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REGULATION OF MITOCHONDRIAL PROTEIN SYNTHESIS.

The City University of New York, Ph.D., 1976
Health Sciences, pathology

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REGULATION OF MITOCHONDRIAL PROTEIN SYNTHESIS

by

Nader G. Ibrahim

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Mount Sinai School of Medicine of the City University of New York.

- 1975 -

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Scinces in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

Sept 26, 1975
Date

Chairman of Examining Committee

Sept 26, 1975
Date

Executive Officer

The City University of New York

ABSTRACT

REGULATION OF MITOCHONDRIAL PROTEIN SYNTHESIS

by

Nader G. Ibrahim

Advisor: Dr. Diana S. Beattie

Amino acid incorporation *in vitro* by isolated yeast mitochondria could be supported by either an external ATP-regenerating system or by the respiratory-chain linked synthesis of ATP. In either system, addition of chloramphenicol, erythromycin or ethidium bromide caused an 80-90% inhibition of incorporation, actinomycin D and cordycepin caused a 20% inhibition, while rifamycin or cycloheximide had no effect.

The rate of amino acid incorporation *in vitro* by isolated mitochondria was not subject to glucose derepression. In contrast, the rate of cycloheximide-insensitive protein synthesis into mitochondrial membranes *in vivo* was lowered over 90% in repressed cells. Analysis of the labeled mitochondrial membranes by sodium dodecylsulfate gel electrophoresis indicated that the products of mitochondrial protein synthesis were qualitatively identical, but quantitatively different, in glucose-repressed and derepressed cells. For example, a two-fold increase in labeling into both the two

peaks of highest molecular weight as well as the peak of lowest molecular weight was observed in mitochondrial membranes obtained from repressed cells as compared to mitochondrial membranes obtained from partially and fully derepressed cells.

The rate of amino acid incorporation in vitro as well as the labeling of mitochondrial membranes in vivo was stimulated 50-100% in partially derepressed yeast cells which had been grown in medium containing chloramphenicol for three hours, washed, and allowed to grow for one hour in fresh medium without chloramphenicol. The stimulation of labeling in vivo resulted in a three-fold increase of labeling into the peak of lowest molecular weight with a concomitant decrease of labeling into the peaks of high molecular weight suggesting that the accumulation of mitochondrial proteins synthesized in the cytoplasm, when chloramphenicol is present in the medium, may stimulate the synthesis of certain specific mitochondrial proteins. Furthermore, growth of the cells in the presence of cycloheximide, which would result in a depletion of the pool of mitochondrial proteins synthesized in the cytoplasm, followed by growth in fresh medium without cycloheximide, resulted in a 50% decrease in the incorporation rate in vitro.

Polysomes consisting of 2-8 monosomes were isolated from yeast mitochondria by lysing the mitochondria with Triton X-100 and centrifugation in a 20-40% linear sucrose gradient. When yeast spheroplasts were pulse-labeled with

[^{3}H]leucine in the presence of cycloheximide to block cytoplasmic protein synthesis, radioactivity which was trichloroacetic acid precipitable was present mainly in the polysome region. Incorporation of leucine was blocked by erythromycin, a specific inhibitor of mitochondrial protein synthesis. Release of radioactivity to the top of the gradient resulted from treating labeled polysomes with either puromycin or ribonuclease (in the latter case with the breakdown of polysomes) indicating that the radioactivity was present in nascent polypeptide chains.

Yeast cells were grown in chloramphenicol for 3 hours, in fresh medium for 1 hour and then pulse-labeled with either [^{3}H]leucine or [^{14}C]formate. Three parameters showed a 2-fold increase in cells grown in chloramphenicol prior to pulse labeling: the polysome to monosome ratio, the amount of labeled precursor incorporated into proteins, and the rate of polypeptide chain initiation as judged by the formation of f-Met-puromycin. Conversely, these parameters were all decreased approximately 50% in cells treated with cycloheximide prior to pulse labeling.

Mitochondria were also isolated from cells previously grown in chloramphenicol or cycloheximide and incubated <u>in vitro</u> with [^{3}H]leucine under optimal conditions. Acid precipitable radioactivity in the polysome region was increased 3-fold in mitochondria from cells grown previously in chloramphenicol and decreased 75% in those grown in cycloheximide. Furthermore, chain initiation was demonstrated

in the isolated mitochondria by formation of f-Met-puromycin. The rate of chain initiation *in vitro* was increased 2-fold in mitochondria isdated from chloramphenicol-treated cells.

These results suggest that products of cytoplasmic protein synthesis which accumulate in yeast cells grown in chloramphenicol and which are depleted in yeast cells grown in cycloheximide may act to regulate mitochondrial protein synthesis.

Ribosomes with a sedimentation coefiicient of 55S were isolated from rat liver mitochondria. When incubated with supernatant factors from *Escherichia coli*, these ribosomes catalyze poly(U)-dependent polyphenylalanine synthesis at rates comparable to those obtained with ribosomes isolated from yeast mitochondria. Protein synthesis on ribosomes isolated from both rat liver and yeast mitochondria was inhibited to the same extent by carbomycin, chloramphenicol and erythromycin over a wide concentration range of drug. Hence, mitoribosomes from yeast or rat liver do not differ in their sensitivity to these inhibitors of protein synthesis.

FORWARD

The auther wishes to express his sincer gratitude to Professor : Diana S. Beattie for her guidence and encouragement in the development and completion of my dissertation. Her kindeness will not be forgotten.

My thanks go to Professor P.G.Katsoyannis for his assistence in making the necessary equipment available to me.

Lastly , with love and thanks i acknowledge my wife Amal , Mom and Dad for their great encouragement .

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PURPOSE OF THIS INVESTIGATION

The overall objective of this dissertation is to gain a greater understanding of the factors which regulate the formation of the mitochondrial membrane. This process requires that proteins synthesized on cytoplasmic ribosomes be integrated into the mitochondrial membrane in conjunction with the 8-10 hydrophobic proteins synthesized on the unique mitochondrial ribosomes. Furthermore, synthesis of these proteins is presumably under the control of two distinct and different genetic systems, one mitochondrial and the other cytoplasmic, which are both involved in the formation of the mitochondrial membrane. Hence, a mechanism must exist in the cell to control both the expression of these two genetic systems as well as the synthesis of proteins at two intracellular sites such that the different enzyme complexes of the inner mitochondrial membrane are assembled in a coordinated manner.

In the present study, several parameters which regulate mitochondrial protein synthesis have been investigated:

1. Optimal conditions to measure amino acid incorporation *in vitro* in yeast mitochondria were established. The effects of glucose repression on rates of mitochondrial protein synthesis were measured both *in vivo* and *in vitro*. Likewise, the products of mitochondrial protein synthesis in glucose repressed and derepressed cells were compared.

2. A stimulation of mitochondrial protein synthesis both *in vitro* and *in vivo* was observed when products of cytoplasmic protein synthesis had accumulated. Possible mechanisms for this stimulation were explored by isolating polysomes from yeast mitochondria.

3. Ribosomes active in poly(U)-dependent polyphenylalanine synthesis were isolated from both yeast and rat liver mitochondria. The inhibitory effects of various antibiotics on protein synthesis on the isolated ribosomes were compared.

LITERATURE REVIEW

All aerobic eucaryotic cells contain in their cytoplasm mitochondria which are a major source of the cell's total energy supply. While the number of mitochondria per cell appears to be relatively constant for any given cell type, the size and shape of the organelle may vary from spherical to thread-like.

A mitochondrion has two membranes, an outer and an inner membrane, which can be separated by the use of detergents or osmotic shock (121). The outer membrane contains certain enzymes which are not present in the inner membrane such as monoamine oxidase. Furthermore, the outer membrane has a higher cholesterol and phosphatidyl inositol content than the inner membrane. The latter has inward folds called cristae in which the enzymes of the respiratory chain and those involved in ATP synthesis are located. Enclosed within the cristae of the inner membrane is the so-called matrix in which the enzymes of the tricarboxylic acid cycle, fatty acid oxidation, and most of the soluble co-factors are located.

The first indication that mitochondrial DNA existed was obtained by Chevreoment, et al. (29,30), by means of Feulgen staining and autoradiography. Four years later, the universal occurrence of mitochondrial DNA located in the matrix was established by electron microscopy visuali-

zation (89). Prior to this observation, investigators in genetic and cell differentiation had questioned both the ability of mitochondria to replicate and the presence of DNA in the organelle. Ephrussi and Hottinguer's (43) observation that a respiratory mutant in yeast showed cytoplasmic (non-Mendelian) inheritance, led them to suggest the existence of genetic material in mitochondria. In 1953, Slonimski (132), reported cytoplasmic petite mutants of yeast lacking cytochromes $\underline{aa_3}$, $\underline{b}$, and $\underline{c_1}$. Mounolou *et al.* (88), reported that these mutants had lost all of their mitochondrial DNA. Sherman and Ephrussi (126), studied the genetics and biochemistry of these mutants and demonstrated deficiences in their respiratory enzymes. Faye *et al.* (46) established that certain petites result from deletion and numerous repetitions of genes within mitochondrial DNA molecules. A complete loss of mitochondrial DNA was not observed in certain petite mutants while in others no mitochondrial DNA was present.

VanBruggen, *et al.* (147), demonstrated that mitochondrial DNA of animal cells was present in covalently closed circular duplex molecules similar to those observed in bacteria. However, the molecular size of mitochondrial DNA was shown to vary among different species. Mitochondrial DNA of animal cells is about 5 μ in length while that of lower eucaryotes is about 25 μ. The reader is referred to Borst (20), for further details.

Mitochondrial DNA has been distinguished from nuclear DNA by differences in the kinetics of thermal denaturation and renaturation by DuBuy, et al. (40), who observed that mitochondrial DNA from mouse brain renatured more rapidly than the corresponding nuclear DNA. Mitochondrial DNA was also distinguished by its circularity (19), and by its unique buoyant density (129). The mechanism of replication of mitochondrial DNA during cell division in Neurospora crassa has been studied in detail by Reich and Luck (108). There is good evidence that mitochondria are able to synthesize DNA and that mitochondrial DNA is stable from generation to generation, indicating at least some autonomy of the genetic system. Wintersberger (153), and Halder et al. (55), reported that DNA synthesis occurred in isolated mitochondria.

Nass (90), observed mouse fibroblasts to contain two to six rings of homogeneous DNA molecules per mitochondrion. Clayton, et al. (32) also observed more than two DNA molecules per mitochondrion. Borst, et al. suggested (21) that the presence of several molecules of DNA per mitochondrion may be due to doubling of DNA occurring before mitochondrial division or budding. In the chloroplast, another partly self-governing cell organelle, there are approximately 20 copies of chloroplast DNA molecules per organelle (151).

The Functions Performed by Mitochondrial DNA:

(1) Transcription of Mitochondrial DNA to RNA: Both biochemical and genetic studies have indicated that mito-

chondrial DNA cannot code for the synthesis of all mitochondrial proteins. The molecular weight of mitochondrial DNA, 10-11 x 10^6, corresponds to about 15,000 base pairs and therefore encodes for 5,000 amino acids. Sinclair and Stevens (129) calculated that this would be equivalent to the maximum weight of protein (about 600,000 daltons) that can be encoded for by one molecule of mitochondrial DNA. Lehninger (74) calculated that the maximum particle weight of the respiratory assembly including the coupling factors was about 2,000,000 daltons. However, these values do not include many important enzymes such as those involved in RNA or DNA synthesis and the Krebs cycle. Therefore, let us examine the functions of mitochondrial DNA as a template molecule for RNA molecules to direct protein synthesis.

Roodyn, et al. (111) demonstrated that even after five washings a significant amount of RNA is maintained in the mitochondrial fraction. Wintersberger (154) isolated three species of RNA from yeast mitochondria with buoyant densities of 23S, 16S, and 4S. Barnett, et al. (10) demonstrated the presence of tRNA's for eighteen different amino acids in purified Neurospora crassa mitochondria. Kalf (64), as well as Neubert and Helg (92), showed that [C^{14}]UTP can be incorporated in vitro into mitochondrial RNA. This incorporation was inhibited by Actinomycin D in swollen mitochondria. These studies strongly suggested that the incorporation of added nucleotide into RNA by isolated mitochondria was due to the presence of a DNA-dependent RNA polymerase. Küntzel

and Schafer (68) reported the isolation of RNA polymerase from Neurospora. Incorporation studies in vitro indicated that the enzyme resembles the bacteria enzyme in its sensitivity to rifampicin and in its resistance to α-amanitin but differs from the RNA polymerases which have been characterized in nuclei of eucaryotic cells. The major products of mitochondrial RNA polymerase in isolated mitochondria were shown to be ribosomal RNA and tRNA's. In addition, the presence of heterogeneous RNA's suggest that mRNA's of mitochondrial origin may exist (47,48,38).

The question then arose whether these mitochondrial RNA's are transcribed from mitochondrial DNA or imported into the mitochondria from the cytoplasm. The answer to this question was provided by hybridization experiments of labeled rRNA's and tRNA's with mitochondrial DNA. In addition, mitochondria contain tRNA species that differ from the corresponding tRNA's found in the cell sap. The most important difference is the presence in mitochondria of a tRNA for formyl methionine not present in the cell sap (56). At least 14 additional tRNA's which are present in Neurospora mitochondria do not occur in the cell sap (45). That these differences are not merely due to secondary alterations of cell sap tRNA imported into mitochondria, has been amply demonstrated by hybridization studies showing at least 4 mitochondrial tRNA's from rat (91) and at least 14 mitochondrial tRNA from yeast have been shown to hybridized with mitochondrial DNA (106).

Base composition differences permit the separation of mitochondrial DNA into light and heavy strands. The work of Attardi et al. with HeLa cells (4), and that of Borst and Aaij (22) with rat liver mitochondrial showed that mitochondrial RNA hybridizes efficiently with heavy strand DNA, whereas less than 1% of the input counts are bound to light strand DNA. Dawid (36) confirmed these results in Xenopus laevis mitochondria, where 97% of the isolated mitochondrial RNA hybridized with the heavy strand. Saturation experiments at a high RNA/DNA ratio showed a maximum of 1.5% hybridization with the light strand as compared to 100% with the heavy strand. However, with a uridine pulse of very short time, the ratio of radioactive RNA which hybridized to the light and heavy strands was that expected for equal transcription of both strands. It is difficult to conceive that both an RNA molecule of one sequence and RNA molecules of the complementary sequence would be functional. It is instead necessary to assume that many of these mitochondrial RNA molecules are not involved in translation.

(2) Translation of Mitochondrial RNA: Mitochondrial DNA carries the genetic information to synthesize a limited number of mitochondrial proteins. Simpson and McLean (128), and McLean et al. (84), reported that mitochondria could incorporate radioactive amino acids into skeletal muscle mitochondria in vivo and in vitro. Roodyn (114) and Kroon (67), also reported amino acid incorporation in isdated mitochondria.

Their data were confirmed in rat liver mitochondria by Beattie, et al. (11). In a subsequent study, Beattie et al. (12), excluded the possibility that contaminating bacteria were responsible for this protein synthesizing activity. Certain conditions were described as necessary to retain amino acid incorporating activity by isolated mitochondria. For example, mitochondria must be intact and capable of a good rate of oxidation phosphorylation (13,157). A recent study in our laboratory (17), outlined the optimal conditions for amino acid incorporation by rat liver mitochondria. In spite of that, some doubts remained that mitochondria can synthesize protein. Hochberg et al., (59), contended that mitochondrial protein synthesis is an artifact. However, studies in our laboratory (61), showed that only freshly prepared intact mitochondria incorporate radioactive amino acids into protein, and that boiled or acid precipitated mitochondria do not incorporate amino acid into protein.

Since the discovery of mitochondrial protein synthesis, extensive research has been devoted to demonstrate the presence of mitochondrial ribosomes. André and Marinozzi (1), demonstrated the presence of ribosomal-like particles in rat liver mitochondria by electronmicroscopy. Ribosomes were first isolated from Neurospora and shown to consist of 73S particles with subunits of 55S and 35S, (69,109). Later studies demonstrated that yeast mitochondrial ribosomes also sediment at 72S to 75S (133,118,119,52).

O'Brien and Kalf (93,94), succeeded in isolating rat liver mitochondrial ribosomes which appeared to have a sedimentation coefficient of 55S, and hence called mini-ribosomes. The data reported by O'Brien et al. caused some confusion due to the low sedimentation value of 55S. Their work was subsequently confirmed by Rabbitts and Work (105) who reported a 55S ribosomal particle in chick mitochondria. Similarly, Swanson and Dawid (134) described a 60S ribosomal particle with subunits of 43S and 32S in mitochondria isolated from Xenopus laevis. The isolation of ribosomes sedimenting at 60S from HeLa cell mitochondria was reported by Attardi and Ojala (5). The term miniribosome is misleading as Sacchi et al. (117), and DeVries (37), have recently reported that the 55S ribosomes contain about twice as much protein as RNA and have a molecular weight about equal to that of bacterial ribosomes. Therefore, the 55S ribosome is not a "miniribosome".

The most conclusive proof that the rat liver mitochondria ribonucleoprotein particles sedimenting at 55S are the actual mitochondrial ribosomes came from Beattie's group in 1973 (Section 4 in this thesis). The isolated rat liver mitochondrial ribosomes were active in poly(U) dependent phenylalanine synthesis which was inhibited by chloramphenicol and erythromycin. Saccone's group (53), independently reported the same result.

Composition and Nature of Proteins Synthesized by Mitochondria:

Approximately 85-90% of mitochondrial protein is coded by nuclear genes, synthesized on cytoplasmic ribosomes and subsequently imported into mitochondria (16,82,139). These proteins include mitochondrial DNA polymerase, (27), RNA polymerase (9,155), mitochondrial ribosomal protein (77,35), the enzymes of the citric acid cycle and of fatty acid oxidation (152), cytochrome c (52), the mitochondrial F_1-ATPase (65, 140), and the oligomycin sensitivity conferring factor which is associated with ATPase (141), the mitochondrial soluble proteins (12), the mitochondrial polypeptide elongation factors (123), the four small subunits of cytochrome oxidase (120), and cytochrome c_1 (115).

The hypothesis that synthesis of mitochondria largely depends on events occurring extramitochondrially was confirmed by the observations of Perlman and Mahler (98) who demonstrated that a mitochondrial-like structure can be synthesized in yeast in the absence of either functional mitochondria DNA or the mitochondrial system for protein synthesis. This structure contains an outer membrane, and an abnormal inner membrane. Petite mitochondria lacking mitochondrial DNA also apparently possess an outer membrane and a distinct inner membrane which does not differ greatly from that observed in wild type mitochondria (145,83). These findings also suggested that mitochondrial DNA has the capacity to encode for only a limited number of proteins ex-

clusively present in the inner membrane. Labeling of inner membrane proteins *in vivo* in the presence of cycloheximide or in isolated mitochondria was demonstrated by Beattie's group in 1968 (13).

Isolated rat liver mitochondria incorporate amino acids only into the mitochondrial membrane residue after extraction with dilute acetic acid (15). Approximately 15% of the insoluble membrane protein of yeast mitochondria was labeled *in vivo* in the presence of cycloheximide (122). The values obtained for *Neurospora* ranged from 8-15% (58). Beattie, (15) and Coote and Work (34), showed that the proteins synthesized on mitochondrial ribosomes, both *in vivo* and *in vitro* belong to a class of hydrophobic protein firmly associated with the inner membrane.

Electrophoresis of these proteins in sodium dodecyl sulfate (SDS) gel electrophoresis reveals from 6-10 bands (124,72,18,110), with molecular weights ranging from about 10,000-45,000. An identical banding pattern is observed when mitochondria are labeled *in vivo* in the presence of cycloheximide (62,73) or *in vitro* (61), suggesting that the products of protein synthesis in isolated mitochondria are identical to those synthesized *in vivo*. The mitochondrially made polypeptides are very hydrophobic, and soluble only in detergents of high alkalinity or acidity (142,125). Some are extractable by organic solvent and appear to be proteolipids (24).

Different approaches have been used to demonstrate the

gene product of mitochondrial DNA. One of these approaches has been the use of selective inhibitors (15). The development of techniques for the isolation of mitochondrial enzyme complexes as combined with the use of these inhibitors has increased our knowledge of the products of mitochondrial protein synthesis. The second approach has been a genetic one (144,97). Results obtained by genetic techniques confirmed those obtained biochemically as to the number of proteins synthesized within mitochondria and the relationship of the two systems for assembly of the enzyme complexes of the mitochondrial inner membrane.

(1) Cytochrome Oxidase Complex: In early studies several groups (31,28,116) showed that the assembly of a functional cytochrome oxidase in yeast requires polypeptides synthesized on both mitochondrial and cytoplasmic ribosomes. The same phenomenon was observed in animal tissue by Kadenbach (63). Purified cytochrome oxidase has been shown to contain seven polypeptides. The heaviest peptides (those with molecular weights of 42,34, and 23,000 daltons) are mitochondrially synthesized, while the four subunits of lower molecular weight are cytoplasmically synthesized (142,104).

(2) Oligomycin Sensitive ATPase Complex: The complex enzyme which catalyzes the terminal phosphorylation step in the respiratory chain consists of the F_1-ATPase, and OSCP which can be synthesized in the absence of a functional mitochondrial system plus a "membrane factor" containing four

polypeptides which are mitochondrially synthesized (143).

(3) Cytochrome b: The biosynthesis of cytochrome b on mitochondrial ribosomes has been demonstrated in Neurospora crassa (150). The purified cytochrome has a molecular weight of about 30,000, while that purified from yeast has a molecular weight of about 40,000 (75).

Sensitivity of Mitochondrial Protein Synthesis to Various Inhibitors:

The similarity between mitochondrial and bacterial protein synthesis in contrast to cytoplasmic protein synthesis has been discussed (16). However, certain differences between the mitochondrial and bacterial systems emerge in the subunit exchange (73), in the stimulatory effects of NH_4Cl in a cell-free system (52) and in response to fusidic acid (50). The size difference is, of course, well known. These experiments indicate that mitochondrial protein synthesis differs sufficiently from both bacterial and cytoplasmic protein synthesis to put it in a separate class.

(1) Sensitivity of Protein Synthesis at the Translational Level: It has been well-documented that D(-)threo-isomers of chloroamphenicol inhibit protein synthesis in bacteria and also in isolated mitochondria (12). In bacteria, it has been shown that the drug binds exclusively to the 50S subunit of the monosomes and inhibits peptidyl transferase (148). The macrolides are another group of antibiotics which inhibit protein synthesis both in vivo and in vitro in bacteria (149). The macrolides consist of a large lactone ring, which

is substituted with one or more sugar residues (25). Erythromycin binds to the 50S ribosomal subunits of bacteria and prevents peptide bond formation by preventing binding of tRNA's carrying large peptide moeities. Erythromycin competes with chloramphenicol for binding sites on 50S ribosomes and also with lincomycin. However, in reciprocal experiments binding of erythromycin was not inhibited by chloramphenicol (96). These observations suggest that a partial overlap may exist in the binding sites for these different durgs. Carbomycin is a strong inhibitor of peptide bond formation and causes extensive breakdown of polyribosomes (42). Lincomycin is a non-macrolide antibiotic that acts in a similar fashion as erythromycin (71).

Towers et al., (136,137), studied the effects of the antibiotics erythromycin and lincomycin on rat liver and yeast mitochondria. They concluded that the inability of erythromycin to inhibit protein synthesis in rat liver mitochondria while inhibiting protein synthesis in yeast mitochondria may result because of phylogenetic differences between liver and yeast mitochondrial ribosomes. However, our data has suggested that the difference in inhibitory action results from an impermeability of the membranes of rat liver mitochondria to erythromycin. The details of this argument are presented in section four of this manuscript.

Emetine is a specific inhibitor of cytoplasmic protein synthesis without influence on mitochondrial protein synthesis.

However, emetine at higher concentrations inhibits amino acid incorporation by rat liver mitochondria. Emetine inhibits elongation, rather than initiation of peptide chain in reticulocyte extracts (78). Cycloheximide, an antibiotic inhibiting eucaryotic ribosome specifically (127), did not inhibit either protein synthesis in intact mitochondria or in mitochondrial extracts (12). Similarly, protein synthesis in chloroplasts is insensitive to cycloheximide (60). Lodish, et al. (78), concluded that since cycloheximide does not, at any concentration, function as a selective inhibitor of initiation, it is therefore better regarded as an inhibitor of peptide chain elongation. Puromycin, is a structural analogue of aminoacyl adenosine, the 3' terminus of amino-acyl tRNA (87), and causes release of immature nascent peptide chains from ribosomal bound tRNA. Because of the marked similarities between the puromycin reaction and the ribosomal peptide bond-forming reaction, this reaction is used for studying the synthesis of individual peptide bonds, especially in measuring the rate of initiation. These antibiotics are powerful tools for identifying the biosynthetic origin of mitochondrial protein and assembly process and have been used extensively.

(2) Sensitivity of Protein Synthesis to Inhibitors of Transcription: Actinomycin D is a widely used antibiotic which binds to DNA so as to selectively inhibit the capacity of the DNA to serve as a template for RNA synthesis (107). Kroon (66)

utilized Actinomycin D to show that mitochondrial protein synthesis is dependent on mitochondrial transcription. Ethidium bromide is a phenanthridine trypanocide which inhibits DNA and RNA synthesis (138). Ethidium bromide has the advantage of selectively inhibiting mitochondrial RNA synthesis without affecting nuclear RNA synthesis (158). The drug induces the conversion of yeast cells to respiration deficient p^- mutant (113,132). The effects of ethidium bromide are virtually instantaneous and may be ascribed to the short lifespan of mitochondria mRNA (54). In addition, mitochondrial DNA is rapidly degraded after treatment with the drug (99).

Rifampicin is a semi-synthetic derivative of the naturally occuring rifamycin B. Rifampicin selectively blocks bacterial RNA polymerase (57). The drug has been found to inhibit both the RNA polymerase of mitochondria (49), and that of chloroplasts (2). Intact mitochondria appear to be impermeable to rifampicin, suggesting that they are probably unaffected by the antibiotic *in vivo*. Studies utilizing the inhibitors of transcription:rifampicin, ethidium bromide, and Actinomycin D have been interpreted to suggest the existence of mRNA synthesized within mitochondria.

Search for the Origin of Mitochondria mRNA:

The origin of mitochondria mRNA has long been the subject of debate. Swanson's (135), experiment demonstrated the uptake of poly(U) by isolated mitochondria and its sub-

sequent translation. This experiment indicated that the poly(U) must have been transported across the mitochondrial membrane. Furthermore, the translation of exogenous poly(U) did not affect the incorporation of other amino acids into protein. Swanson concluded from this experiment that mitochondria had the capability to transport extraneous mRNA across the mitochondrial membrane where it was translated on mitochondrial ribosomes. This experiment, however, does not prove that all mitochondrial mRNA's are imported from the cytoplasm as no uptake of rRNA's, tRNA's was observed. These have a high degree of secondary structure which would be expected for natural mRNA. Dawid (36) suggested that the main function of the mitochondrial system for protein synthesis was the translation of imported mRNA since the mitochondrial DNA of animal cells has a genetic information content probably not exceeding 15,000 base pairs.

Dubin (39) demonstrated the possible existence of mRNA by isolating rapidly labeled heterogeneous RNA from rat liver mitochondria. Küntzel and Blossey (70) were able to transcribe mitochondrial DNA *in vitro* using an RNA polymerase from *E. coli*. The resulting mRNA was translated into two polypeptides with apparent molecular weights of 10,000 and 12,000 daltons. These data suggest that in *Neurospora crassa* the information for these protein(s) is coded on mitochondrial DNA and synthesized by endogenous mitochondrial polyribosomes. Support for the idea that mitochondrial DNA has the capacity to transcribe mitochondria mRNA is based on the following:

(1) The number of different proteins identified as products of mitochondrial protein synthesis could be coded by mitochondrial DNA. Information for these proteins plus the genes for mitochondria rRNA and tRNA's does not exceed the coding capacity of mitochondrial DNA; (2) Ethidium bromide blocks the increase in cytochrome oxidase activity in yeast undergoing glucose derepression (99). This result would not be anticipated if the mRNA for these proteins were synthesized in the nucleus; (3) Cells have an efficient mechanism to transport mitochondrial proteins synthesized in the cell sap through the mitochondrial membrane. All of these experiments suggest that it is unlikely that mitochondrial mRNA was previously synthesized in the nucleus. However, the data should be interpreted with caution. Evidence in part three of this thesis suggests that mRNA imported into mitochondria is quantitatively unimportant, if it occurs at all, in yeast. The only means to prove that mitochondrial mRNA is transcribed from mitochondria DNA is by isolating mitochondrial polysomes and demonstrating that the mRNA present in these studies hybridizes to mitochondrial DNA.

Experimentally, isolation and characterization of mitochondrial polysomes has proven to be a formidable task (81,33, 101). These structures carry nascent polypeptide chains and are dissociated into monosomes by relatively mild treatment with ribonuclease. On the other hand, Ojala et al., (95), isolated HeLa mitochondrial polysome-like structures which were sensitive to ribonuclease only after treatment with

pronase. Michel and Neupert (85,86), described polymeric ribosomes from Neurospora crassa mitochondria which were resistant to ribonuclease. They suggested that these structures were merely aggregates which were stabilized by the interaction of the hydrophobic nascent chains rather then by mRNA; however, they did not demonstrate the disaggregation of the polysome-like structures by proteolytic digestion. It is obvious from the above data that the isolation of large quantities of polysomes has not been achieved.

Isolating a unique species of mRNA by specific immunoprecipitation of polysomes with antibodies to ATPase and cytochrome b is under study by Beattie's group. Such techniques have been successfully used by Schimke's group. An alternative approach for isolating mitochondrial RNA involves the specific binding of stretches of poly(A). Perlman et al., (100), described a heterogeneous RNA fraction, in HeLa cells containing poly(A). Synthesis of this RNA was inhibited by ethidium bromide, but not by camptothecin, a specific inhibitor of RNA synthesis in the nucleus. Attardi et al.. (6) also independently reported the isolation of an heterogeneous RNA, containing covalently bound poly(A) from HeLa cell mitochondria. Recently, however, Groot, et al. (54b.) reported that yeast mitochondrial mRNA does not contain poly(A). This finding may have been due to the effect of various ribonucleases on their preparation and should be confirmed by other groups.

The Interrelationship Between the Mitochondrial and Nuclear Genetic System:

Biochemical and genetic approaches have advanced our understanding of the relationship between the mitochondrial and nuclear genetic systems. There is no doubt that these two closely coordinated, though distinct, systems influence each other through their protein products. Clark-Walker and Linnane (31) demonstrated that aerobic adaptation and appearance of cytochrome oxidase, cytochromes aa_3, b, and c_1 can be prevented by chloramphenicol. They concluded the necessity of the mitochondrial protein synthetic system for the information of three major enzymes in the inner membrane. Chen and Charalampous (28) have reported that the induction of functional cytochrome oxidase in yeast undergoing the anaerobic to aerobic transition appears to involve protein synthesis by both the cytoplasmic and the mitochondrial systems.

Proteins synthesized in the cytoplasm might require a component synthesized in the mitochondria for proper assembly into a functional enzyme complex of the inner membrane. Inter-system co-ordination between mitochondria and cytoplasm required for the assembly of mitochondria has also been reported by Tzagoloff (141) and Lin et al. (76). When yeast cells were grown in chloramphenicol for 3 hours followed by an hour or more in cycloheximide, large increases in the activity of oligomycin-sensitive ATPase and coenzyme QH_2-cytochrome c reductase were observed. These data suggest

that proteins made in the cytoplasm when mitochondrial protein synthesis is blocked by chloramphenicol proteins may accumulate and act to stimulate the synthesis of mitochondrial proteins.

Investigators have begun to probe the regulation of the interaction between the mitochondrial and nuclear systems. Barath and Küntzel (8) attempted to follow the selective inhibitors approach in investigating the regulation of mitochondrial protein synthesis in Neurospora crassa. They incubated cells with chloramphenicol for fourteen hours or ethidium bromide for ten hours. They found that after washing out the inhibitors there was a 2-fold increase in mitochondrial ribosomal protein and in methionyl-RNA-transformylase. Since these proteins are specified by nuclear genes, these data might indicate control of the nuclear gene by "repressor-like" mitochondrial products which normally act to inhibit specific nuclear genes. However, it is difficult to determine whether the effect of prolonged treatment with ethidium bromide resulted in a drop in total cellular ATP levels which, in turn, may stimulate synthesis of specific protein in the cytoplasm. Ethidium bromide may induce decreased transcription of mitochondrial mRNA from mitochondria DNA or transcription of mitochondrial rRNA. Whatever the effect of ethidium bromide, a relationship does exist between the mitochondria and cytoplasm.

Recently, Poyton and Groot (102,103), reported that in anaerobic yeast cells cytochrome oxidase subunits, which are made on the cytoplasmic ribosomes, will be assembled into the inner membrane after the mitochondrial subunits are made.

Their assembly has been found to require oxygen. That is, the initial response to oxygen was made by the mitochondria and hence oxygen is regulating the assembly of cytochrome oxidase at the mitochondrial translational level. Whatever the initial response to oxygen, their data also suggested that co-operation must exist between the mitochondrial and cytoplasmic products to form functional cytochrome oxidase.

Ebner et al.,(41), have isolated a number of respiratory deficient mutants of Saccharomyces cerevisiae. These mutants resulted from a single nuclear gene which effects the synthesis of mitochondrial protein. These data suggest that proteins coded in the nucleus are necessary for the synthesis of one mitochondrially-made subunit of cytochrome oxidase. How can a nuclear gene mutation cause the loss of one polypeptide made on mitochondrial ribosomes unless there is some close co-ordination between the two systems. Ono et al. (97), chracterized different nuclear mutants, one of which is P 494-1. The data show clearly that the alteration or loss of nuclearly coded cytoplasmically made protein results in the loss of protein(s) made by the mitochondria. Therefore, the P 494-1 may be a regulatory mutant.

We reported in our laboratory, as have others such as Sebald (125) and Tzagoloff et al. (142), that the accumulation of cytoplasmic protein in certain conditions stimulates mitochondrial protein synthesis. Current studies in this manuscript show that the stimulation of mitochondrial protein by the cytoplasmic product can be observed on isolated mitochondrial polysomes.

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II. FORMATION OF THE YEAST MITOCHONDRIAL MEMBRANE: EFFECTS OF GLUCOSE REPRESSION ON MITOCHONDRIAL PROTEIN SYNTHESIS

LITERATURE REVIEW

An asynchronous process for the assembly of the mitochondrial membrane was suggested (1). The activities of the various enzymes and enzyme complexes of the respiratory chain as well as oligomycin-sensitive ATPase appeared in the mitochondrial membrane at different times during the growth cycle. One explanation for these kinetics, as well as the long lag times observed prior to increase in some enzymic activities during growth, might be that glucose represses mitochondrial protein synthesis. Such repression might result from an effect on the rate of mitochondrial protein synthesis, as measured by amino acid incorporation _in vitro_, and/or on the specific products of mitochondrial protein synthesis, as measured by pulse-labeling _in vivo_ in the presence of cycloheximide and analysis by sodium dodecylsulfate gel electrophoresis.

This suggestion was made attractive by the reports that three different enzyme complexes of the mitochondrial membrane, oligomycin-sensitive ATPase (2), cytochrome oxidase (3), and a membrane protein enriched in cytochrome _b_ (4), contain proteins synthesized on the chloramphenicol-sensitive mitochondrial ribosomes. Hence, the different time sequence in which these three enzyme complexes appeared in the mitochondrial membrane during glucose derepression might result because of a differential effect of glucose on the intramitochondrial synthesis of one or more of their

component proteins.

The results obtained suggested that amino acid incorporation *in vitro* supported either by an external ATP-regenerating system or by the respiratory-chain-linked synthesis of ATP proceeds at an identical rate in mitochondria obtained from both repressed and derepressed yeast cells. In contrast, the extent of labelling *in vivo* was greatly decreased in mitochondria obtained from glucose repressed cells. Analysis of the products of mitochondrial protein synthesis by sodium dodecylsulfate gel electrophoresis indicated that glucose repression had almost no qualitative effect on the bands labeled in the presence of cycloheximide. However, certain significant quantitative differences in the labeling of the various bands were observed as the cells underwent glucose derepression.

MATERIAL AND METHODS

Growth of Yeast and Preparation of Mitochondria:

A diploid strain of *Saccharomyces cerevisiae* was grown with either 1% glucose or 5% glucose as an energy source in the medium previously described by Kim and Beattie (1). The cells were harvested by centrifugation for 10 min at 1000 x g, washed once with water and once with 0.25 M mannitol, 0.01 M Tris, pH 7.4, and 0.001 M EDTA and then broken in a Bronwill mechanical shaker at 4000 rev./min for 20 s. The combined supernatants, after washing the beads, were

centrifuged at 600 x g for 10 min three times to remove unbroken cells and nuclei. The supernatant was then centrifuged at 17,000 x g for 10 min and the pellet containing mitochondria washed three times with mannitol-Tris-EDTA medium and recentrifuged for 10 min at 17,000 x g. The final mitochondrial pellet was resuspended in mannitol-Tris-EDTA to a final concentration of 6-8 mg/ml protein. The above steps were performed under strictly sterile conditions; all glassware and solutions were autoclaved prior to use.

Amino-Acid-Incorporation Studies:

Mitochondria were incubated in a medium containing 90 mM KCl, 5.0 M phosphate pH 7.6, 50 mM bicine buffer (N-tris(hydroxymethyl)methylglycine), pH 7.6, 10 mM $MgCl_2$ 1 mM EDTA, 22.5 µg of a complete amino acid mixture minus leucine as described by Roodyn et al. (5), 1.0 µCi per ml of uniformly labeled L-[^{14}C]leucine, 2.0 mM ATP and 2.0 mg mitochondrial protein in a final volume of 2.0 ml. Phosphoenolpyruvate, pyruvate kinase or respiratory substrates were added in the concentrations indicated in the legends to the tables. After 15 min at 30°C in a metabolic shaker, the incubation was terminated by the addition of 10 mM unlabeled leucine followed by precipitation with 5% trichloroacetic acid. The labeled proteins were prepared for counting (6) and counted in a scintillation counter with an efficiency for ^{14}C of 90%. Protein concentration was determined by the method of Lowry et al. (7).

Labeling in vivo:

Yeast cells were harvested as described above by centrifugation at 1000 x g and washed once with water. The cells were suspended to a final concentration of 250 mg/ml in 0.05 M phosphate buffer, pH 7.4, containing 0.1% glucose. Cycloheximide (100 µg/ml) was added and the culture incubated for 15 min prior to addition of µCi per ml [^{3}H]leucine. After incubation for 15 or 30 min at 30°C, 10 mM unlabeled L-leucine was added and the incubation continued for 15 min. The cells were then harvested and mitochondria prepared as described above.

A similar procedure was used to label cells for gel electrophoresis except that the concentration of [^{3}H]leucine was increased to 75 µCi per ml and the cells incubated for 30 min prior to the addition of unlabeled leucine.

Gel Electrophoresis:

Mitochondria were suspended in mannitol-Tris-EDTA at a concentration of 10 mg/ml and sonicated for 15 s at maximum output in a Branson sonifier. The membranes were isolated by centrifugation at 150,000 x g for 30 min. The pellets were extracted with five volumes of 5% trichloroacetic acid and the precipitated proteins dissolved in a solvent containing 10% glycerol (w/v), 1% sodium dodecylsulfate, 1% mercaptoethanol, 0.01 M phosphate buffer, pH 7.0, and 0.002% bromphenol blue at a concentration of 1.0 mg per ml. The protein solution was heated at 70°C for 20 min (2) and

gel electrophoresis was carried out on 10% polyacrylamide gels 9-cm long at room temperature with a constant current of 3.0 mA per gel. The gels were sliced into 1.0-mm sections with a Gilson Aliquogel fractionator and dissolved by heating with 0.5 ml of 30% hydrogen peroxide at 50°C for 24 h. 5 ml toluene containing Triton X-100 (8) were then added and the radioactivity determined in a Packard scintillation counter with an efficiency of 27% for ^{3}H. Over 95% of the counts put on each gel were recovered in the slices.

Gels were calibrated with cytochrome c, α-chymotrypsinogen, ovalbumin and bovine serum albumin (9).

Materials:

Nucleotides, oligomycin, antimycin A, atractyloside, rifamycin, cordycepin, chloramphenicol, erythromycin and cycloheximide were obtained from Sigma; actinomycin D from Merck, Sharpe and Dohme; and uniformly labeled L-[^{14}C]leucine (300 mCi/mmol) and L-[4,5-^{3}H]leucine (50 Ci/mmol) from Amersham Searle.

RESULTS

The medium described by Lamb et al. (10) was used in our initial attempts to study amino acid incorporation by mitochondria from derepressed yeast cells which had been grown for 22-24 h in 5% glucose. The very low incorporation rates obtained prompted us to try our medium developed for

amino acid incorporation with rat liver mitochondria (11). The incorporation rates observed with yeast mitochondria under these conditions were comparable to those obtained with liver mitochondria, although somewhat lower. Addition of 2.0 mM ATP resulted in maximal rates of incoporation in the presence of an ATP-regenerating system (Table 1). Exogenous phosphate was not essential for incorporation under these conditions. The substitution of NaCl for KCl in the medium resulted in a 50% decrease in the incorporation rate; however, substitution of NH_4Cl for KCl had no effect. No significant difference in the incorporation rate was observed when mitochondria were prepared in a medium containing either sucrose or sorbitol in place of mannitol. However, when the incorporation was supported by ATP and succinate, higher rates were obtained with mitochondria prepared in mannitol or sorbitol compared to those prepared in sucrose (Table 1).

The addition of the respiratory substrates, succinate, glutamate or ethanol to the ATP-regenerating system increased the incorporation rate 1.5 to 2-fold (Table 2). Furthermore, the presence of succinate and ATP alone resulted in an incorporation rate equal to that observed when succinate had been added to the ATP-regenerating system. Exogenous phosphate was necessary under these conditions.

Amino acid incorporation supported by the ATP-regenerating system was inhibited 15-25% by addition of antimycin A, atractyloside or oligomycin. When the ATP-regenerating sys-

TABLE 1

Optimal Conditions for Amino Acid Incorporation
By Isolated Yeast Mitochondria

CONDITIONS	Radioactivity incorporated in	
	ATP-regenerating	ATP-succinate
	counts x min^{-1} x mg^{-1}	
Control (2.0 mM ATP)	783	1550
-ATP	100	-
+0.2 mM ATP	320	-
+5.0 mM ATP	314	-
-Phosphate	755	-
+10 mM Phosphate	711	-
+NH_4Cl	780	-
+NaCl	390	-
Prepared in sorbitol	676	1470
Prepared in sucrose	737	1060

Yeast mitochondria were prepared in mannitol-Tris-EDTA medium as described in Methods. Amino acid incorporation was measured in a medium containing 90 mM KCl, 50 mM bicine, pH 7.6, 10 mM $MgCl_2$, 1 mM EDTA, 22.5 µg of an amino acid mixture, 5.0 mM phosphate, pH 7.6, 2.0 mM ATP, 1.0 µCi of [^{14}C]leucine and 2.0 mg mitochondrial protein in a final volume of 2.0 ml. The ATP-regenerating system also contained 5.0 mM phosphoenolpyruvate and 2.0 µg pyruvate kinase, while the ATP-succinate system contained 10.0 mM succinate. For the experiments with NH_4Cl and NaCl the KCl in the medium was replaced with either 90 mM NH_4Cl or 90 mM NaCl. For the experiments in which the mitochondria were prepared in sorbital or sucrose, mannitol was replaced with either sucrose or sorbital in the preparation medium.

TABLE 2

Effect of Inhibitors of Respiration and Oxidative Phosphorylation on Amino Acid Incorporation

Conditions	Radioactivity Incorporated	
	counts x min^{-1} x mg^{-1}	% control
ATP, phosphoenolpyruvate, pyruvate kinase	1070	100
+Succinate	2200	205
+Glutamate	1800	168
+Ethanol	1500	140
+Oligomycin	800	75
+Atractyloside	900	84
+Antimycin A	790	74
-Pyruvate kinase	315	30
ATP, succinate	2300	-
ATP, succinate, phosphoenolpyruvate, pyruvate kinase	2200	100
+Oligomycin	850	39
+Antimycin A	450	20
+Atractyloside	2300	104

Amino acid incorporation was assayed as described in the legend to Table 1 with the following additions where indicated: 2.0 mM ATP, 5.0 mM phosphoenolpyruvate, 2 µg/ml pyruvate kinase, 10 mM succinate, 10 mM glutamate, 50 mM ethanol, 10 µg/ml oligomycin, 50 µM atractyloside, 5 µg/ml antimycin A.

tem was supplemented with succinate, antimycin A and oligomycin caused inhibitions of 80 and 61%, respectively, while atractyloside had no effect.

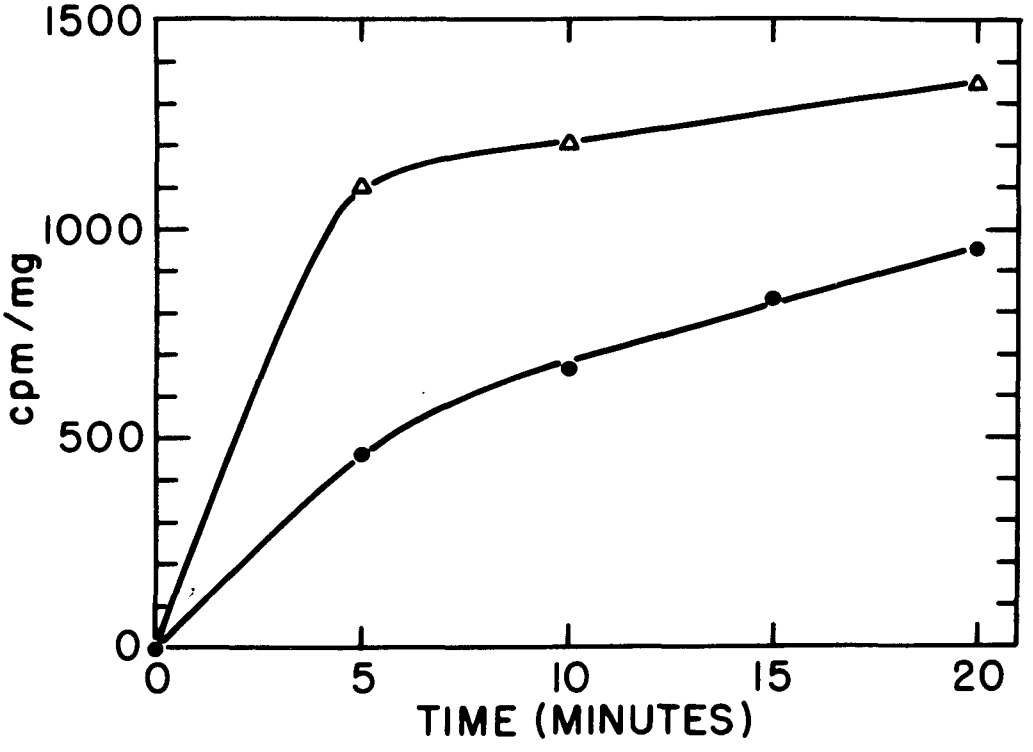
The incorporation increased throughout a 20-min incubation in the ATP-regenerating system (Fig. 1). In contrast, when either succinate or ethanol was added, the incorporation rate increased very rapidly in the first 5 min and then more slowly over the next 15 min. In all subsequent experiments, the time of incubation was 15 min.

The specific inhibitors of mitochondrial protein synthesis (12) chloramphenicol and erythromycin, inhibited amino acid incorporation by isolated yeast mitochondria, while cycloheximide, a specific inhibitor of protein synthesis on cytoplasmic ribosomes (12), had no effect (Table 3). The inhibitors of RNA synthesis, cordycepin (13) and actinomycin D caused a 16 to 19% inhibition of the incorporation rate, while rifamycin even at a concentration of 5 µg/ml had no significant effect. In contrast, addition of 0.1-0.5 µg/ml ethidium bromide to the incubation mixture caused a 70-95% decrease in the incorporation rate.

The products of mitochondrial protein synthesis <u>in vitro</u> were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis. Mitochondria (1 mg/ml) were incubated for 10 min with 25 µCi/ml of [^{3}H]leucine. Unlabeled L-leucine (10 mM) was added and the incubation continued for 20 min. The mitochondria were then reisolated by centrifugation at 12,000 x g for 10 min, washed once in mannitol-Tris-EDTA

FIG. 1. Time course of amino acid incorporation into isolated yeast mitochondria. Amino acid incorporation was assayed as described in the legend to Table 1 either in the presence of an ATP-regenerating system (●——●) or ATP-succinate (Δ——Δ). A zero time value of 45 counts/min was subtracted from each point.

FIG. 1



-A 43‡

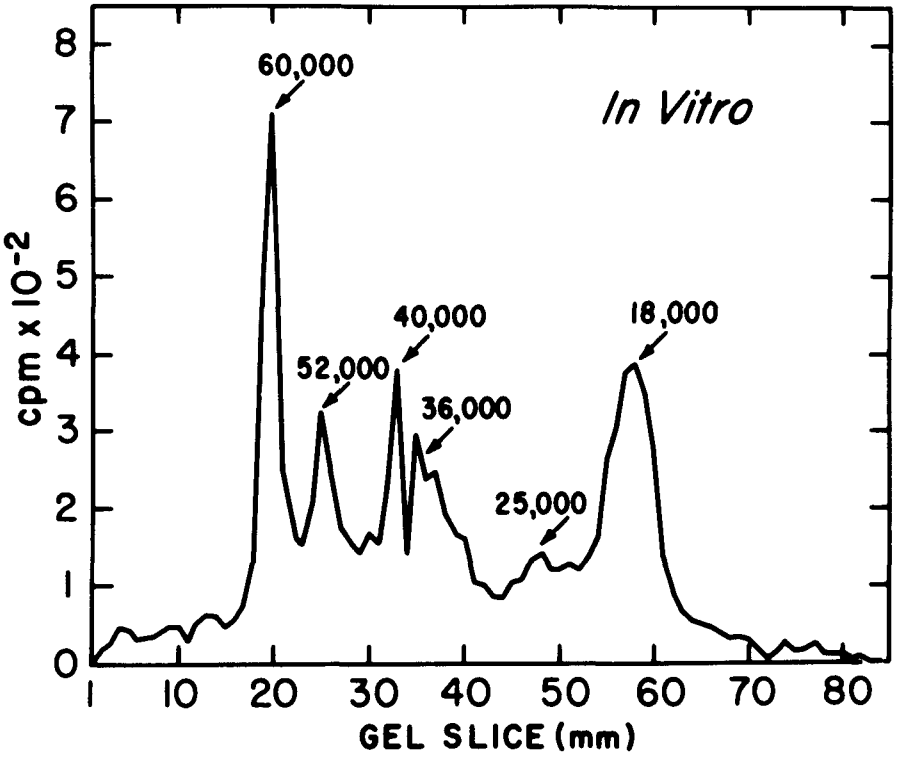
TABLE 3

Effect of Inhibitors of RNA and Protein Synthesis on Amino Acid Incorporation by Isolated Yeast Mitochondria

Conditions	Concentration of Inhibitor	Radioactivity Incorporated	Inhibition
	μM	counts x min^{-1} x mg^{-1}	%
Control		1660	
+Ethidium bromide	0.25	493	70
	1.3	71	96
	2.5	50	97
+Cordycepin	2.0	1630	2
	8.0	1530	8
	40	1340	19
+Actinomycin D	0.1	1640	1
	1.0	1400	16
+Erythromycin	14	995	40
	68	83	95
	μg/ml		
+Rifamycin	1	1640	1
	5	1560	6
	mM		
+Chloramphenicol	0.15	332	80
	0.31	232	86
+Cycloheximide	3.5	1670	NO

FIG. 2. Sodium dodecylsulfate gel electrophoresis profile of mitochondrial membrane labeled in vitro. Mitochondria were prepared from yeast cells which had been grown for 16 h in 1% glucose and incubated with ATP-succinate as described in Table 1 with 25 μCi/ml [^{3}H]leucine. After 10 min of incubation, 10 mM unlabeled L-leucine was added and the incubation continued for 20 min. Submitochondrial particles were prepared and 100 μg protein were electrophoresed on a 9-cm gel for 16 h as described in the text. Specific activity of the final preparation subjected to gel electrophoresis was 108,000 counts x min^{-1} x mg^{-1}. Recovery from the gel was 95%. The molecular weights indicated for each peak in the figure were calculated according to Weber and Osborne (9).

FIG. 2


60,000
In Vitro
40,000
18,000
52,000
36,000
25,000
cpm x 10-2
0
1
2
3
4
5
6
7
8
1
10
20
30
40
50
60
70
80
GEL SLICE (mm)

medium containing 10 mM unlabeled L-leucine and submitochondrial particles prepared. After electrophoresis, the mitochondrial membranes separated into 6-7 clearly differential peaks with molecular weights ranging from 60,000 to 18,000 (Fig. 2). These labeled peaks appear similar to those observed when the mitochondrial membranes were labeled *in vivo* (Fig. 3); however, the relative heights of the peaks differ significantly when the labeling *in vitro* is compared to the labeling *in vivo*. For example after labeling *in vitro* the peak of 18,000 molecular weight is very broad and contains significantly more radioactivity when compared to the higher molecular weight peaks than does the 18,000 molecular weight peak observed after labeling *in vivo*. Furthermore, the peak of 60,000 molecular weight contains significant radioactivity on membranes labeled *in vitro*.

The possibility that the ability of mitochondria to synthesize protein is subject to glucose repression was tested by measuring the extent of amino acid incorporation in mitochondria isolated from yeast cells harvested after 9,16 and 22 h during growth in 5% glucose. As indicated by the rate of oxygen uptake and cytochrome oxidase activity (Table 4), the cells were still repressed after 9 h of growth. After 16 h of growth, the cells were at late log phase but still undergoing derepression, while after 22 h of growth, the cells were at early stationary phase and

FIG. 3. Sodium dodecylsulfate gel electrophesis profile of mitochondrial membranes obtained from yeast cells labeled *in vivo* in the presence of cycloheximide as described in the legend to Table 5, with 40 µCi/ml [^{3}H]leucine. Cells were obtained after (A) 9 h (early log phase) (B) 13 h (late log phase); or (C) 22 h (stationary phase) of growth in 5% glucose. Gel electrophoresis was performed as described in legend to Fig. 2. Specific activities of final membrane preparation was 65,000 counts x min^{-1} x mg^{-1} for the 9-h cells, 68,000 counts x min^{-1} x mg^{-1} for the 13-h grown cells and 250,000 counts x min^{-1} x mg^{-1} for the 22-h cells. The numbers at the top of the gels refer to 10^{-3} x molecular weights for each peak.

FIG. 3

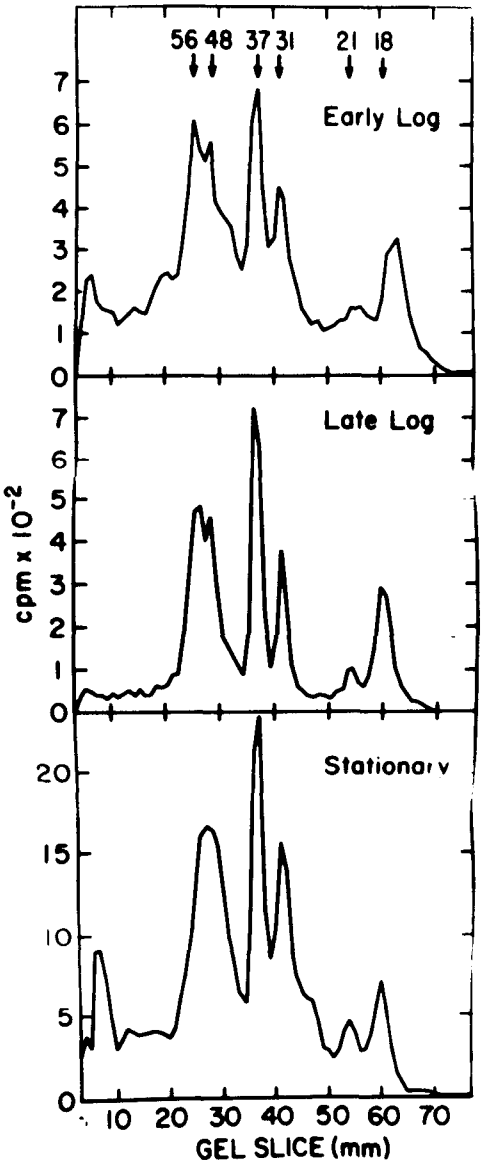

56 48 37 31 21 18
Early Log
Late Log
Stationary
cpm x 10⁻²
GEL SLICE (mm)

TABLE 4

Effect of Glucose Repression on Amino Acid Incorporation *In Vitro* and the Respiratory Chain

Time of Cell Growth	Incorporation		Oxygen uptake (NADH)	Cytochrome Oxidase
	ATP-regenerating	ATP-succinate		
h	counts x min^{-1} x mg^{-1}		nmol O x min^{-1} x mg protein^{-1}	k x min^{-1} x mg protein^{-1}
9	893	1530	25	1.7
16	764	1430	120	2.4
22	875	1350	280	6.0

Amino acid incorporation was measured as described in the legend to Table 1. Oxygen uptake was measured with a Clarke electrode in a medium containing 0.3 M mannitol, 10 mM Tris, pH 7.5, 10 mM phosphate, pH 7.5, 1.2 mM NADH and 2.0 mg mitochondrial protein. Specific activity is nmol of oxygen uptake x min^{-1} x mg mitochondrial protein^{-1}. Cytochrome oxidase was assayed in 1.0 ml containing 40 µM reduced cytochrome *c*, 10 mM phosphate, pH 7.0 and mitochondria. Specific activity is expressed as initial first-order rate constant k x min^{-1} x mg mitochondrial protein^{-1}.

almost derepressed [cf. (1)]. The rate of amino acid incorporation was nearly identical in mitochondrial preparations from all three cultures whether supported by an external ATP-regenerating system or by respiratory chain-linked phosphorylation.

The possibility exists, however, that mitochondrial protein synthesis in vivo may be lowered in yeast cells subject to glucose repression. The rate of labeling in vivo into repressed (9-h), partially derepressed (16-h) and derepressed (22-h) yeast cells was measured in the presence or absence of cycloheximide. The labeling was linear for at least 30 min under these conditions. As the yeast cells underwent derepression, the mitochondrial membranes were labeled at an increasing rate such that the label in membranes from cells after 22 h of growth was 16-fold greater than that in membranes from cells after 9 h of growth whether cycloheximide was present or not. In addition, it should be noted that the concentration of cycloheximide used in this experiment inhibited 98% incorporation into the post-mitochondrial supernatant fraction while inhibiting that into the mitochondrial membranes approximately 75% (Table 5).

Despite the increasing rate of mitochondrial protein synthesis in vivo as the yeast cells underwent derepression, the labeled mitochondrial membrane proteins from both repressed and derepressed cells appeared to migrate into six similar peaks after sodium dodecylsulfate gel electro-

TABLE 5

Effect of Glucose Repression on Labeling In Vivo of Mitochondrial Membranes

Time of Cell Growth	Radioactivity in sub-mitochondrial particles		Radioactivity in post-mitochondrial supernatant	
	Control	+cyclo-heximide	Control	+cyclo-heximide
h	counts x min^{-1} x mg^{-1}			
9	933	347	-	-
13	-	1060	-	-
16	-	6060	-	-
22	14,700	5600	1640	65

Yeast cells obtained after growth in 5% glucose for the time indicated were washed, and incubated in phosphate buffer, pH 7.4, containing 0.1% glucose. Where indicated cycloheximide (100 µg/ml) was added and the culture incubated for 15 min prior to addition of 1 µCi/ml of [^{3}H]leucine. After incubation for 30 min, 10 mM unlabeled L-leucine was added and the incubation continued for 15 min. The cells were harvested and submitochondrial particles prepared as described under Methods. The post-mitochondrial supernatant is the 17,000 x g supernatant after the first mitochondrial centrifugation for 10 min.

phoresis (Fig. 3). The ratio of counts in the peaks, however, does vary as glucose derepression occurs. For example, in the repressed cells (early log phase), the two highest molecular weight peaks were labeled to a greater extent relative to the other peaks when compared to the gel pattern of the partially derepressed cells (late log phase). A direct comparison of the counts in these two gels can be made as exactly the same number of counts was added to each gel and the electrophoresis performed at the same time. In stationary phase cells, the peak of molecular weight 37,000 was labeled to a greater extent and the peak of molecular weight 18,000 was labeled to a lesser extent relative to the other peaks when compared to the gel patterns of membranes obtained from cells at the early or late log phase. Furthermore, the peak of molecular weight 18,000 became increasingly less broad as growth proceeded suggesting a greater homogeneity of the proteins comprising the peak. In the stationary phase cells, the two highest molecular weight peaks migrated as one peak in this experiment, although in some experiments they were resolved. These three gels are representative of at least four experiments at each time in which similar trends were noted. It should be stressed that six peaks were always obtained at all times studied during glucose derepression.

Tzagoloff (2) has reported that mitochondrial protein synthesis is stimulated when cells are allowed to accumulate

products of cytoplasmic protein synthesis by growth for various times in chloramphenicol. Perhaps, the increased labeling of mitochondrial membranes *in vivo* might be a reflection of an increased rate of mitochondrial protein synthesis. To test this possibility, yeast cells were grown for 13 h in 5% glucose at which time the culture was divided into three equal parts. To one was added chloramphenicol (4 mg/ml), to another was added cycloheximide (20 µg/ml), while the third was used as the control. After another 3 h of growth, all three cultures were harvested, washed and transferred to an equal volume of fresh medium containing 0.8% glucose. A portion of the cells from each culture were used at this time to prepare mitochondria while the remaining cells were allowed to grow for another one to two hours. The presence of either inhibitor of protein synthesis in the growth medium caused significant decreases in the rate of amino acid incorporation by isolated mitochondria *in vitro* despite the extensive washing of both the cells and the mitochondrial pellets prior to the incorporation studies (Table 6). After 1 h of growth in fresh medium without inhibitor, the incorporation measured in both the ATP-regenerating and the ATP-succinate systems was nearly 50% higher in mitochondria isolated from cells which had been preincubated in chloramphenicol. A higher rate of incorporation was also observed in mitochondria from cells after 2 h of growth in fresh medium; however, these higher rates were not observed

TABLE 6

Effects of Growth in Inhibitors on Amino Acid Incorporation by Isolated Yeast Mitochondria

Condition of cells	Radioactivity in					
	ATP-regenerating			ATP-succinate		
	Control	chloram-phenicol	cyclo-heximide	Control	chloram-phenicol	cyclo-heximide
	counts x min^{-1} x mg^{-1}					
After growth in inhibitors	874	337	490	1603	337	457
One-hour Recovery	837	1200	583	2010	2920	1510
Two-hour Recovery	1042	1344	-	2130	2910	-

Partially derepressed yeast cultures were grown for 3 h in the presence or absence of chloramphenicol (4 mg/ml) or cycloheximide (10 µg/ml). The cells were washed and transferred to an equal volume of fresh medium containing 0.1% glucose and allowed to grow for 1 or 2 h. Amino acid incorporation was assayed as described in the legend to Table 1.

when the cells had been allowed to grow for 5 h after the preincubation in chloramphenicol. In contrast, after 1 h in fresh medium, amino acid incorporation *in vitro* was derepressed nearly 30% in mitochondria isolated from cells which had been preincubated in cycloheximide.

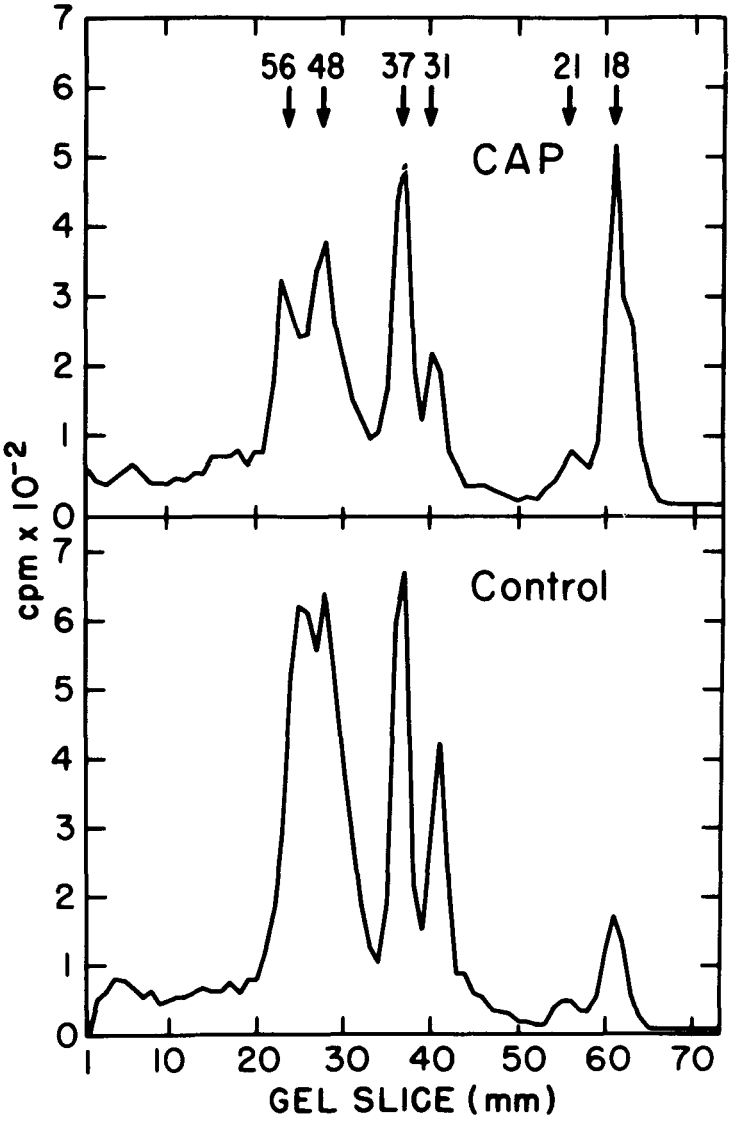
Yeast cells obtained after 3 h of growth in chloramphenicol followed by 1 h of growth in fresh medium were labeled *in vivo* with [^{3}H]leucine in the presence of cylcoheximide. Control cells were similarly labeled and the mitochondrial membranes from both cultures analyzed by sodium dodecylsulfate gel electrophoresis (Fig. 4). Exactly the same number of counts were added to both gels so that the radioactivity in the different peaks can be compared directly. It is apparent that the counts in the four peaks of molecular weights 56,000 to 31,000 are much lower in the membranes from the cells preincubated in chloramphenicol while the counts in the peak of molecular weight 18,000 are stimulated nearly 3-fold.

DISCUSSION

Before attempting to study the effects of glucose repression on mitochondrial protein synthesis, it was necessary to establish optimal conditions for the study of amino acid incorporation by isolated yeast mitochondria. Our results indicate that maintaining osmolarity in the incubation medium with 90 mM KCl or NH_4Cl yielded higher rates of in-

FIG. 4. Sodium dodecylsulfate gel electrophoresis profiles of mitochondrial membranes obtained from yeast cells treated as described in Table 6. Partially derepressed yeast cultures were grown in the presence of chloramphenicol (4 mg/ml) for 3 h, washed and transfered to an equal volume of fresh medium containing 0.1% glucose and allowed to grow for 1 h (A). Control cells were incubated without chloramphenicol, washed and allowed to grow for 1 h in fresh medium (B). The cells were then harvested and labeled in the presence of cycloheximide with 30 μCi/ml [^{3}H]leucine. Mitochondrial membranes were prepared as described in Methods and subjected to gel electrophoresis as described in the legend to Fig. 2. Final specific activity for both preparations was 110,000 counts x min^{-1} x mg^{-1}. The numbers at the top of the gels refer to 10^{-3} x molecular weights for each peak.

FIG. 4



corporation than maintaining osmolarity with sorbitol, sucrose or NaCl. The incorporation rate supported by ATP and succinate with or without phosphoenolpyruvate and pyruvate kinase was nearly double that with the ATP-regenerating system alone. Amino acid incorporation in the ATP-succinate supported system was inhibited by blocking electron transport with antimycin A or phosphorylation with oligomycin indicating tht the respiratory chain-linked synthesis of ATP provides the source of energy for the incorporating process. In contrast to these results, Lamb et al. (10) reported earlier that maximum incorporation rates in vitro were obtained with an external ATP-regenerating system; however, Grivell and Roodyn (14) observed that an ATP-regenerating system could only support amino acid incorporation when supplemented with succinate. The lack of atractyloside inhibition of amino acid incorporation supported by the external ATP-regenerating system suggests that the transport of ATP into the mitochondria by the atractyloside-sensitive adenine translocase (15) may be limited, although yeast mitochondria have been shown to contain an adenine translocase similar to that of liver mitochondria (16). However, a comparison of the properties of amino acid incorporation by isolated yeast and liver mitochondria suggests that the adenine translocase may differ in the two mitochondria. First, amino acid incorporation in vitro by isolated rat liver mitochondria proceeds at

an identical rate in the presence of the ATP-succinate or the external ATP-regenerating system (11). Secondly, incorporation supported by the latter system is inhibited 60-70% by atractyloside suggesting that the ATP must be first transported across the mitochondrial membrane by the atractyloside-sensitive adenine translocase prior to incorporation.

Amino acid incorporation by isolated yeast mitochondria was inhibited by antibiotics which are selective for mitochondrial ribosomes, e.g., chloramphenicol and erythromycin (12,16) but not by cycloheximide, the inhibitor of protein synthesis on cytoplasmic ribosomes (17). Low concentrations of the inhibitros of RNA synthesis, cordycepin (13) and actinomycin D, caused a 20% decrease in the incorporation rate. This inhibition presumably represents the turnover of mRNA synthesized in the mitochondria during the 15 min of incubation. Very low concentrations of ethidium bromide, known to intercalate in mitochondrial DNA and prevent transcription (18,19), also completely blocked the incorporation process; however, it is unlikely that the inhibition of protein synthesis by ethidium bromide can be correlated with its effects on RNA synthesis. A much better explanation of the immediate and complete block of amino acid incorporation *in vitro* might be the binding of ethidium bromide to mitochondrial tRNAs as proposed by Perlman and Penman (20) and demonstrated with *Escherichia coli* tRNAs by Lurquin *et al.* (21).

The rate of amino acid incorporation was not significantly decreased in mitochondria obtained from glucose repressed cells when measured with the ATP-regenerating system, or when succinate was present and respiratory-chain-linked synthesis of ATP provided the necessary energy for protein synthesis. This observation differs from that of Yang and Criddle (22), who reported that amino acid incorporation by isolated yeast mitochondria in the presence of an external ATP-regenerating system and oligomycin was decreased 90% in mitochondria obtained from repressed cells when compared to mitochondria from derepressed cells. Their incorporation rates, however, were very low and showed no sensitivity to ethidium bromide. In contrast, Murray and Linnane (23) recently reported that the rate of incorporation *in vitro* in mitochondria from log phase cells was high compared to that observed in mitochondria obtained from late log or stationary phase. No explanation for these differences is apparent.

In contrast, the rate of mitochondrial protein synthesis *in vivo*, as measured by the labeling of mitochondrial membranes in yeast cells incubated with radioactive leucine in the presence of cycloheximide, was decreased in glucose-repressed cells. A similar finding was previously reported by Kellerman *et al.* (24). These results suggest that glucose must repress some ancillary cellular function which results in a lowered rate of mitochondrial

protein synthesis in the intact cell, as the ability of isolated mitochondria to synthesize proteins is not lowered in mitochondria obtained from glucose-repressed cells. Perhaps, the lowered amount of DNA present in glucose-repressed cells (25) is the cause of the lowered labeling.

The mitochondrial membrane proteins labeled in vivo in the presence of sufficient cycloheximide to block 98% cytoplasmic protein synthesis separated after sodium dodecylsulfate gel electrophoresis into at least six clearly discernible peaks of molecular weights ranging from 56,000 to 18,000. Labeled peaks of similar mobility were observed in membranes both from glucose-repressed cells and from derepressed cells despite the lowered rates of labeling in the repressed cells. Quantitatively, however, significant differences in the ratio of counts in the various peaks were observed, suggesting that glucose does not effect the absolute repression of synthesis of any one membrane protein within the mitochondrion, but in some complex way, may act to control the rate of synthesis of some mitochondrial proteins. Similarly, the complete protein profile of the mitochondrial membrane after either gel electrophoresis (26) or isoelectric focusing (27) was reported to be qualitatively similar in glucose repressed and derepressed cells. Quantitatively, however, the pattern appeared clearly different in both studies suggesting that glucose repression may play a role in controlling the synthesis of the mitochondrial mem-

brane. Furthermore, anaerobic growth of yeast cells resulted in an approximate 20% inhibition of the labeling *in vivo* of one major band of the mitochondrial membrane (28). Oxygen stimulated the rate of labeling of this band, while glucose prevented the oxygen stimulation.

Experimental evidence obtained in several laboratories (2,29,30) has lead to the suggestion that synthesis of certain mitochondrial proteins is stimulated by products of cytoplasmic protein synthesis. Similarly, the absence of products of cytoplasmic protein synthesis resulted in a significant decrease in the rate of mitochondrial protein synthesis (31). In the present study, the rate of amino acid incorporation *in vitro* was greatly decreased in mitochondria obtained from cells grown in either chloramphenicol or cycloheximide. One explanation for the large decrease observed after growth in chloramphenicol is that some of the drug present in the mitochondria, perhaps bound to the mitochondrial ribosome, is not removed despite the extensive washing of the mitochondrial pellet. However, the rate of incorporation was significantly increased in mitochondria obtained from cells allowed to grow for 1 to 2 h in fresh medium suggesting that the products of cytoplasmic protein synthesis which had accumulated during growth in chloramphenicol may actually stimulate mitochondrial protein synthesis. The rate of labeling *in vivo* was similarly stimulated in cells which had been preincubated in

chloramphenicol. The stimulation of mitochondrial protein synthesis observed in vivo did not result from an increased synthesis of all of the peaks observed after gel electrophoresis. The labeling of the high molecular weight peaks was significantly lowered while the labeling of the peak of lowest molecular weight was stimulated 3 to 5-fold. Moreover, when the products of cytoplasmic protein synthesis were depleted by growth of cells in cycloheximide, the rate of amino acid incorporation by isolated mitochondria was inhibited 40 to 60%. Growth for 1 h in fresh medium was not sufficient to cause a reversal of this inhibition. Presently, studies are in progress to determine which enzyme complexes, if any, of the mitochondrial membrane are affected by this stimulation of mitochondrial protein synthesis.

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III. THE REGULATION OF MITOCHONDRIAL PROTEIN SYNTHESIS AT THE POLYRIBOSOMAL LEVEL

LITERATURE REVIEW

The biogenesis of a functional mitochondrial respiratory chain is controlled by two distinct genetic systems, one mitochondrial and one cytoplasmic, as well as two separate systems for protein synthesis (1,2). The vast majority of mitochondrial proteins are synthesized on cytoplasmic ribosomes and transferred into the mitochondria in a subsequent step (3,4). Integration of these proteins into the inner mitochondrial membrane requires the presence of 8-10 hydrophobic proteins which are synthesized on mitochondrial ribosomes. Extensive studies with purified enzymes have indicated that the formation of three enzyme complexes of the inner membrane; cytochrome oxidase (5,6) oligomycin-sensitive ATPase (6) and cytochrome b of complex III (7,8) requires the coordinated synthesis of proteins in both the cytoplasm and mitochondria. Under normal growth conditions, mitochondrial membrane assembly proceeds in an orderly manner such that a constant level of enzymes is maintained suggesting that a mechanism exists to control the synthesis of mitochondrial proteins at the two different intracellular sites.

Recently, evidence has accumulated to suggest that protein synthesis on mitochondrial ribosomes measured both *in vitro* and *in vivo* may be controlled by proteins synthesized in the cytoplasm (9-11). Although the mechanism by which cytoplas-

mic proteins may affect mitochondrial protein synthesis remains unclear, several reports have suggested that the synthesis of specific mitochondrial proteins is stimulated when cytoplasmically made proteins have accumulated (8,9).

In the present study, possible mechanisms for the regulation of mitochondrial protein synthesis have been investigated by studying polysomes isolated from yeast mitochondria. When yeast cells were grown in chloramphenicol to allow cytoplasmically made proteins to accumulate, a 2-fold increase in the polysome to monosome ratio was observed. Furthermore, increased labeling in the polysome region was observed using either radioactive leucine or formate both *in vitro* and *in vivo*. These results suggest that proteins synthesized in the cytoplasm may control mitochondrial protein synthesis either by stimulating chain initiation or by causing increased formation of specific mitochondrial messenger RNAs.

MATERIALS & METHODS

Yeast cells were grown in 5% glucose as described previously (12) and harvested after 11-13 h. The conversion of yeast cells to spheroplasts was performed as previously described (13) with some minor modifications which improved the yield of functional mitochondrial polyribosomes. The cells were not incubated in thioglycolic acid or any other thioreagent because this treatment resulted in a decrease in the activity of polysomes. After the cells were harvested,

they were washed with sterile, distilled water. Then, 1 g wet weight of cells was resuspended in 1 ml of 2.0 M Sorbitol and 1 ml of 1.0 M citrate-phosphate buffer, pH 5.8. Subsequently, 1 ml of glusulase was added per 5 g wet weight of cells. The cells were incubated in a Dubnoff water bath shaker at 33°C until 70% of the cells were converted to spheroplasts as determined by the change in absorbancy at 600 nm (13). The digestion was stopped by the addition of an equal volume of ice-cold 1 M Sorbitol. The spheroplasts were recovered by centrifugation at 1500 x g for 10 min. The pellet was washed gently with semisynthetic medium containing 0.1% peptone, 0.1% yeast extract, 0.1% KH_2PO_4, 0.16% $(NH_4)_2SO_4$, 0.1% $MgSO_4 \cdot 6H_2O$ in 0.9 M Sorbitol (Medium A).

Isolation of Mitochondria:

The spheroplasts were lysed by suspension in 0.25 M Sorbitol containing TMK buffer (50 mM Tris·HCl, pH 7.4, 10 mM $MgCl_2$, 30 mM KCl) to a final concentration of 1 g/10 ml. In some experiments the breakage was achieved by using a Waring blender at low speed for 15 sec. The homogenate was centrifuged at 1500 x g for 10 min, 3 times to ensure that there was no contamination of the mitochondrial pellet with nuclei or spheroplast membranes, although centrifugation at this speed did result in the loss of some mitochondria. The supernatant was centrifuged at 13,000 x g for 10 min. The mitochondria were washed four times with very gentle homogenization to avoid damage to the mitochondrial membrane. All manipulations

were performed at 0-4°C unless otherwise noted.

Isolation of Mitochondrial Polyribosomes; Sucrose Density Gradient Centrifugation:

The mitochondria were suspended by homogenization in TMKH buffer (50 mM Tris·HCl pH 7.6, 14 mM $MgCl_2$, 300 mM KCl supplemented with Heparin at 500 µg/ml). The mitochondria at a concentration of 10-15 mg/ml were lysed by addition of Triton X-100 to a final concentration of 1.7% for 5-10 min at 0-4°C. The suspension was centrifuged twice at 27,000 x g for 10 min. The clear yellow supernatant free of mitochondrial membranes was layered on a 20-40% sucrose gradient prepared in TMKH buffer containing 100 µg/ml of Heparin. This was followed by centrifugation for 3.75 h at 95,000 x g in a SW27 rotor at 0-4°C. The distribution of polysomes was determined by puncturing the bottom of the tubes, collecting 1 ml fractions and recording the absorbance at 260 nm.

Discontinuous sucrose density gradient:

The clear yellow supernatant containing the crude mitochondrial polyribosomes (2-3 ml) was layered on a discontinuous sucrose gradient containing 1.5 ml of 2.5 M sucrose and 3 ml of 1.2 M sucrose as described by Schimke (14) and centrifuged for 2.5 h at 170,000 x g in a SW50.1 rotor. The opalescent polysomes which band at the boundary of the two sucrose layers were isolated in 0.5-0.7 ml of sucrose solution by puncturing the side of the tubes with a sterile sy-

ringe right below the band. The polysomes were dialyzed at least 4 h against the TMKH buffer containing 40 µg/ml Heparin buffer in an ice-water bath using dialysis tubing which had been boiled in 5% $NaHCO_3$, containing 0.1 mM EDTA to inactivate ribonuclease activity and chelate any metal ions) and then washed thoroughly with sterilized distilled water. After the dialysis the preparation was centrifuged for 5 min at 27,000 x g. The clear supernatant was then used to study amino acid incorporation in a cell-free system or alternately layered on a continuous 20-40% sucrose density gradient as described above. Freshly prepared polysomes were used in all experiments.

Labeling Procedures; Incorporation of [^{14}C]formate:

Cells were converted to spheroplasts and incubated in Medium A supplemented with 0.5% glucose and 1% ethanol as carbon source. After 1 h incubation, cycloheximide (80 µg/ml) was added and the incubation continued for 15 min. Labeled formate was added to a final concentration of 20 µCi/ml and the incubation continued for 5 min at 30°C. The reaction was terminated by the addition of 10 mM unlabeled formate and 4 mg/ml of chloramphenicol. Mitochondria were isolated and polysomes prepared as described above.

Alternately, the spheroplasts were incubated for 15 min as described above with cycloheximide plus 0.5 mM puromycin prior to addition of [^{14}C]formate. After incubation for 10 min at 30°C, 10 mM unlabeled formate and 4 mg/ml of chlor-

amphenicol were added. The isolated mitochondria were treated with 0.1 M Na acetate, pH 5.5, to extract f-Met-puromycin exactly as described by Leder and Brusztysn (15).

Labeling of the Nascent Polypeptide Chain on Mitochondrial Polysomes:

Spheroplasts were suspended and incubated in semi-synthetic medium as described above. Cycloheximide (80 µg/ml) was added for 15 min prior to addition of [^{3}H]leucine and the incubation continued for 2.5-5 min. Under these conditions, the specific activities obtained were low due to the presence of unlabeled amino acids in the peptone. Therefore, the procedure was modified so that the spheroplasts were incubated for 1 h and then reisolated by centrifugation at 1500 x g for 10 min. The pellet was resuspended in 0.9 M Sorbitol, 50 mM phosphate buffer, pH 6.8. Cycloheximide (80 µg/ml) was added 15 min prior to [^{3}H]leucine as described above. The incorporation was stopped by addition of 5 mM unlabeled leucine and chloramphenicol (4 mg/ml).

Labeling of Isolated Mitochondria In Vitro:

Yeast mitochondria were prepared from spheroplasts in 0.44 M Sorbitol, 14 mM $MgCl_2$, 10 mM Tris, 10 mM KCl, pH 7.6. Amino acid incorporation was measured in a medium containing 90 mM KCl, 50 mM Bicine pH 7.6, 10 mM $MgCl_2$, 1 mM EDTA, 22.5 µg of an amino acid mixture minus leucine, 5.0 mM phosphate, pH 7.6, 2.0 mM ATP, 10 mM succinate, 5 mM

phosphoenolpyruvate, 2 μg/ml pyruvate kinase, 2 mg of mitochondrial protein and [^{3}H]leucine (10.0 μCi/ml) for 5 min. In the studies of initiation all amino acids were added at the same concentration as described above except puromycin was added before [^{14}C]formate (20 μCi/ml) and the incubation continued for 5 minutes. Extraction of f-Met-puromycin was described above.

Polysomes isolated by the cushion method were labeled *in vitro* in a medium consisting of 10 mM Tris·HCl, pH 7.6, 80 mM KCl, 10 mM Mg acetate, 5 μM phenylalanine, 25 μM tyrosine, an amino acid mixture minus leucine, 30 μM GTP, 1 mM ATP, 6 μM mercaptoethanol 5 mM phosphoenolpyruvate, 20 μg pyruvate kinase and 250 μg of an S-100 fraction from *E. coli*. Labeling was performed with either [^{14}C]phenylalanine, [^{3}H]leucine or an amino acid mixture labeled with ^{3}H.

Measurement of Radioactivity:

Trichloroacetic acid insoluble material was measured as follows: Mitochondrial polysome fractions at 1 ml/tube were mixed with 50 μg BSA and 2 ml of 15% trichloroacetic acid. The tubes were agitated and left at room temperature for about 30 min. The subsequent steps of washing the acid insoluble material on Millipore filter nitrocellulose (0.45 μ millipore) was described previously (13).

Sterilization Procedures:

All glassware was washed with chromic acid (cleaning

solution), rinsed with distilled water and autoclaved. When possible, sterilized plastic disposable tubes and pipettes were also used. All solutions which came in contact with the polysomes were autoclaved and 500 µg/ml of Heparin was added. In addition, treatment of the solutions with diethylpyrocarbonate (500 µl/l) followed by heating the solution at 100°C to let the CO_2 and ethanol escape improved the polysome yield appreciably.

Materials:

Puromycin dihydrochloride, cycloheximide, chloramphenicol, Ribonuclease T_1, Ribonuclease-A (Bovine Pancreas) Heparin (163 USP units/mg) were obtained from Sigma; sucrose (ribonuclease free) was obtained from Schwarz-Mann and diethylpyrocarbonate from Eastman. Uniformly labeled [^{14}C]phenylalanine (485 mC_i/mmole), [^{14}C]formate (56 mC_i/mmole) and a ^{3}H-amino acid mixture were obtained from New England Nuclear; [4,5-^{3}H]leucine (53 C_i/mmole) from Amersham-Searle and uniformly labeled [^{14}C]leucine (312 mC_i/mmole) from Schwarz-Mann.

Glusulase obtained from Endo laboratories or prepared in our laboratory were equally effective. Snails, Otala lactea were obtained from the Scozzaro Brooklyn Fish Market. The snails were starved in the cold room for one day and then cracked. The intestinal tracts were removed, collected in an ice-bath and diluted with an equal volume of cold water. The mixture was centrifuged 2 times at 13,000 x g for 20 min. The clear red supernatant was collected and fractionated with

$(NH_4)_2SO_4$. The 55-70% $(NH_4)_2SO_4$ fraction was collected and resuspended in citrate phosphate buffer, at pH 5.8, and dialyzed through colloidin membrane at 0-5° for about 6-9 h.

The glusulase enzyme prepared in the laboratory or obtained commercially was recycled after the yeast cells were converted to spheroplasts by an ammonium sulfate precipitation. A batch of enzyme retained activity for 8 experiments.

RESULTS

Isolation of Mitochondrial Polysomes:

Mitochondrial polysomes from yeast have not been well-characterized to date largely due to the difficulties inherent in their isolation (16). Several possibilities exist to explain some of these problems. First, the products of mitochondrial protein synthesis are extremely hydrophobic and the resulting interactions of these proteins during the isolation procedures may cause the polysomes to aggregate (17). As a result, the nascent chains on the polysomes would sediment during the gradient centrifugation. Secondly, the presence of endogenous as well as exogenous ribonucleases will cause the destruction of the polysomes by breaking down the messenger RNA's. To overcome the latter difficulty, all solutions were sterilized and Heparin, an inhibitor or ribonuclease, was added. In addition, the presence of high concentrations of Mg^{2+} and KCl in the polysome buffer were important for the stabiliza-

tion of the polysomes during isolation. These conditions resulted in the isolation of mitochondrial polysomes consisting of 2-8 monosomes plus the monosome sedimenting at 74S as indicated by absorbancy measurements at 260 nm (Figure 5 A).

Treatment of the Triton X-100 solubilized fraction with 40 mM EDTA caused a disappearance of all the material absorbing at 260 nm from the polysome and monosome regions of the gradient (Figure 6). The ribosomes were dissociated into the two subunits sedimenting at 30S and 50S, similar to those values previously reported by Schmitt (18). Both the monosome and the two subunits of E. coli ribosomes were used as markers for the gradient to determine the sedimentation value of the yeast mitochondrial ribosome subunits. When yeast spheroplasts were incubated for 15 min with cycloheximide, a specific inhibitor of cytoplasmic protein synthesis, followed by a 2-5 min pulse label with [^{3}H]leucine prior to the preparation of polysomes from isolated mitochondria, trichloroacetic-precipitable radioactivity was observed mainly in the polysome region of the gradient (Figure 5 A). Ribonuclease digestion caused degradation of the polysomes to a single monosome peak with release of radioactivity to the top of the gradient (Fig. 5B). Furthermore, addition of 5 mM puromycin to the spheroplasts during the incubation with [^{3}H]leucine caused a release of radioactivity from the polysome region to the top of the gradient, suggesting that the [^{3}H]leucine counts in the polysome region do in-

FIG. 5. Sedimentation profile of mitochondrial polysomes before and after treatment with ribonuclease. Saccharomyces cerevisiae were converted to spheroplasts and incubated with cycloheximide and 20 μC_i/ml of [^{3}H]leucine. The incubation was stopped by the addition of 5 mM unlabeled L-leucine and 4 mg/ml of chloramphenicol. Mitochondrial polysomes were prepared as described under Methods. The crude mitochondrial polysomes (10 A260 units) were layered on a 20-40% continuous sucrose gradient and centrifuged at 95,000 x g for 3.75 hours. One ml fractions were collected from the gradient. (A) Control (B) Crude polysomes (10 A260 units) were incubated with 10 µg/ml of ribonuclease for 10 min at 4° before layering on the sucrose gradient. (●——●) O.D. at 260 nm (o----o) counts/min.

FIG. 5

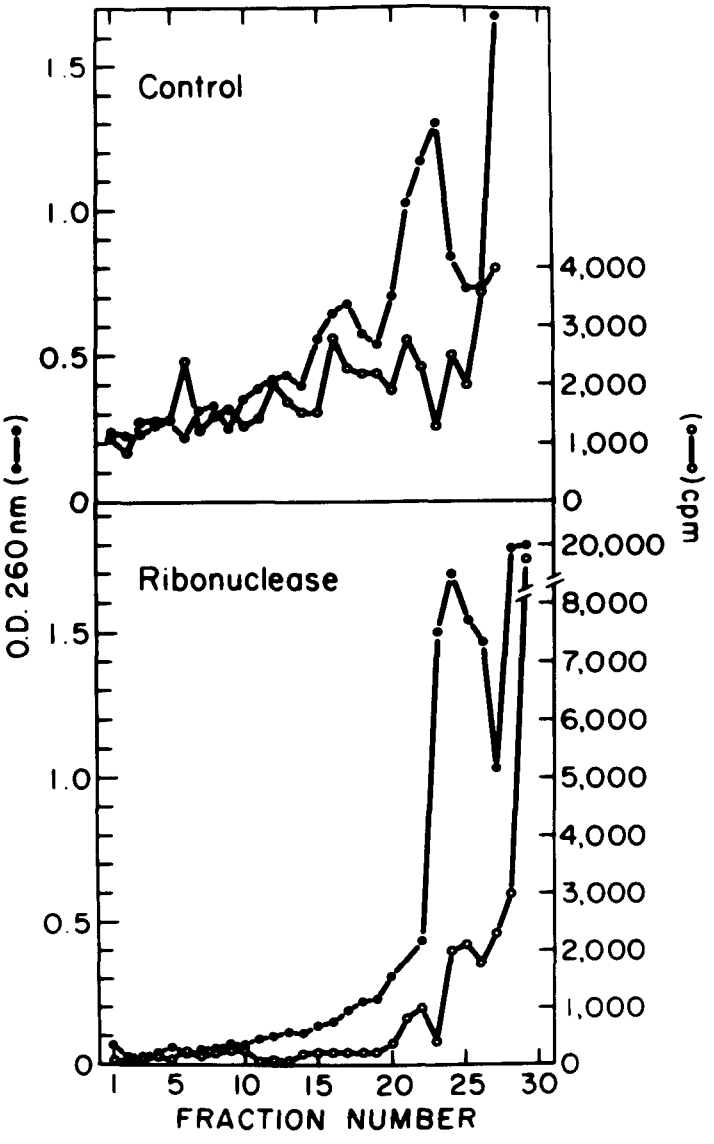

Control
Ribonuclease
O.D. 260nm
cpm
1.5
1.0
0.5
0
4,000
3,000
2,000
1,000
20,000
8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000
1
5
10
15
20
25
30
FRACTION NUMBER

FIG. 6. Sedimentation pattern of mitochondrial polysomes after treatment with EDTA. The crude mitochondrial polysomes (13 A260 units) were incubated with 50 mM EDTA for 10 min at 4° and layered on a 10-30% continuous sucrose gradient containing 50 mM EDTA and centrifuged at 95,000 x g for 6 hours. Subunits of E. coli ribosomes were used as markers at 30S and 50S. (●——●) control (o----o) EDTA.

FIG. 6

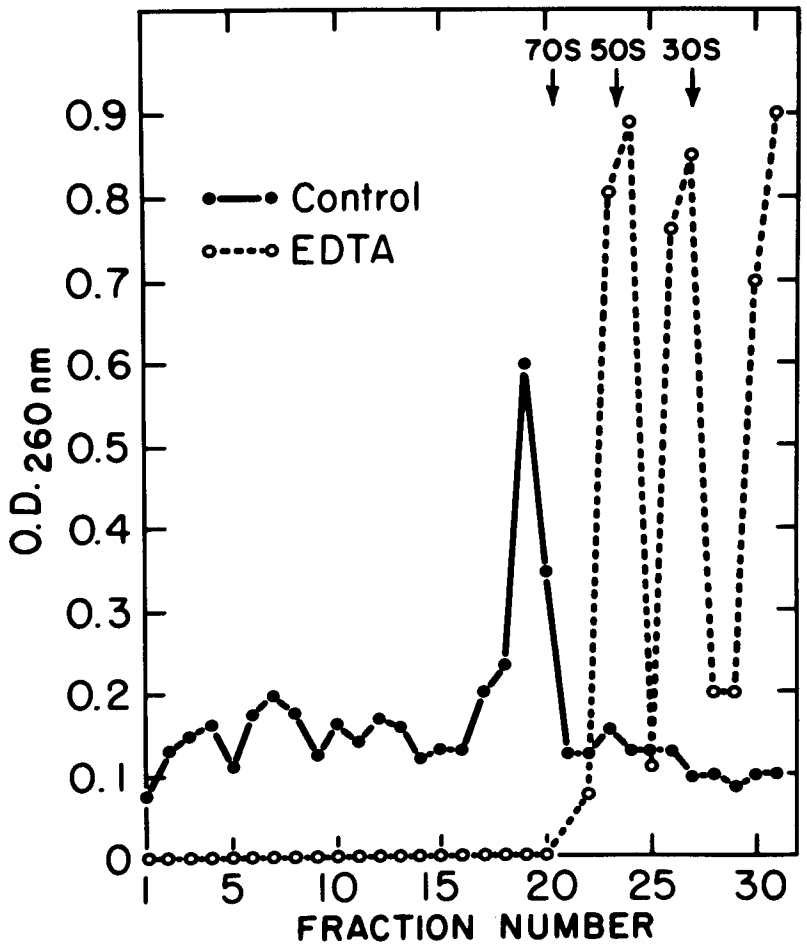
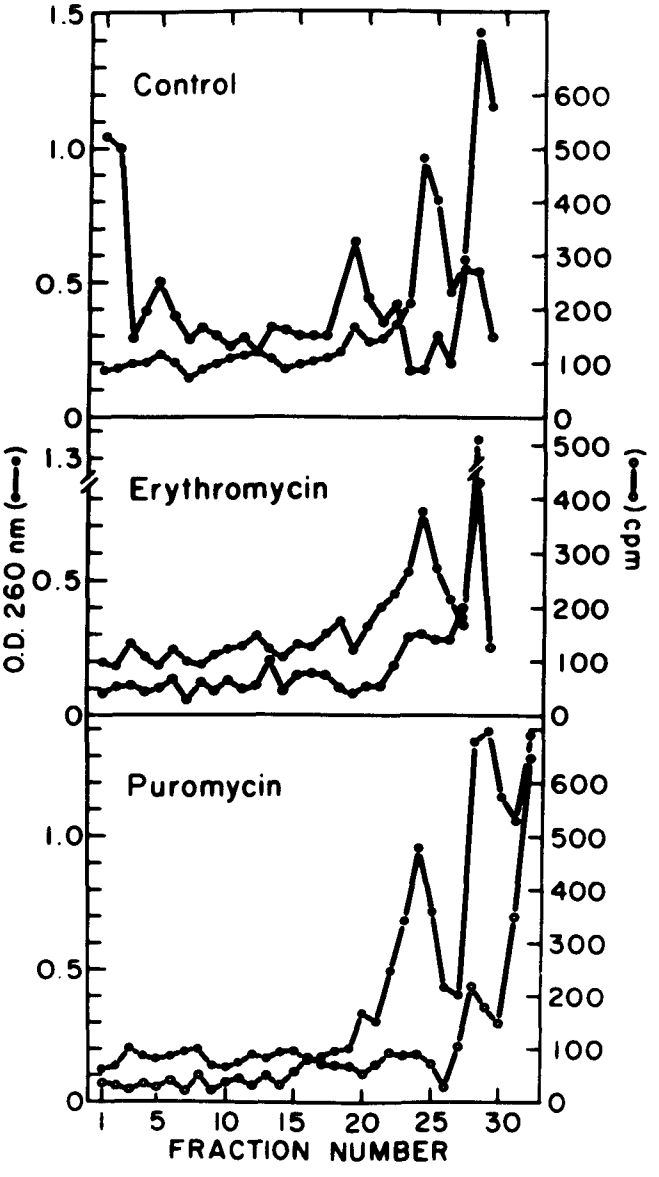

70S 50S 30S
Control
EDTA
O.D. 260 nm
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
5
10
15
20
25
30
FRACTION NUMBER

FIG. 7. Typical mitochondrial polysome profiles after treatment with various inhibitors. Spheroplasts were incubated with 10 μC_i/ml of [^{3}H]leucine for 5 min and mitochondria prepared as described in the legend to Figure 5. (Top) Control. (Bottom) Spheroplasts were incubated for 3 min with [^{3}H]-leucine and then 5.0 mM puromycin was added and the incubation continued for 2 min. (Middle) Spheroplasts were incubated with 2 mg/ml of erythromycin 15 min prior to addition of [^{3}H]leucine. (●——●) O.D. at 260 nm (o----o) counts/min.

FIG. 7


Control
Erythromycin
Puromycin
O.D. 260 nm (—•—)
cpm (—•—)
FRACTION NUMBER
1.5
1.0
0.5
1.3
0
600
500
400
300
200
100
1
5
10
15
20
25
30

deed represent nascent polypeptide chains (Figure 7, Bottom). All labeling in the polysome region was completely inhibited by addition to the incubation medium of erythromycin, a specific inhibitor of mitochondrial protein synthesis; however, the absorbance at 260 nm increased under these conditions (Figure 7, Middle). These results indicate that the polysomes isolated in these experiments are mitochondrial polysomes and do not contain significant amounts of contaminating cytoplasmic polysomes.

Labeling of Mitochondrial Polysomes with [^{3}H]leucine:

Tzagoloff (9) first reported that mitochondrial protein synthesis *in vivo* was increased in yeast cells which were allowed to accumulate products of cytoplasmic protein synthesis by growth of the yeast in chloramphenicol for various periods of time. Subsequently, we reported that the rate of amino acid incorporation *in vitro* in isolated mitochondria was stimulated nearly 100% in partially glucose derepressed yeast cells which had been grown for 3 hours in medium containing chloramphenicol, washed, and allowed to grow for one hour in fresh medium without chloramphenicol (10). These results suggested that the products of cytoplasmic protein synthesis which had accumulated during growth in chloramphenicol may actually have stimulated mitochondrial protein synthesis. Furthermore, growth of the cells in cycloheximide which would deplete the pool of mitochondrial proteins synthesized in the cytoplasm, followed by growth for one hour in

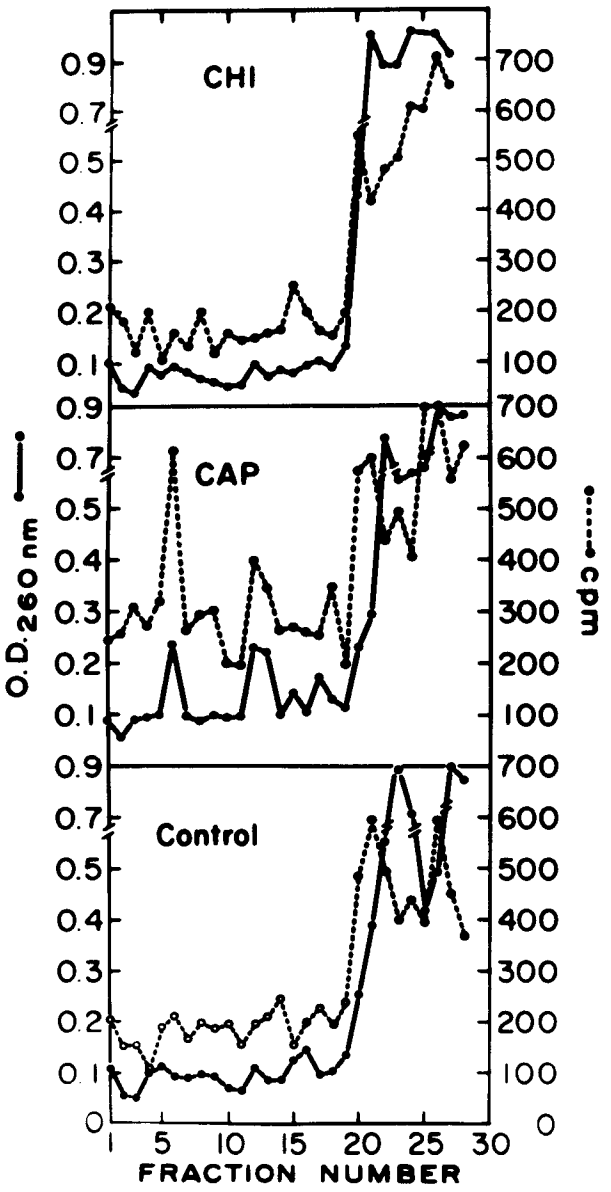
fresh medium without cycloheximide resulted in a 50% decrease in the incorporation *in vitro*.

To gain further insight into the mechanism by which cytoplasmically-made proteins may regulate mitochondrial protein synthesis, labeled mitochondrial polysomes were isolated from yeast cells grown for 3 hours in the presence of both chloramphenicol and cycloheximide followed by 1 hour in fresh medium. The data presented in Figure 8 indicates that the polysome to monosome ratio determined by the absorbance at 260 nm was increased nearly 2-fold in mitochondria isolated from the cells grown in chloramphenicol as compared to controls. Similarly, the acid precipitable radioactivity in the polysome region due to [^{3}H]leucine was increased 2-fold. Conversely, the polysome to monosome ratio decreased 60% in mitochondria isolated from yeast cells grown in cycloheximide. A corresponding decrease in the radioactivity due to [^{3}H]-leucine in the polysome region was also observed (Figure 8). The polysome region contained 75% as much RNA as did the monosome region in the cells preincubated in chloramphenicol as compared to 40% in control cells and 25% in cells previously grown in cycloheximide.

Polysomes were also isolated from mitochondria which had been incubated with [^{3}H]leucine *in vitro* under optimal conditions (10). Although yeast mitochondria prepared by mechanical breakage were used in our previous studies of amino acid incorporation, it was necessary to use mitochondria

FIG. 8. Typical mitochondrial polysome profiles labeled in vivo after growth of yeast in various inhibitors. Yeast cells were grown in 5% glucose for 12 hours and then divided into three culture flasks. One flask contained 4 mg/ml chloramphenicol, one flask contained 80 µg/ml cycloheximide and one served as the control. After three hours of growth, the cells were harvested, washed twice and resuspended in fresh medium without inhibitors for 1 hour. The cells were converted to spheroplasts and resuspended in 10% of the original medium prior to labeling with 10 μC_i/ml of [^{3}H]leucine as described in legend to Figure 5. (●——●) O.D. at 260 nm (o----o) counts/min.

FIG. 8


CHI
CAP
Control
O.D.260 nm
cpm
FRACTION NUMBER
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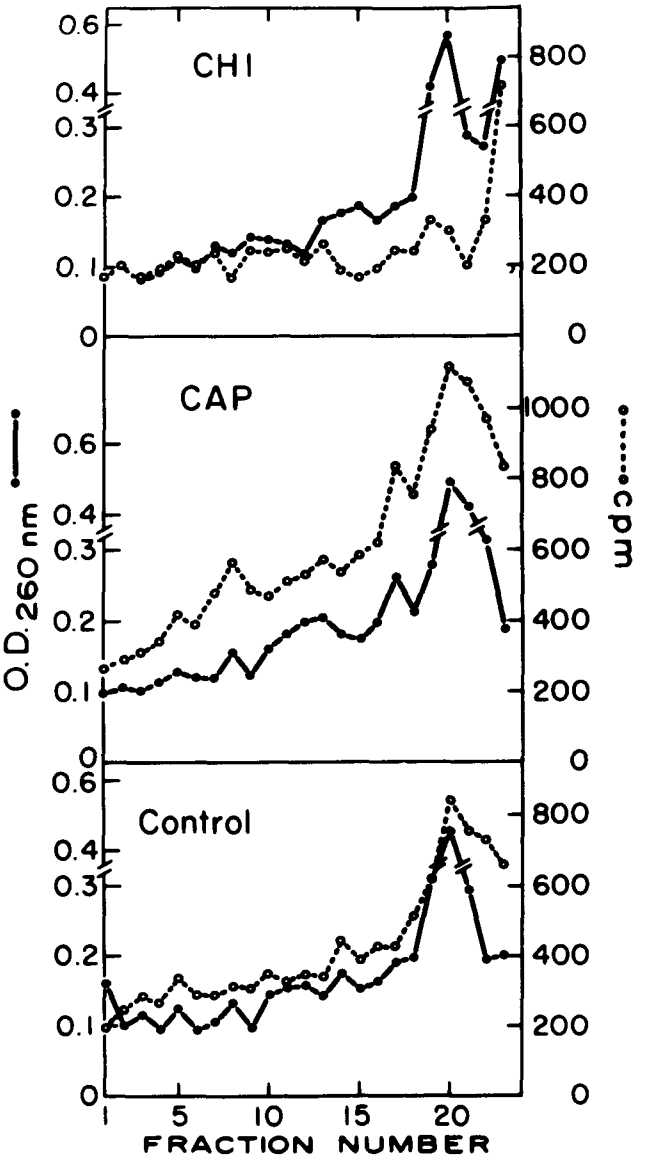
0.7
0.5
0.4
0.3
0.2
0.1
0
700
600
500
400
300
200
100
1
5
10
15
20
25
30

isolated from spheroplasts prepared by enzymatic digestion to obtain reproducible polysome profiles. After the incubation *in vitro* with [^{3}H]leucine, the mitochondria were washed twice in medium containing 10 mM leucine and then lysed with Triton X-100. Radioactivity due to [^{3}H]leucine was observed predominantly in the polysome region of the gradient (Figure 9). Mitochondria isolated from yeast cells grown for three hours in either chloramphenicol or cycloheximide followed by 1 hour in fresh medium were also incubated with [^{3}H]leucine. The acid-precipitable radioactivity in the polysome region was increased nearly 3-fold in mitochondria isolated from cells grown in chloramphenicol and decreased nearly 75% in mitochondria isolated from cells grown in cycloheximide (Figure 9). Similar changes in the polysome to monosome ratio were also observed. It should be noted that the changes observed *in vitro* were greater than those obtained when cells were labeled *in vivo*.

The results of both these experiments provide further evidence that proteins made in the cytoplasm may act to control mitochondrial protein synthesis at the polysomal level. The increased radioactivity in the polysome region suggests that more nascent polypeptide chains are present on the mitochondrial polysomes isolated from cells which had been grown in chloramphenicol and hence accumulated cytoplasmically-made proteins. These cytoplasmic proteins may act to promote the association of free ribosomes with messenger RNA resulting

FIG. 9. Typical mitochondrial polysome profiles labeled in vitro after growth of yeast in various inhibitors. Mitochondria were isolated from spheroplasts which had been grown in the presence of chloramphenicol or cycloheximide as described in the legend to Figure 8. The mitochondria were incubated with 10 μC_i/ml of [^{3}]leucine in the medium described under Methods containing 80 µg/ml of cycloheximide. Polysomes were prepared as described in the legend to Figure 1. (●——●) O.D. at 260 nm (o----o) cpm.

FIG. 9


CHI
CAP
Control
O.D. 260 nm
cpm
0.6
0.4
0.3
0.2
0.1
0
800
600
400
200
0
1000
1
5
10
15
20
FRACTION NUMBER

in the observed increase in the ratio of polysomes to monosomes which may be responsible for the increased rates of protein synthesis previously observed both *in vitro* and *in vivo*.

Labeling of Mitochondrial Polysomes with [^{14}C]formate:

One possible explanation for the increased rates of mitochondrial protein synthesis might be that proteins made in the cytoplasm act by stimulating the initiation of protein synthesis on mitochondrial ribosomes. Previous work has demonstrated that the reassociation of mRNA with ribosomes involves specific initiation factors (19). This possibility was investigated by labeling spheroplasts with [^{14}C]formate and isolating polysomes. It has been well-established that the initiation of protein synthesis in mitochondria involves formyl-methionyl tRNA (20). As seen in Figure 10 considerable radioactivity due to [^{14}C]formate was observed in the polysome region of the gradient. A 50% increase in the labeling due to formate was observed in mitochondrial polysomes isolated from yeast cells grown previously in chloramphenicol as compared to the controls.

The rate of chain initiation can also be measured by studying the formation of f-Met-puromycin from [^{14}C]formate and puromycin as described in Methods. The [^{14}C]formate present as f-Met-puromycin after incubating spheroplasts for 10 min with the radioactive formate was appreciable (Table 7). A 45% increase in labeling was observed in spheroplasts ob-

FIG. 10. Mitochondrial polysomes labeled with formate. Spheroplasts were prepared from yeast cells grown in chloramphenicol as described in the legend to Figure 8. The spheroplasts with 20 μC_i/ml of [^{14}C]-formate for 5 min. The reaction was terminated by the addition of 10 mM sodium formate and 4 mg/ml chloramphenicol. Polysomes were prepared as described in legend to Figure 5. (●——●) O.D. at 260 nm (o——o) cpm.

FIG. 10

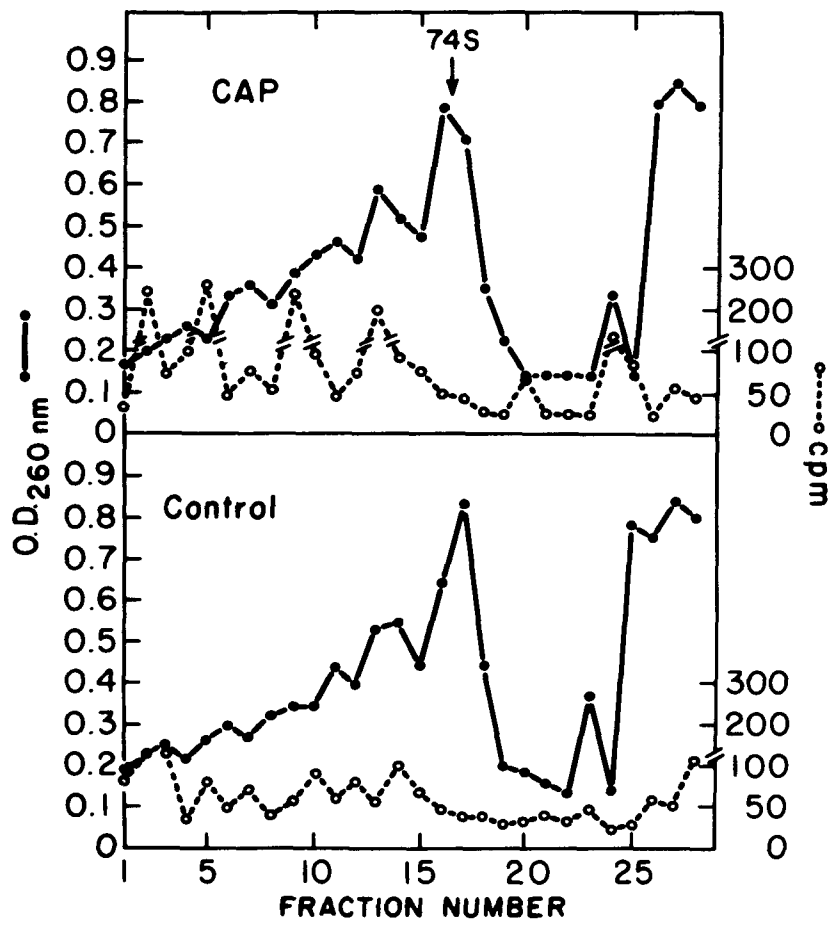

74S
CAP
Control
O.D.260 nm
cpm
FRACTION NUMBER
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
300
200
100
50
0
1
5
10
15
20
25

TABLE 7

Formation of f-Met-puromycin by Yeast Mitochondria *In Vivo* and *In Vitro*

In Vivo	f-Met-puromycin counts/min/mg
Control	8580
Chloramphenicol-grown	12400
Cycloheximide-grown	6490
In Vitro	
Control	1220
Chloramphenicol-grown	1866
Cycloheximide-grown	988

Spheroplasts prepared from cells grown in chloramphenicol and cycloheximide as described in the legend to Figure 8 were incubated for 15 minutes with 80 µg/ml of cycloheximide. Then, 0.5 mM puromycin was added 1 minute prior to addition of 20 μC_i/ml of [^{14}C]formate and incubated for 10 minutes at 30°C. The reaction was terminated by addition of 10 mM unlabeled sodium formate and 4 mg/ml chloramphenicol. Isolation of f-Met-puromycin was as described in Methods. Isolated mitochondria were incubated with 0.5 mM puromycin and 20 μC_i/ml of [^{14}C]-formate for 5 mintues.

tained from yeast cells previously grown in chloramphenicol while a 25% decrease was observed in cells previously grown in cycloheximide. These results are in good agreement with the labeling of polysome in vivo with [^{14}C]formate (Figure 10).

Likewise isolated mitochondria were incubated in vitro with [^{14}C]formate and puromycin prior to isolation of f-Met-puromycin. Labeling of f-Met-puromycin with [^{14}C]formate was also observed when mitochondria were incubated in vitro indicating that initiation of protein synthesis does occur in isolated mitochondria (Table 7). Again, 53% more f-Met-puromycin was formed in mitochondria from yeast cells previously grown in chloramphenicol while 20% less f-Met-puromycin was formed in mitochondria from cells grown in cycloheximide. These results indicate that the rate of chain initiation on mitochondrial polysomes is increased when proteins previously synthesized in the cytoplasm have accumulated.

Protein Synthesis with Isolated Polysomes:

Polysomes were also isolated from yeast mitochondria by the discontinuous sucrose gradient cushion method (14). As seen in Figure 11, a large increase in the polysome to monosome ratio was observed when the mitochondria were isolated from yeast cells which had been previously grown in chloramphenicol. The tRNAs which obscure measurement at 260 nm in the monosome region of the gradient were eliminated when polysomes were prepared by this method.

Polysomes isolated by the cushion method were also in-

FIG. 11. Sedimentation profile of mitochondrial polysomes isolated by discontinuous sucrose gradient centrifugation. Spheroplasts were obtained from cells grown in chloramphenicol as described in the legend to Figure 8. The crude polysomal fraction containing 33 A260 units was layered on a discontinuous sucrose gradient as described in Methods and centrifuged for 2.5 hours at 170,000 x g. The polysomes banding at the boundary were removed and 10 A260 units layered on a 20-40% continuous sucrose gradient as described in Methods.

FIG. 11

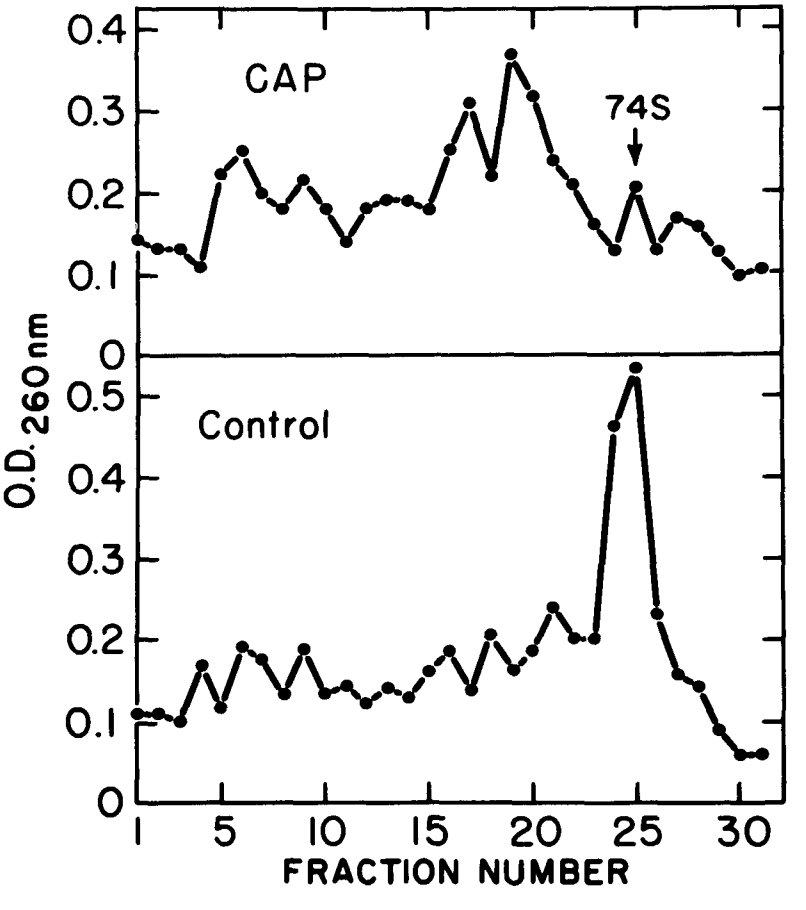

CAP
74S
Control
O.D. 260nm
0.4
0.3
0.2
0.1
0
0.5
0.4
0.3
0.2
0.1
0
1
5
10
15
20
25
30
FRACTION NUMBER

TABLE 8

Protein Synthesis by Isolated Polysomes

	% Control*
+RNase (0.01 μg)	28
+RNase (0.1 μg)	16
+EDTA (50 mM)	10
+Chloramphenicol (4 mg/ml)	27
+Puromycin (0.5 mM)	40
+Cycloheximide (80 μg/ml)	99
-S-100	10
-Polysomes	5
-ATP regenerating system	16

*Control value was 500-2000 cpm per 1 A260 unit.

Polysomes were prepared by the discontinuous sucrose gradient method. One A260 unit was incubated in 0.25 ml of a medium containing 10 mM Tris-Cl, pH 7.6, 80 mM KCl, 10 mM magnesium acetate, 5 μm phenylalanine, 25 μM of an amino acid mixture, 30 μM GTP, 1 mM ATP, 6 mM mercaptoethanol, 5 mM phosphoenolpyruvate, 20 μg of pyruvate kinase, 100 μg of an S-100 fraction from E. coli and 1 μC_i of [^{3}H]leucine or the ^{3}H-labeled amino acid mixture.

TABLE 9

Protein Synthesis *In Vitro* by Mitochondrial Polysomes Isolated from Cells Grown in Chloramphenicol

Source of Polysomes	Counts/min/tube
Control	3890
Chloramphenicol-grown	10100

Mitochondrial polysomes were isolated by discontinuous sucrose gradient centrifugation from cells grown in chloramphenicol as described in the legend to Figure 8. The polysomes were dialyzed 6 hours against TMKH buffer containing 40 µg/ml of Heparin. One A260 unit was incubated in 0.25 ml of the medium described in the legend to Table 8 containing 1 μC_i of [^{3}H]amino acid mixture for 20 minutes at 30°.

cubated *in vitro* with radioactive amino acids to study peptide chain elongation of the nascent chains already present on the polysomes. The incubation medium was essentially that used to study protein synthesis by isolated ribosomes in a cell-free system with some minor modifications (13). In some experiments [^{3}H]leucine was used as a precursor while in others a ^{3}H-amino acid mixture was used. Essentially identical results were obtained with either precursor. The rate of amino acid incorporation was linear for 40 min and was dependent on the presence of ATP and an ATP-regnerating system (Table 8). The incorporation was inhibited 60% by chloramphenicol and 40% by puromycin but was completely resistant to cycloheximide. Low concentrations of ribonuclease completely blocked the reaction as did treatment with EDTA. In the experiments reported in Table 8 , an S-100 fraction isolated from *E. coli* was used. Similar results were obtained with an S-100 fraction from yeast mitochondria.

A comparison of the rate of amino acid incorporation was also made using polysomes isolated from yeast cells which had been previously grown in chloramphenicol (Table 9). In order to make such a comparison, an identical amount of material absorbing at 260 nm was added to each incubation tube. The amount of amino acid incorporation into acid-insoluble material was more than double in the polysomes from the cells in which cytoplasmically-made proteins had

accumulated by growth in chloramphenicol. These results undoubtedly reflect the greater percentage of ribosomes associated with mRNA and hence polysomes in the cells pre-treated with chloramphenicol.

DISCUSSION

Several groups have observed a stimulation of mitochondrial protein synthesis under conditions when proteins synthesized in the cytoplasm were allowed to accumulate (9,10,21). Experimentally, either yeast cells (9,10) or Neurospora (21) are grown for short periods of time in sufficient chloramphenicol to block mitochondrial protein synthesis. Although the actual rates of mitochondrial protein synthesis are increased whether measured in vivo in the presence of cycloheximide or in vitro using isolated mitochondria, this stimulation appears specific. Labeling of certain unique mitochondrial proteins is increased under these conditions (9,11).

In the present study, we have attempted to examine mitochondrial protein synthesis at the polyribosomal level in order to obtain an insight into mechanisms which control the overall process. The results obtained indicate that there is a 2-fold increase in the labeling of nascent polypeptide chains present on mitochondrial ribosomes isolated from yeast cells previously grown in chloramphenicol compared to control cells. The increased polysome to monosome ratio

in the mitochondria from these cells is also a reflection of an increased activity in protein synthesis. The rate of initiation measured by [^{14}C]formate labeling of the polysomes or by f-Met-puromycin formation was also doubled when proteins made in the cytoplasm had accumulated. By contrast, chain initiation, labeling of nascent chains with radioactive leucine and the polysome to monosome ratio were decreased more than 50% in mitochondria isolated from cells which had been grown in cycloheximide for 3 hours. Under these conditions, the pool of cytoplasmically-made proteins would be severely depleted. Furthermore, it should be noted that these effects were also observed in isolated mitochondria. Hence, the cytoplasmically-made proteins which have presumably accumulated must still be present in the isolated mitochondria. Alternately, the effect of these proteins on some process, such as transcription, is still manifest.

Several possible mechanisms exist to explain how cytoplasmically-made proteins may control mitochondrial protein synthesis. First these proteins may affect the rate of translation on stable mRNA's in the mitochondria as has been proposed for the regulation of protein synthesis in mammalian cells (22). In both HeLa cells (22) and reticulocytes (23) translation appears to be controlled by factors which affect chain initiation. Nonspecific changes in components which affect polypeptide chain initiation would alter the relative translation of different mRNA's present in the cell

or organelle. The regulation of hemoglobin synthesis in cell-free extracts of reticulocytes has been shown to involve only initiation. Neither the rate of chain elongation on the polysomes nor release of completed chains from the polyribosomes is changed under conditions in which the overall rate of protein synthesis varies considerably (24).

The data obtained in the present study could also be explained by a control of chain initiation on mitochondrial ribosomes by proteins made in the cytoplasm. Such a translational control mechanism would be expected if mitochondria contain long-lived mRNAs. Furthermore, the factors which have been shown to control initiation in mammalian systems are specific so that translational of only certain mRNAs is enhanced.

Alternately, the rate of mitochondrial protein synthesis may be regulated at the transcriptional level as is the case in bacteria (25,26). According to this mechanism, products of cytoplasmic protein synthesis would function as activators to the transcription of certain specific mRNAs from mitochondrial DNA. Such controls would function effectively in a system containing mRNAs with a rapid turnover. In this context, it is of considerable interest that a protein synthesized in the cytoplasm and coded for in the nucleus appears to be absolutely necessary for the synthesis of one subunit of cytochrome oxidase in the mitochondria (27). Perhaps, this protein acts by regulating the transcription of a certain specific mRNA for this subunit in the mitochondrion.

An alternative interpretation for the increase in protein synthesis and polysome content observed after growth in chloramphenicol was previously proposed by Hoober and Blobel (28) to explain similar data obtained with chloroplast ribosomes. Immediately after growth of Chlamydomonas for 10 to 60 minutes in chloramphenicol, a decrease in the 68S monosomes was observed combined with an increase in polysomal material. Perhaps, chloramphenicol causes polysomes to accumulate by preventing chain elongation but not chain initiation. Although this explanation cannot be completely ruled out at the present time, the results of several experiments make it seem unlikely. In our experiments the cells were allowed to grow for one hour in fresh medium after washing out the chloramphenicol. Mitochondria isolated at this time are fully capable of active protein synthesis. Furthermore, decreased rates of protein synthesis and polyribosome content were observed after growth in cycloheximide which specifically blocks cytoplasmic protein synthesis.

In this paper, we have also reported the isolation of intact mitochondrial polysomes which are active in protein synthesis in the absence of exogenous mRNA. Either labeled leucine or an amino acid mixture were incorporated into the nascent polypeptide chains present on the isolated polysomes. The sensitivity of this reaction to chloramphenicol and its resistance to cycloheximide indicate that the isolated mitochondrial polysomes are not significantly contaminated with

cytoplasmic ribosomes. Polysomes active in protein synthesis were previously obtained from Euglena mitochondria (29).

The polysome to monosome ratio was dramatically increased when mitochondrial polysomes were isolated from yeast cells which had been previously grown in chloramphenicol. Such an increase is a reflection of greater protein synthetic actitivity and might result from either a larger mRNA pool or increased rates of polypeptide chain initiation. The 3-fold greater rate of leucine incorporation observed in the isolated polysomes from the chloramphenicol-treated cells indicates that there are indeed a significantly greater number of nascent chains on these polysomes. Polysomes isolated by this method and active in protein synthesis may be useful for the isolation and characterization of mitochondrial mRNAs.

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IV. THE SENSITIVITY OF RAT LIVER AND YEAST MITOCHONDRIAL RIBOSOMES TO INHIBITORS OF PROTEIN SYNTHESIS

LITERATURE REVIEW

Several groups have shown that the mitochondrial ribosome from animal cells has a sedimentation coefficient in the range of 55 to 60S (1). The observation that labeled nascent protein chains were associated with these particles in lysates of purified mitochondria suggested that these small ribosomes were actually the ribonucleoprotein particle involved in mitochondrial protein synthesis (2-5). More convincing evidence for the role of this small ribosome in protein synthesis was obtained by Swanson and Dawid (6), who demonstrated the poly(U)-dependent synthesis of polyphenylalanine using the 60S mitochondrial ribosome obtained from Xenopus eggs; however, the sensitivity of this protein synthesis to inhibitors was not examined. Subsequently, DeVries et al. (7) reported the isolation of rat liver mitochondrial ribosomes which retained peptidyltransferase activity, indicating that the 55S ribosome was able to catalyze this critical reaction of protein synthesis sensitive to inhibition by certain antibiotics. Recently, we reported the isolation of rat liver mitochondrial ribosomes highly active in poly(U)-dependent polyphenylalanine synthesis in a cell-free system (8). Greco et al. (9) also have suceeded in the isolation of ribosomes from rat liver mitochondria which are active in protein synthesis.

A phylogenetic difference between the mitochondrial

ribosome of mammals and yeast has been claimed on the basis of sensitivity to erythromycin (10,11). Amino acid incorporation by isolated yeast mitochondria is strongly inhibited by the macrolides erythromycin, carbomycin, and lincomycin (12), while incorporation by intact rat liver mitochondria is completely resistant to erythromycin and lincomycin but is inhibited by very low concentrations of carbomycin. This difference has been attributed by Linnane's groups (10,11) to a change in the mitochondrial ribosome during evolution so that the 55S ribosomes of mammalian mitochondria can still bind erythromycin, although peptide bond formation is not affected. In contrast, Kroon and DeVries (13) have suggested that the lack of sensitivity of mammalian ribosomes to erythromycin results from a selective permeability barrier in the mitochondrial membrane since erythromycin inhibited incorporation in swollen liver mitochondria. The present study was undertaken in an attempt to distinguish clearly between these two alternatives. Protein synthesis on isolated ribosomes from both rat liver and yeast mitochondria was inhibited to the same extent by three different inhibitors: carbomycin, chloramphenicol, and erythromycin. The binding of [^{14}C]chloramphenicol to yeast mitochondrial ribosomes was prevented to the same extent by erythromycin, carbomycin and lincomycin as was the binding to Escherichia coli ribosomes; however, all three antibiotics were less effective inhibitors of the binding of [^{14}C]chloramphenicol to rat liver mitochondrial ribosomes.

MATERIALS AND METHODS

Growth of Escherichia coli and Preparation of Ribosomes:

An E. coli mutant (Q_{13}) which is low in ribonuclease (obtained from F.L. Chang, Temple University, Philadelphia, Pa.) was grown in a liquid medium containing 8 g of nutrient broth, 5 g of peptone, 5 g of NaCl and 1 g of glucose per liter. The cells were harvested at late log phase and ground vigorously with alumina in a chilled mortar and pestle in the cold room. As the cells underwent breakage, additional alumina was added slowly. After 10 min of grinding 60 ml of freshly prepared buffer containing 10 mM Tris-Cl, pH 7.8, 14 mM magnesium acetate, 60 mM KCl, and 6 mM mercaptoethanol were added to the mortar. The subsequent preparation of ribosomes and S-100 fraction was exactly as described by Nirenberg (14).

Preparation of Ribosomes from Rat Liver Mitochondria:

Rat liver mitochondria were prepared from 40 to 60 Sprague-Dawley rats weighing 125 to 150 g in 0.34 M sucrose containing 5 mM Tris-Cl, pH 7.6, by the procedure of O'Brien and Kalf (2). Sterility of glassware and solution was maintained at all steps. The mitochondrial pellet obtained after seven washes at 3500 x g was resuspended at a concentration of 5 mg per ml in Medium A (0.1 M KCl, 5 mM Tris-HCl, pH 7.6, and 30 mM $MgCl_2$). The mitochondria were lysed by the addition of 0.1 volume of 10% Triton X-100. Deoxyribonuclease, free of ribonuclease, then was added at a fin-

al concentration of 5 μg per ml and the solution was kept on ice for 5 min. The suspension then was centrifuged for 10 min at 60,000 x g in the Spinco 30 rotor. The resultant supernatant was layered on 2 ml of Medium A containing 24% sucrose and centrifuged for 3 hours at 230,000 x g in the Spinco 65 rotor. The crude ribosomal pellet was resuspended in 2 ml of Medium A containing 6 mM mercaptoethanol and centrifuged at 3,000 x g for 5 min. The supernatant was layered on a 10 to 30% sucrose gradient containing Medium A supplemented with 6 mM mercaptoethanol. After 16 hours at 20,000 x g in the Spinco SW 27 rotor, 1 ml fractions were collected and the absorbance at 260 nm was measured for each fraction.

The fraction corresponding to the mitochondrial ribosomes was diluted with medium and centrifuged at 230,000 x g for 3 hours in the Spinco 65 rotor. The pellet containing ribosomes was resuspended in the same medium. The absorbance ratio (A_{260}:A_{280}) of the purified ribosomes was 1.1:1.4.

Preparation of Yeast Mitochondrial Ribosomes:

A diploid wild type strain of Saccharomyces cerevisiae was grown in liquid medium (15) containing 1% glucose as carbon source. The cells were harvested at late log phase and suspended in water at a concentration of 1 g per ml. Then, 0.5 ml of thioglycolic acid (57 mg per ml) and 0.5 ml of 0.1 M EDTA were added per ml of medium and the pH of the suspension was adjusted to pH 7.0 with NaOH. The suspension was incubated at 33° for 30 min with gentle stirring

and then centrifuged at 3,000 x g for 5 min. The pellet was resuspended at a concentration of 1 g per ml of cells in a medium containing 1.8 M sorbitol, 0.25 M citrate-phosphate buffer, and 1.0 ml of Glusulase (Endolaboratories) per 3 g of cells as described by Kovacs et al. (16). The mixture was incubated at 33° for 10 to 20 min with gentle stirring. The conversion to spheroplasts was monitored by measuring the absorbance at 600 nm. The digestion was stopped by chilling the suspension as soon as the turbidity decreased to a value 20% of the initial value. The spheroplasts were collected by centrifugation at 3,000 x g for 10 min. The cells were washed twice with medium containing 1.5 M sorbitol, 10 mM Tris-malate, pH 6.7, and 0.1 mM EDTA. The protoplasts then were resuspended in Medium B (0.44 M mannitol, 5 mM Tris-Cl, pH 6.7, and 0.1 mM EDTA) at a concentration of 0.2 g per ml. The suspension was dispersed two times in a Waring Blendor at high speed for 20 s. The cellular debris and nuclear fragments were removed by two centrifugations at 2,000 x g for 5 min. Mitochondria were sedimented by centrifugation at 12,000 x g for 10 min and the pellet was washed four times by resuspension in Medium B. The mitochondrial ribosomes were isolated as described by Grivell et al. (17).

Preparation of Mitochondria for Amino Acid Incorporation:

Rat liver mitochondria were prepared under sterile conditions in 0.25 M sucrose containing 10 mM Tris-Cl, pH 7.4, and 1.0 mM EDTA as previously described. Inner membrane-matrix

fractions were prepared with digitonin (18). Yeast mitochondria were prepared from cells in late log phase in Medium C (0.25 M mannitol, 10 mM Tris-Cl, pH 7.4, and 1.0 mM EDTA) as described previously (19).

Protein Synthesis:

Amino acid incorporation was assayed in rat liver mitochondria (20) and yeast mitochondria (19) by previously described methods. Cell-free protein synthesis was assayed by the method described by Ibrahim and Beattie (8).

Binding of [^{14}C]Chloramphenicol to Ribosomes:

Radioactive chloramphenicol was added at a final concentration of 3.0 μM to a 2.0 ml suspension of ribosomes (0.5 mg per ml) in 0.2 M KCl containing 10 mM magensium acetate and 10 mM Tris-Cl, pH 7.6. In the experiments with rat liver mitochondria, the concentration of magensium acetate was raised to 20 mM. After a 5 min incubation at 4°, the suspension was centrifuged at 150,000 x g for 80 to 100 min. The pellet was rinsed four times with 2.0 ml of buffer and then suspended in 1.0 ml of water for determination of radioactivity. In the controls, 0.3 mM unlabeled chloramphenicol was present during the incubation and 3.0 μM [^{14}C]chloramphenicol was added immediately before centrifugation. The radioactivity in these tubes provided a correction for the [^{14}C]chloramphenicol present in fluid trapped in the ribosomal pellet (21).

Materials:

Sucrose (ribonuclease-free) was obtained from Mann, poly(U) from Miles Laboratories; erythromycin, chloramphenicol and other nucleotides from Sigma; lincomycin and sparsomycin from Upjohn; rifampicin and valinomycin from Calbiochem and actinomycin D from Merck, Sharpe and Dohme. Uniformly labeled [^{14}C]leucine (270 mCi per mmole) was obtained from Amersham-Searle, 1,2-[^{14}C]chloramphenicol (5 mCi per mmole) and uniformly labeled [^{14}C]phenylalanine (460 mCi per mmole) from New England Nuclear. Carbomycin was a generous gift from Pfizer.

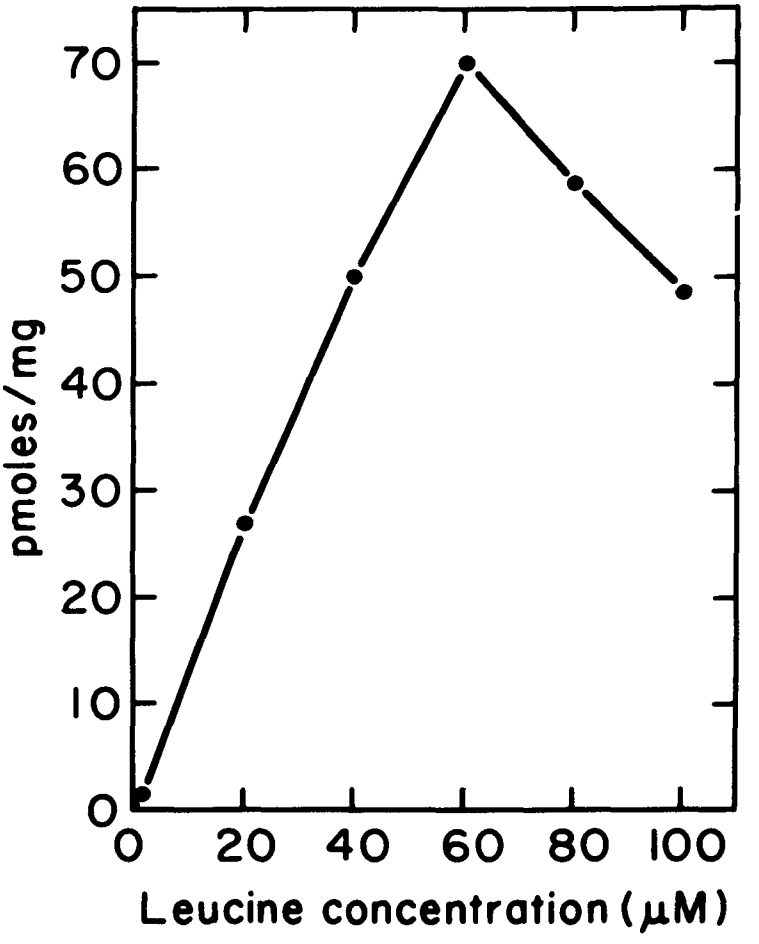
RESULTS

Effects of Antibiotics on Amino Acid Incorporation by Isolated Mitochondria:

To study the effect of various inhibitors of mitochondrial protein synthesis, it is necessary to study amino acid incorporation under optimal conditions. In a previous study (20) we explored the ionic and energy requirements for maximum rates of incorporation. The concentration dependence of leucine also has been investigated to ensure that the substrate amino acid is not rate-limiting for a reaction occurring on the mitochondrial ribosome. As seen in Fig. 12, an optimal rate of amino acid incorporation was observed at a leucine concentration of 60 μM. A slight decrease in the incorporation rate was observed at higher concentrations of leucine.

FIG. 12. Effect of leucine concentration on amino acid incorporation by rat liver mitochondria. Incubation medium contained in a final volume of 2 ml: 50 mM Bicine (N, N-bis(2-hydroxyethyl) glycine obtained from Sigma) buffer, pH 7.6, 90 mM KCl, 10 mM $MgCl_2$ 1 mM EDTA, 5 mM phosphate, pH 7.6, 5 mM P-enolpyruvate, 2 mM ATP, 10 µg of pyruvate kinase, 22.5 µg of a complete amino acid mixture leucine, the appropriate amount of leucine containing 0.5 μC_i of uniformly labeled [^{14}C]leucine and 6 to 8 mg of mitochondrial protein. After 20 min at 30° in a metabolic shaker, the reaction was terminated by the addition of trichloroacetic acid to a final concentration of 5% and the proteins prepared for counting as previously described (23).

FIG. 12


70
60
50
40
30
20
10
0
pmoles/mg
0
20
40
60
80
100
Leucine concentration (μM)

The effects of various inhibitors of protein synthesis on amino acid incorporation by rat liver and yeast mitochondria are listed in Table 10. Low concentrations of chloramphenicol, carbomycin, and sparsomycin inhibited completely the incorporation by intact mitochondria, while a 0.75 mM concentration of emetine inhibited by nearly 60% the incorporation rate. In contrast, the addition of erythromycin and lincomycin had no effect on amino acid incorporation by intact rat liver mitochondria.

One possible explanation for the inability of certain antibiotics to inhibit protein synthesis is that the mitochondrial membrane acts as a permeability barrier to the drug. The inner membrane-matrix fraction prepared by use of digitonin (18) has altered permeabilities to many substances and yet is still active in amino acid incorporation (22). Both macrolides, erythromycin and lincomycin, caused a 70% inhibition of amino acid incorporation in the inner membrane-matrix fraction. In addition, emetine was an effective inhibitor of incorporation in this fraction at much lower concentrations than in intact mitochondria. Similar inhibitory effects of erythromycin, carbomycin, and emetine were obtained when the inner membrane-matrix fraction was incubated in the presence of valinomycin when maximum rates of amino acid incorporation are obtained (20).

The various inhibitors also were tested in rat liver mitochondria which had been suspended in 2.5 mM sucrose at 0° at

TABLE 10

Effect of Various Antibiotics on Amino Acid Incorporation by Rat Liver and Yeast Mitochondria

Inhibitor	Mitochondria (40.0)	Inner membrane (48.6)	Inner membrane + valinomycin (191)	Hypotonic mitochondria (8.6)	Yeast mitochondria (36.3)
µg/ml	%Inhibition				
Chloramphenicol					
20	44		69	40	75
60	84		82	65	86
Carbomycin					
2	47	42	33		
20	78	73			
30	90	80	81		
Erythromycin					
25	0	19	18	0	40
50	0	42	41	0	90
100	0	67	70	0	
Lincomycin					
10	0	10		0	
25	0	34		0	
500	0	59		64	
Emetine					
150	0	52			11
300	29	75	78		35
500	58	82	82		
Sparsomycin					
0.1	35				
1.0	90				

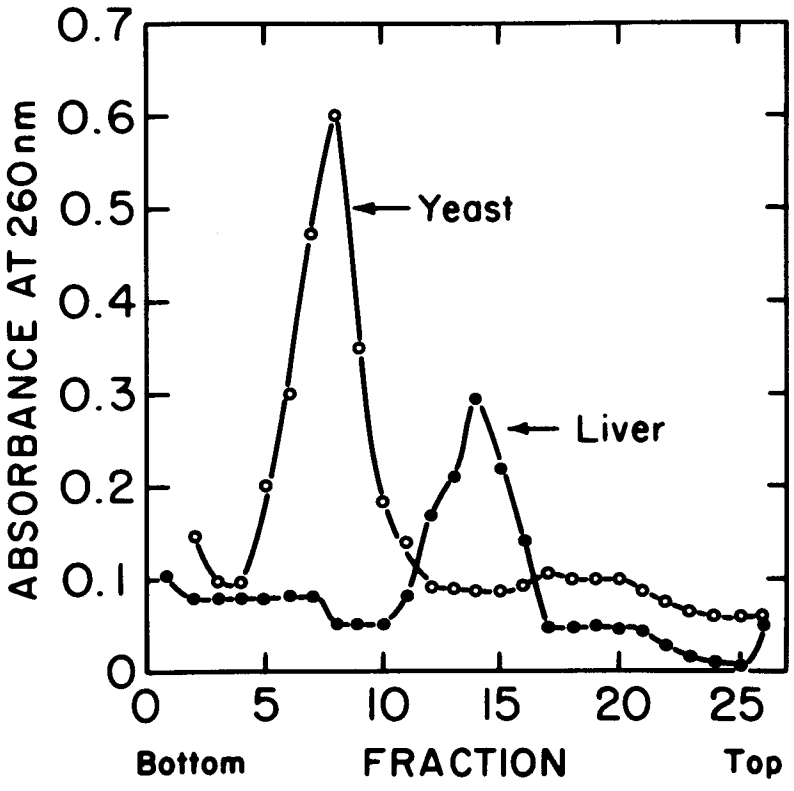
Intact rat liver or yeast mitochondria, the inner membrane-matrix fraction from rat liver mitochondria and liver mitochondria treated with hypotonic sucrose, were incubated as described in the legend to Fig. 12 with the various inhibitors. A small zero time control was substracted from each value. The numbers in parentheses represent the control values in picomoles per mg.

a concentration of 10 mg per ml for 20 min before addition to the incubation medium (10,13). Treatment with hypotonic sucrose may increase the permeability of the mitochondrial membrane to certain drugs (13). As seen in Table 10, the incorporation rate in mitochondria treated this way was only 25% of that observed in mitochondria resuspended in 0.25 M sucrose. Chloramphenicol inhibited amino acid incorporation by these mitochondria to the same extent as in the intact mitochondria, while erythromycin and lincomycin had no effect.

These results indicate that protein synthesis on rat liver mitochondrial ribosomes has retained sensitivity to the macrolides erythromycin and lincomycin. However, it is imperative that amino acid incorporation be studied in mitochondria which have been treated so as to lose some permeability barriers to various drugs and yet maintain an incorporation rate equal to or greater than that of the intact mitochondria. The effects of the various inhibitors also were tested on amino acid incorporation by isolated yeast mitochondria. The data of Table 10 indicate that much lower concentrations of erythromycin inhibit protein synthesis in isolated yeast mitochondria than are effective with the inner membrane-matrix fraction obtained from rat liver mitochondria. For example, addition of 50 μg per ml of erythromycin caused a 90% inhibition in yeast mitochondria but only a 40% inhibition in the inner membrane fraction. The maximum inhibition by erythromycin observed with liver mitochondria was only 70%

FIG. 13. Sucrose density gradient centrifugation of ribosomes isolated from rat liver and yeast mitochondria as described under "Experimental Procedure." The yeast mitoribosomes (o——o) sediment at 74S, while the rat liver mitoribosomes (●——●) sediment at 55S.

FIG. 13


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE AT 260nm
Yeast
Liver
0
5
10
15
20
25
Bottom
FRACTION
Top

even with much greater concentrations of erythromycin.

This difference in sensitivity to the macrolides of rat liver and yeast mitochondria may result because a change in the properties of the mitochondrial ribosome has occurred so that certain macrolides are less effective inhibitors of protein synthesis, as suggested by Linnane's group. An alternative explanation might be that the inner membrane-matrix fraction has retained some permeability barriers to these drugs so that the internal concentration of the drug is much lower than that of the medium. A clear choice between these two explanations can be obtained only by comparing the antibiotic sensitivities of isolated ribosomes from both types of mitochondria in a cell-free system for protein synthesis.

The sedimentation profile of ribosomes extracted from rat liver mitochondria is compared with that of ribosomes extracted from yeast mitochondria (Fig. 13). The rat liver mitoribosomes sediment at 55S with almost no contaminating material of higher buoyant density. As discussed in a previous publication (8), the concentration of Mg^{2+} in the gradient was maintained at 20 mM to prevent dissociation of the 55S ribosomes to the two subunits of 39S and 28S. As seen in Fig. 13, the yeast mitoribosomes sediment at 74S and clearly can be differentiated from the rat liver mitoribosomes in the gradient. As seen in Fig. 14, the liver mitoribosomes also can be distinguished from the bacterial 70S ribosomes. Tubes 16 to 20 of the gradient of Fig. 14 were pooled and this fraction was used for all further studies

FIG. 14. Sucrose density gradient centrifugation of ribosomes isolated from rat liver mitochondria and Escherichia coli as described under "Experimental Procedure." The rat liver mitoribosomes sediment (●——●) at 55S, while the E. coli ribosomes (o——o) sediment at 70S. The shaded area represents the tubes which were pooled for the studies of rat liver mitochondria.

FIG. 14

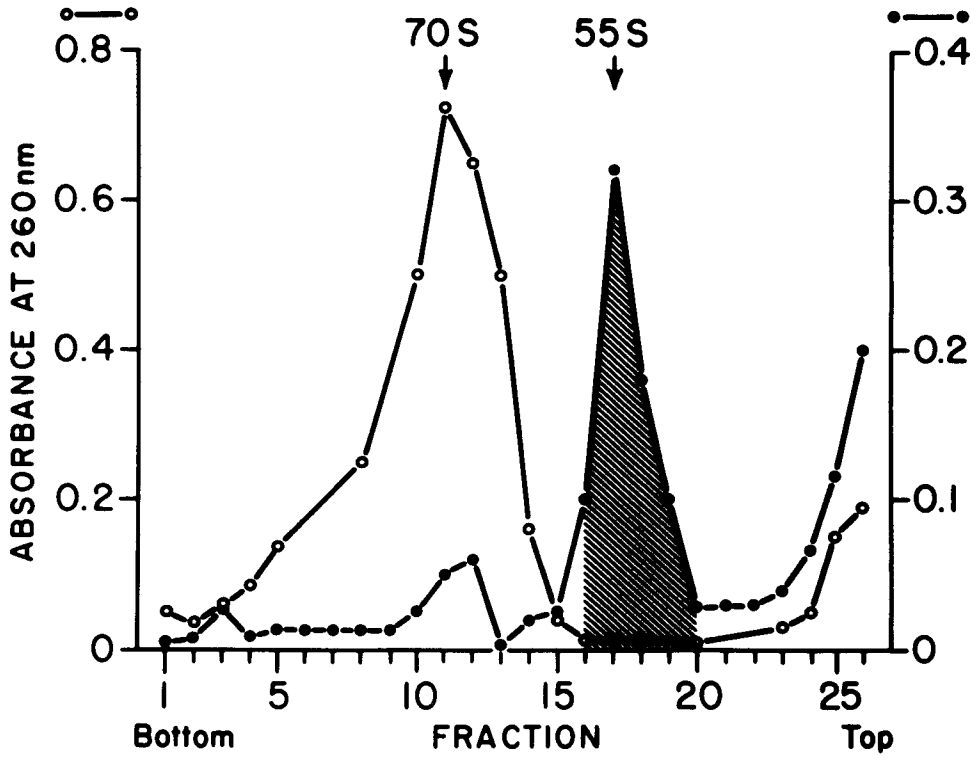

70S
55S
0.8
0.6
0.4
0.2
0
0.4
0.3
0.2
0.1
0
ABSORBANCE AT 260nm
1
5
10
15
20
25
Bottom
FRACTION
Top

TABLE 11

Optimal Conditions for Poly(U)-directed Protein Synthesis on Mitochondrial Ribosomes

	Liver	Yeast
	pmoles/mg RNA/15 min	
Complete (plus *Escherichia coli* S-100)...	835	554
Minus ribosomes	44.6	
Minus poly(U)	9	
Minus supernatant factors	75	
Minus ATP, P-enolpyruvate, and pyruvate kinase	8	
Plus mitochondrial S-100 (in place of *E. coli* S-100)	458	84

Ribosomes isolated from either rat liver or yeast mitochondria were incubated in 0.25 ml of medium containing 10 mM Tirs-HCl, pH 7.8, 50 mM KCl, 20 mM magnesium acetate, 25 µM amino acid mixture (18), 30 µM GTP, 1 mM ATP, 6 mM mercaptoethanol, 5 mM P-enolpyruvate, 25 µg of pyruvate kinase, 1 mM dithiothreitol, 250 µg of poly(U), and 50 µM [^{14}C]phenylalanine in the presence of 250 µg of a supernatant fraction from *E. coli* (11). Mitochondrial ribosomes were added at a concentration of 100 µg of RNA. After 15 min at 30°, the proteins were precipitated with 5% trichloroacetic acid and prepared for counting. A small zero time value of 10 pmoles per mg of RNA per 15 min was substracted from each value.

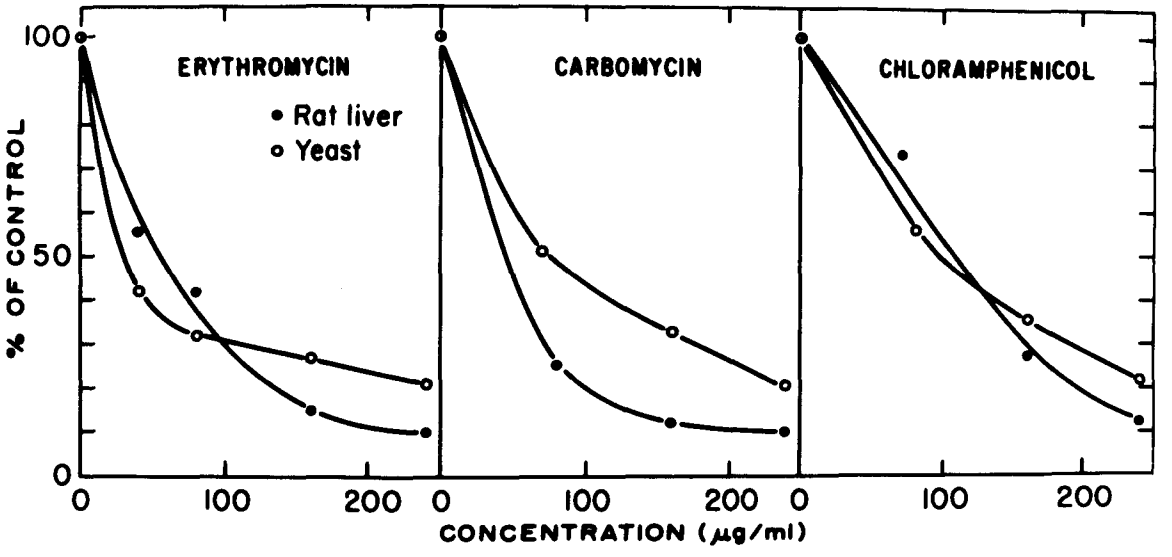
involving rat liver mitochondrial ribosomes. Hence, the slight amount of contaminating material of bouyant density greater than 55S was not used. It is unlikely that this material represents bacterial contamination since our mitochondrial preparations contain less than 100 bacterial colonies per ml of a mitochondrial suspension containing at least 10 mg of protein.

Both liver and yeast mitoribosomes isolated in this way were highly active in cell-free protein synthesis measured with poly(U) and phenylalanine. The extent of incorporation was dependent on the addition of poly(U), supernatant factors, an energy source and ribosomes (Table 11). The S-100 fraction obtained from Escherichia coli was more effective in supporting protein synthesis in the cell-free system than was a similar fraction obtained from the respective mitochondria. Both Grivell et al. (17) and Scragg (22) had previously observed that optimal rates of protein synthesis on mitoribosomes from yeast were obtained with a supernatant fraction from E. coli.

The inhibitory effects of different concentrations of erythromycin, carbomycin, and chloramphenicol were tested in the cell-free system with both rat liver and yeast mitoribosomes. As seen in Fig. 15, polyphenylalanine synthesis on the two different ribosomes was inhibited to the same degree by all concentrations of the three inhibitors. The one slight exception is carbomycin which at low concentra-

FIG. 15. Inhibition of polyphenylalanine synthesis in a cell-free system by erythromycin, carbomycin, or chloramphenicol. Ribosomes isolated from rat liver (●——●) or yeast (o——o) mitochondria were incubated as described in the legend to Table 11 with different concentrations of the inhibitors. Control value for ribosomes from rat liver mitochondria was 356 pmoles per mg of RNA and that from yeast mitochondria was 302 pmoles per mg of RNA.

FIG. 15


100
50
0
% OF CONTROL
ERYTHROMYCIN
CARBOMYCIN
CHLORAMPHENICOL
• Rat liver
o Yeast
0
100
200
CONCENTRATION (μg/ml)

tions was a more effective inhibitor of protein synthesis on the liver mitoribosomes than on the yeast mitoribosomes.

The binding of radioactive chloramphenicol to the purified ribosomes obtained from both liver and yeast mitochondrial ribosomes was compared to that obtained with E. coli ribosomes. As seen in Table 12, the amount of chloramphenicol quantitatively bound to each type of ribosome varied over a 2.5-fold range but was in the same order of magnitude. Addition of either erythromycin or carbomycin almost completely prevented the binding of chloramphenicol to the ribosomes from E. coli or yeast mitochondria. Lincomycin blocked by 75% the binding of chloramphenicol to the yeast mitochondrial ribosomes, but only by 43% that to the E. coli ribosomes. The effect of the different macrolides on the binding of chloramphenicol to rat liver mitoribosomes indicated that these ribosomes do differ from yeast mitoribosomes. Carbomycin was the only antibiotic which effectively prevented the binding of chloramphenicol to the ribosomes isolated from rat liver mitochondria. The effects of lincomycin were similar to those observed with E. coli ribosomes; however, erythromycin was a very ineffective inhibitor of chloramphenicol binding to liver mitochondrial ribosomes.

DISCUSSION

The present study was undertaken to determine whether protein synthesis on rat liver mitochondrial ribosomes is

TABLE 12

Binding of [^{14}C]chloramphenicol to Isolated Ribosomes: Inhibition by Macrolides

Source of ribosomes	*Escherichia coli*		Yeast Mitochondria		Rat liver mitochondria	
	Cpm/mg[a]	%Inhibition	Cpm/mg[a]	%Inhibition	Cpm/mg[a]	%Inhibtion
Control	472		1130		694	
+Carbomycin	176	-63	184	-84	228	-67
+Lincomycin	270	-43	288	-75	456	-34
+Erythromycin ...	78	-84	8	-99	600	-14

The binding of [^{14}C]chloramphenicol (3.0 µM) to isolate ribosomes was tested as described by Vazquez (21) and under "Experimental Procedure." Carbomycin, erythromycin, and lincomycin were added to a final concentration of 0.09 mM. Each value is corrected for a small control value obtained when 3.0 mM unlabeled chloramphenicol was present with the labeled chloramphenicol during the incubation.

sensitive to the macrolides, erythromycin, and lincomycin, as is protein synthesis on yeast mitochondrial ribosomes. Initially, the inhibitors were tested both in intact rat liver mitochondria and in an inner membrane-matrix fraction prepared by the use of digitonin. The latter fraction had been shown previously (23) to catalyze amino acid incorporation at rates similar to those obtained with intact liver mitochondria provided that ATP and a regenerating system were used as a source of energy. An exogenous energy source also has advantages in inhibitor studies, as an interference of the drug with ATP synthesis by oxidative phosphorylation can be ruled out. Both erythromycin and lincomycin inhibited amino acid incorporation only in the inner membrane-matrix fraction, while carbomycin inhibited incorporation equally well in the intact mitochondria. These results are in essential agreement with those of Kroon and DeVries (13) but in direct contrast to those reported by Towers et al. (10,11) with rat liver mitochondria and Williams and Birt (24) with blowfly muscle mitochondria. Both of these groups used mitochondria presumably made permeable to the drugs by treatment with hypotonic solutions and observed no inhibition by erythromycin or lincomycin. We have observed that mitochondria treated by incubation in hypotonic sucrose have been damaged so that the incorporation rates are only 25% of these obtained with the digitonin inner membrane fraction. For meaningful inhibition studies, it is essential to study amino acid incorporation under optimal conditions.

Inhibitor studies with isolated yeast mitochondria revealed that the amount of erythromycin necessary to inhibit the incorporation rate was almost one-half that needed for comparable inhibitions of incorporation by the inner membrane fraction. Furthermore, the maximum inhibition of incorporation in this fraction was approximately 70% even when the concentration of erythromycin was increased significantly. This differential sensitivity might result because of a change in the mitochondrial ribosome so that erythromycin is not bound as tightly to the ribosome and is hence a less effective inhibitor; alternatively, the inner membrane may still be somewhat impermeable to these drugs.

The isolation of mitochondrial ribosomes highly active in protein synthesis in a cell-free system has demonstrated clearly that there is no difference in the response of rat liver and yeast mitoribosomes to the inhibitors, chloramphenicol, carbomycin, and erythromycin. Polyphenylalanine synthesis by both ribosomes was inhibited to the same extent over a wide range of inhibitor concentrations. Lincomycin, however, was not an effective inhibitor of protein synthesis in the cell-free system with either ribosome. Hence, we can conclude that the decreased sensitivity of amino acid incorporation by the inner membrane fraction to erythromycin results from a permeability barrier to the drug in the inner membrane as the isolated ribosomes respond identically to all three inhibitors.

The activity of rat liver mitochondrial ribosomes in the cell-free system varied somewhat in different preparations. When the synthetic activity of a preparation was less than 200 pmoles per mg of RNA, erythromycin, carbomycin, and chloramphenicol were all much less effective inhibitors of protein synthesis. In order to ensure maximum rates of protein synthesis, ribosomes were frozen at -70° immediately after preparation and only thawed once prior to use. The source of supporting enzymes was also critical for maximum rates of protein synthesis. The supernatant factors obtained from E. coli supported protein synthesis on both rat liver and yeast mitochondrial ribosomes to a much greater extent than the corresponding supernatant factors obtained from either mitochondria. The E. coli S-100 fraction was also frozen at -70° immediately after preparation and only thawed once prior to use. Furthermore, it was imperative that at least 1 mg per ml of S-100 be added to each incubation tube.

The method we have used for the isolation of mitochondrial ribosomes active in protein synthesis differs significantly from that recently reported by Greco et al. (9). For example, they prepare mitochondria from the post-nuclear supernatant by centrifugation 10,000 x g. We have observed that the isolation of mitochondria at centrifugal forces greater than 7,000 x g, while it increases the yield of mitochondria substantially, resulted in significant contaminating material sedimenting at 80S. Furthermore, the medium used

in our studies to lyse the mitochondria as well as the sucrose gradient contained a 20 mM concentration of Mg^{2+} concentration of 10 mM; however, O'Brien (25) had demonstrated previously that high concentrations of Mg^{2+} prevented the dissociation of the mitochondrial monosome to its two subunits. Despite these differences the 55S mitochondrial ribosome isolated by Greco et al. (9) from the sucrose gradient possessed similar activity as our preparation of 55S ribosomes in the cell-free system for protein synthesis.

One significant difference between the ribosomes isolated from liver and yeast mitochondria was observed when the effects of the different macrolides on the binding of chloramphenicol was tested. Both erythromycin and carbomycin almost completely prevented the binding of radioactive chloramphenicol to yeast mitochondrial ribosomes, while only carbomycin effectively blocked the binding of chloramphenicol to liver mitochondrial ribosomes. In addition, it should be noted that the inhibitory effects of carbomycin were significantly lower with the liver mitoribosomes than with the yeast mitoribosomes. Erythromycin, however, only blocked by 15% the binding of chloramphenicol, despite the fact that protein synthesis on the same preparation of ribosomes was inhibited 70% by erythromycin. Hence, the affinity of the 55S rat liver mitoribosomes for erythromycin may be much lower than the affinity of yeast mitoribosomes. A similar conclusion was reached by DeVries et al. (26) who studied the peptidyltransferase reaction of isolated rat liver mitoribosomes. Very high con-

centrations of erythromycin were necessary to reverse the chloramphenicol inhibition of the reaction. Extensive studies (27) with E. coli ribosomes have indicated that chloramphenicol, lincomycin, and erythromycin act at closely related sites on the 50S subunit; however, significant differences in the binding sites for the three different antibiotics were observed. Perhaps the binding sites for these antibiotics to liver mitochondrial ribosomes also may have significant differences which may be responsible for the inability of the macrolides to prevent the binding of chloramphenicol to these ribosomes.

Another significant difference in the response of 55S bovine mitochondrial ribosomes to the antibiotic lincomycin as compared to bacterial ribosomes was recently reported (28). Nearly 100-fold higher concentrations of lincomycin were necessary to inhibit partially the "fragment reaction" catalyzed by the 55S ribosomes than were necessary to inhibit completely the reaction catalyzed by the 70S bacterial ribosomes.

The results of this paper indicate that mitochondrial protein synthesis, especially when assayed in the inner membrane-matrix fraction, is sensitive to emetine confirming several previous reports (29-31). Originally, Grollman (32) had observed that emetine, a compound which is structurally similar to cycloheximide, also resembled cycloheximide in its mode of action on 80S cytoplasmic ribosomes.

Furthermore, he reported that emetine was not an inhibitor of protein synthesis on bacterial ribosomes. The inhibition of mitochondrial protein synthesis by emetine may suggest a similarity between cytoplasmic and mitochondrial ribosomes not shared by bacterial ribosomes. In addition, these results suggest that emetine should be used with some caution as a selective inhibitor of cytoplasmic protein synthesis in studies of mitochondrial biogenesis in whole cells (33).

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ABBREVIATIONS

A	adenosine
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
ATPase	adenosine triphosphatase
c	cytosine
cpm	counts per minute
°C	degree centrigrade
dalton	The mass of 1 hydrogen atom.
DNA	deoxyribonucleic acid
DTE	dithioerythreitol
EDTA	ethylenediamine tetracetric acid
F_1	soluble adenosine triphosphatase
f-Met-tRNA	formyl-methionyl-transfer RNA
G	guanosine
g	force of gravitation
gm	gram
hr	hours
i.m.	inner membrane
M	molar (mole/liter)
mg	milligram
met	methionine
min	minutes
ml	milliliter
mM	millimolar (millimole/liter)
mRNA	messenger ribonucleic acid
M.W.	molecular weight

n	normal
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
nm	nanometer
OSCP	oligomycin sensitivity conferring protein
poly(A)	poly(adenylic acid)
poly(U)	poly(uridylic acid)
pMoles	picomoles
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
rRNA	ribosomal RNA
tRNA	transfer ribonucleic acid
r.p.m.	revolutions per minute
s	seconds
SDS	sodium dodecylsulfate
S	sedimentation coefficient (Svedberg units)
T	thymidine
TCA	trichloroacetic acid
Tris	tris(hydroxymethyl)aminomethane
U	uridine
UDP	uridine diphosphate
μ	micron
μC_i	microcurie
μg	microgram
x g	times gravity