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MITOCHONDRIAL PROTEIN SYNTHESIS: A DESCRIPTION OF THE OPTIMAL CONDITIONS FOR STUDY *IN VITRO*, DRUG SENSITIVITIES, AND PRODUCTS

by

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ABSTRACT

MITOCHONDRIAL PROTEIN SYNTHESIS: A DESCRIPTION OF THE OPTIMAL CONDITIONS FOR STUDY IN VITRO, DRUG SENSITIVITIES, AND PRODUCTS

by

JAMES P. BURKE

Advisor: Dr. Diana S. Beattie

The concentrations of KCl, phosphate, leucine, and ATP necessary to obtain maximum rates of leucine incorporation into protein by isolated rat liver mitochondria or inner membrane-matrix fractions in the presence of an ATP regenerating system were determined. The substitution of NaCl or NH_4Cl for part or all of the KCl resulted in decreased rates of amino acid incorporation. Almost identical incorporation rates were observed when ATP or ADP and succinate replaced ATP and the regenerating system. Under these conditions, antimycin A caused a 90% inhibition, suggesting that ATP was largely synthesized by respiratory chain linked phosphorylations. In contrast, atractyloside inhibited 70% the incorporation when ATP and the regenerating system were present, suggesting that the ATP necessary for protein synthesis under these conditions had been previously transported across the membrane by the atractyloside sensitive adenine translocase.

Chloramphenicol, sparsomycin, and carbomycin inhibited amino acid incorporation by intact mitochondria, while lincomycin, erythromycin, and emetine had no effect. However, amino acid incorporation in isolated inner membrane-matrix fractions was found to be inhibited by lincomycin, erythromycin, and emetine indicating that the mitochondrial membrane was a permeability barrier to these drugs and prevented their inhibitory effects on protein synthesis. Inhibitors of RNA synthesis, such as cordycepin and rifampicin, caused significant inhibition of protein synthesis, indicating a relatively short half-life of mitochondrial mRNA.

Rat liver mitochondria were incubated *in vitro* with (^{14}C)leucine and fractionated by several methods including extraction with acetic acid and Lubrol, extraction with phosphate, pH 11.0, freezing and thawing with KCl, and sonication. Separation of the insoluble membrane fractions by sodium dodecyl sulfate gel electrophoresis revealed three radioactive bands, corresponding to molecular weights of 40,000, 27,000, and 20,000. Electrophoresis for longer periods of time at higher concentrations of acrylamide revealed eight labeled bands, ranging in molecular weights from 48,000 to 12,000. Labeling of the bands was completely inhibited by chloramphenicol but was unaffected by cycloheximide.

Liver mitochondria were labeled *in vivo* by intraperitoneal injection of (^{3}H)leucine fifteen minutes after sufficient injection of cycloheximide to block microsomal protein synthesis. Gel electrophoresis of membrane fractions prepared

as described above revealed the same profile as those labeled *in vitro*. The results suggest that isolated rat liver mitochondria incorporate amino acid into the same protein species which are synthesized *in vivo* by mitochondria.

Approximately 16% of the total leucine incorporated into protein by isolated rat liver mitochondria *in vitro* could be extracted by chloroform:methanol. Gel electrophoresis of the chloroform:methanol extract revealed counts in several bands but the majority of counts were in the 40,000 molecular weight region. Lyophilization of the submitochondrial particles before extraction with chloroform:methanol yielded a fraction which separated after gel electrophoresis into three broad bands in the low molecular weight region of 14,000 to 10,000 with insignificant counts in the higher molecular weight regions.

Yeast cells were pulse labeled *in vivo* with (^{3}H)leucine in the presence of cycloheximide and the submitochondrial particles extracted with chloroform:methanol. The extract separated after gel electrophoresis into four labeled bands ranging in molecular weights from 52,000 to 10,000. Preincubation of the yeast cells with chloramphenicol prior to the pulse labeling caused a six-fold stimulation of labeling into the band of lowest molecular weight suggesting that the accumulation of mitochondrial proteins synthesized in the cytoplasm, when chloramphenicol is present, may stimulate the synthesis of certain specific mitochondrial proteins.

FOREWORD

The author wishes to express his sincerest gratitude to Dr. Diana S. Beattie for her initiation of the problem and guidance in this research. He also wishes to acknowledge the help of Dr. Robert Stuchell, who aided in the growth and isolation of yeast cells.

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I. PURPOSE OF THIS INVESTIGATION

Various aspects of mitochondrial biogenesis have intrigued investigators since mitochondria were first discovered in the 19th Century. For many years, this problem was studied by cytologists using both light and electron microscopy; however, the discovery of nucleic acids unique to mitochondria plus the observation that mitochondria could synthesize protein has been the stimulus for much biochemical research in this area during the past ten years. In the present study, several parameters of mitochondrial biogenesis have been examined:

1. Optimal conditions to measure mitochondrial protein synthesis in vitro have been determined. A maximum rate of protein synthesis was necessary for meaningful studies in the following areas.

2. The effect of various antibiotics on amino acid incorporation by isolated mitochondria has been studied. In this way, it was hoped to compare the mitochondrial ribosome of liver and yeast in terms of the response to different inhibitors with a known mechanism of action.

3. The number and nature of the proteins synthesized by the mitochondria has been investigated using several fractionation procedures and gel electrophoresis. These studies would enable us to make an estimate of the expression of the mitochondrial genome.

4. The synthesis of several proteolipids by isolated

mitochondria has been established. These very hydrophobic proteins may play a crucial role in the membrane formation.

II. INTRODUCTION

A. Conditions to Study Mitochondrial Protein Synthesis *In Vitro*

Investigators in many laboratories have published conditions necessary to study amino acid incorporation into protein by isolated mitochondria (1). The various published incubation media, however, differ both qualitatively and quantitatively (2,3,4). In one of the earliest studies, Roodyn, Reis, and Work (2) reported that washed rat liver mitochondria could incorporate amino acids into protein if incubated aerobically in the presence of oxidizable substrate, inorganic phosphate, AMP, magnesium ions, NAD^+, and a full complement of amino acids. The system was slightly stimulated by ribonuclease, did not require cell sap or pH 5 enzymes, and was dependent upon the maintenance of oxidative phosphorylation in the mitochondria. Replenishment of the medium during incubation considerably improved the duration of incorporation. Significant bacterial contamination was observed in the final mitochondrial fraction; however, these authors claimed that this contamination contributed negligible amounts to the observed incorporation which continued for three hours. In contrast, Sandell, Low, and Von Der Decker (3) reported

that amino acid incorporation by mitochondria isolated under sterile conditions was active for approximately 15 minutes at 30°C. Furthermore, the ability of these preparations to incorporate amino acids into protein was extremely low, although it could be enhanced somewhat by addition of EDTA. These workers concluded that isolated rat liver mitochondria, when free from contaminating bacteria, and obtained from adult rats, were not able to catalyze the incorporation of amino acids into protein at a reasonable rate.

This problem was reinvestigated by Wheeldon and Lehninger (5) who demonstrated that isolated mitochondria do indeed possess the ability to incorporate amino acids under conditions in which the contribution of contaminating bacteria had been minimized. They reported that protein synthesis in isolated rat liver mitochondria proceeds at a maximum rate in a medium containing 2.0 mM ATP, an ATP generating system, $MgCl_2$, KCl and L-proline at pH 7.5. ADP could not replace ATP, while AMP was inhibitory. Protein synthesis was linear for an hour.

Beattie, Basford, and Koritz (6) also reported that mitochondrial preparations essentially free of bacterial contamination (less than 100 bacteria per ml. of incubation medium) could incorporate amino acids into protein in the presence of adenine nucleotides, Mg^{++}, phosphate, a complete amino acid mixture, EDTA, and succinate. In a subsequent study (4), this group also demonstrated that an inner membrane-matrix fraction isolated by a digitonin procedure (7) could incorporate amino acids into protein. The requirements for this system

paralleled those for intact mitochondria except that an external ATP generating system was necessary. Bicine or Tricine buffer was found to be superior to Tris in both systems.

Recently, Hamberger, et al. (8) observed that leucine incorporation by heart and brain mitochondria from rabbit was critically dependent on the concentration of ATP and the buffer employed in the incubation media. Bicine buffer yielded optimal rates of amino acid incorporation while Tris and other buffers were found to depress leucine incorporation into protein. Also, NH_4^+ was found to produce a striking but transitory stimulation of the incorporation rate. Coote and Work (9), also reported that high concentrations of K^+ and NH_4^+ were needed to stimulate the incorporation of (^{14}C)leucine into protein of rat liver mitochondria. Under their experimental conditions, ATP produced by oxidative phosphorylation was more effective in supporting protein synthesis than ATP generated externally from phosphoenolpyruvate and pyruvate kinase; however, they did not detect any stimulation of the incorporation rate upon addition of excess proline, which had been previously reported to stimulate mitochondrial protein synthesis under some conditions (8).

In a recent study, Williams and Birt (10), have examined protein synthesis in mitochondria isolated from the Australian sheep blow fly (Lucinae cuprana). They reported that solutions containing NaCl or KCl were superior to sucrose as isolation media for the mitochondria. Magnesium ion was found to be an obligatory requirement for amino acid incorporation

and was most effective at a concentration of 18 mM. This was contrasted with results obtained from non-sterile systems (5) where considerable incorporation (20-25% of optimal) was obtained without magnesium ion. EDTA at a concentration of less than 2 mM produced a slight variable stimulation but was always found to inhibit strongly above 2 mM (e.g. 86% at 10 mM). Incorporation was maximum at a concentration of 0.1 mM leucine. The pH optimum for amino acid incorporation varied from 6.8 to 7.6; however a different pH optimum was observed in the ATP generating system as compared to a substrate supported system. The ratio of potassium to sodium ions in their incubation medium was found to affect the rate of amino acid incorporation into protein. As the ratio of potassium to sodium increased, incorporation increased markedly. In a subsequent paper (11), Williams and Birt also reported an absolute requirement for ADP or ATP for amino acid incorporation. Oxidizable substracts (pyruvate, D,L-glycerolphosphate, proline) or an ATP-generating system increased incorporation above that observed with adenine nucleotides alone.

In a similar study, Beattie and Ibrahim (12) recently reported that nearly identical rates of incorporation could be obtained if the necessary ATP was either generated by oxidative phosphorylation or by the addition of an external ATP generating system. In the latter case, it was first necessary for the ATP to be transported across the inner mitochondrial membrane by the adenine translocase (13). The addition of substances such as valinomycin (plus potassium), low

concentrations of calcium, or uncouplers of oxidative phosphorylation which effect the adenine translocase such that a higher concentration of intramitochondrial ATP results, caused a large increase in the rate of *in vitro* labeling.

The need for additional amino acids other than the radioactive amino acid varies from system to system and may reflect the presence of different pool sizes of amino acids in the various mitochondrial preparations. Beattie *et al*. (4) reported that the need for an amino acid mixture could be partially replaced by serine, proline, or methionine, suggesting that these amino acids might be rate limiting in rat liver mitochondria. As mentioned earlier, Wheeldon and Lehninger (5) reported only a need for added proline.

The major problem in evaluating the need for various components to obtain rates of mitochondrial protein synthesis *in vitro* is the complexity of the process. For example, there are many energy dependent steps in the overall sequence in which an external amino acid is incorporated into a newly formed mitochondrial protein. Some contingent factors might be:

1. Energy dependent transport of amino acids across mitochondrial membranes.
2. Intramitochondrial oxidation of amino acids.
3. Intramitochondrial transamination reactions.
4. ATP dependent activation of amino acids.
5. Energy dependent reactions of peptide bond formation.

Thus, it is possible that many of the substances required

for studies of amino acid incorporation *in vitro* may be involved in ancillary processes in the mitochondria rather than in the actual protein synthesizing steps.

B. Effect of Inhibitors of Protein Synthesis

In studies of mitochondrial biogenesis, it is necessary to assess the contributions of two systems for protein synthesis, namely that in the mitochondria and that in the cytoplasm, to the formation of the mitochondrial membrane. Various specific inhibitors of protein synthesis have been utilized as important tools to determine the site of synthesis of the different mitochondrial proteins. The observations made with isolated mitochondria made possible the use of these inhibitors in later studies in whole cells.

Chloramphenicol was one of the first specific inhibitors of mitochondrial protein synthesis to be studied. Eisenstadt and Brawerman (14) first reported that chloramphenicol inhibited amino acid incorporation by isolated mitochondria and chloroplasts, as well as by bacterial systems. An inhibition of mitochondrial protein synthesis by chloramphenicol was subsequently reported by Kroon (15) as well as Firkin and Linnane (16). Linnane *et al*. (17), in 1966, made the important observation that chloramphenicol inhibits the normal development of mature mitochondria, when glucose-repressed yeast were undergoing derepression. This observation demonstrated the potential use of these specific inhibitors

in studies of mitochondrial membrane synthesis. Furthermore, the specific inhibitory effects of chloramphenicol suggested that the mitochondrial ribosome must resemble that of bacteria, as Vazquez (18) has demonstrated that chloramphenicol binds specifically to the 50S subunit of the 70S bacterial ribosome and not with the 80S ribosomes of the microsomes. Later studies did confirm that the mitochondrial ribosome did not resemble that of the cytoplasm. For example, cycloheximide has been shown to inhibit completely protein synthesis in mammalian ribosomes isolated from the cytoplasm (19). The mechanism of cycloheximide inhibition is thought to result from prevention of the association of peptide synthetase with the 80S ribosomes (20). Beattie et al. (4) demonstrated that cycloheximide does not inhibit amino acid incorporation by isolated rat liver mitochondria. A similar observation was reported in yeast mitochondria (21). Hence, the selective inhibitors of chloramphenicol and cycloheximide have made possible many subsequent studies.

Chloramphenicol has been reported to inhibit amino acid incorporation in nearly all types of mammalian and non-mammalian mitochondria. One exception was reported by Gordon and Deanin (22), who found that amino acid incorporation by rat brain mitochondria was not affected by chloramphenicol. However, Cunningham and Bridgers (23) subsequently reported that chloramphenicol did inhibit amino acid incorporation by rat brain mitochondria if sufficient potassium

was in the medium. They concluded that the high sodium concentration in the medium of Gordon and Deanin might possibly have blocked the binding of chloramphenicol to the ribosome and thus prevented its inhibitory action. Since the incorporation of radioactive amino acids into protein is diminished when ammonium or sodium ions replaced potassium (1), such an explanation is highly plausible. Chloramphenicol was inhibitory when the sodium and ammonium ions were present, but to a lesser degree than that observed when potassium was present (1).

A partial inhibition of protein synthesis in the intact synaptosome by both chloramphenicol and cycloheximide has been reported by Morgan and Austin (24). They concluded that this dual inhibition resulted from a mitochondrial (cycloheximide-insensitive) and a non-mitochondrial (cycloheximide-sensitive) system for protein synthesis within the synaptosome. Hence, it appears that brain mitochondria do indeed resemble other mitochondria in their sensitivity to chloramphenicol.

Other antibiotics also completely inhibit mitochondrial protein synthesis. Beattie et al. (4) reported that puromycin inhibits amino acid incorporation by rat liver mitochondria. This result indicated that protein synthesis in mitochondria must involve a binding of amino acyl-t-RNA to the mitochondrial ribosome, similar to the mode of action in a bacterial system. The discovery of specific mitochondrial t-RNA's (25), activating enzymes (26), and mitochondrial polyribosomes (27,28, 29), confirmed this suggestion. The inhibition of mitochon-

drial incorporation by the drug sparsomycin, has also been reported (30). Tada and Trakatellis (30) showed that sparsomycin inhibits protein synthesis by combining with petidyl transferase on the 50S subunit of the ribosome.

The macrolide antibiotics, erythromycin, lincomycin, oleandomycin, and carbomycin interfere with bacterial protein synthesis at the level of the ribosomes (31). These antibiotics all bind to the large subunit of the ribosome and prevent either translocation (erythromycin) (32) or peptide bond formation (lincomycin) (33). Amino acid incorporation by isolated yeast mitochondria (34) is inhibited by all these antibiotics suggesting that the ribosomes of the yeast mitochondria must be similar to those of bacteria. The effects of erythromycin on protein synthesis in yeast mitochondria are of significant importance, since it has been noted that erythromycin sensitivity is a maternally inherited characteristic and hence probably under control of mitochondrial DNA (35,21).

The situation regarding the effects of these drugs on mitochondrial ribosomes from animal cells is still unclear. In an early study, Firkin and Linnane (36) reported that erythromycin did not inhibit amino acid incorporation by isolated rat liver mitochondria. In contrast, erythromycin did inhibit mitochondrial protein synthesis if the whole rat liver is pre-incubated with the drug. These workers hypothesized that during the course of evolution the mitochondrial ribosome may have changed in such a way as to lose its sensitivity to erythromycin. Several reports have indicated that the mito-

chondrial ribosomes from the animal cells are characterized by a sedimentation value of 55S (37,38,39). However, enough work has not yet been done on the physical characteristics of these particles to justify their identification as very small or mini-ribosomes.

Kroon and DeVries have also investigated the sensitivity of amino acid incorporation by rat liver mitochondria to erythromycin and other macrolides (40). They observed no inhibition with either erythromycin or lincomycin on amino acid incorporation by intact, isolated mitochondria. Inhibition, however, did occur when the mitochondria were "swollen" in a hypotonic sucrose solution prior to the incorporation studies. Erythromycin, oleandomycin, carbomycin, and lincomycin were all found to be effective inhibitors in varying degrees. These workers concluded that the seeming insensitivity of rat liver mitochondria protein synthesis to the macrolides was due to the fact that the mitochondrial membranes were impermeable to these drugs.

Firkin and Linnane (36) also investigated the effect of lincomycin on rat liver mitochondria that were subjected to sonic vibrations and hence permeable to the drug. They observed no inhibition by lincomycin; however, the conditions used by the two groups to isolate the mitochondria as well as the conditions used during incubation are so strikingly divergent that it is difficult to draw any meaningful results. It is apparent, however, that the specific activity in pmoles of amino acid per mg. of protein, reported by Linnane et al.

(36) is approximately 10% of that of Kroon and DeVries. The concentration of leucine must not be rate limiting if one is to obtain any valid interpretation of studies with inhibitors.

In a subsequent series of studies (41,42,43) Linnane's group has attempted to respond to the argument of Kroon and DeVries. In one study, Hela cells were grown in the presence of the antibiotics mikamycin, carbomycin, spiromycin, erythromycin, and paromycin (41). Mikamycin and carbomycin selectively inhibited mitochondrial protein synthesis in the intact cells, erythromycin and paromyc had no effect, while spiromycin exerted some minor effects on both protein synthesis and cytochrome formation. At high concentrations, all of these showed a secondary effect - direct and immediate slowing of all growth.

Later (42) Linnane's group attempted to examine the effect of these inhibitors on amino acid incorporation by mitochondria whose membranes have been damaged extensively so as to permit access of the inhibitors to the mitoribosomes. Two methods were used to make the membranes more permeable including the hypotonic method of Kroon and Devries (40). Chloramphenicol, mikamycin, carbomycin, and spiromycin strongly inhibited mitochondrial protein synthesis, while erythromycin, lincomycin, and paromycin had little or no effect on the incorporation rates.

The inhibitory effect of carbomycin on mitochondrial protein synthesis in rat liver was modified by the simultaneous addition of either erythromycin or lincomycin. A

decrease in the degree of inhibition and an increase in the half-maximal inhibitory concentration of carbomycin was observed in the presence of erythromycin or lincomycin (43). The authors concluded that the above effects could be rationalized in terms of a competitive model where the binding of a non-inhibitory antibiotic (erythromycin) to a ribosomal site prevents inhibition by exclusion of the inhibitory antibiotic (carbomycin).

C. Nature of Proteins Synthesized by the Mitochondria

Although it has been established that isolated mitochondria incorporate amino acids into protein, the products of mitochondrial protein synthesis have not yet been clearly defined. The major impediment to the purification of these proteins has been their extreme insolubility in most normal solvent systems. Basically, two approaches have been employed to study this problem. In the first, mitochondria are incubated in vitro with radioactive amino acids, reisolated, washed, and then fractionated according to various schemes, and analyzed using such techniques as gel electrophoresis and liquid scintillation counting. Such preparations may then be compared with similar preparations labeled in vivo to determine if there is any correlation between the two phenomena. In the second method, well characterized enzymes of the inner membrane, such as cytochrome oxidase or ATPase have been isolated from yeast or Neurospora cells which were first labeled

in vivo with radioactive amino acids in the presence of sufficient cycloheximide to block completely cytoplasmic protein synthesis. In this way it could be determined if these enzyme complexes contained any proteins synthesized within the mitochondria.

1. Fractionation of Particulate Membrane Fractions

In an early study, Roodyn (44) labeled rat liver mitochondria *in vitro* and subsequently isolated the labeled proteins by solubilization of the membrane with the non-ionic detergent, Triton X-100. The bulk of the radioactivity was in a lipid-rich insoluble component which was thought to be derived from the mitochondrial membrane. Treatment of this lipid-rich fraction with butan-1-ol gave a protein fraction which had the properties of a "structural protein." The term "structural protein" was first applied to a protein fraction isolated by Criddle *et al.* (45) by fractionation of mitochondria with cholate, deoxycholate, sodium dodecyl sulfate, and ammonium sulfate. A "structural protein" fraction prepared in this way contained over 22% of the total radioactivity of the intact mitochondria. The isolation of a similar "structural protein" fraction containing radioactivity was confirmed by other groups (46,47,5).

The intramitochondrial localization of the proteins synthesized within the mitochondria was determined by use of fractionation procedures which separate mitochondria into

discrete morphological entities. Digitonin has been used to separate the inner and the outer mitochondrial membranes (48), as well as procedures involving osmotic shock and sonication (49). Inner membrane fractions obtained by both these procedures have been shown to contain proteins labeled by the mitochondria *in vitro* (4,50). Beattie *et al.* (4) reported that a "structural protein" fraction and other membranous proteins of the inner membrane accounted for the majority of the radioactivity, while almost no radioactivity was present in the outer membrane fraction or the soluble proteins of the matrix.

In addition, inner membranes which were isolated using digitonin were able to incorporate amino acids into proteins (4). Thus, it was established, with some degree of certainty that mitochondrial protein synthesis is selectively involved in some manner in the biogenesis of the inner mitochondrial membrane.

The "structural protein" fraction which contained labeled mitochondrial proteins after an *in vitro* incubation is no longer thought to contain a single protein. Haldar *et al.* (47) using the method of Richardson *et al.* (51) for the preparation of "structural protein," examined this fraction obtained from rat liver mitochondria labeled *in vitro* with radioactive amino acids by disc gel electrophoresis. On analysis, the fraction was found to contain four major protein bands, several of which contained radioactivity. Further analysis indicated that one of the labeled protein bands was

similar in electrophoretic mobility to that of Racker's coupling factor-4 (52).

New attempts were initiated in the hope of attaining a more homogenous preparation of these insoluble membrane fractions. Lenaz et al. (53) utilized several procedures, such as extraction with trichloroacetic acid-methanol, 8 M urea, and dilute solutions of hydrochloric or acetic acid. "Structural protein" fractions prepared by these methods were found to separate into four major bands on disc gel electrophoresis. Beattie et al. (54) extracted liver mitochondria, previously labeled in vitro with radioactive leucine by the methods of Lenaz et al. (53) to obtain fractions containing "structural protein." In addition, "structural protein" was prepared from labeled mitochondria by extraction with acetic acid (55). The "structural protein" fraction prepared by acid treatment did not contain any radioactivity, while "structural proteins" prepared by detergents (51) were labeled with approximately 40% of the total counts of the intact mitochondria. However, the radioactivity in this fraction was not associated with the bands visualized after disc gel electrophoresis but was retained at the origin. Hence, these radioactively labeled proteins appeared to be a contaminant of the "structural protein" preparation.

In 1970, Yang and Criddle (56) analyzed "structural protein" fractions of yeast mitochondria incubated in vitro with radioactive amino acids. These fractions were further extracted into four major subfractions using the methods of Richardson

et al. (51) and Lenaz et al. (53). The proteins of these subfractions were then solubilized by reduction and carboxymethylation or sulfonation in the presence of sodium dodecyl sulfate and separated on disc gel electrophoresis. One predominant component CS-1, in contrast to results mentioned previously, was found to contain 50-60% of the total radioactivity incorporated into the insoluble membrane proteins. This incorporation of radioactive amino acids into CS-1 was found to be strongly inhibited by chloramphenicol. The CS-1 fraction had a specific radioactivity four times that of the unfractionated mitochondria.

Beattie et al. (54) reported that isolated rat liver mitochondria incorporate amino acids almost quantitatively into a membranous residue remaining after extracting the mitochondria with dilute acetic acid (1.4% final concentration). This fraction contained less than 10% of the total mitochondrial protein and had a specific activity eight times that of the unfractionated mitochondria. At that time this increase in specific activity was the highest yet reported. However, the material was extremely heterogenous, with at least twelve discernable bands on gel electrophoresis. In addition, many of the counts remained at the origin.

The conclusion, (57,58) that the products of mitochondrial protein synthesis represent less than 10% of the total mitochondrial protein, has been substantiated by several groups. Sebald et al. (57) concluded that the products of mitochondrial protein synthesis account for only 5% of the total mito-

chondrial protein in Neurospora. Similar results were found by Henson et al. (58) on studies of mitochondrial biogenesis in yeast. Thus it became apparent that the classical "structural protein" which had been calculated to represent approximately 30% of the total protein of the mitochondria could not be synthesized intramitochondrially.

Electrophoresis on polyacrylamide gels containing the detergent sodium dodecyl sulfate (59,60,61) has permitted the analysis and molecular weight determination of monomeric proteins as well as the quantitative proportions of different polypeptides in complex mixtures. In the past three to four years, this technique has been applied to the problem of mitochondrial biogenesis in an attempt to elucidate the number and molecular weights of proteins synthesized by the mitochondria. Sebald et al. (57,62) separated proteins synthesized by N. crassa mitochondria on the basis of both charge and size and observed four major bands and six minor ones. They observed the same number of bands in vitro or in vivo and concluded that the same proteins were synthesized under the two conditions. Swank et al. (63) also examined the products of mitochondria protein synthesis from the same organism. They concluded that wild type Neurospora crassa synthesized three major proteins, comprising 10% of the total mitochondrial protein, with molecular weights of 33,500, 27,500, and 17,500 in relative proportions of 64:20:16. Minor proteins, comprising approximately 1% of the total, revealed weights of 25,000, 21,000, and 11,000.

Yang and Criddle (56), using fractionation techniques described previously, found that in Saccharomyces carlsbergensis four bands were radioactively labeled with molecular weights of 40,000, 26,500, 20,000, and 15,000. In contrast, experiments in vivo have yielded conflicting results. Thomas and Williamson (64) observed six radioactive bands in yeast mitochondria with molecular weights ranging from 48,000 to 11,000. However, three of the bands were near the limits of detection in their system. Weislogel and Butow (65) using a temperature sensitive yeast mutant, have observed seven to eight labeled bands ranging in molecular weights from 45,000 to 13,000. Likewise, Groot et al. (66) have reported eight labeled bands in yeast mitochondria with molecular weights ranging from 94,000 to 13,000.

In studies involving rat liver and cerebral cortex, Bosmann (67) reported that mitochondria synthesized four distinct proteins, each of which is a glycoprotein. One of the peaks was a low molecular weight glycoprotein with a relative mobility similar to that of insulin.

Studies on the nature of proteins synthesized by the mitochondria have also been published by Coote and Work (9). Using BHK-21 hamster cells, they observed that the proteins synthesized by mitochondria present in whole cells were indistinguishable from those synthesized by isolated mitochondria. Examination on SDS-gel electrophoresis of both hamster cell mitochondria and rat liver mitochondria revealed at least ten proteins coded for by the mitochondrial genome, with

molecular weights ranging from 50,000 to 14,000.

2. Analysis of Well Characterized Enzymes of the Inner Mitochondrial Membrane

a. Yeast Mitochondrial ATPase

The subunit proteins of various membrane-bound enzymes and enzyme complexes have been analyzed to determine which proteins are products of the mitochondrial system for protein synthesis. The rutamycin (or oligomycin) sensitive ATPase of yeast mitochondria is a constituent of the inner membrane. The entire complex has been extracted from the membrane with Triton X-100 and further purified by passage through sucrose or glycerol gradients (68). Analysis on SDS-gel electrophoresis has revealed that the ATPase complex contains eight to nine different molecular weight species, five of which are subunits of the soluble ATPase, (F_1), one of which corresponds to the oligomycin-sensitivity-conferring factor (OSCP), while the remaining components are part of a lipoprotein fraction which modulates the catalytic and physical properties of F_1 (68). Using inhibitors of both mitochondrial and cytoplasmic protein synthesis, Tzagoloff et al. (68,69), demonstrated that the mitochondria may synthesize four subunits of the rutamycin-sensitive ATPase which are necessary both for conferral of rutamycin sensitivity and for integration of F_1 and OSCP into the membrane during the assembly of the ATPase complex. Mitochondria were not in-

volved in the synthesis of any subunits of F_1 or OSCP.

b. Cytochrome Oxidase

The appearance of cytochromeoxidase activity in yeast cells during aerobic adaption requires products of both mitochondrial and cytoplasmic translation (70,71). Chen and Charalampous reported that both cytoplasmic and mitochondrial precursors of cytochrome oxidase were present in yeast undergoing the anaerobic-aerobic transition (72).

In the recent studies of Tzagoloff (73,74), inhibitors of mitochondrial and cytoplasmic protein synthesis were used to demonstrate the intracellular site of subunits of cytochrome oxidase. The purified enzyme contains six subunits of molecular weights (35,000, 24,500, 22,000, 14,500, 12,000, 9,500). Antibodies to the pure enzyme were employed to isolate cytochrome oxidase from yeast cells given a pulse of labeled amino acids in the presence of cycloheximide. The three large molecular weight subunits of the enzyme contained label under these conditions indicating that these proteins are synthesized in the mitochondria. Similar studies by Mason and Schatz (75) also established that the three large polypeptides of cytochrome oxidase are synthesized on the mitoribosomes. The difference in molecular weights of the six subunits of the two groups may be due to the difference in the electrophoretic conditions employed.

c. Complex III of the Respiratory Chain

Mahler and Perlman (76) have examined the effect of ethidium bromide, a selective inhibitor of mitochondrial transcription, on various mitochondrial enzymes as well as cytochrome content in Sacchromyces cerevislae. Ethidium bromide treated cells were grown on galactose, a fermentable, but weakly repressing energy source. The increase in activity of NADH-cytochrome c reductase as well as cytochrome oxidase was inhibited in the ethidium bromide treated cells while succinate dehydrogenase activity increased at the same rate as the control cells. The actual increase in cytochrome $\underline{a}$-$\underline{a}_3$ content was also inhibited completely by addition of ethidium bromide, while synthesis of cytochrome b was inhibited partially. The rate of synthesis of cytochromes c and $\underline{c}_1$ appeared normal.

Kim and Beattie (77) recently reported that chloramphenicol inhibited the increase of NADH cytochrome c reductase activity in yeast cells undergoing glucose derepression. Subsequently, Beattie et al. (78) observed that chloramphenicol caused an inhibition of coenzyme QH_2-cytochrome c reductase activity, a measure of the cytochrome $\underline{b}$-$\underline{c}_1$ region of the respiratory chain. Similarly, Weiss has reported that a membrane fraction containing cytochrome b contained two subunits synthesized on the mitoribosomes (79).

The above observations suggest that some of the membrane proteins of the $\underline{b}$-$\underline{c}_1$ complex must also be synthesized within the mitochondria.

D. Synthesis of Proteolipids by Mitochondria

In 1951, Folch and Lees (80) described the presence in brain tissue of a protein material insoluble in water and aqueous solvents and soluble in chloroform:methanol mixtures. To designate this new chemical class, they coined the term proteolipid to indicate that these compounds were lipoproteins which retained some of the physical properties of lipids but differed from other known lipoproteins which were soluble in water and insoluble in chloroform:methanol.

The various proteolipids isolated by extraction with chloroform:methanol all exhibit several unique properties. In a biphasic system chloroform:methanol:water, 8:4:3 (v/v/v) these proteins are present quantitatively in the lower chloroformic phase, and completely absent from the upper methanol: water phase (81). Optical rotary dispersion studies of proteolipid fractions (82,83), indicate that these proteins possess a high helical content which remains unchanged even during delipidation. After all the lipids have been removed, the resulting apoprotein becomes soluble in water while retaining its solubility in chloroform. The increasing solubility in water is paralleled by a decrease in α-helix content to below the detectable limits (<10%).

All of the proteolipid fractions described are resistent to digestion by pepsin, trypsin, papain, and erepsin (81). This resistence is not due to the presence of lipids since this property persists in the delipidated apoprotein. The only proteolytic enzyme known to attack proteolipids is

pronase, although the total extent of this digestion has not been explored.

The nature of the bond between protein and lipid is still unknown. After exhaustive dialysis against acidic chloroform:methanol, the majority of lipids are removed, indicating that most of the protein-lipid bonds are ionic. However, after delipidation, approximately two to three percent of the original fatty acids remain. These fatty acids show a consistent pattern of about sixty percent palmitic, twenty-five percent oleic, and ten percent stearic acids (81). Stoffyn and Folch (84) have established conclusively that these fatty acids are bound to the protein by an ester bond since they do not react either with diazomethane or with sodium borohydride to produce corresponding alcohols. Amino acid analysis of proteolipid fractions have indicated a high content of hydrophobic amino acids, and a negligible amount of acidic or basic amino acids. The excessive amounts of hydrophobic amino acids may confer the property of lipid solubility on the protein.

The proteins synthesized by mitochondria are known to be very hydrophobic and insoluble in many solvents. The fractionation with organic solvents of the mitochondrial membrane offered a new approach to the solubilization and purification of these proteins (85,86). In 1971, Kadenbach reported (86) that a proteolipid fraction could be obtained from rat liver mitochondria extracted with chloroform:methanol. This fraction consisted of a single polypeptide of low molecular

weight (about 2000). Although this report was later retracted (87), others have reported the existence of mitochondrial proteins soluble in chloroform:methanol (88,89).

Cattell et al. (90) obtained from ox heart mitochondria a proteolipid fraction which bound dicyclohexylcarbodiimide (DCCD), an inhibitor of oxidative phosphorylation with a similar action as oligomycin. Mitochondria were treated with radioactive DCCD, extracted with chloroform:methanol, and the extract chromatographed on Sephadex LH-20. Several protein peaks containing radioactivity were eluted; however, gel electrophoresis revealed that 90 to 100% of the radioactivity in each peak was associated with a protein of small molecular weight.

The possibility that proteolipids might be synthesized by yeast mitochondria has been investigated by two procedures. Murray and Linnane (89) reported that protein extracted with chloroform:methanol accounts for up to 3% of the total yeast mitochondrial protein. The proportion of radioactive label solubilized in chloroform:methanol was dependent on the growth phase of the yeast cells. Murray and Linnane concluded that the proteolipid protein was a major product of mitochondrial protein synthesis, but not the sole product. Similar results have been obtained using rat liver mitochondria (87). Isolated rat liver mitochondria incorporated radioactive amino acids into two types of proteins, approximately one third of the incorporated radioactivity being soluble in chloroform: methanol. The rate of incorporation into the chloroform:

methanol soluble proteins was different from the rate of incorporation into insoluble proteins, suggesting that two different systems for protein synthesis may exist in mitochondria.

In contrast, Tzagoloff and Akai (85) have reported that the majority of counts incorporated into yeast mitochondria *in vivo* in the presence of cycloheximide are extractable into chloroform:methanol. Gel electrophoresis of this extract revealed one peak of 7800 molecular weight, which Tzagoloff has claimed is the main product of mitochondrial protein synthesis. The apparent discrepancies between Tzagoloff and the above-mentioned reports may be due to two factors. One is that the extraction procedures of the various groups are different. Second, and probably the most important, Tzagoloff uses yeast which have been grown for seventeen hours in cycloheximide. The viability of cells grown for such extreme periods of time in the absence of protein synthesis has never been satisfactorily demonstrated.

III. MATERIALS AND METHODS

A. Preparation of Mitochondria

1. Rat Liver Mitochondria

Rat liver mitochondria were prepared under sterile conditions in a medium containing 0.25 M sucrose, 0.01 M Tris-

Cl, pH 7.8, and 0.001 M EDTA (sodium salt) by the method of Beattie (91). This yielded a pellet 3% contaminated with microsomal protein as determined by glucose 6-phosphatase activity.

2. 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 (77). The cells were harvested by centrifugation for ten minutes 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 RPM for 20 seconds. The combined supernatants, after washing the beads, were centrifuged at 600 x g for ten minutes three times to remove unbroken cells and nuclei. The supernatant was then centrifuged at 17,000 x g for ten minutes and the pellent containing mitochondria washed three times with mannitol-Tris-EDTA medium and recentrifuged for ten minutes at 17,000 x g. The final mitochondrial pellet was resuspended in mannitol-Tris-EDTA to a final concentration of 6-8 mg/ml of protein. The above steps were performed under strictly sterile conditions; all glassware and solutions were autoclaved prior to use.

B. Amino Acid Incorporation Studies

1. *In Vitro Labeling of Rat Liver Mitochondria*

Amino acid incorporation into mitochondrial protein *in vitro* was determined using mitochondria obtained under sterile conditions (6). The four times washed liver mitochondrial pellet was resuspended in 0.25 M sucrose to a final concentration of 6-8 mg/ml of protein and incubated in a medium containing 50 mM Bicine buffer, pH 7.6, 90 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 5 mM phosphate, pH 7.6, 22.5 µg of a complete amino acid mixture minus leucine, as described by Roodyn *et al.* (2), 0.25 μC_1 per ml of uniformly labeled (^{14}C)leucine, 5 mM phosphoenolpyruvate, 2 mM ATP, and 10 µg of pyruvate kinase. In other experiments involving the ionic strength of the medium, various concentrations of KCl, NH_4Cl and phosphate were added as indicated in the legends to the tables. Also, in some of the fractionation studies, higher concentrations of (^{14}C)leucine and (^{3}H)4,5-L-leucine were utilized as indicated in the appropriate tables and figures. After 30 minutes incubation 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 proteins were then prepared for counting. When fractionation studies were employed, the precipitation with trichloroacetic acid was omitted and the mitochondria were reisolated at 12,000 x g for ten minutes. The mitochondrial pellet was then washed two times in sucrose containing unlabeled leucine (10 mM, final concentration) prior to fractionation.

2. In Vivo Labeling of Yeast Mitochondria

Yeast cells were harvested as described above by centrifugation at 1,000 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 fifteen minutes prior to addition of 50-60 μC_i/ml of (^{3}H)4,5-L-leucine. After incubation for thirty minutes at 30°C, 10 mM unlabeled leucine was added and the incubation continued for fifteen minutes. The cells were then harvested and mitochondria prepared as described above.

3. In Vivo Studies of Amino Acid Incorporation in Rat Liver

Cycloheximide, dissolved in 0.9% saline, was injected intraperitoneally to adult male rats weighing less than 150 g at a dosage of 5 mg/100 g body weight. Fifteen minutes later the animals received 500 μC_i of (^{3}H)4,5-L-leucine by intraperitoneal injection. The animals were killed after two hours. Liver mitochondria were prepared and fractionated as described in the following sections.

4. In Vitro Labeling of Yeast Mitochondria

Mitochondria were incubated in a medium containing 90 mM

KCl, 50 mM phosphate, pH 7.6, 50 mM Bicine buffer, 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. (2), 0.5 μC_i per ml of uniformly labeled (^{14}C)leucine, 2.0 mM ATP, 5.0 mM phosphoenolpyruvate, 2.0 µg of pyruvate kinase and 2.0 mg of mitochondrial protein in a final volume of 2.0 ml. After 15 min at 30°C in a metabolic shaker, the incubation was terminated by addition of 10 mM leucine followed by precipitation with 5% trichloroacetic acid. Labeled proteins were prepared for counting as described in section G.

C. Fractionation Procedures

1. Acetic Acid-Lubrol

Reisolated mitochondria were brought to a concentration of 5 mg/ml and extracted with 1.4% acetic acid (final concentration), for thirty minutes at 0°C as described by Zahler et al. (55). The insoluble residue was removed by centrifugation at 80,000 x g for one hour. The pellet was washed once with the original volume of 1.4% acetic acid and recentrifuged at 100,000 x g for one hour. The resulting pellet was resuspended to a concentration of 10 mg/ml in isolation medium and the non-ionic detergent Lubrol was added at a concentration of 1 mg/10 mg protein. The suspension was centrifuged at 80,000 x g for one hour. The resulting pellet was washed with distilled H_2O and recentrifuged at 80,000 x g for one hr.

2. KCl Extractions

The washed mitochondrial pellet after incubation was resuspended in 0.4 N KCl, frozen, thawed, and centrifuged at 12,000 x g for ten minutes. The resulting pellet was resuspended in 0.4 N KCl, frozen, thawed, and recentrifuged at 17,000 x g for ten minutes.

3. Sonication

The washed mitochondrial pellet was resuspended in isolation medium to a final concentration of 1-2 mg/ml. Sonication was employed at maximum output of an Ultrasonic Model W185D sonifier for fifteen seconds. The sonicated mitochondria were centrifuged at 164,000 x g for thirty minutes.

4. Phosphate Extraction

The method of Coote and Work was employed (9). The washed mitochondrial pellet, reisolated after an incubation, was resuspended at a concentration of 10 mg/ml in 0.05 M phosphate (pH 11.0) and centrifuged immediately at 25,000 x g for thirty minutes.

5. Inner Membrane Fraction

Inner membrane fractions were prepared as described by Schnaitman and Greenawalt (7). The pellet obtained by centrifuging the digitonin treated mitochondria was washed twice by resuspending in the same volume of sucrose-Tris-EDTA and centrifuging at 9000 x g. The resulting pellet was resuspended to a concentration of 6-8 mg/ml and used further for amino acid incorporation studies.

D. Fractionation of Proteolipids

1. Method 1

Submitochondrial particles containing 5 mg of protein were suspended to 1 ml in isolation medium and extracted with 9 ml of chloroform:methanol (2:1,v/v) for thirty minutes at room temperature. The extracts were centrifuged at maximum speed in a clinical centrifuge. The lower layer was washed once with 5 ml of H_2O and three times with 10 ml of chloroform:methanol:water (3:48:47) (92). In some experiments, the initial chloroform:methanol extract was used without the washes.

2. Method 2

Submitochondrial particles containing 5 mg of protein were suspended in 1 ml of isolation medium and extracted with 10 ml of 90% methanol. The pellet after centrifugation at 1000 x g was then extracted with 2 ml of chloroform: methanol (2:1, v/v) and incubated at 50°C for thirty minutes. The mixtures were centrifuged at 1000 x g and the residue extracted a second time with chloroform:methanol (85).

3. Method 3

Submitochondrial particles were lyophilized and then

extracted with 10 ml of chloroform:methanol (2:1, v/v). The chloroform:methanol soluble fraction was used as such (93).

E. Preparation of Extracts and Residues for Counting and Gel Electrophoresis

The extracts and residues from all methods were dried under a stream of nitrogen at 50°C and dissolved in 1% SDS for counting and protein determination, or in the medium used for gel electrophoresis (85). Polyacrylamide gel electrophoresis was performed on 10 cm gels as described by Tzagoloff (94).

F. Gel Electrophoresis

1. Preparation of Membrane Fractions for Electrophoresis

The various membranous fractions were resuspended in isolation medium at a concentration of 1-2 mg/ml, sonified at maximum output for fifteen seconds, and centrifuged at 164,000 x g for thirty minutes. The pellets were extracted with five volumes of 5% trichloroacetic acid. The precipitated proteins were washed three times with distilled H_2O and dissolved in a solvent system containing 10% glycerol (w/v), 1% SDS, 1% mercaptoethanol, 0.01 M phosphate buffer (pH 7.1), and 0.002% Bromphenol Blue at a protein concentration of 2 mg/ml (94). To insure monomerization, the protein

solution was heated at 70°C for twenty minutes. The proteins were separated on 7 or 10 cm gels according to the method of Weber and Osborn (61). The electrophoresis was carried out at room temperature at either of two conditions:

(1) 8 ma/gel (7%) for approximately 3-4 hours.

(2) 3 ma/gel (10%) for approximately 16-24 hours.

Gels were stained overnight with 2% Coomassie Blue in methanol-acetic acid-H_2O (5:1:5) and were destained electrophoretically in the same solvent system. The gels were scanned at 540 nm in a Gilford spectrophotometer, equipped with scanning equipment.

2. Gel Electrophoresis of Yeast Mitochondria

Mitochondria were suspended in mannitol-Tris-EDTA at a concentration of 10 mg/ml and sonicated for 15 seconds at maximum output in a Bronson Sonifier. The membranes were isolated by centrifugation at 150,000 x g for thirty minutes. The remaining steps were similar to those described for rat liver mitochondria in section F1.

3. Standards and Calibration

Gels were calibrated according to the method of Weber and Osborn (61). Standards used were insulin (B chain), cytochrome c, chymotrypsinogen, trypsin, pepsin, alcohol dehydrogenase, ovalbumin, and bovine serum albumin.

4. Counting of Gels

Gels were sliced into 1.0 mm or 2.0 mm slices, depending on the experiment, with a Gilson Model B-100 Aliquogel Gel Slicer, and 0.5 ml of hydrogen peroxide was added automatically. The gels were heated overnight at 50°C or until completely dissolved. Ten ml of Bray's solution (95) or five ml of Toluene counting solution (96) were added, depending on the type of counting vials utilized. The radioactivity was determined in a Packard Scintillation Counter with an efficiency for ^{14}C of 77% and ^{3}H of 25% in Bray's solution and 90% for ^{14}C and 27% for ^{3}H in the Toluene counting solution. In the case of the double label experiment, the amount of crossover of ^{14}C into the ^{3}H channel was calculated and subtracted from the total ^{3}H counts.

G. Protein Analysis

Protein concentrations were determined by the method of Lowry et al. (97) or by the Biuret method described by Gornall et al. (98). Proteins were prepared for counting by previously described methods (6).

H. Materials

Uniformly labeled (^{14}C)L-leucine (250 mC_1/mmole) and (^{3}H)4,5-L-leucine (30 C_1/mmole) were obtained from New England

Nuclear. ATP, PEP, oligomycin, pyruvic kinase, SDS, antimycin A, atractyloside, cordycepin, chloramphenicol, erythromycin, cycloheximide, emetine, and the standards used for gel calibration were obtained from Sigma. Lincomycin and sparsomycin were obtained from Upjohn; actinomycin D was obtained from Merck, Sharpe, and Dohme; Rifampicin and vallnomycin were obtained from Pfizer; Chloropromazine was obtained from Smith, Kline, and French Laboratories. Acrylamide, Bisacrylamide, tetramethylethylenediamine, and DDC were obtained from Eastman. Allylisopropylacetamide was obtained from Hoffman-La Roche. Lubrol was gift of ICI American, Inc. Triton X-100 was purchased from Rohm and Haas.

IV. RESULTS

A. KCl Concentration

In previous studies of amino acid incorporation by isolated rat liver mitochondria (4,54), a solution of 0.154 M KCl was added to the incubation medium to bring the final volume to 2 ml. The final concentration of KCl obtained in this manner was generally 50-60 mM KCl. As seen in Table I an optimal rate of amino acid incorporation was obtained at 90 mM KCl under all conditions; however, raising the concentration of KCl from 60 to 90 mM only increased 20% the incorporation rate. The omission of KCl and substitution of sucrose to maintain the molarity resulted in a 75% decrease in the

TABLE I

Effect of KCl concentration, phosphate and NH_4Cl on Amino Acid Incorporation.

Conditions	cpm/mg		
	60	90	120 mM KCl
No phosphate	726	782	622
5 mM phosphate	995	1180	1060
+ 10 mM NH_4Cl	844	990	938

Rat liver mitochondria (2-3 mg) were incubated for 30 min at 30°C in 2 ml of a medium containing 50 mM Bicine, pH 7.6, 10 mM $MgCl_2$, 1 mM EDTA, 22.5 µg of an amino acid mixture, 2 mM ATP, 5 mM PEP, 2.0 µg of pyruvic kinase and the concentration of KCl, phosphate, (pH 7.6) and NH_4Cl as indicated.

incorporation rate. The addition of 5 mM phosphate increased the rate of amino acid incorporation approximately 50% at all concentrations of KCl. The addition of 10 mM NH_4Cl was slightly inhibitory.

B. Effect of phosphate and Mersalyl

The optimal concentration of phosphate for amino acid incorporation by intact rat liver mitochondria was 5 mM (Table II). Higher concentrations were slightly inhibitory. In the absence of phosphate, the rate of incorporation was lowered nearly 30%. Likewise, addition of mersalyl, a specific inhibitor of phosphate transport, caused a 30% inhibition of the incorporation rate. The requirement for phosphate was more pronounced when ATP was generated from succinate and ADP by the respiratory chain. The incorporation rate in the absence of phosphate was decreased 60%. The addition of mersalyl caused a 70% inhibition in the presence of ADP and succinate. In studies with the inner membrane-matrix fraction, the omission of phosphate from the medium only resulted in a 13% decrease in amino acid incorporation. Mersalyl also caused a 16% inhibition.of the incorporation of the fraction.

C. Effect of Ammonium Ions

Several groups (8,9,99) have reported stimulation of amino

TABLE II

Effect of Phosphate on Amino Acid Incorporation By Intact Mitochondria and the Inner Membrane-Matrix Fraction.

Phosphate Concentration	Incubation Conditions		
	ATP-PEP		ADP-Succinate
	Mitochondria	Inner Membrane	Mitochondria
	cpm/mg		cpm/mg
None	1240	1930	735
5 mM	1700	2210	1835
10 mM	1500		
20 mM	1200		
5 mM and Mersalyl	1220	1850	566

Mitochondria or the inner membrane-matrix fraction were incubated as described in the legend to Table I with the addition of 90 mM KCl.

Final concentration of mersalyl was 3 mM.

acid incorporation when NH_4^+ ions are present in the incubation medium. The addition of 10 mM NH_4Cl to the medium containing 90 mM KCl, 5 mM phosphate, and the ATP generating system, appeared to cause a slight inhibition (Table I). In the second series of experiments (Figure 1), various concentrations of NH_4Cl were substituted for the KCl in the medium while the ionic strength was maintained at a constant value. A 30% decrease in the rate of incorporation was observed when the ionic composition was 30 mM NH_4Cl and 60 mM KCl as compared with 90 mM KCl alone. Amino acid incorporation proceeded at a rate nearly 40% lower when KCl was completely omitted and replaced with NH_4Cl at a concentration of 90 mM. Amino acid incorporation by the inner membrane-matrix fraction was also inhibited by NH_4^+ ions, but not to the same extent. The replacement of KCl with NH_4Cl resulted in only a 23% decrease in the incorporation rate.

D. Effect of Sodium Ions

As seen in Figure 2, the addition of 10 mM NaCl to the incubation medium containing 90 mM KCl caused a 20% inhibition of the incorporation. The substitution of increasing amounts of NaCl for KCl at a constant ionic strength resulted in a greatly diminished rate of amino acid incorporation. The complete replacement of KCl with NaCl resulted in a 65% decrease in the incorporation rate. Similar results were obtained with the inner membrane-fraction.

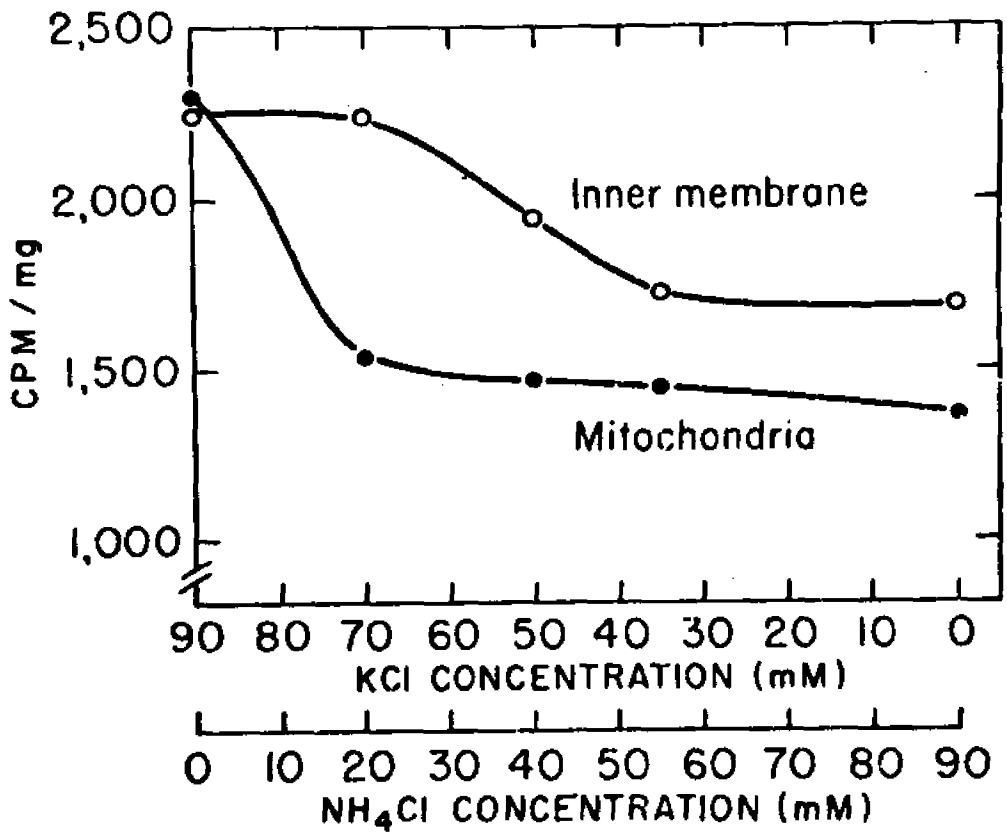

Figure 1. The effect of substituting NH_4Cl for KCl in the incubation medium for amino acid incorporation. Mitochondria (●——●) or the inner membrane-matrix fraction (o——o) were incubated as described in the Legend to Table 1.

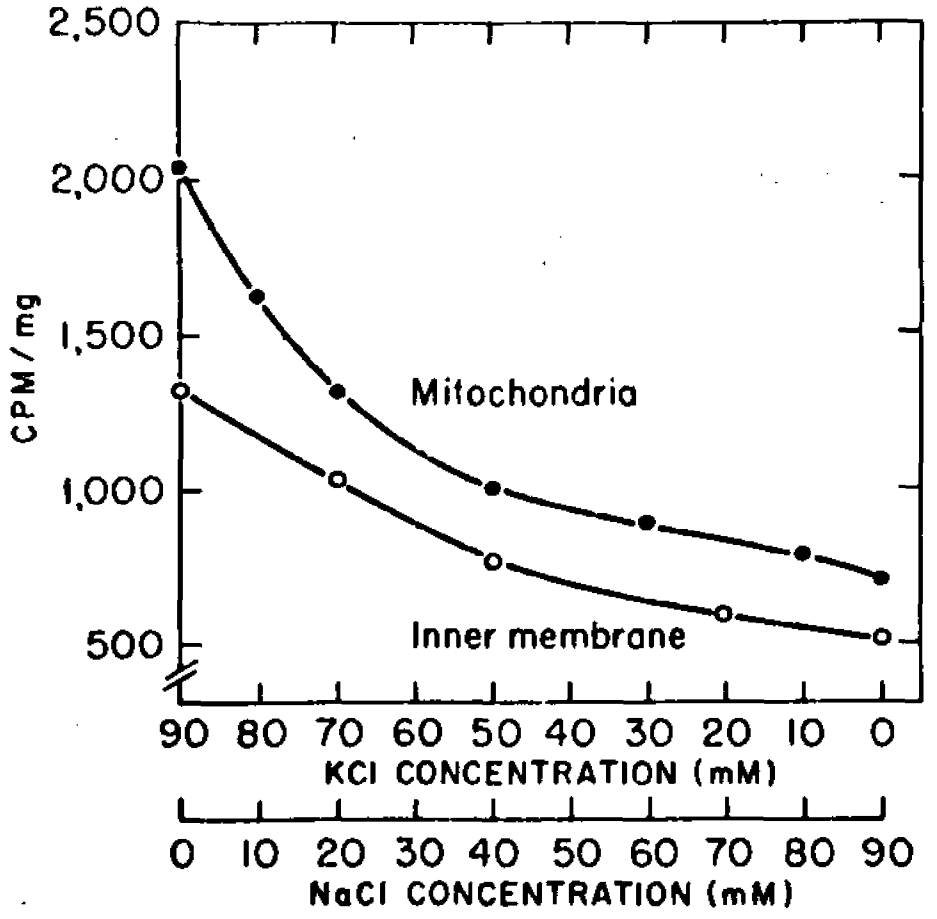

Figure 2. The effect of substituting NaCl for KCl in the incubation medium for amino acid incorporation. Mitochondria (●——●) or the inner membrane matrix fraction (○——○) were incubated as described in the Legend to Table I.

E. ATP Concentration

The optimal concentration of ATP for amino acid incorporation in the presence of phosphoenolpyruvate and pyruvic kinase was 2 mM with both intact liver mitochondria and the isolated inner membrane-matrix fraction (Figure 3). In the absence of exogenous ATP, the rates observed with liver mitochondria decreased to 10% of that observed when 2 mM ATP was present. The incubation medium was thus standardized to contain 90 mM KCl, 5 mM phosphate, and 2 mM ATP in all further experiments. The potassium salts of nucleotides and substrates were routinely used and KOH was used to neutralize all solutions.

F. Effect of ATP Regenerating System and Succinate Plus ADP on Amino Acid Incorporation

As can be seen in Table III, identical rates of amino acid incorporation were obtained using either the ATP regenerating system or succinate plus ATP. The amino acid incorporation rate measured in the presence of ATP and PEP or ATP and succinate were both inhibited by atractyloside, the inhibitor of the adenine translocase (13). However, atractyloside, caused a 60-70% inhibition in the ATP-PEP system but much less inhibition in the ATP-succinate system. In the ATP-succinate system, antimycin A caused a 90% inhibition of the incorporation rate as compared to a 30% inhibition

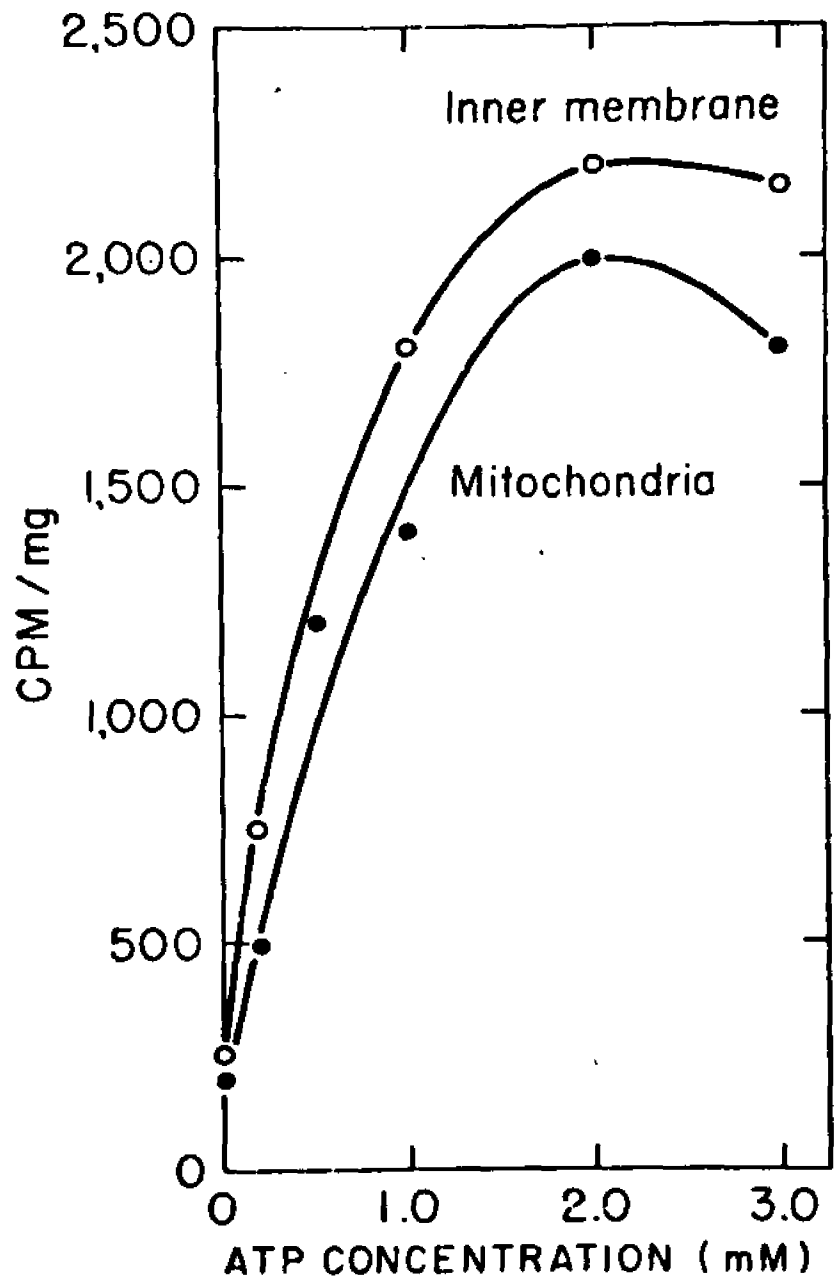

Figure 3. Concentration curve for ATP in amino acid incorporation. Mitochondria (●——●) or in the inner membrane matrix fraction (o——o) were incubated as described in the Legend to Table I.

TABLE III

Effect of Inhibitors on Amino Acid Incorporation when ATP is Generated Under Different Conditions.

Additions	cpm/mg	
	ATP-PEP	ATP-Succinate
None	2170	1920
+ atractyloside (50 µM)	883	1270
+ oligomycin (5 µg/ml)	2260	1090
+ antimycin A (2.5 µg/ml)	1360	131

Mitochondria were incubated as described in the legends to Tables I and II.

Final concentrations were 2.0 mM ATP, 5.0 mM PEP, and 10 mM succinate.

when ATP-PEP was present. Oligomycin was found to cause a 50% inhibition in the presence of ATP-succinate, but in the ATP-PEP regenerating system, there was a slight stimulation. As previously reported by Beattie and Ibrahim (12) addition of valinomycin caused a 2-fold increase in the incorporation rate.

G. Inhibitors

To study the effect of various inhibitors of mitochondrial protein synthesis, it is necessary to study amino acid incorporation under optimal conditions. The substrate concentration, i.e. leucine, should not be rate-limiting in studies of reactions occurring on the ribosomes. Kroon et al. (40) have reported that the maximum rate of incorporation (expressed as pmoles of amino acid incorporated/mg protein) required a final leucine concentration of 40-60 μM. Our studies also indicate that the maximum incorporation rate occurs at a leucine concentration of 60 μM (Figure 4). A slight decrease in incorporation was observed at higher concentrations of leucine. A 40 μM concentration of leucine was used in the studies with antibiotics described below.

The effects of various inhibitors of protein synthesis on amino acid incorporation by rat liver mitochondria in vitro are listed in Table IV. Chloramphenicol, carbomycin, and spersomycin inhibited severely protein synthesis by intact rat liver mitochondria. Emetine, at extremely high concen-

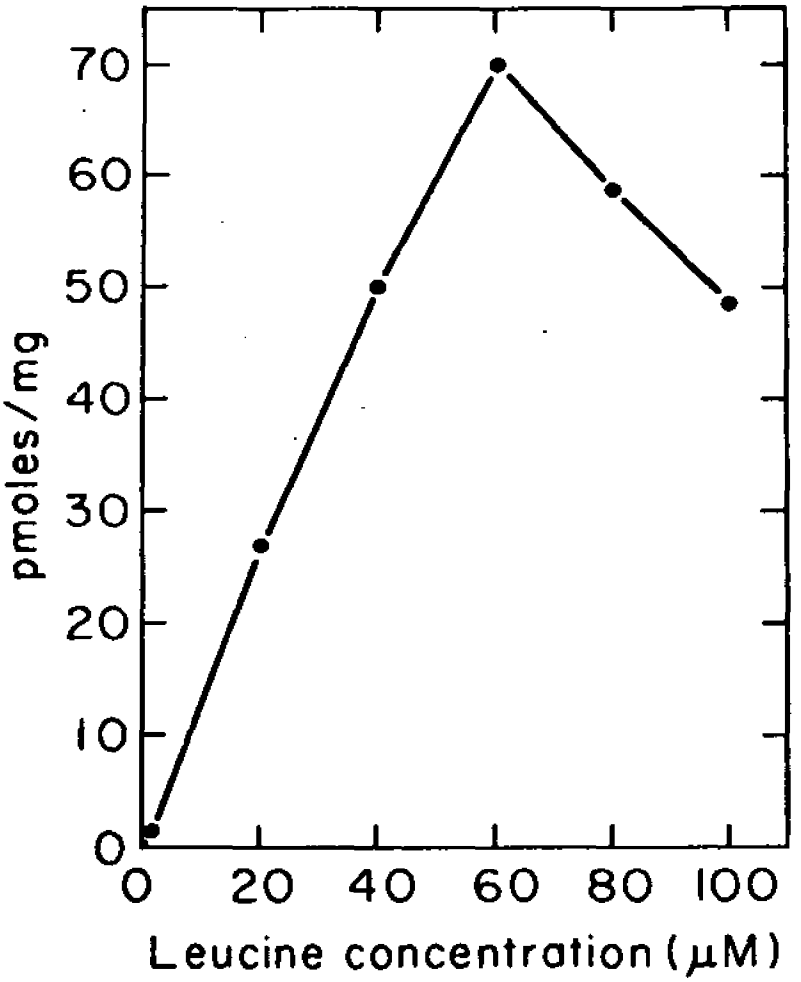

Figure 4. Effect of leucine concentration on amino acid incorporation. Mitochondria were incubated as described in Tables I and II.

TABLE IV

Effect of Various Inhibitors of Protein Synthesis on Amino Acid Incorporation *In Vitro*.

Control		Mito.	I.M.	I.M.+Val.	Linnane	Yeast
		cpm/mg				
		1674	2036	7990	360	1520
	µg/ml	% Inhibition				
Chloramphenicol	(20)	44	-	69	40	75
	(60)	64	-	82	65	86
Carbomycin	(2)	47	42	33	-	-
	(20)	78	73	-	-	-
	(30)	90	80	81	-	-
Erythromycin	(25)	0	19	-	0	40
	(50)	0	42	-	0	90
	(100)	0	67	-	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	-	-	-	

Rat liver mitochondria and inner membrane fractions were incubated as described in Tables I and II. Yeast mitochondria were incubated as described in Materials and Methods. Mitochondria in some experiments were treated with hypotonic sucrose according to the method of Linnane (42). A final concentration of 40 µM leucine was used in the incubations. The final concentration of valinomycin was 0.5 µg/ml; I.M. - Inner Membrane; Val. - valinomycin.

trations (500 µg/ml), was also inhibitory (58%). In contrast, erythromycin and lincomycin had no effect on the incorporation rate in intact mitochondria.

The inability of certain antibiotics to inhibit protein synthesis in intact mitochondria might be due to a permeability barrier to the drug in the mitochondrial membrane rather than to a change in the ribosome itself. The inner membrane-matrix fraction prepared by the digitonin method of Schnaitman and Greenawalt (7) has certain altered permeabilities to various substances. Hence, the various antibiotics were studied in isolated inner membrane fractions which have retained the ability for amino acid incorporation. As seen in Table IV, erythromycin and lincomycin inhibited mitochondrial protein synthesis 67% and 59% respectively at the maximum concentrations used. In addition, emetine was a much more effective inhibitor (82% inhibition) of amino acid incorporation in the inner membrane fractions than in whole mitochondria (58% inhibition). Similar inhibitions were observed in the inner membrane fractions incubated with valinomycin in which the highest rates of amino acid incorporation were observed.

Amino acid incorporation was studied in mitochondria treated with hypotonic sucrose as described by Linnane et al. (42). Mitochondria were suspended in 0.0025 M sucrose at 0°C at a concentration of 10 mg/ml for twenty minutes before addition to the incubation medium. As seen in Table IV, the rate of incorporation in mitochondria treated in this way

was only 25% of that observed in mitochondria resuspended in 0.25 M sucrose. Chloramphenicol inhibited amino acid incorporation in the hypotonic mitochondria to the same extent as in the intact mitochondria, while erythromycin and lincomycin were found to have no effect. It should be noted that mitochondria isolated from yeast were also inhibited by chloramphenicol (86%), erythromycin (90%), and to a lesser degree, emetine (35%). However, the concentration of erythromycin which inhibits incorporation 90% in the yeast mitochondria only inhibits incorporation in the inner membrane fraction 42%. The maximum inhibition of the incorporation rate by erythromycin in liver mitochondria is only 67%.

The effects of inhibitors known to act at the level of RNA synthesis, actinomycin D, cordycepin, rifampicin, were also tested in the inner membrane-matrix fraction. As seen in Table V, actinomycin D caused a slight inhibition of mitochondrial protein synthesis (16%), while cordycepin (34%), and rifampicin (54%) were more effective. Preincubation of the inner membrane with the antibiotics had little effect, precluding any problems of permeability.

The drug chloropromazine has been shown to inhibit protein synthesis in brain tissue of rats (100, 101). The effect of this drug was investigated in intact rat liver mitochondria. A concentration curve of its effect is shown in Figure 5. A significant inhibition (38%) is shown at a final concentration of 10^{-6} M while even greater inhibitions were observed at higher concentrations of the drug.

TABLE V

Effect of Inhibitors of RNA Synthesis on Mitochondrial Protein Synthesis.

	Normal Incubation			15 min. Pre-Incubation		
	Conc.	cpm/mg	% Inhib.	Conc.	cpm/mg	% Inhib.
Control	-	396	0	-	387	0
Actinomycin D	2 µg/ml	334	16	2 µg/ml	340	11
Cordycepin	10 µg/ml	263	34	10 µg/ml	299	23
Rifampicin	10 µg/ml	190	52	10 µg/ml	196	49

Inner membrane fractions were incubated as described in Tables I and II.

A final concentration of 40 µM leucine was used during the incubations.

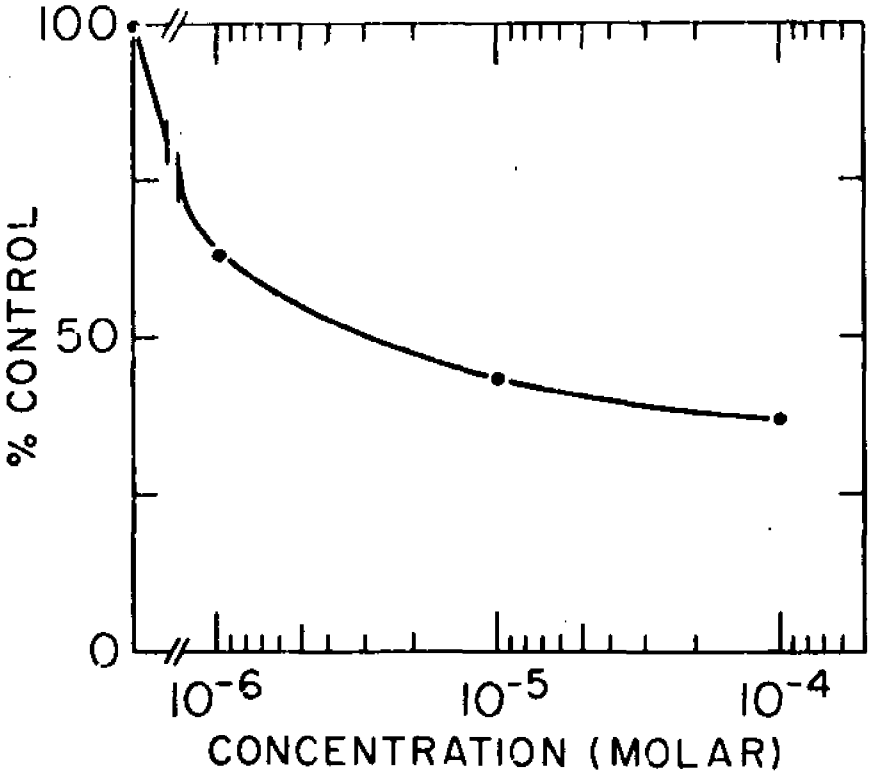

Figure 5. Effect of chloropromazine concentration on amino acid incorporation. Mitochondria were incubated as described in Tables I and II.

H. Protein Synthesized by the Mitochondria

Beattie *et al.* (54) previously reported that mitochondria *in vitro* incorporate amino acids into a heterogenous membrane fraction obtained by extracting mitochondria with acetic acid (55). In the present study, the acid insoluble membranous fraction was further extracted with the non-ionic detergent Lubrol. As seen in Table VI, the final pellet obtained after extraction with acetic acid and Lubrol had a six-fold increase in specific activity as compared to the intact mitochondria and contained 13% of the total mitochondrial protein. Analysis of this preparation by SDS-polyacrylamide gel electrophoresis revealed ten or more distinct protein bands (Figure 6). Radioactivity, however, was observed in only three labeled peaks of approximate molecular weights of 40,000, 27,000, and 20,000. The same profile was obtained whether the Lubrol pellet was extracted directly with trichloracetic acid or sonicated prior to extraction and then dissolved in the electrophoresis solvent system. It should be noted that no radioactivity was observed in any of the low molecular weight protein bands observed in the densitometric tracing of the same gel.

The addition of valinomycin has been shown to stimulate two-fold the rate of amino acid incorporation in isolated rat liver mitochondria (12). Gel electrophoresis of a mitochondrial preparation labeled in the presence of valinomycin revealed an identical pattern of labeling. Hence, the stimu-

TABLE VI

Distribution of Radioactivity After Fractionation With Acetic Acid-Lubrol.

FRACTION		PROTEIN	TOTAL
	cpm/mg	mg	cpm
Mitochondria	4,100	50	205,000
Proteins Soluble in Acetic Acid	74	28	2,070
Residue After Acetic Acid Extraction	14,200	12	170,400
Lubrol Pellet	25,100	6.5	163,150

Mitochondria obtained from one liver were incubated for 30 min in 25 ml of incubation medium containing 0.25 μC/ml of (^{14}C)leucine as described under Materials and Methods. After incubation, the mitochondria were reisolated at 12,000 x g in 0.25 M sucrose containing 10 mM unlabeled L-leucine and fractionated with acetic acid and Lubrol.

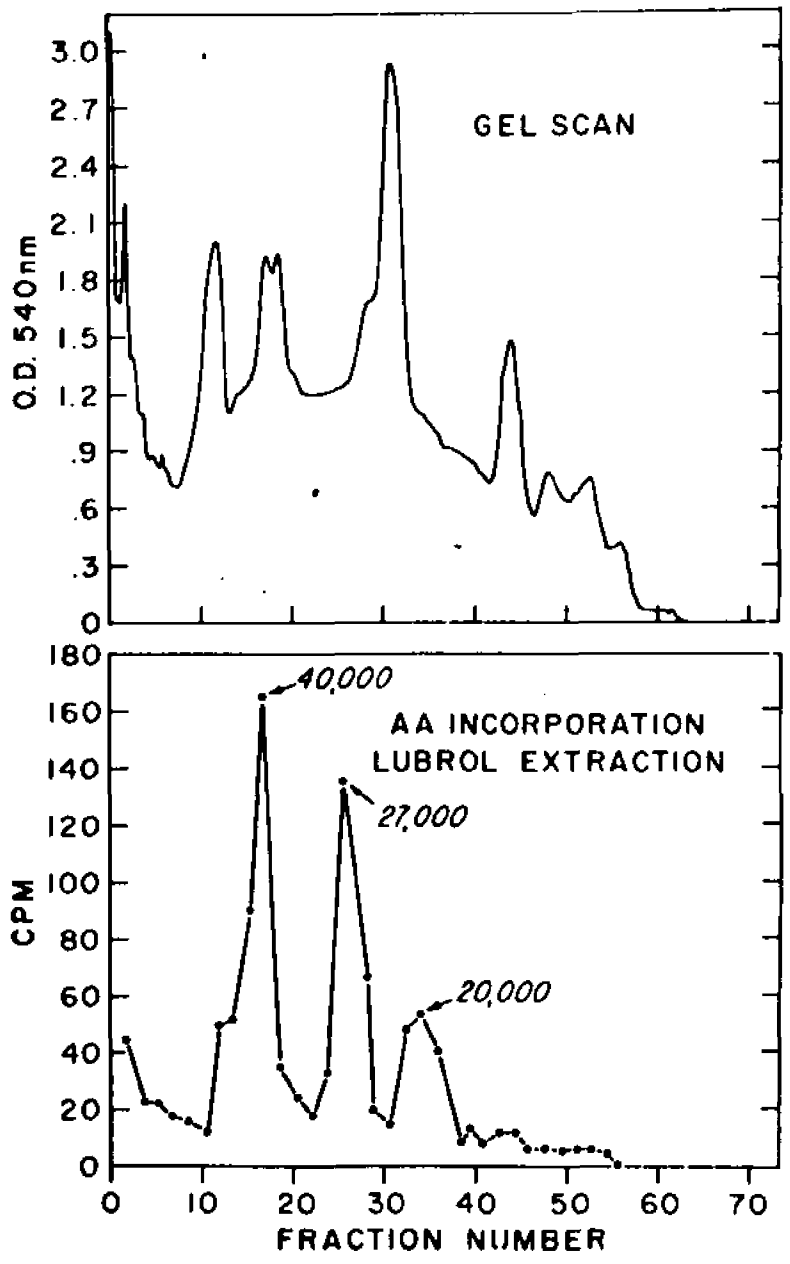

Figure 6. Electrophoretic pattern of radioactivity and optical density scan of acetic-acid Lubrol fractionated proteins. Lubrol protein fraction (Table VI) was prepared for electrophoresis as described in Materials and Methods. Approximately 100 µg of protein was added to the gels. Upper box shows optical scan of the stained analytical gel. The lower box shows actual levels of (^{14}C)leucine in the fractions.

lation observed by valinomycin reflects a general increase in the rate of protein synthesis.

The effects of the inhibitors cycloheximide and chloramphenicol on the labeling pattern were tested to determine whether the incorporation observed resulted solely from mitochondrial protein synthesis and not from microsomal contamination in the mitochondrial preparation. After incubation with the drugs, the mitochondria were reisolated and fractionated with acetic acid and Lubrol as described in Materials and Methods. The amount of radioactivity, neither specific activity nor total radioactivity, was unaffected by incubation with cycloheximide, but was decreased by nearly 85% with chloramphenicol (Table VII). Furthermore, there was no increase in specific activity after fractionation of the mitochondria incubated with chloramphenicol with a concomitant loss of 90% of the total radioactivity. Gel electrophoresis revealed that the same three protein bands were labeled in the cycloheximide treated preparation as compared with the control (Figure 7). No radioactive label was present in any part of the gel after electrophoresis of the chloramphenicol treated preparation. Thus it can be concluded that the three labeled peaks in the Lubrol-treated preparation are indeed products of the mitochondrial protein synthesizing system and were not due to contamination of the mitochondrial pellet by the endoplasmic reticulum.

To rule out the possibility that fractionation with Lubrol

TABLE VII

Effect of Cycloheximide and Chloramphenicol on Distribution of Radioactivity.

FRACTION	CONTROL		CYCLOHEXIMIDE		CHLORAMPHENICOL	
	cpm/mg	Total Counts	cpm/mg	Total Counts	cpm/mg	Total Counts
Mitochondria	810	81,000	750	75,000	140	14,000
Acetic Acid Pellet	2,610	45,600	2,200	44,000	124	2,480
Lubrol Pellet	5,110	40,800	4,920	34,400	185	2,040

Mitochondria from three livers were incubated either in the absence (Control), or presence of cycloheximide (1.6 mM), or chloramphenicol (0.47 mM) for 30 minutes in incubation medium containing 0.25 μC/ml of (^{14}C)leucine. The three preparations were reisolated and fractionated with acetic acid and Lubrol.

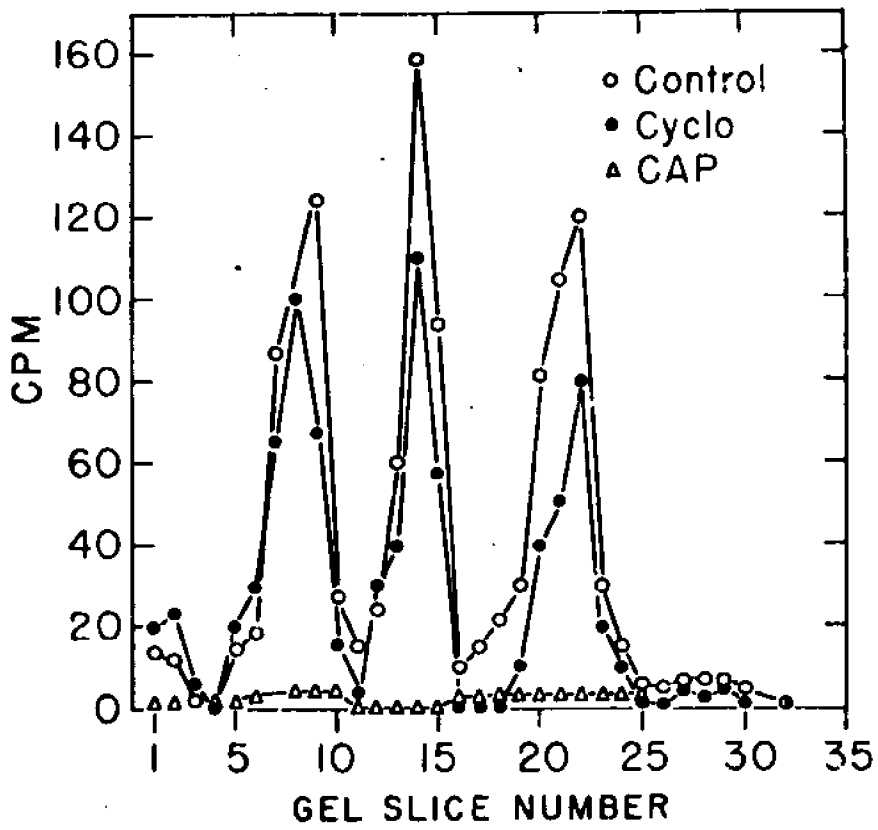

Figure 7. Electrophoretic patterns of radioactivity in the gels of the experiment described in Table VII. Lubrol fractions of control, cycloheximide, and chloramphenicol treated mitochondria were prepared for electrophoresis as described in Materials and Methods. Approximately 100 µg was added to each gel. Control, (o——o); Cycloheximide; (•——•) Chloramphenicol, (Δ——Δ).

did not solubilize some proteins synthesized by the mitochondria, other fractionation procedures were tested: sonication, extraction with KCl, and extraction with phosphate according to the method of Coote and Work (9). Extraction with KCl or sonication yielded an approximately three-fold increase in specific activity with negligible loss in total radioactivity; however, extraction with phosphate resulted in a nearly ten-fold increase in specific activity, no loss of radioactivity, and loss of 90% of the total mitochondrial protein (Table VIII). Gel electrophoresis of the three insoluble membranous fractions obtained by these procedures revealed a very different profile both in terms of number of proteins present in the fraction and in the relative amounts of each protein (Figure 8). As seen in Figures 9, 10, and 11, all three preparations contained three labeled peaks in approximately the same position as previously observed in the Lubrol preparation. A major difference in the pellet obtained after phosphate extraction was a loss of radioactivity in the protein band of 20,000 molecular weight as compared to other preparations (Figure 9).

The possibility existed that the time of electrophoresis was not sufficient to obtain full resolution of all the proteins synthesized by the mitochondria. Furthermore, it was possible that insufficient radioactivity was present in the membranes to discern any peaks with lower radioactivity. Rat liver mitochondria, incubated <u>in vitro</u> as previously described, were sonicated and the pellet containing sub-

TABLE VIII

Distribution of Radioactivity After Fractionation With Phosphate, Sonication, or KCl.

FRACTION		PROTEIN	TOTAL COUNTS
	cpm/mg	mg	cpm
Method 1			
Intact Mitochondria	1,450	50	72,700
Phosphate Pellet	14,800	4.5	66,600
Method 2			
Intact Mitochondria	2,500	50	125,000
Sonicated Pellet	7,770	15	116,500
Method 3			
Intact Mitochondria	9,610	24.2	232,560
KCl Pellet	32,800	6	196,800

In method 1, mitochondria from one liver was incubated for 30 min in 25 ml of incubation medium containing 0.25 µC/ml of (^{14}C)leucine. After incubation mitochondria were reisolated as previously described and extracted with 0.05 M PO_4 according to the method of Coote and Work (9).

In method 2, mitochondria from one liver was incubated in 25 ml of incubation medium containing 0.50 µC/ml of (^{14}C)leucine. Reisolated mitochondria were sonicated as described in Materials and Methods.

In method 3, mitochondria from one liver was incubated in 25 ml of incubation medium containing 1.5 µC/ml of (^{14}C)leucine. Reisolated mitochondria were extracted with KCl according to Beattie et al. (54).

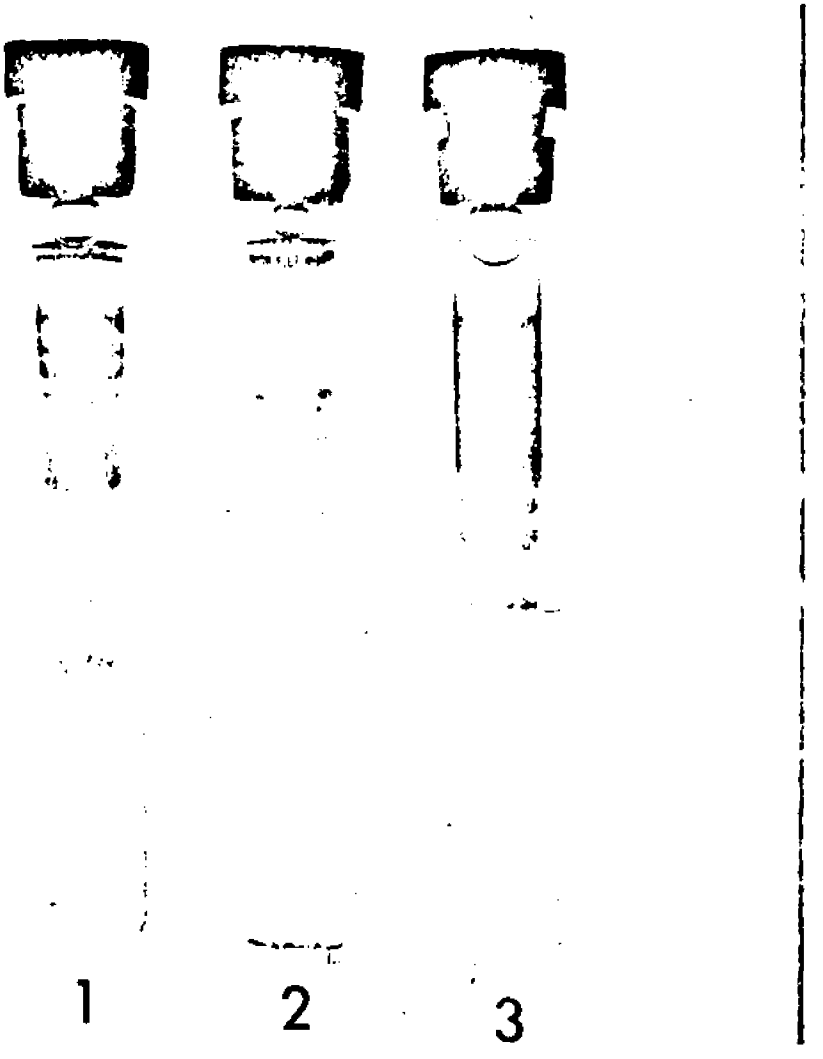

Figure 8. Polyacrylamide gel profile of insoluble fraction prepared by (1) phosphate extraction (2) sonication and (3) KCl extraction. Approximately 100 µg were added to each gel.

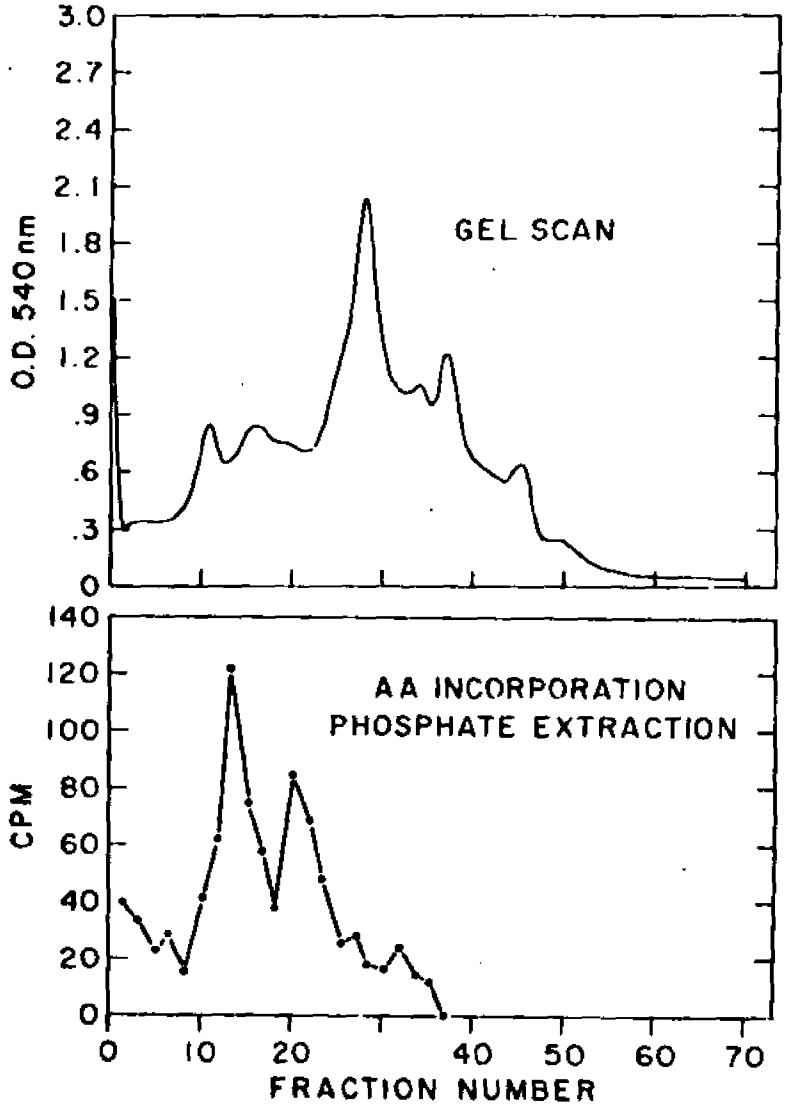

Figure 9. Electrophoretic pattern of radioactivity and optical density scan of phosphate extracted pellet prepared according to method of Coote and Work (9). Proteins were prepared for electrophoresis as described in Materials and Methods. Upper box shows an optical scan of the stained gel and lower box shows levels of radioactivity (^{14}C)leucine in the fractions of the gel.

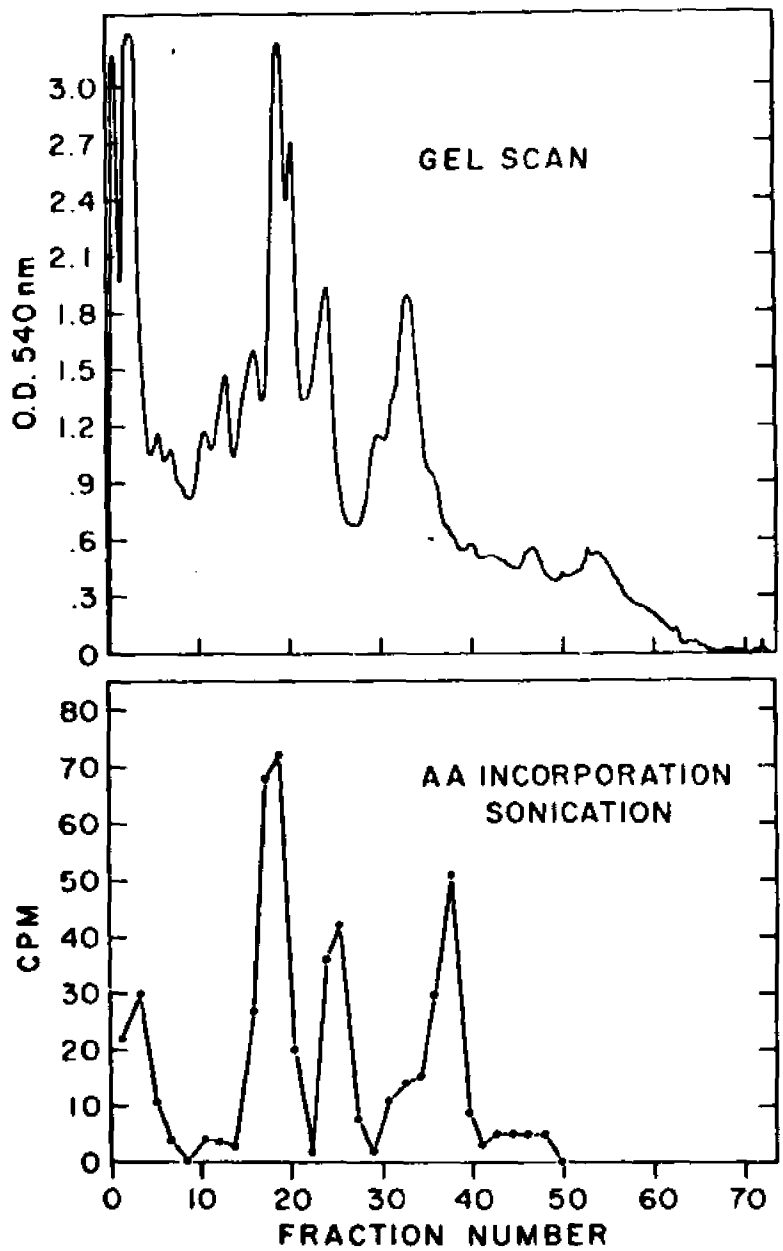

Figure 10. Electrophoretic pattern of radioactivity and optical density scan of the sonicated pellet fractionated as described in Materials and methods. Electrophoresis was carried out as described previously. Upper box is an optical density scan of the gel, and the lower box is a profile of the radioactivity in each slice.

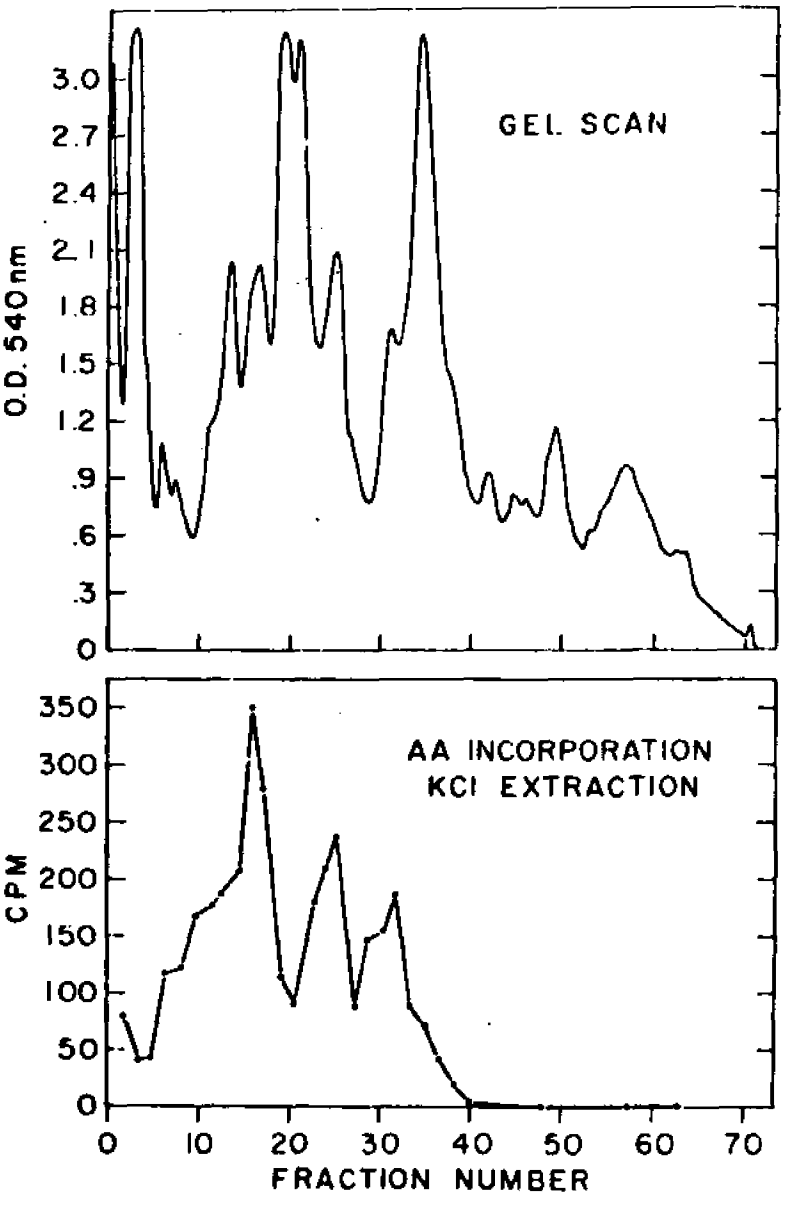

Figure 11. Electrophoretic pattern and optical density scan of proteins of insoluble KCl fraction extracted according to the method of Beattie et al. (54). Sample was prepared for electrophoresis as previously described. Upper box is an optical density scan of the gel, and the lower box is a profile of the radioactivity in each slice.

mitochondrial particles (SMP) was analyzed on gels containing a final concentration of either 10% or 12% acrylamide for sixteen and twenty-four hours respectively. The results are shown in Figures 12 and 13. As seen in Figure 12, electrophoresis of the SMP on 10% gels for sixteen hours revealed radioactivity in three peaks of molecular weights of 40,000, 27,000, and 20,000 as seen in the shorter gel. In addition, two peaks of molecular weights between 12,000 and 16,000 were also apparent. After electrophoresis of the SMP on 12% gels for twenty-four hours, the two peaks of highest molecular weight were resolved more fully into at least five peaks, while there seems to be at least three molecular weight peaks in the molecular weight range below 20,000.

The proteins labeled after an in vitro incubation of isolated mitochondria were compared to the products of mitochondrial synthesis in vivo. Liver mitochondria were obtained from rats injected with (^{3}H)leucine after treatment with sufficient cycloheximide to block microsomal protein synthesis more than 97% (102) and fractionated with acetic acid and Lubrol. An approximate seven-fold increase in specific activity was observed in the Lubrol pellet with a recovery of 62% of the total radioactivity and less than 10% of the total protein (Table IX). An aliquot of the Lubrol preparation was thoroughly mixed with an equivalent aliquot of a Lubrol pellet prepared from mitochondria after an in vitro incubation with (^{14}C)leucine. Approximately 150 µg of this mixture was analyzed by gel electrophoresis

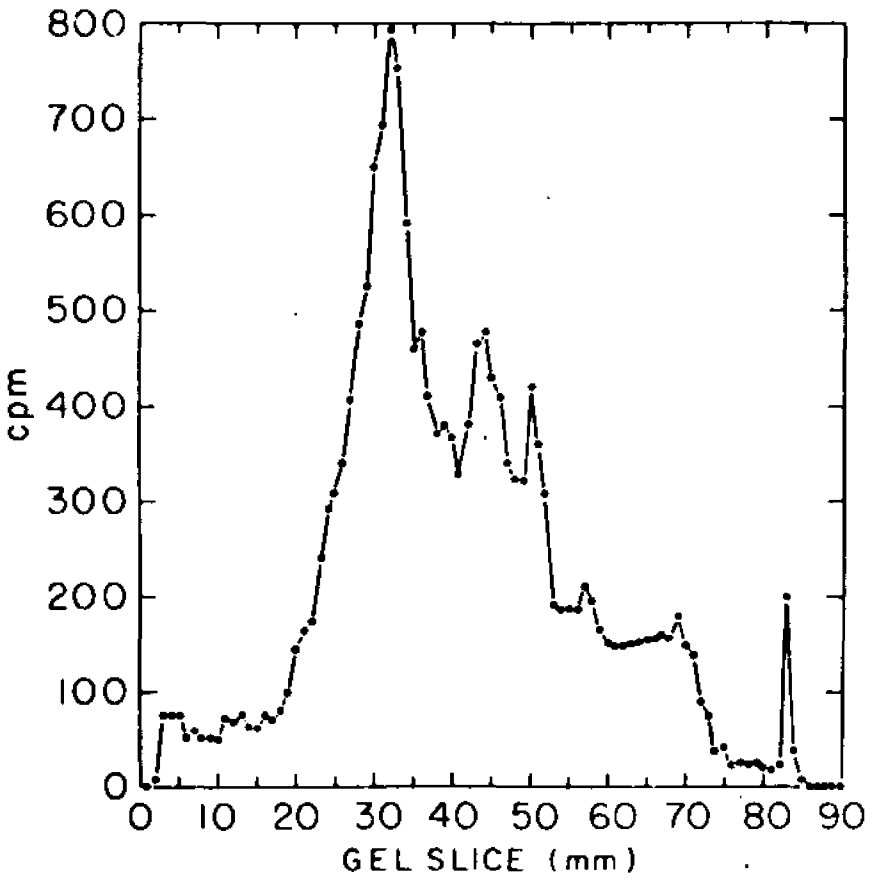

Figure 12. Electrophoretic pattern of radioactivity of a sonicated pellet fractionated as described in Materials and Methods. Proteins in the sonicated pellet were prepared for electrophoresis as described previously. The concentration of acrylamide was 10%, and the time of electrophoresis was 16 hours. The specific activity of the sonicated pellet was 140,000 cpm/mg. Approximately 150 µg was added to the gel.

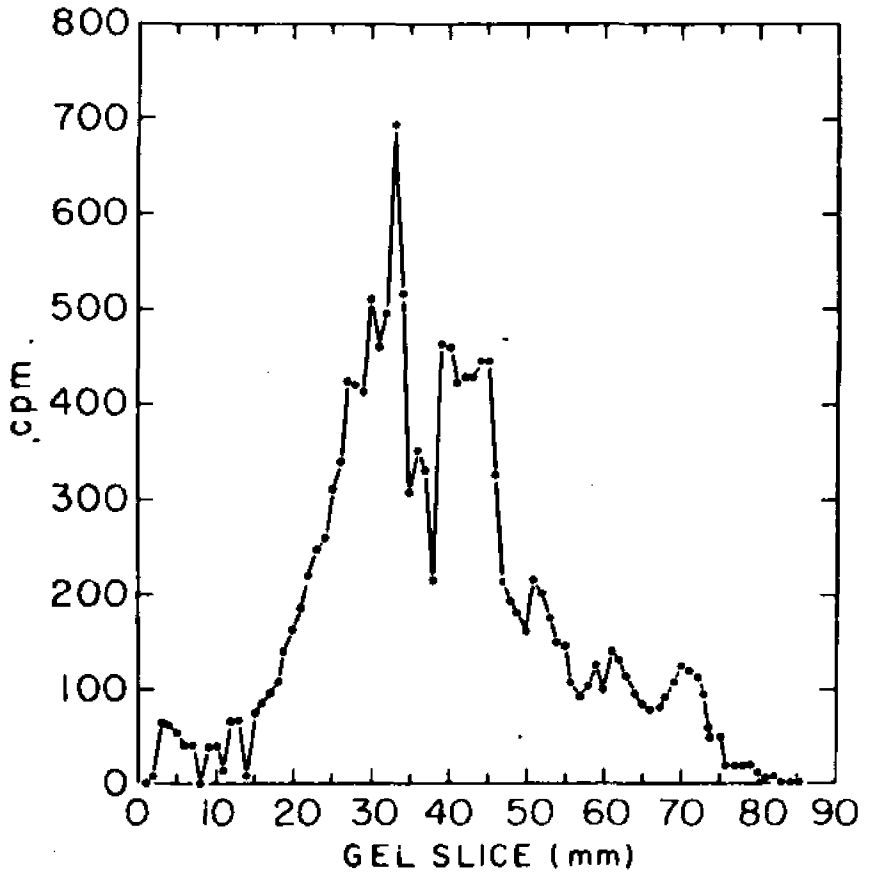

Figure 13. Electrophoretic pattern of radioactivity of a sonicated pellet fractionated as described in Materials and Methods. Proteins were prepared for electrophoresis as previously described. The concentration of acrylamide was 12% and the time of electrophoresis was 24 hours. The specific activity of the pellet was 109,000 cpm/mg. Approximately 150 µg was added to the gel.

TABLE IX

Distribution of Radioactivity After Acetic Acid-Lubrol Fractionation of Liver Mitochondria Labeled *In Vivo*.

FRACTION		PROTEIN	TOTAL COUNTS
	cpm/mg	mg	cpm
Intact Mitochondria	842	69.6	58,600
Acetic Acid Pellet	3,660	10.1	36,860
Lubrol Pellet	5,730	6.1	34,950

Cyclohexamide (50 mg/kg body weight dissolved in 0.9% NaCl) was injected intraperitoneally into a rat 15 minutes before the intraperitoneal injection of 500 μCi of (^{3}H)leucine. Two hours later the rat was sacrificed, liver excised, and mitochondria isolated as described in Materials and Methods. The reisolated mitochondria was extracted with acetic acid and Lubrol in the same manner as in the *in vitro* studies.

(Figure 14). The bands labeled with (^{3}H)leucine were found to have the same relative mobilities as those labeled with (^{14}C)leucine.

The possibility of a precursor-product relationship or incomplete synthesis of polypeptide chains during an *in vitro* incubation was investigated by use of short time incubations. The bands labeled after a ten minute incubation were compared with those obtained after a ten minute incubation followed by a chase with unlabeled leucine. An identical increase in specific activity was observed in the Lubrol pellet from mitochondria obtained after both incubation conditions (Table X). Gel electrophoresis of both preparations revealed similar labeled peaks with relatively the same mobilities as those observed after the usual thirty minute incubation (Figure 15); however, the ratio of counts in the three peaks was slightly different in the pellet obtained after a ten minute incubation. The highest molecular weight peak contained fewer counts than the 27,000 molecular weight peak, a reversal of the situation observed after a thirty minute incubation. This result may be due to different rates of synthesis of these proteins. However, the observation that only three bands were labeled in relatively the same position on the gel in both preparations, seems to preclude any precursor-relationship.

Beattie and Stuchell (103) reported that the administration of allylisopropylacetamide (AIA) to rats caused an increase of δ-aminolevulinic acid (ALA) synthetase activity in

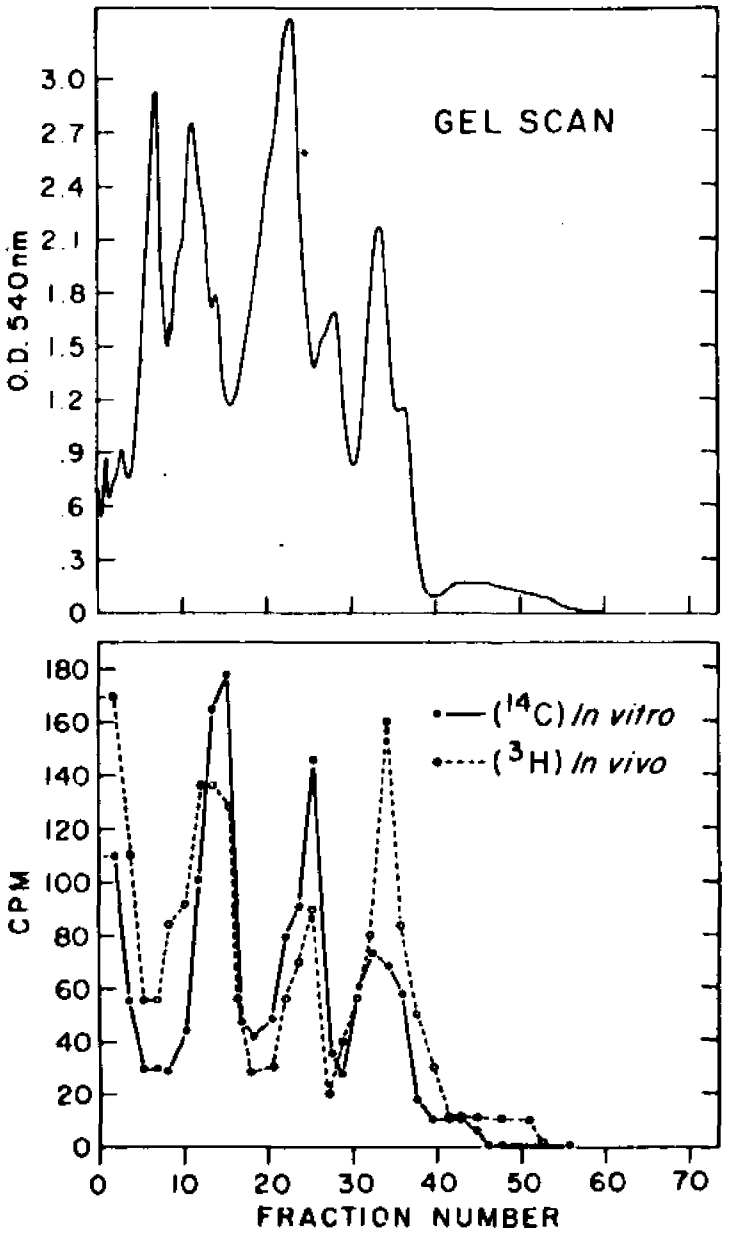

Figure 14. Analysis of samples of an *In vitro* (Table VI) and an *In vivo* (Table IX) preparation subjected to acetic-acid-Lubrol fractionation. Equal aliquots of the two samples were mixed thoroughly, and approximately 150 µg of the mixture were added to the gels (o——o) (^{3}H)leucine (*In vivo*); (•——•) (^{14}C)leucine (*In vitro*).

TABLE X

Effect of Short-Time Incubations on Distribution of Radioactivity.

FRACTION		PROTEIN	TOTAL COUNTS
	cpm/mg	mg	cpm
Experiment 1			
Intact Mitochondria	5,560	40	222,400
Acetic Acid Pellet	29,000	-	-
Lubrol Pellet	37,000	3.4	125,800
Experiment 2			
Intact Mitochondria	16,800	40	672,000
Acetic Acid Pellet	77,000	6	462,000
Lubrol Pellet	96,000	3.5	336,000

In experiment 1, mitochondria from one liver was incubated for 10 minutes in 20 ml of incubation medium containing 1.25 µC/ml (^{14}C)leucine and 0.5 µg/ml of valinomycin and chased for 20 minutes with 10 mM unlabeled L-leucine. After the incubation, the mitochondria were reisolated and fractionated with acetic acid-Lubrol.

In experiment 2, mitochondria from one liver was incubated for 10 minutes in a metabolic shaker in 20 ml of isolation medium containing 2 µC/ml of (^{14}C)leucine and 0.5 µg/ml of valinomycin. After incubation the mitochondria were reisolated and fractionated with acetic acid-Lubrol.

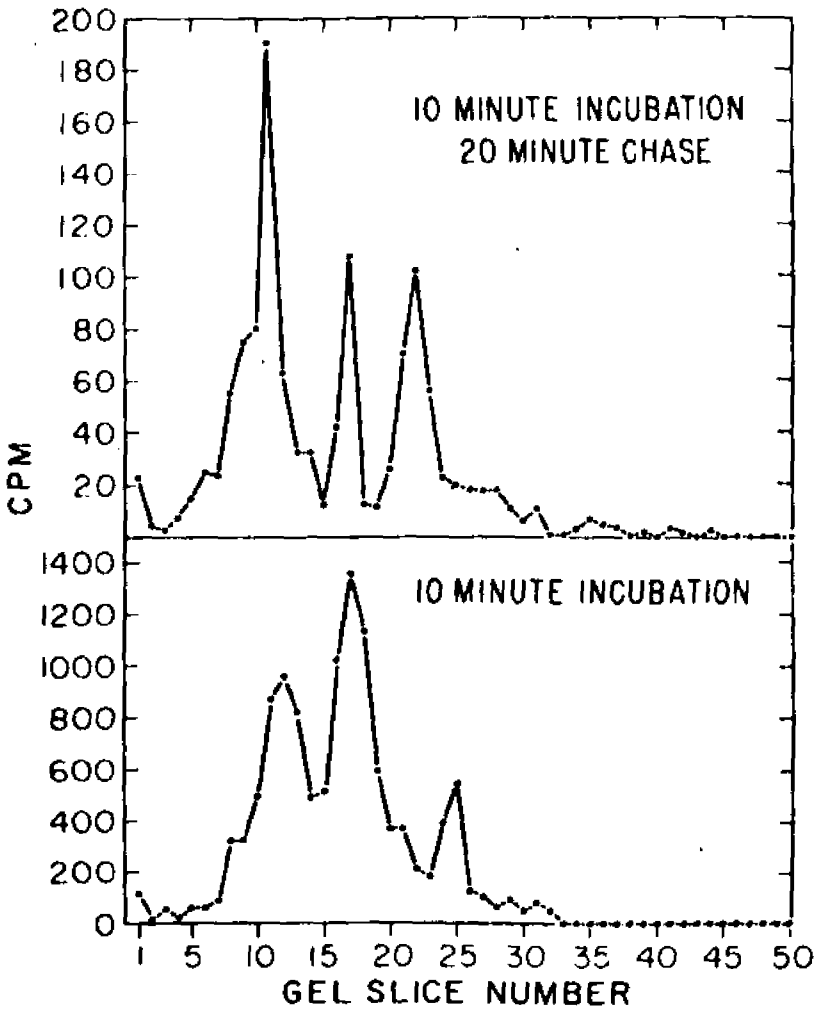

Figure 15. Electrophoretic patterns of radioactivity of the two experiments described in Table X. Upper box represents 10 minute incubation with (^{14}C)leucine followed by a 20 minute chase while the lower box represents the ten minute incubation. Electrophoresis was run as previously described.

rat liver mitochondria. In a subsequent study, Beattie (104) observed that liver mitochondria obtained from animals after AIA treatment had a 50-60% greater rate of amino acid incorporation *in vitro* as compared to mitochondria from control animals. It was of interest to learn whether this increased rate of protein synthesis was due to a stimulation of labeling in all the bands observed or in a selective stimulation of certain proteins. Dietyl 1,4-Dihydro-2,4,6-trimethylpyridine-3,5-dicarboxylate (DDC) was injected into rats to induce ALA synthetase activity (105). Amino acid incorporation was studied in the mitochondria obtained from rats injected with DDC sixteen hours prior to sacrifice. The incorporation rate was stimulated nearly two-fold compared to that of the control. However, the radioactive profile was similar to that of the control, indicating that the increased rate of protein synthesis in the mitochondria of porphyric rats is non-specific.

1. Proteolipids

Less than 10% of the protein of the submitochondrial particles and 16% of the radioactivity were extracted by neutral chloroform:methanol after an incubation of liver mitochondria *in vitro* with (^{3}H)leucine (Table XI, Method 1). The loss of total radioactivity observed after this method of extraction resulted partly from the difficulty in recovering all of the residue protein when the chloroform: methanol extracts were centrifuged, and alternately, from

TABLE XI

Extraction of Labeled Mitochondrial Proteins by Chloroform:Methanol.

	Specific Activity	Protein	Total Radio-Activity	% of Total
	cpm/mg	mg	cpm	
Method 1				
Particles	92,600	5.0	463,000	100
C:M Extract	173,000	0.42	72,600	16
Residue	46,400	3.5	162,000	35
Method 2				
Particles	111,000	5.0	555,000	100
Methanol Extract	-	-	122,000	25
C:M Extract	269,000	0.3	80,800	7
Residue	63,800	4.4	281,000	68

Isolated mitochondria were incubated with 20 μC_1/ml of (^{3}H)leucine (50 C_1/mmole) and fractionated as described in Materials and Methods.

the possible loss of radioactive proteins into the washes of the lipid fraction. Extraction with methanol prior to chloroform:methanol (Method 2) removed about 25% of the total radioactivity. These counts may represent free leucine not present in peptide linkage (85), or polypeptides which are not soluble in methanol. As a result, the chloroform: methanol extract obtained by this method of extraction contained only 8% of the total radioactivity. The specific activity of the chloroform:methanol extract, after both extraction procedures, was double that of the submitochondrial particles (Table XI) or four times greater than that of intact mitochondria.

Incorporation into the proteolipid fraction was inhibited to the same extent as the intact mitochondria by chloramphenicol (70%) and carbomycin (80%), both known to be specific inhibitors of protein synthesis on mitochondrial ribosomes (Table XII). These results suggest that the radioactivity present in the proteolipid fractions represents products of the mitochondrial system for protein synthesis.

The proteins of the submitochondrial particles labeled <u>in vitro</u> after SDS-gel electrophoresis revealed seven peaks (Figure 16). The major radioactive proteins were present in that portion of the gel corresponding to a molecular weight of 40,000 with a shoulder at 48,000. Significant radioactivity was also present in peaks of molecular weights of 28,000 and 20,000, while minor labeling was observed in three peaks of lower molecular weight. The proteolipid fractions (extract),

TABLE XII

Effect of Inhibitors on Proteolipid Synthesis.

FRACTION	cpm/mg		
	Intact Mito	C:M Extract	Residue
Control	15,400	65,000	20,200
+chloramphenicol	4,520	20,300	4,840
%Inhibition	71	69	76
+carbomycin	3,080	12,300	4,240
%Inhibition	80	81	79

Mitochondria were incubated with 5 $\mu C/_1 ml$ of (^3H)leucine with 100 µg/ml of chloramphenicol and 30 µg/ml of carbomycin and fractionated as described in Materials and Methods.

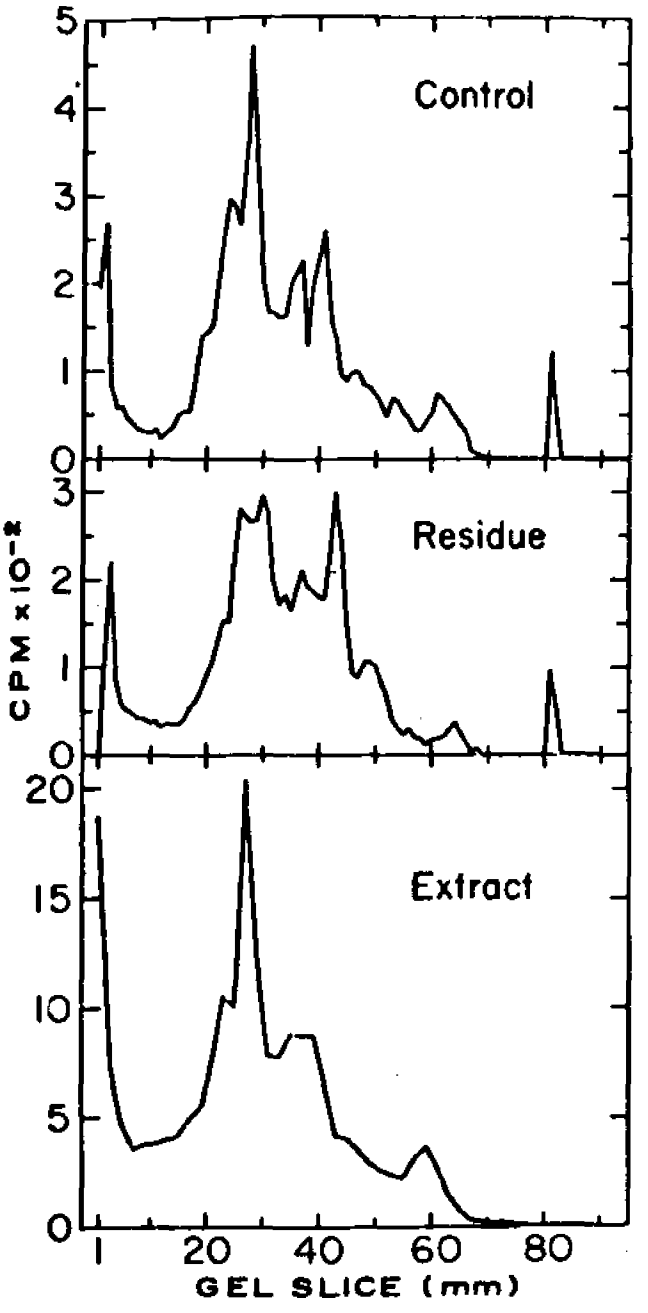

Figure 16. SDS-gel electrophoresis of 100 µg of submitochondrial particles (CONTROL), 50 µg of the washed chloroform:methanol extract (EXTRACT) and 100 µg of the residue after chloroform:methanol extraction. Specific activities of the fractions are presented in Table XI (Method 1). Recovery from the gel 95%. The gels were cut into 1 mM slices and counted (85).

prepared by Method I, separated into a sharp peak of 40,000 molecular weight with a slight shoulder and two very broad peaks of low molecular weight. In the residue after extraction with chloroform:methanol, labeling of the 40,000 molecular weight peak was diminshed, while an almost complete loss of radioactivity in the two lowest molecular weight peaks was observed. The magnitude of labeling of the other peaks was unchanged by extraction with chloroform:methanol. Extraction with acidic chloroform:methanol also removed 20% of the counts, but the extract did not separate into clearly defined peaks after gel electrophoresis.

The possibility remained that the method of Linnane (89), involving four washes of the chloroform:methanol extract may have caused the loss of some of the proteolipids synthesized by the mitochondria. Two alternative approaches were tested: a single extraction of the SMP with chloroform: methanol without the water washes and lyophilization of the submitochondrial particles prior to extraction with chloroform:methanol (93). Extracts from these two procedures were dried under a stream of nitrogen and prepared for gel electrophoresis as described in Materials and Methods. The radioactive profiles are shown in Figures 17,18, and 19. The gel pattern of the extract which was not washed (Figure 18) looks similar to that in the washed chloroform:methanol extract (Figure 17); however, the 10,000 molecular weight peak appears to contain more radioactivity in the non-washed extract. In contrast, only three broad peaks were observed

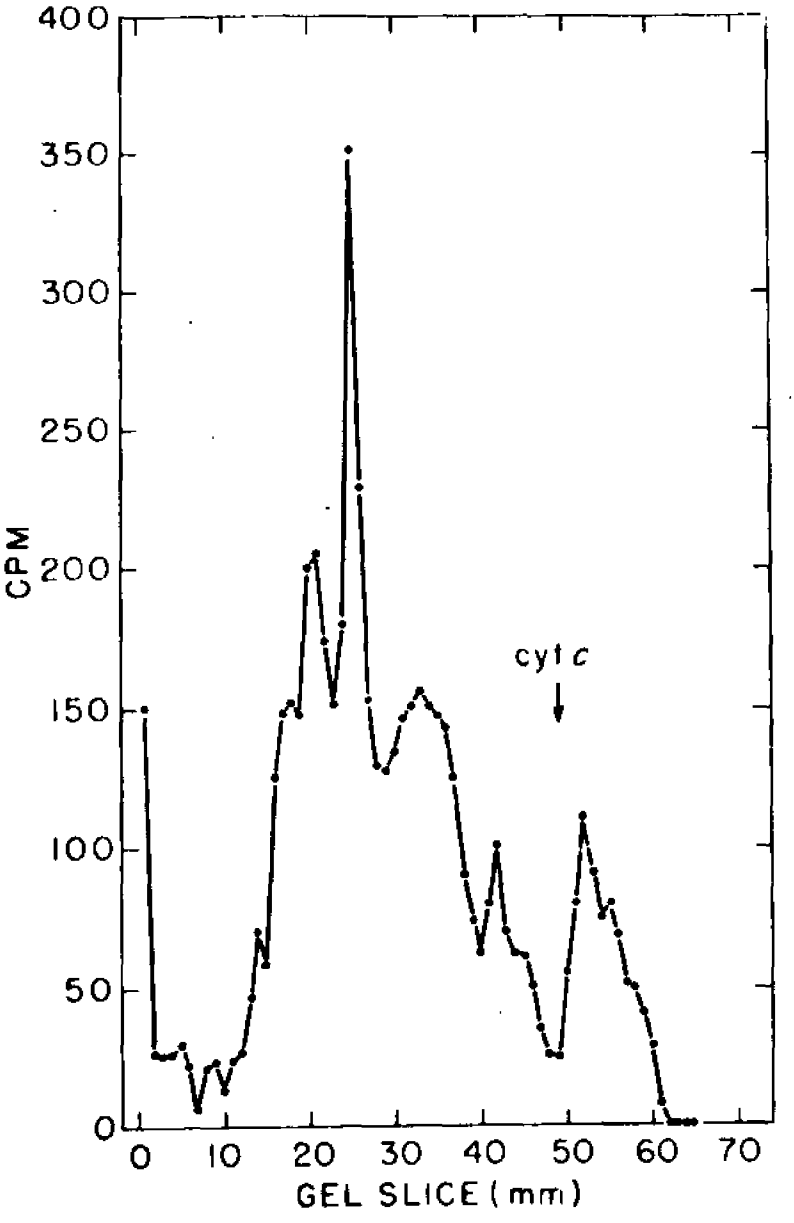

Figure 17. Electrophoretic pattern of radioactivity of washed chloroform:methanol extract. Extract was prepared for electrophoresis as described in Materials and Methods. The specific activity of the chloroform:methanol extract was 130,000 cpm/mg. 50 µg was added to the gel.

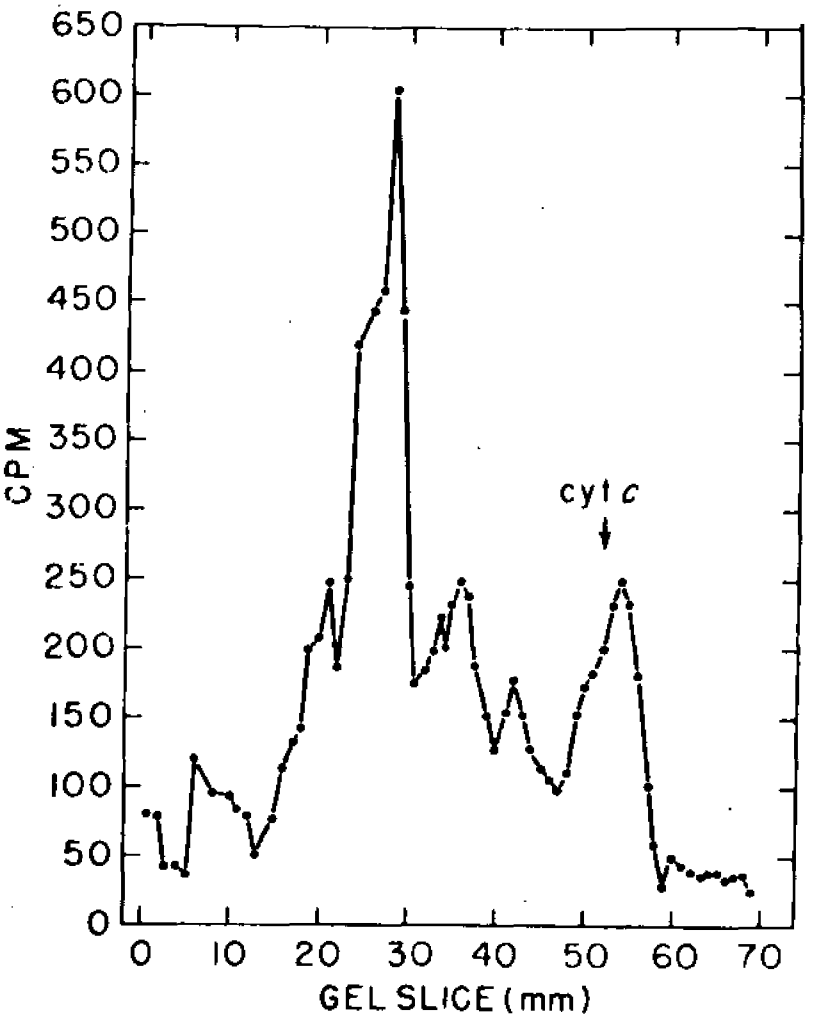

Figure 18. Electrophoretic pattern of radioactivity of non-washed chloroform:methanol extract. The extract was prepared for electrophoresis as previously described. The specific activity of the chloroform:methanol extract was 100,000 cpm/mg. 100 µg was added to the gel.

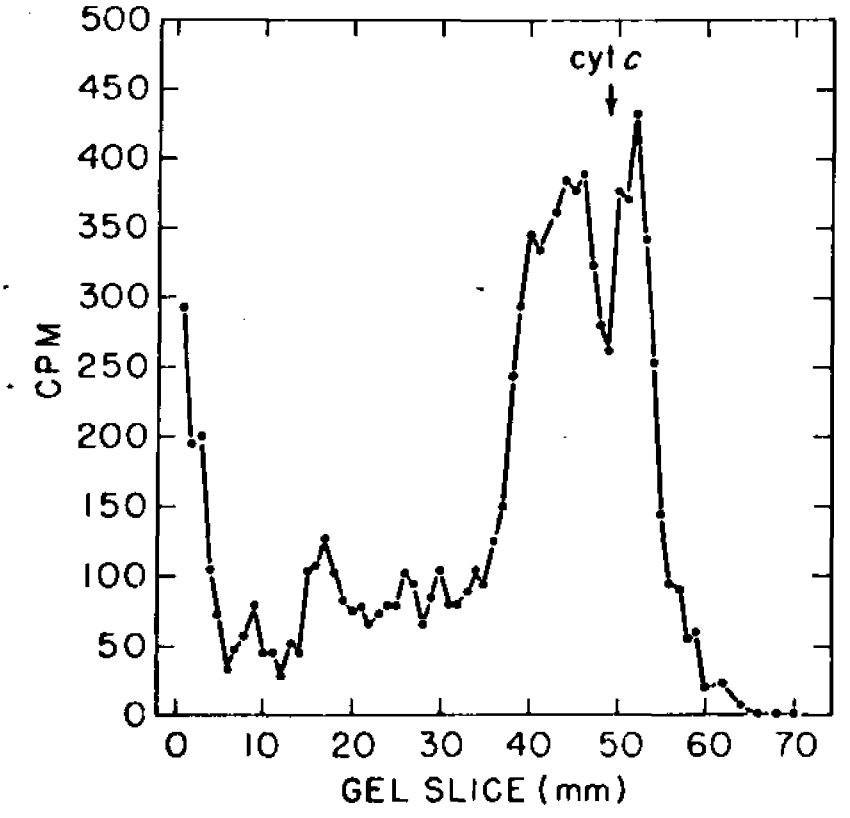

Figure 19. Electrophoretic pattern of chloroform:methanol extract from lyophilized submitochondrial particles prepared according to Method 3 (93). Extract was prepared for electrophoresis as previously described. The specific activity of the chloroform:methanol extract was 98,000 cpm/mg. 100 µg was added to the gel.

in the low molecular weight region of 14,000 to 10,000, in the chloroform:methanol extract of the lyophilized mitochondrial membranes (Figure 19). Insignificant counts were observed in the region of the gel corresponding to molecular weights greater than 14,000.

In a previous study, Ibrahim et al. (106) reported that the rate of yeast mitochondrial protein synthesis measured both in vitro and in vivo was stimulated when cells were allowed to accumulate products of cytoplasmic protein synthesis by growth of the cells for various times in chloramphenicol. It was hoped that this increased rate of labeling of mitochondrial membranes in vivo might be a reflection of an increased rate of proteolipid synthesis. To test this possibility, yeast cells were grown for thirteen hours in 5% glucose at which time the culture was divided into two equal parts. To one was added chloramphenicol (4 mg/ml), while the other was used as the control. After another three hours of growth, both cultures were harvested, washed, and incubated in vivo as described in Materials and Methods. Submitochondrial particles were prepared from both preparations and extracted with chloroform:methanol (2:1, v/v). A stimulation was observed in the specific activity of the mitochondrial membranes obtained from cells grown for three hours in chloramphenicol compared to that of the control (Table XIII). In both preparations approximately 13% of the total radioactivity in the membraneswas extracted with chloroform:methanol and less than 10% of the protein. The gel profile of the membranes revealed a stimulation of the low

TABLE XIII

Extraction of Yeast Submitochondrial Particles Grown in Chloramphenicol for Three Hours and Transferred to Fresh Medium.

	Specific Activity	Protein	Total Radio-Activity	% of Total
	cpm/mg	mg	cpm	
Control				
Submitochondrial Particles	64,295	12	731,546	
C:M Extract	125,000	0.8	100,000	13.6
Chloramphenicol Treated				
Submitochondrial Particles	100,728	8	805,824	
C:M Extract	198,000	0.5	99,000	12.2

Partially derepressed yeast cultures were grown for three hours in the presence or absence of chloramphenicol. The cells were washed, harvested, and incubated *in vivo* as described in Materials and Methods.

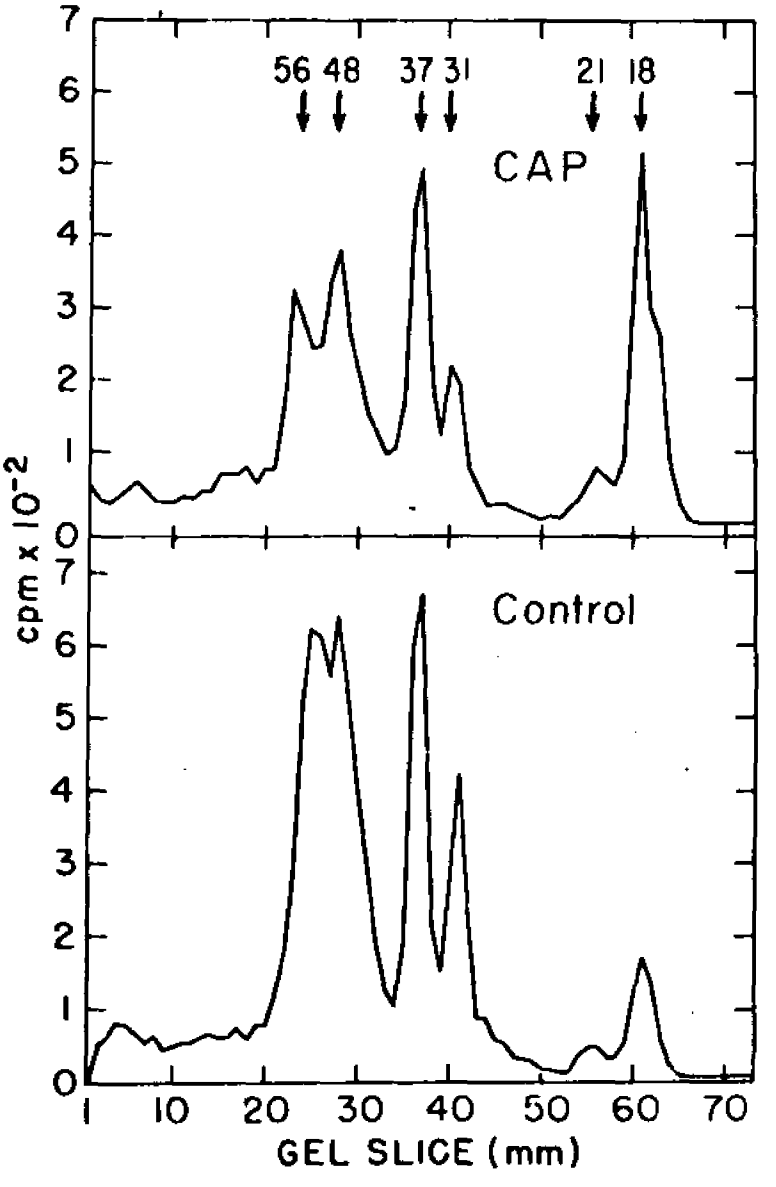

Figure 20. Sodium dodecylsulfate electrophoresis profiles obtained from yeast cells treated as described in Table XIII. Mitochondrial membranes were prepared as described in Materials and Methods and subjected to gel electrophoresis.

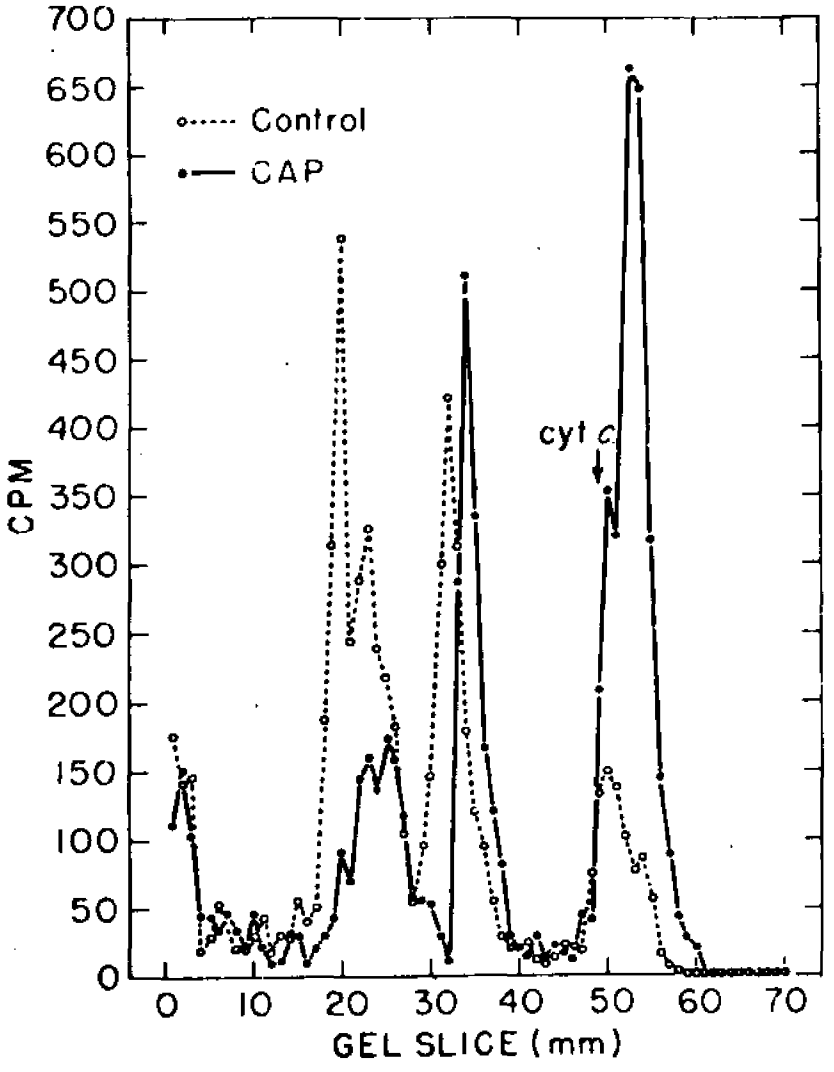

Figure 21. Electrophoretic patterns of chloroform:methanol extracts of submitochondrial particles from yeast cells treated as described in Table XIII. (o——o)- control; (•——•)- chloramphenicol treated.

molecular weight protein in the cells preincubated in the chloramphenicol (Figure 20). In addition, an almost six-fold stimulation was observed in the band of low molecular weight in the chloroform:methanol extract (Figure 21). Furthermore, less radioactivity was present in the two bands of highest molecular weight.

V. Discussion

Previous studies have indicated that the substitution of sucrose for KCl to maintain osmolarity in the medium resulted in a decrease in the rate of amino acid incorporation (4). Subsequently, Haldar and Freeman (107) confirmed that decreasing amounts of sucrose in the medium increased the incorporation rate. Several groups have reported that further addition of NaCl (108) or NH_4Cl (99) to the medium containing KCl resulted in a stimulation of the rate of amino acid incorporation. The stimulatory effects of NH_4^+ were later confirmed (8,9). The results of the present study, however, indicate that potassium is the preferred cation for studies of amino acid incorporation in rat liver mitochondria incubated with either ATP and the regenerating system, PEP and pyruvic kinase, or with ATP and succinate. Replacement of KCl with either NH_4Cl or NaCl resulted in decreased rates of incorporation. Furthermore, the substitution of either of these ions for potassium while maintaining a constant ionic strength caused a decrease in the incorporation rate.

Cunningham and Bridgers (23) reported no difference in incorporation by rat liver mitochondria when NaCl replaced KCl in the incubation medium; however, the rates of incorporation were significantly lower than those reported here.

The need for potassium has been shown previously to be essential for protein synthesis in other systems. In 1969, Coleman (109) reported that in the presence of 0.05 M Tris buffer, pH 7.6, maximum amino acid incorporation into proteins by gently disrupted cells of B. amyloliquefaciens were achieved in the presence of 0.01 M-Mg^{+2} and 0.07-0.1 M-K^{+}. It thus appears that a similar situation exists in the mitochondrial protein synthesizing system.

A stimulation of amino acid incorporation was observed in the presence of low concentrations of phosphate. When phosphate was omitted, an approximate 60% decrease in the incorporation rate was observed in the presence of succinate and ADP where ATP produced by the respiratory chain provided the necessary energy for protein synthesis. In contrast, a 30% decrease in the incorporation rate was observed in the ATP-PEP regenerating system when phosphate was removed. One possible role of phosphate in this system might be as a specific anion for the uptake of substrates or transport of ATP across the inner mitochondrial membrane.

The data presented here suggests that high intramitochondrial levels of ATP result in enhanced rates of amino acid incorporation. The need for 2 mM concentrations of exogenous ATP during an incubation in which no more than 7-40

pmoles of leucine are incorporated into protein (110), suggests that ATP may be involved in processes other than amino acid incorporation in peptide bond formation. Perhaps ATP may play a role in maintaining the proper membrane conformation. In support of this idea, Linnane and Haslam (111) have suggested that the mitochondrial ribosome may be an integral part of the inner mitochondrial membrane. Similarly, Perlman and Mahler (112) have suggested that changes in the membrane may affect the mutational effects of ethidium bromide.

Mitochondria prepared by extensive washings with sucrose can support amino acid incorporation to approximately the same extent whether ATP is generated by the respiratory chain or by an external ATP-regenerating system consisting of ATP, PEP and pyruvate kinase. A decrease in activity was observed when ATP without PEP was added to the incubation medium. Sometimes variations were observed from one preparation to another; however, in many different experiments the rate of amino acid incorporation was approximately the same whether ADP and succinate were added to the incubation medium or ATP and PEP. It thus appears that rat liver mitochondria as prepared in our laboratory can incorporate amino acids into protein using either an external source of ATP plus an ATP regenerating system or ATP synthesized by the respiratory chain. This is in direct contrast to the report of Coote and Work (9) in which much higher rates of incorporation were obtained when ATP was generated by oxidative phosphorylation.

In the presence of succinate and either ATP or ADP, the necessary ATP was largely generated by the respiratory chain as indicated by the 90% inhibition by antimycin A. In the presence of the ATP-regenerating system, the majority of the ATP must have been transported across the mitochondrial membrane as indicated by the 70% inhibition by atractyloside. However, some of the incorporation in the presence of exogenous ATP and the regenerating system were still dependent on oxidative phosphorylation as indicated by the 30-40% inhibition by antimycin A. It thus appears that mitochondrial protein synthesis occurs in the matrix of the mitochondria, and that intramitochondrial ATP, either generated by respiratory chain linked phosphorylation or transported across the inner membrane, provides the energy necessary for amino acid incorporation.

The inhibitory effects of atractyloside in the presence of ATP and the regenerating system do not agree with previous studies which indicated that atractyloside stimulated amino acid incorporation (113) or had no effect (110). Perhaps the incorporation in these studies was soley dependent on ATP synthesized within the mitochondria and not by the transport of exogenous ATP into the mitochondria. It is interesting in this context that addition of respiratory substrates reversed the atractyloside inhibition observed in the ATP-PEP system (12). Presumably these additional substrates might generate sufficient ATP by respiration using the endogenous adenine nucleotide pools.

The optimal conditions for the study of various antibiotics on mitochondrial protein synthesis were investigated. It was found that maximum incorporation in terms of pmoles of leucine/mg protein was attained at a leucine concentration of 40-60 μM. At this concentration, the radioactive amino acid was not rate limiting and one could thus evaluate the effect of the antibiotic on peptide bond synthesis at the ribosome.

The effects of certain antibiotics on protein synthesis in rat liver mitochondria were investigated at a final concentration of 40 μM leucine. Erythromycin and lincomycin had no inhibitory effect on protein synthesis in whole mitochondria; however, significant inhibition by these antibiotics was observed when the mitochondria were either first preincubated with the drugs or fractionated with digitonin to form inner membranes and then incubated with radioactive leucine. When incorporation was tested in the hypotonic medium described by Firkin and Linnane (36) no significant inhibition by erythromycin or lincomycin was observed. However, the rate of incorporation, as measured in pmoles of amino acid/mg of protein, is extremely low, such that any true effect on mitochondrial protein synthesis could possibly be obscured. Our results seem to confirm the suggestion of Kroon and DeVries (40) that the lack of inhibition of incorporation by lincomycin and erythromycin in intact mitochondria is due to the impermeability of the inner membrane to the antibiotic.

An alternative explanation for the lack of inhibitory

effect of the macrolides has been proposed by Linnane's group (43). They have suggested that during evolution a protein or proteins of the mammalian mitochondrial ribosome may have changed in such a way that the ribosome retained the ability to bind the antibiotic but protein synthesis was unaffected. One difficulty in deciding between these two alternatives is that the concentration of erythromycin needed to inhibit protein synthesis in rat liver mitochondria was almost double that needed for a similar inhibition in yeast mitochondria. Hence, the mammalian mitochondrial ribosome may have lost some of its sensitivity to erythromycin during evolution. The only way to establish definitely the sensitivity of these ribosomes is to test the effect of erythromycin on isolated ribosomes which are active in protein synthesis. Ibrahim and Beattie (114) recently reported that low concentrations of erythromycin (0.1 mM) or carbomycin inhibited protein synthesis on isolated rat liver ribosomes which were highly active in catalyzing poly (U)-directed polyphenylalanine synthesis. Most important, however, was the observation that these drugs inhibited protein synthesis on yeast mitochondrial ribosomes to the same extent as the liver mitochondrial ribosomes.

Inhibitors which act at the level of transcription were also tested for their effect on amino acid incorporation by rat liver mitochondria. Actinomycin D, a potent inhibitor of nuclear RNA synthesis, was found to have negligible effect on protein synthesis in rat liver mitochondria. In contrast,

both rifampicin and cordycepin, which are potent inhibitors of mitochondrial transcription, significantly inhibited amino acid incorporation. These results suggest a relatively short half life for mRNA in the mitochondria.

Chloropromazine is a drug which specifically affects the central nervous system. Goertz, Emmerich, and Kerstein (115) reported that chloropromazine inhibits up to 30% protein synthesis *in vitro* assayed with either polysomes or ribosomes from rat brain or liver. The inhibition was reported to result from the inhibition of the aminoacylation of the tRNA. These workers also examined the effect of chloropromazine on aminoacylation of *E. coli* tRNA and mammalian tRNA and observed a slight difference in kinetics, magnesium dependency, and extent of inhibition. In the mammalian system, chloropromazine does not appear to interfere directly with tRNA or the aminoacylating enzyme, but interacts with the complex of amino acid synthetase and tRNA. In our studies, the degree of inhibition of mitochondrial protein synthesis was found to be significant (38%) at a concentration of the antibiotic of 10^{-6} M.

The non-ionic detergent Lubrol has been shown by Schnaitman and Greenawalt (7) to be effective in solubilizing mitochondrial matrix proteins. In the present study, this detergent was coupled with the acetic acid extraction used previously (54) to obtain a fraction with a significant increase in specific activity over that of the unfractionated mitochondria. In addition, it was hoped that a more homogenous

preparation might result for analytical studies on SDS-polyacrylamide gel electrophoresis. A 5-7 fold increase in specific activity was observed in the pellet obtained after this fractionation procedure as well as an 87% loss of total protein. Gel electrophoresis revealed three radioactive peaks corresponding to molecular weights of 40,000, 27,000, and 20,000.

To determine whether some proteins labeled by the mitochondria during the in vitro incubation were solubilized by the acetic acid-Lubrol fractionation scheme, other techniques, which remove much less of the total protein, were employed. The insoluble residues obtained after KCl extraction, sonication, or phosphate extraction were analyzed on gel electrophoresis and found to contain three radioactive peaks with approximately the same relative mobilities as found in the Lubrol preparation. In these preparations, a greater recovery of the original radioactivity was achieved.

In order to determine if the time of electrophoresis (4½ hours) was optimal for good resolution, longer gels were run with more concentrated acrylamide (10% and 12%) and for longer times (16 and 24 hours). The majority of the radioactivity after incubation in vitro still remained in the three large molecular weight peaks; however, some significant radioactivity was observed in 2-3 smaller molecular weight peaks. Twenty-four hour gel electrophoresis revealed some resolution of the 40,000 and 27,000 peaks into two bands each. Thus one can conclude from these analytical studies that rat

liver mitochondria are capable of synthesizing 7-8 proteins of molecular weights ranging from 50,000 to 10,000. It is apparent that the same number of proteins are synthesized by mammalian mitochondria as by mitochondria from simpler eukaryotes which possess several times more mitochondrial DNA.

Studies of amino acid incorporation are always subject to the criticism that incubation studies may be inadequate for maximum labeling of certain proteins or enzyme complexes. Hence, the products of mitochondrial synthesis in vivo obtained in animals treated with cycloheximide were compared to the products of the in vitro incorporation. Co-electrophoresis of aliquots of a Lubrol preparation labeled in vitro with (^{14}C)leucine and one labeled in vivo with (^{3}H)leucine revealed that the three peaks labeled in vitro migrated approximately to the same position on the gel as those labeled in vivo. Although the ratio of counts in the peaks appeared to differ in the two preparations, it is apparent that the in vitro labeling is an accurate reflection of mitochondrial protein synthesis in vivo.

Coote and Work (9) reported that rat liver mitochondria incorporate amino acids into ten to twelve radioactively labeled peaks. The discrepancy between their study and the work presented here may be due to the methods of extraction and the length of time of electrophoresis. One conclusion may be that the methods of isolation of these proteins from particulate fractions are not totally reliable in determing the exact number of products of mitochondrial protein syn-

thesis. It is apparent, however, that the number of proteins synthesized is small and that three proteins (40,000, 27,000, and 20,000) are synthesized to a greater extent.

The results described in this thesis suggest that a number of proteins synthesized by the mitochondria are proteolipids, proteins which are soluble in chloroform:methanol and insoluble in water. These proteolipids, however, represent less than 20% of the total radioactivity incorporated into mitochondrial membranes in vitro. The specific inhibitors of mitochondrial protein synthesis, chloroamphenicol and carbomycin, inhibited labeling of the proteolipid fraction to the same extent as the submitochondrial particles, indicating that they are products of mitochondrial protein synthesis.

Gel electrophoresis of the chloroform:methanol extract revealed 3-4 labeled peaks of molecular weights ranging from 10,000 to 40,000. A substantial loss of radioactivity from the large molecular weight peak of the residue after extraction with chloroform:methanol was also observed (Figure 16). The same gel profile was revealed when the chloroform:methanol extract had been dialyzed against acidic chloroform:methanol for seven days to remove phospholipids.

The existence of proteolipids was first described by Folch (80) in 1951, and their existence confirmed in mitochondria by Tzagoloff (85), Kadenbach (87), and Linnane (89). Tzagoloff has reported (87) that a major product of mitochondrial protein synthesis in yeast is a proteolipid of

low molecular weight (7800). The various procedures used in preparation of submitochondrial particles and the subsequent fractionation schemes are so varied that a comparison of the results reported by various groups is extremely difficult. To determine if our extraction procedure resulted in the loss of certain proteolipids synthesized by the mitochondria, two other procedures were utilized and compared to the original extraction procedure. In the chloroform:methanol extract that was not washed prior to preparation for gel electrophoresis, very little difference was observed from our original profile, with the possible exception of a slight increase in radioactivity in the low molecular weight peak (10,000). Gel electrophoresis of the chloroform:methanol extract of the lyophilized membranes indicated that the bulk of the radioactivity migrated into the region representing molecular weights of 14,000 to 10,000. Almost no radioactivity was observed in the high molecular weight region. A possible explanation for this observation is that the high molecular weight proteolipid may be more hydrophilic than those of lower molecular weight. Hence, after lyophilization, when all aqueous medium has been removed, they are not extracted into the chloroform:methanol phase.

Tzagoloff has reported (68) that the synthesis of certain mitochondrial proteins is stimulated by products of cytoplasmic protein synthesis. Similarly, the absence of products of cytoplasmic protein synthesis has been reported to cause a significant decrease in the rate of mitochondrial

protein synthesis (116). In our laboratory, the rate of amino acid incorporation both *in vitro* and *in vivo* was significantly increased in mitochondria obtained from cells grown for three hours in chloramphenicol and then allowed to grow for 1-2 hours in fresh medium, suggesting that the products of cytoplasmic protein synthesis which had accumulated during growth in chloramphenicol, may actually stimulate mitochondrial protein synthesis (106). The increased rate of labeling observed under these conditions does not result from an increased rate of synthesis of all of the labeled bands after electrophoresis, but from an increase in labeling of the low molecular weight protein. Furthermore, labeling of the low molecular weight proteolipid of the chloroform:methanol extract was also stimulated nearly six-fold in yeast cells grown in chloramphenicol compared to control cells. Perhaps, proteolipids are essential for the subsequent integration of several proteins into the mitochondrial membrane. The accumulation of mitochondrial proteins in the cytoplasm when mitochondrial protein synthesis has been blocked by chloramphenicol could possibly stimulate an increased synthesis of proteolipids. The low molecular weight protein (10,000) observed in the chloroform:methanol extracts of yeast mitochondria in our studies could possibly correspond to that reported by Tzagoloff (85). However, our results also indicate that other proteolipids may also be synthesized by yeast mitochondria. The existence of several proteolipids in the whole homogenates of rat liver, kidney, and brain has

been reported by Folch (117).

ABBREVIATIONS

AMP - adenosine-5'-monophosphate

ADP - adenosine-5'-diphosphate

ATP - adenosine-5'-triphosphate

AIA - allylisopropylacetamide

ALA - δ-aminolevulinic acid

ATPase - adenosine triphosphatase

DDC - diethyl-1,4-dihydro-2,4,6-trimethylpyridine-3,5-dicarboxylate

DCCD - dicyclohexylcarbodiimide

DNA - deoxyribonucleic acid

EDTA - ethylenediamine tetraacetate

F_1 - soluble adenosine triphosphatase

NAD - nicotinamide adenine dinucleotide

OSCP - oligomycin sensitivity conferring protein

PEP - phosphoenolpyruvate

mRNA - messenger ribonucleic acid

SMP - submitochondrial particles

tRNA - transfer ribonucleic acid

Tris - Tris (hydroxymethyl) aminomethane

Poly (U) - polyuridylic acid

VAL. - Valinomycin

a.a - amino acid

conc. - concentration

cpm. - counts per minute

ABBREVIATIONS (continued)

cm. - centimeter(s)

mm - millimeter(s)

nm - nanometer(s)

g - gram(s)

mg - milligram(s)

µg - microgram(s)

M - molar

mM - millimolar

µM - micromolar

pMoles - picomoles

N - normality

x g - times gravity

S - Svedberg unit

ml - milliliter

°C - degree Centigrade

r.p.m. - revolutions per minute

sec. - second(s)

min - minute(s)

hr. - hour(s)

I.M. - inner membrane

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