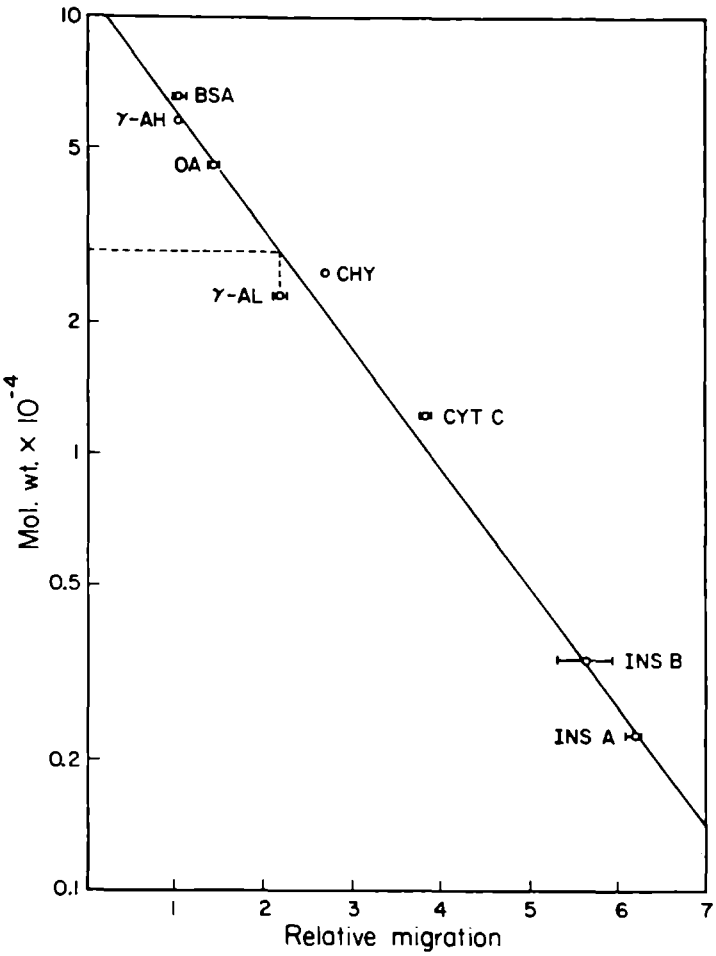

10
5
2
1
0.5
0.2
0.1
Mol. wt. x $10^{-4}$
BSA
γ-AH
OA
CHY
γ-AL
CYT C
INS B
INS A
1
2
3
4
5
6
7
Relative migration

FIGURE 7: Electrophoretic patterns in SDS-urea polyacrylamide gels of fractions of human placenta mitochondria containing cytochrome c oxidase at various stages of the purification procedure. Slot 1: purified beef heart cytochrome c oxidase (Capaldi and Hayashi, 1972). Slots 2-6: fractions of human placenta mitochondria; slot 2: fraction $P_4$; slot 3: Sephadex G-25 eluate; slots 4,5: DEAE-cellulose 0.10 M $NaPO_4$ eluate; slot 6: sucrose gradient purified cytochrome c oxidase. Samples in slots 2-4 and in slots 5 and 6 derive from different preparations and were run in separate gels. Roman numerals near slots 1 and 6 indicate the subunits of beef heart and human placenta cytochrome c oxidase, respectively.

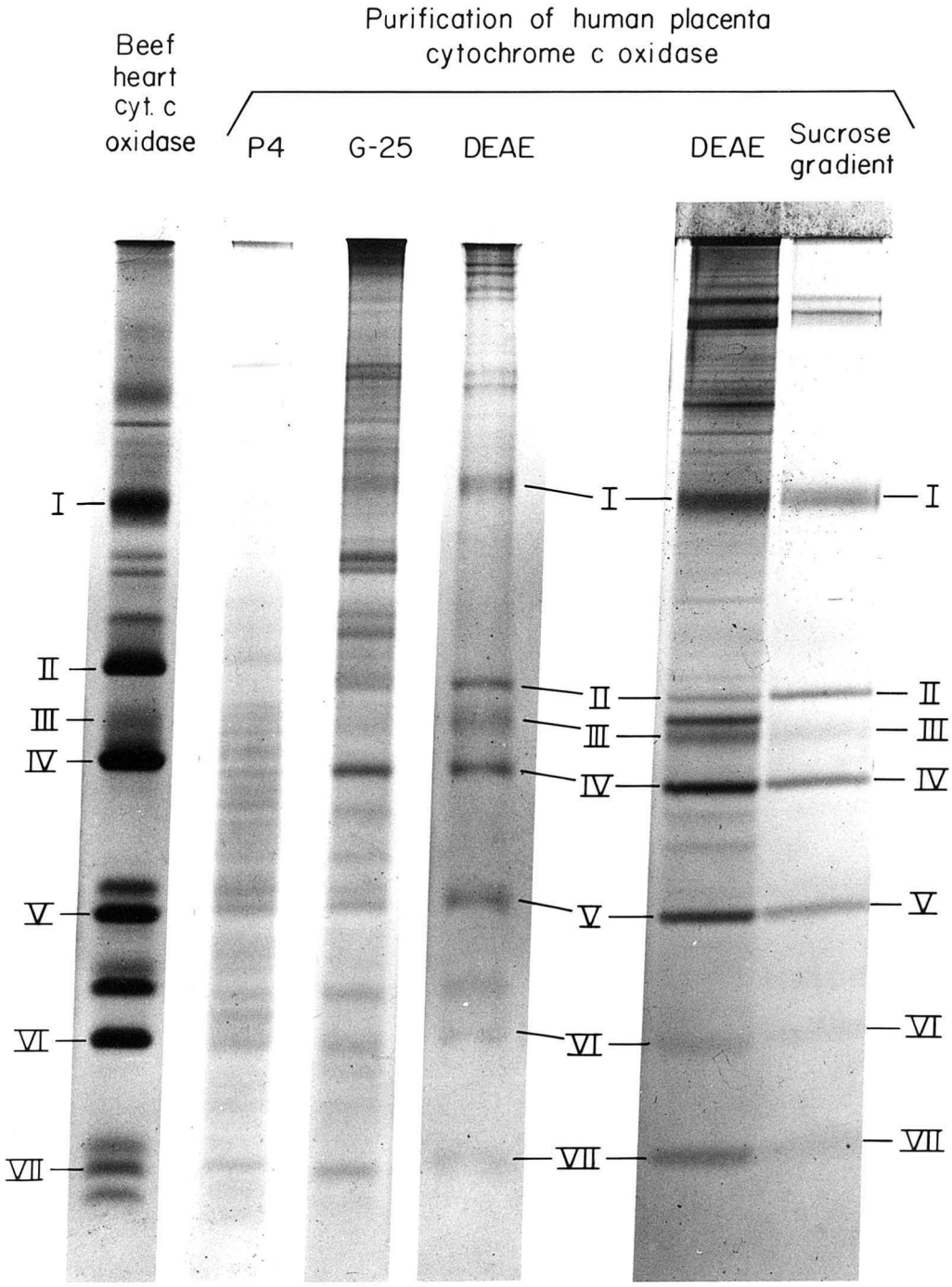
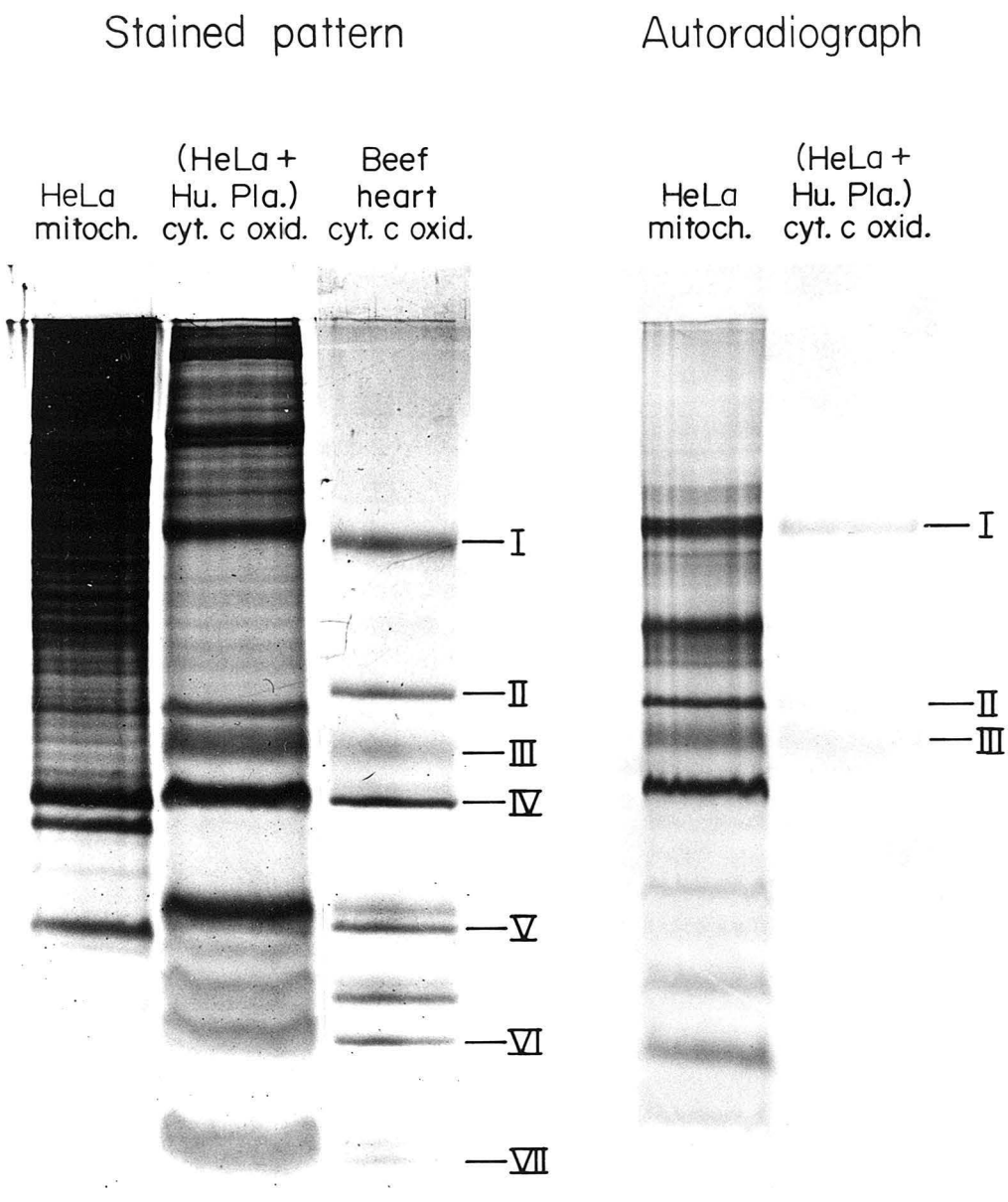

Purification of human placenta cytochrome c oxidase
Beef heart cyt. c oxidase
P4
G-25
DEAE
DEAE
Sucrose gradient
I
II
III
IV
V
VI
VII

FIGURE 8: Stained pattern and autoradiograph, after SDS-urea polyacrylamide gel electrophoresis, of cytochrome c oxidase isolated from a mixture of [$^{35}$S] methionine-labeled HeLa cell mitochondria and an excess of unlabeled human placenta mitochondria, and run in parallel with a sample of total [$^{35}$S]methionine-labeled mitochondrial proteins and with a sample of beef heart cytochrome c oxidase [purified by the Kuboyama et al. (1972) procedure]. The HeLa cell mitochondrial sample was from cells labeled with [$^{35}$S]methionine for 2 h in the presence of cycloheximide and chased as described in the text.


Stained pattern
Autoradiograph
HeLa mitoch.
(HeLa + Hu. Pla.) cyt. c oxid.
Beef heart cyt. c oxid.
HeLa mitoch.
(HeLa + Hu. Pla.) cyt. c oxid.
I
II
III
IV
V
VI
VII
I
II
III

# Chapter 6

## Update and Outlook for the Future

The results presented in the preceding chapters have dealt with various aspects of the protein synthesizing capability of HeLa cell mitochondria. The ultimate goals of the investigations were to understand some of the mechanisms of membrane and organelle biogenesis. Although the actual work was necessary as a foundation for further work which may be continued, the central questions of biogenesis were not reached. Speculations on where future work should be directed in this system should consider relevant data accumulated from other systems. A brief summary of the available information which was not described in Chapter 1 will follow.

**Mitochondrial 4S RNA Species**

The situation involving the mitochondrial 4S RNA species has made significant progress since 1977 when Chapter 2 was written. The general problem relates to the number of tRNA species necessary for the mitochondrial protein synthesizing system to translate its messages and the accompanying problem of where these tRNA species are coded. The minimum number of species necessary to translate all 61 amino acid specifying codons is 32 where Crick's "wobble" hypothesis is assumed, although estimates of the true number utilized in cellular translation systems indicates many more (Crick, 1966).

Progress in the study of mitochondrial 4S RNA has taken place primarily from two approaches: further resolution or mapping of RNA species and sequence analysis of specific species. The total number of 4S RNA species found on the mitochondrial genome has climbed steadily from 13 up to a very soft 31 species (Wu et al., 1972; Van Ommen et al., 1977). Techniques have included electron microscopic mapping, hybridization of charged tRNA species to the DNA and two-dimensional chromatography. Since the amino acid incorporation in Chapter 2 was presented, further studies using electron microscopy have identified 21 different

species on the Xenopus laevis mitochondrial genome with weak evidence for as many as five more. Thus, in one animal system, some evidence exists for as many as 26 species with the potential for additional sites in a small region not investigated (Ohi et al., 1978). Investigation of rat liver tRNA species has found specificity for amino acids not previously discovered in mammalian cell mitochondria and additional isoacceptors which would presumably indicate at least 21 individual tRNAs (Aujame and Freeman, 1979).

In the lower eukaryotes, two-dimensional electrophoresis has resolved about 25 discrete species in Neurospora (de Vries et al., 1978). Another investigation in yeast using hybridization and gross over-interpretation of the data has estimated a soft 24-31 4S RNA species. Because hybridizations were performed on individual DNA restriction fragments, a very rough map was derived (Van Ommen et al., 1977).

Much more reliable investigations on yeast have made use of deletion mapping, analysis of isoaccepting tRNA species or two-dimensional electrophoretic separation. The studies have shown specificity for 19 out of the 20 common amino acids and evidence for 27 discrete 4S RNA species. The deletion mapping study localized tRNA sites by specificity and a continuation study showed a number of isoaccepting species sufficient to boost the total to 27, although some of the species may be from the same gene (Martin et al., 1977a; Martin and Rabinowitz, 1978). The mapping did not, however, confirm some of the hypothesized species localized by the above hybridization study, and some regions were not searched. Interestingly, the distribution of 4S RNA sites about the mitochondrial genome in animal cells (Xenopus and human) is relatively even while the yeast tRNA sites are clustered in two areas. Consequently, although the informational content in terms of numbers of 4S RNA sites appear similar, the distributions of the information are quite different. The investigation separating 4S RNAs by two-dimensional

electrophoresis resolved 26 distinct species which hybridize to mitochondrial DNA but did not indicate amino acid specificity or show origin from distinct genes (Martin et al., 1977b).

The deletion mapping investigation unexpectedly found that the glutamine-tRNA is initially mischarged with glutamic acid. Thereafter, an amino-transferase activity converts the glutamic acid to a glutamine. Failure to demonstrate a similar mechanism for asparagine leaves it as the sole remaining amino acid in yeast without a demonstrated tRNA (Martin et al., 1977a).

The amino acid incorporating capability of the mitochondrial translation system was answered partially by Chapter 2 in which virtually all of the 20 common amino acids were utilized. The unambiguous and conclusive evidence is sequence information of known mitochondrially synthesized polypeptides. Such data have become available in sequence of the yeast oligomycin-sensitive ATPase subunit 9 (Wachter et al., 1977). Some additional sequence information is available for portions of the beef heart cytochrome c oxidase subunit II, which is also certainly mitochondrially synthesized (Buse and Steffens, 1976). The combination of these two sequences indeed utilizes all 20 of the common amino acids.

This utilization of all amino acids does not bear on whether all the tRNA species are coded on the mitochondrial genome, and the possibility of import does remain. Though careful studies have not yet identified the minimum 32 necessary to translate all 61 amino acid specifying codons, the 26 found is not unreasonably short. Furthermore, the available data for the one known translated sequence indicate a far from random codon utilization in yeast, with AT rich codons usually selected when possible (Macino and Tzagoloff, 1979a,b; Hensgens et al., 1979 ). Consequently, the possibility exists that the translation machinery utilizes a less than full complement of codons allowing translation with fewer than normal tRNA species.

Thus, these experiments are unable to answer exactly how many tRNA species are actually utilized by the translation apparatus and where they are coded. Complete polypeptide and genome sequence alignment will eventually become available and should ultimately resolve whether all possible codons are utilized and exactly where and how many tRNA species are contained within the genome. Where unusual or inconsistent data arise, careful and directed study of the biology involved will yield results which would otherwise likely be missed.

**A Quick Comment on Evolutionary Relationships**

Besides the problems related to number and location of the tRNA species utilized in the mitochondrial translation apparatus, other interesting biological information can arise from study of the tRNA species. In particular, sequence information has uncovered some useful information.

Essentially, three mitochondrial tRNA sequences have been published including the Neurospora initiator formyl-methionine and tyrosine tRNAs and the yeast phenylalanine tRNA (Heckman et al., 1978, 1979; Martin et al., 1978). The mitochondrial species have their own unique features and show sequence homologies to both the cytoplasmic and prokaryotic forms, but with greater homology to the prokaryotic species.

The other observation arises from the disagreement of the polypeptide and nucleotide sequence alignment of the oligomycin-sensitive ATPase subunit 9. The nucleotide sequence indicates a leucine residue where the protein sequence indicates a threonine (Macino and Tzagoloff, 1979a,b; Hensgens et al., 1979). Closer analysis of the threonine isoaccepting tRNA species has shown that one does not recognize any of the threonine codons (Macino and Tzagoloff, 1979c). It may be suggestive of the possibility that this tRNA recognizes the leucine codon

and inserts a threonine. As this is the only CUA codon in the sequence, perhaps it may turn out that the "universal" code is different in the mitochondrial translation system (i.e., CUA translates to threonine instead of leucine). In any case, some unusual phenomenon is involved.

It is assumed, both in this work and by most of the investigators in the field, that a generality exists such that observations in mitochondria of one organism almost always apply to all others. The similarities are stressed while the differences are de-emphasized. The two observations derived from sequence analysis relate to the question of organelle origin and evolution, though neither of the observations has much discriminative significance.

It has been assumed, with the exception of the oligomycin-sensitive ATPase subunit 9 where it has been clearly shown to be untrue, that the informational content encoded in mitochondrial DNA is functionally invariant (Sebald, 1977; Sebald et al., 1979). There is evidence for its truth and might be more appropriately applied when comparing within the animal kingdom, but is nevertheless an assumption.

Other differences, which may be minor, also exist between mitochondria of different organisms. Considerably different genome sizes and nucleotide compositions exist between higher plants, protozoa, lower eucaryotes and animal cells (Borst and Flavell, 1976). There also exists considerable diversity in the sizes of mitochondrial ribosomes isolated from different species and in the size and composition of the ribosomal RNA species (O'Brien, 1976). The 4S RNA gene distributions in yeast and animal cells are quite different, as noted above. And when tRNA sequence comparisons are made between Neurospora and yeast, there are differences in relatively invariant regions of the molecules.

Interestingly, the yeast transcription picture has included splicing in at least two different genes, the large ribosomal RNA and the cytochrome b subunit

(Borst et al., 1977; Bos et al., 1978). Splicing has also been found in the large ribosomal RNA in Neurospora mitochondria (Mannella et al., 1979). There has been no evidence for any similar phenomenon in animal cell mitochondrial transcription.

Thus, generalizations regarding universality in mitochondrial structure and function should be viewed in proper perspective, keeping in mind that the systems may have evolved under strict functional evolutionary pressures but allowing considerable flexibility in its accomplishment.

**Recent Work on the Cytochrome c Oxidase and the Other Respiratory Chain Complexes**

Considerable progress has taken place in the study of the respiratory chain complexes, particularly regarding their structure and biogenesis. Much of the initial work involved the cytochrome oxidase complex, but the same techniques have been applied to the cytochrome $bc_1$ and oligomycin-sensitive ATPase complexes. After purification and characterization of each complex, the next step has been to prepare antibodies against the intact complex and each of the individually purified subunits. The resulting subunit specific reagents are useful for investigation of the structure and biogenesis of the complex.

Among the enzyme complexes in the mitochondrial respiratory chain, genetic data have strongly implicated mitochondrial gene products in and only in the cytochrome $bc_1$, cytochrome oxidase and oligomycin-sensitive ATPase complexes (Tzagoloff et al., 1975, 1976a,b; Foury and Tzagoloff, 1976). Consequently, the NADH and succinate dehydrogenases are not of particular interest here. The purification and subunit characterization of the cytochrome oxidase have been discussed in Chapter 5, but much work has also focused on the cytochrome $bc_1$ and oligomycin-sensitive ATPase complexes (Rieske, 1976; Sebald, 1977).

The cytochrome $bc_1$ complex (Complex III) has been purified and characterized to contain seven or eight subunits in yeast and beef heart (Das Gupta and Rieske, 1973; Katan et al., 1976a; Bell and Capaldi, 1976; Marres and Slater, 1977). The studies have shown that the complex contains one mitochondrially synthesized subunit which is the sole polypeptide having the extreme hydrophobicity characteristic of both integral membrane and mitochondrially synthesized proteins. This protein is the heme containing cytochrome b (Katan et al., 1976b). Other workers have broken down the complex to study the cytochrome b and $c_1$ apoproteins alone (Weiss, 1972, 1976; Weiss and Ziganke, 1974; Lorenz et al., 1974; Lin and Beattie, 1978; Lin et al., 1978; Beattie et al., 1979; Ross and Schatz, 1976a,b)

The oligomycin-sensitive ATPase from various sources has likewise been characterized to contain about 10 distinct polypeptides (Tzagoloff and Meagher, 1971; Capaldi, 1973; Jackl and Sebald, 1975). The oligomycin-sensitive ATPase consists of three subcomponents, the $F_1$ ATPase, which contains the phosphorylating activity, the oligomycin-sensitivity conferring protein (OSCP) and the membrane component. Further studies have shown the $F_1$ ATPase and OSCP to be cytoplasmically synthesized and 2-4 of the membrane component polypeptides are mitochondrially synthesized (Tzagoloff, 1969, 1970; Tzagoloff and Meagher, 1972; Tzagoloff et al., 1972; Jackl and Sebald, 1975; Sebald, 1977). This membrane component contains the DCCD-binding protein whose site of synthesis appears to differ between yeast and Neurospora (Sebald et al., 1979).

The orientation of the cytochrome oxidase subunits within the membrane has been investigated using the techniques of [$^{35}$S]-diazobenzylsulfonate, antibody binding and iodination. The exposed peripheral membrane subunits are reacted from the outer, inner and both compartments (Eytan and Schatz, 1975; Eytan et al., 1975; Ludwig et al., 1979; Frey et al., 1978; Chan and Tracy, 1978). Although

the subunit orientations derived from various studies do not agree completely, it is quite clear that the cytochrome oxidase complex is asymmetrically oriented and completely spans the membrane. This is suggested by other observations and is expected if the complex serves as a proton port as hypothesized by the chemiosmotic theory for oxidative phosphorylation. Further studies have been performed to determine the relative positions of various subunits within the complex (Briggs and Capaldi, 1977; Dockter et al., 1978). Subunit V of the complex has even been sequenced (Tanaka et al., 1977, 1979).

Similar subunit orientation studies have been performed on the cytochrome $bc_1$ and oligomycin-sensitive ATPase complexes to determine both membrane orientation and relative orientations within the complex (Smith and Capaldi, 1977; Smith et al., 1978; Satre et al., 1976).

Experiments designed to distinguish between enzymatic and structural subunits of the cytochrome oxidase have reported that two mitochondrially synthesized subunits can be removed while the remaining structure retains its electron transport activity (Phan and Mahler, 1976). However, another study reported that antibody binding to one of these subunits does inhibit activity (Poyton and Schatz, 1975). In the oligomycin-sensitive ATPase complex, the ATP-Pi exchange activity is contained in the cytoplasmically synthesized $F_1$ ATPase while the mitochondrially synthesized proteins are in the membrane-bound portion (Sebald, 1977). The cytochrome b subunit contains a heme but appears also to be membrane bound (Weiss, 1976). The ribosomal protein possibly universally coded by mitochondrial genomes also would have a similar structural role. Since the translation system appears to remain functional in the absence of mitochondrial protein synthesis, it is suggestive of the possibility that this ribosomal protein is not crucial for ribosomal function. Although each complex is an integrated unit whose properties

depend on all components, there is some suggestion that the highly hydrophobic mitochondrially synthesized polypeptides might be predominantly structural rather than enzymatic.

Indeed, it would be expected that the costly evolutionary retention of a seemingly redundant protein synthesizing apparatus must produce polypeptides with some peculiar properties. The predominant mitochondrial contribution to oxidative phosphorylation might suggest that these enzymatic activities require some peculiar type polypeptides requiring retention of their genes in the mitochondrial genome, though there exist cytoplasmic cytochrome species. However, the mitochondrially synthesized subunits are known to be among the most hydrophobic polypeptides known, characteristic of integral membrane proteins and suggesting that they may not be transportable across the membrane. On the other hand, secretory proteins are passed through the outer cell membrane without actually crossing that lipid bilayer, and a similar mechanism might conceivably have been devised throughout evolution if the problem were so simple.

Among the most interesting are studies attempting to determine the mechanism of assembly of these enzyme complexes. An early series of investigations in yeast had indicated an association between cytoplasmic ribosomes and the mitochondrial membranes. It was proposed that these ribosomal binding sites, localized to junctions of the inner and outer membranes, correspond to rough endoplasmic reticulum ribosomes synthesizing secretory proteins which are co-translationally extruded through the membrane (Kellems and Butow, 1972, 1974; Kellems et al., 1974, 1975). However, other studies indicated that there exist free cytoplasmic pools of the cytoplasmically synthesized intramitochondrially-localized polypeptides and that the transport of these species is independent of ribosomal functions (Schwab, 1972; Godinot and Lardy, 1973; Tzagoloff, 1969; Hallermayer et al., 1977; Harmey et al., 1977).

The possibility that both mechanisms are utilized should not be excluded, but more recent evidence supports the latter. All investigations of specific subunits in the cytochrome $bc_1$, cytochrome oxidase and $F_1$ ATPase complexes have indicated that the cytoplasmically synthesized subunits are translated on soluble ribosomes into larger precursors which are released into free cytoplasmic pools and are processed into the mature forms after or upon insertion into the membranes (Coté et al., 1979; Poyton and Kavanaugh, 1976; Poyton and McKemmie, 1976; Maccecchini et al., 1979). This mechanism obviously differs from the signal hypothesis for secretory proteins since here, soluble precursors are post-translationally transported across the membranes. Further study of this phenomenon may yield an additional mechanism for protein insertion into or across lipid bilayers, perhaps analogous to proteins such as complement, diphtheria toxin and cholera toxin (Chua and Schmidt, 1979; Kolb et al., 1972; Boquet and Pappenheimer, 1976; Boquet et al., 1976; Gill, 1976).

Perhaps one of the last obvious directions of pursuit will be to try to identify the functions of the rest of the mitochondrially synthesized polypeptides. After it is shown that the minor species have some real significance, sequence information should be accumulated. Alignment with the DNA sequence would yield a map and sequence homologies with other known proteins might suggest function.

Perhaps a more useful approach would be to produce monoclonal antibodies against each of the species. The technique does not require pure antigen for eliciting a response (remember that mass purification is virtually impossible) but an assay for antibody is quite easy. With the specific binding reagent, mass purification would become possible leading the way to further studies including sequence determination and investigations of post-translational modifications

such as glycosylation. Function might even be identified by in vitro antibody inhibition of activity, and studies on biogenesis could follow.

Certainly, the area of membrane and organelle biogenesis is just beginning to make substantial progress. A considerable amount of work remains and will likely suggest mechanisms applicable to the more complex problems involved in cellular membrane biogenesis and development.

**References**

Aujame, L. and Freeman, K. B. (1979) Mammalian mitochondrial transfer RNAs: chromatographic properties, size and origin. Nuc. Acids Res. **6**, 455-469.

Beattie, D. S., Chen, Y. S., Clejan, L. and Lin, L. F. H. (1979) Electrophoretic behavior of cytochrome b in a partially purified preparation and evidence for high molecular weight associated mitochondrial translation products. Biochemistry **18**, 2400-2406.

Bell, R. L. and Capaldi, R. A. (1976) The polypeptide composition of ubiquinone-cytochrome c reductase (Complex III) from beef heart mitochondria. Biochemistry **15**, 996-1001.

Boquet, P. and Pappenheimer, A. M., Jr. (1976) Interaction of diphtheria toxin with mammalian cell membranes. J. Biol. Chem. **251**, 5770-5778.

Boquet P., Silverman, M. S., Pappenheimer, A. M., Jr. and Vernon, W. B. (1976) Binding of Triton X-100 to diphtheria toxin, crossreacting material 45, and their fragments. Proc. Nat. Acad. Sci. **73**, 4449-4453.

Borst, P., Bos, J. L., Grivell, L. A., Groot, G. S. P., Heyting, C., Moorman, A. F. M., Sanders, J. P. M., Talen, J. L., Van Kreijl, C. F. and Van Ommen, G. J. B. (1977) The physical map of yeast mitochondrial DNA anno 1977. In: Mitochondria 1977. Genetics and Biogenesis of Mitochondria (eds. W. Bandlow et al.), pp. 213-254. Walter de Gruyter, Berlin.

Borst, P. and Flavell, R. A. (1976) Properties of mitochondrial DNAs. In: Handbook of Biochemistry and Molecular Biology, 3rd Edition, Nucleic Acids Vol. 2 (ed. G. Fasman), pp. 363-374. CRC Press, Cleveland.

Bos, J. L., Heyting, C., Borst, P., Arnberg, A. C. and Van Bruggen, E. F. J. (1978) An insert in the single gene for the large ribosomal RNA in yeast mitochondrial DNA. Nature **75**, 336-338.

Briggs, M. M. and Capaldi, R. A. (1977) Nearest-neighbor relationships of the subunits of cytochrome c oxidase. Biochemistry **16**, 73-77.

Buse, G. and Steffens, G. J. (1976) Analysis of the polypeptide chains of cytochrome oxidase from beef heart mitochondria. In: Genetics and Biogenesis of Chloroplasts and Mitochondria (eds. Th. Bucher et al. ), pp. 189-194. Elsevier/North Holland, Amsterdam.

Capaldi, R. A. (1973) On the subunit structure of oligomycin-sensitive ATPase. Biochem. Biophys. Res. Commun. **53**, 1331-1337.

Chan, S. H. P. and Tracy, R. P. (1978) Immunological studies on cytochrome c oxidase: Arrangements of protein subunits in the solubilized and membrane-bound enzyme. Eur. J. Biochem. **89**, 595-605.

Chua, N. H. and Schmidt, G. W. (1979) Transport of proteins into mitochondria and chloroplasts. J. Cell Biol. **81**, 461-483.

Coté, C., Solioz, M. and Schatz, G. (1979) Biogenesis of the cytochrome $bc_1$ complex in yeast mitochondria. J. Biol. Chem. **254**, 1437-1439.

Crick, F. H. C. (1966) Codon-anticodon pairing: The Wobble hypothesis. J. Mol. Biol. **19**, 548-555.

Das Gupta, U. and Rieske, J. S. (1973) Identification of a protein component of the antimycin-binding site of the respiratory chain by photoaffinity labeling. Biochem. Biophys. Res. Commun. **54** 1247-1254.

de Vries, H., De Jonge, J. C., Schneller, J. M., Martin, R. P., Dirheimer, G. and Stahl, A. J. C. (1978) Neurospora crassa mitochondrial transfer RNAs. Biochim. Biophys. Acta **520**, 419-427.

Dockter, M. E., Steinemann, A. and Schatz, G. (1978) Mapping of yeast cytochrome c oxidase by fluorescence resonance energy transfer. J. Biol. Chem. **253**, 311-317.

Eytan, G. D., Carroll, R. C., Schatz, G. and Racker, E. (1975) Arrangement of the subunits in solubilized and membrane-bound cytochrome c oxidase from bovine heart. J. Biol. Chem. **250**, 8598-8603.

Eytan, G. D. and Schatz, G. (1975) Cytochrome c oxidase from bakers' yeast. V. Arrangement of the subunits in the isolated and membrane-bound enzyme. J. Biol. Chem. **250**, 767-774.

Foury, F. and Tzagoloff, A. (1976) Assembly of the mitochondrial membrane system. XIX. Genetic characterization of $mit^-$ mutants with deficiencies in cytochrome oxidase and coenzyme $QH_2$-cytochrome c reductase. Molec. Gen. Genetics **149**, 43-50.

Frey, T. G., Chan, S. H. P. and Schatz, G. (1978) Structure and orientation of cytochrome c oxidase in crystalline membranes. Studies by electron microscopy and by labeling with subunit-specific antibodies. J. Biol. Chem. **253**, 4389-4395.

Gill, D. M. (1976) The arrangement of subunits in cholera toxin. Biochemistry **15**, 1242-1248.

Godinot, C. and Lardy, H. A. (1973) Biosynthesis of glutamate dehydrogenase in rat liver. Demonstration of its microsomal localization and hypothetical mechanism of transfer to mitochondria. Biochemistry **12**, 2051-2060.

Hallermayer, G., Zimmermann, R. and Neupert, W. (1977) Kinetic studies on the transport of cytoplasmically synthesized proteins into the mitochondria in intact cells of Neurospora crassa. Eur. J. Biochem. **81**, 523-532.

Harmey, M. A., Hallermayer, G., Korb, H. and Neupert, W. (1977) Transport of cytoplasmically synthesized proteins into the mitochondria in a cell free system from Neurospora crassa. Eur. J. Biochem. **81**, 533-544.

Heckman, J. E., Alzner-Deweerd, B. and RajBhandary, U. L. (1979) Interesting and unusual features in the sequence of Neurospora crassa mitochondrial tyrosine tRNA. Proc. Nat. Acad. Sci. **76**, 717-721.

Heckman, J. E., Hecker, L. I., Schwartzbach, S. D., Barnett, W. E., Baumstark, B. and RajBhandary, U. L. (1978) Structure and function of initiator methionine tRNA from the mitochondria of Neurospora crassa. Cell **13**, 83-95.

Hensgens, L. A. M., Grivell, L. A., Borst, P. and Bos, J. L. (1979) Nucleotide sequence of the mitochondrial structural gene for subunit 9 of yeast ATPase complex. Proc. Nat. Acad. Sci. **76**, 1663-1667.

Jackl, G. and Sebald, W. (1975) Identification of two products of mitochondrial protein synthesis associated with mitochondrial adenosine triphosphatase from Neurospora crassa. Eur. J. Biochem. **54**, 97-106.

Katan, M. B., Pool, L. and Groot, G. S. P. (1976a) The cytochrome $bc_1$ complex of yeast mitochondria. Isolation and partial characterization of the cytochrome $bc_1$ complex and cytochrome b. Eur. J. Biochem. **65**, 95-105.

Katan, M. B., Harten-Loosbrock, N. V. and Groot, G. S. P. (1976b) The cytochrome $bc_1$ complex of yeast mitochondria. Site of translation of the polypeptide in vivo. Eur. J. Biochem. **70**, 409-417.

Kellems, R. E., Allison, V. and Butow, R. A. (1974) Cytoplasmic type 80S ribosomes associated with yeast mitochondria. II. Evidence for the association of cytoplasmic ribosomes with the outer mitochondrial membrane in situ. J. Biol. Chem. **249**, 3297-3303.

Kellems, R. E., Allison, V. F. and Butow, R. A. (1975) Cytoplasmic type 80S ribosomes associated with yeast mitochondria. IV. Attachment of ribosomes to the outer membrane of isolated mitochondria. J. Cell Biol. **65**, 1-14.

Kellems, R. E. and Butow, R. A. (1972) Cytoplasmic 80S ribosomes associated with yeast mitochondria. I. Evidence for ribosome binding sites on yeast mitochondria. J. Biol. Chem. **247**, 8043-8050.

Kellems, R. E. and Butow, R. A. (1974) Cytoplasmic type 80S ribosomes associated with yeast mitochondria. III. Changes in the amount of bound ribosomes in response to changes in metabolic state. J. Biol. Chem. **249**, 3304-3310.

Kolb, W. P., Haxby, J. A., Arroyave, C. M. and Müller-Eberhard, H. J. (1972) Molecular analysis of the membrane attack mechanism of complement. J. Exp. Med. **135**, 549-566.

Lin, L. F. H. and Beattie, D. S. (1978) Purification and properties of a major cytochrome b peptide from baker's yeast. J. Biol. Chem. **253**, 2412-2418.

Lin, L. F. H., Clejan, L. and Beattie, D. S. (1978) The synthesis of cytochrome b on mitochondrial ribosomes in baker's yeast. Eur. J. Biochem. **87**, 171-179.

Lorenz, B., Kleinow, W. and Weiss, H. (1974) Mitochondrial translation of cytochrome b in Neurospora crassa and Locusta migratoria. Hoppe-Seyler's Z. Physiol. Chem. **355**, 300-304.

Ludwig, B., Downer, N. W. and Capaldi, R. A. (1979) Labeling of cytochrome c oxidase with [$^{35}$S] diazobenzenesulfonate. Orientation of this electron transfer complex in the inner mitochondrial membrane. Biochemistry **18**, 1401-1407.

Maccecchini, M. L., Rudin, Y., Blobel, G. and Schatz, G. (1979) Import of proteins into mitochondria: Precursor forms of the extramitochondrially made $F_1$-ATPase subunits in yeast. Proc. Nat. Acad. Sci. **76**, 343-347.

Macino, G. and Tzagoloff, A. (1979a) Assembly of the mitochondrial membrane system: Partial sequence of a mitochondrial ATPase gene in Saccharomyces cerevisiae. Proc. Nat. Acad. Sci. **76**, 131-135.

Macino, G. and Tzagoloff, A. (1979b) Assembly of the mitochondrial membrane system. The DNA sequence of a mitochondrial ATPase gene in Saccharomyces cerevisiae. J. Biol. Chem. **254**, 4617-4623.

Macino, G. and Tzagoloff, A. (1979c) Assembly of the mitochondrial membrane system. Two separate genes coding for threonyl-tRNA in the mitochondrial DNA of Saccharomyces cerevisiae. Molec. Gen. Genetics **169**, 183-188.

Mannella, C. A., Collins, R. A., Green, M. R. and Lambowitz, A. M. (1979) Defective splicing of mitochondrial rRNA in cytochrome-deficient nuclear mutants of Neurospora crassa. Proc. Nat. Acad. Sci. **76**, 2635-2639.

Marres, C. A. M. and Slater, E. C. (1977) Polypeptide composition of purified $QH_2$:cytochrome c oxidoreductase from beef-heart mitochondria. Biochim. Biophys. Acta **462**, 531-548.

Martin, N. C. and Rabinowitz, M. (1978) Mitochondrial transfer RNAs in yeast: Identification of isoaccepting transfer RNAs. Biochemistry **17**, 1628-1634.

Martin, N. C., Rabinowitz, M. and Fukuhara, H. (1977a) Yeast mitochondrial DNA specifies tRNA for 19 amino acids. Deletion mapping of the tRNA genes. Biochemistry **16**, 4672-4677.

Martin, R. P., Schneller, J. M. and Dirheimer, G. (1977b) Study of yeast mitochondrial tRNAs by two-dimensional polyacrylamide gel electrophoresis: Characterization of isoaccepting species and search for imported cytoplasmic tRNAs. Nuc. Acids Res. **4**, 3497-3510.

Martin, R. P., Sibler, A. P., Schneller, J. M., Keith, G., Stahl, A. J. C. and Dirheimer, G. (1978) Primary structure of yeast mitochondrial DNA-coded phenylalanine-tRNA. Nuc. Acids Res. **5**, 4579-4592.

O'Brien, T. W. (1976) Mitochondrial protein synthesis. In: Protein Synthesis, Vol. 2 (ed. E. H. McConkey), pp. 249-307. Marcel Dekker, New York.

Ohi, S., Ramirez, J. L., Upholt, W. B. and Dawid, I. B. (1978) Mapping of mitochondrial 4S RNA genes in Xenopus laevis by electron microscopy. J. Mol. Biol. **121**, 299-310.

Phan, S. H. and Mahler, H. R. (1976) Studies on cytochrome oxidase. Preliminary characterization of an enzyme containing only four subunits. J. Biol. Chem. **251**, 270-276.

Poyton, R. O. and Kavanaugh, J. (1976) Regulation of mitochondrial protein synthesis by cytoplasmic proteins. Proc. Nat. Acad. Sci. **73**, 3947-3951.

Poyton, R. O. and McKemmie, E. (1976) The assembly of cytochrome c oxidase from Saccharomyces cerevisiae. In: Genetics and Biogenesis of Chloroplasts and Mitochondria (eds. Th. Bucher et al.), pp. 207-214. Elsevier/North Holland, Amsterdam.

Poyton, R. O. and Schatz, G. (1975) Cytochrome c oxidase from baker's yeast. IV. Immunological evidence for the participation of a mitochondrially synthesized subunit in enzymatic activity. J. Biol. Chem. **250**, 762-766.

Rieske, J. S. (1976) Composition, structure, and function of complex III of the respiratory chain. Biochim. Biophys. Acta **456**, 195-247.

Ross, E. and Schatz, G. (1976a) Cytochrome $c_1$ of bakers' yeast. I. Isolation and properties. J. Biol. Chem. **251**, 1991-1996.

Ross, E. and Schatz, G. (1976b) Cytochrome $c_1$ of bakers' yeast. II. Synthesis on cytoplasmic ribosomes and influence of oxygen and heme on accumulation of the apoprotein. J. Biol. Chem. **251**, 1997-2004.

Satre, M., Klein, G. and Vignais, P. V. (1976) Structure of beef heart mitochondrial $F_1$-ATPase arrangement of subunits as disclosed by cross-linking reagents and selective labeling by radioactive ligands. Biochim. Biophys. Acta **453**, 111-120.

Schwab, A. J., Sebald, W. and Weiss, H. (1972) Different pool sizes of the precursor polypeptides of cytochrome oxidase from Neurospora crassa. Eur. J. Biochem. **30**, 511-516.

Sebald, W. (1977) Biogenesis of mitochondrial ATPase. Biochim. Biophys. Acta **463**, 1-27.

Sebald, W., Graf T. and Lukins, H. B. (1979) The dicyclohexylcarbodiimide-binding protein of the mitochondrial ATPase complex from Neurospora crassa and Saccharomyces cerevisiae. Identification and isolation. Eur. J. Biochem. **93**, 587-608.

Smith, R. J. and Capaldi, R. A. (1977) Nearest neighbor relationships of the polypeptides in ubiquinone cytochrome c reductase (Complex III). Biochemistry **16**, 2629-2633.

Smith, R. J., Capaldi, R. A., Muchmore, D. and Dahlquist, F. (1978) Cross-linking of ubiquinone cytochrome c reductase (Complex III) with periodate-cleavable bifunctional reagents. Biochemistry **17**, 3719-3723.

Tanaka, M., Haniu, M., Yasunobu, K. T., Yu, C. A., Yu, L., Wei, Y. H. and King, T. E. (1977) Amino acid sequence of the heme a subunit of bovine heart cytochrome oxidase and sequence homology with hemoglobin. Biochem. Biophys. Res. Commun. **76**, 1014 1019.

Tanaka, M., Haniu, M., Yasunobu, K. T., Yu, C. A., Yu, L., Wei, Y. H. and King, T. E. (1979) Amino acid sequence of subunit V of bovine heart cytochome oxidase, the heme a-containing subunit. J. Biol. Chem. **254**, 3879-3885.

Tzagoloff, A. (1969) Assembly of the mitochondrial membrane system. II. Synthesis of the mitochondrial adenosine triphosphatase, $F_1$. J. Biol. Chem. **244**, 5027-5033.

Tzagoloff, A. (1970) Assembly of the mitochondrial membrane system. III. Function and synthesis of the oligomycin sensitivity-conferring protein of yeast mitochondria. J. Biol. Chem. **245**, 1545-1551.

Tzagoloff, A., Akai, A., Needleman, R. B. and Zulch, G. (1975) Assembly of the mitochondrial membrane system. Cytoplasmic mutants of Saccharomyces cerevisiae with lesions in enzymes of the respiratory chain and in the mitochondrial ATPase. J. Biol. Chem. **250**, 8236-8242.

Tzagoloff, A., Akai, A. and Sierra, M. F. (1972) Assembly of the mitochondrial membrane system. VII. Synthesis and integration of $F_1$ subunits into the rutamycin-sensitive adenosine triphosphatase. J. Biol. Chem. **247**, 6511-6516.

Tzagoloff, A., Foury, F. and Akai, A. (1976a) Assembly of the mitochondrial membrane system. XVIII. Genetic loci on mitochondrial DNA involved in cytochrome b biosynthesis. Molec. Gen. Genetics **149**, 33-42.

Tzagoloff, A., Foury, F. and Akai, A. (1976b) Resolution of the mitochondrial genome. In: The Genetic Function of Mitochondrial DNA (eds. C. Saccone and A. M. Kroon), pp. 155-161. Elsevier, North Holland, Amsterdam.

Tzagoloff, A. and Meagher, P. (1971) Assembly of the mitochondrial membrane system. V. Properties of a dispersed preparation of the rutamycin-sensitive adenosine triphosphatase of yeast mitochondria. J. Biol. Chem. **246**, 7328-7336.

Tzagoloff, A. and Meagher, P. (1972) Assembly of the mitochondrial membrane system. VI. Mitochondrial synthesis of subunit proteins of the rutamycin-sensitive adenosine triphosphatase. J. Biol. Chem. **247**, 594-603.

Van Ommen, G. J. B., Groot, G. S. P. and Borst, P. (1977) Fine structure physical mapping of 4S RNA genes on mitochondrial DNA of Saccharomyces cerevisiae. Molec. Gen. Genetics **154**, 255-262.

Wachter, E., Sebald, W. and Tzagoloff, A. (1977) Altered amino acid sequence of the DCCD-binding protein in the Oli 1 resistant mutant D273-10B/A21 of Saccharomyces cerevisiae. In: Mitochondria 1977. Genetics and Biogenesis of Mitochondria (eds. W. Bandlow et al.), pp. 441-449. Walter de Gruyter, New York.

Weiss, H. (1972) Cytochrome b in Neurospora crassa mitochondria. A membrane protein containing subunits of cytoplasmic and mitochondrial origin. Eur. J. Biochem. **30**, 469-478.

Weiss, H. (1976) Subunit composition and biogenesis of mitochondrial cytochrome b. Biochim. Biophys. Acta **456**, 291-313.

Weiss, H. and Ziganke, B. (1974) Cytochrome b in Neurospora crassa mitochondria. Site of translation of the heme protein. Eur. J. Biochem. **41**, 63-71.

Wu, M., Davidson, N., Attardi, G. and Aloni, Y. (1972) Expression of the mitochondrial genome in HeLa cells. XIV. The relative positions of the 4S RNA genes and of the ribosomal RNA genes in mitochondrial DNA. J. Mol. Biol. **71**, 81-93.