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STUDIES OF THE CONTROL OF MITOCHONDRIAL PROTEIN SYNTHESIS IN TISSUES OF THE RAT

City University of New York PH.D. 1981

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STUDIES OF THE CONTROL OF MITOCHONDRIAL PROTEIN SYNTHESIS IN TISSUES OF THE RAT

by

Ronald W. Rinehart

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

- 1981 -

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

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Executive Officer

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ABSTRACT

Investigations were conducted into the mechanisms by which mitochondrial protein synthesis is governed in tissues of the rat. The historical perspectives leading to the current state of understanding of the role of mitochondrial protein synthesis in the overall process of mitochondrial biogenesis are discussed. A particularly useful tool in these investigations was the examination of the effects of streptozotocin-induced diabetes mellitus upon mitochondria from rat skeletal muscle and kidney. The rate of amino acid incorporation *in vitro* by isolated muscle mitochondria from diabetic animals was decreased by 50-80% from control values. Treatment of diabetic animals with insulin lowered blood glucose levels to control values and restored the rate of muscle mitochondrial protein synthesis *in vitro* to control levels. The rates of muscle mitochondrial protein synthesis *in vitro* were decreased by 25% when the animals were fasted for two days. Comparison by dodecyl sulfate polyacrylamide gel electrophoresis of the translation products synthesized by isolated muscle mitochondria from control and diabetic rats revealed a uniform decrease in the synthesis of all polypeptides in the latter. Aurintricarboxylic acid and pactamycin, inhibitors of peptide chain initiation, blocked protein synthesis *in vitro* by muscle mitochondria to a greater extent in controls than in diabetics, suggesting that diabetic muscle mitochondria are less able to initiate protein synthesis than control muscle mitochondria.

Phenotypic changes observed in diabetic muscle mitochondria included a 36% decrease in the content of cytochrome aa_3 and a 27% decrease in cytochrome b, both of which contain products of mitochondrial translation. State 3 and uncoupler-stimulated respiration (with glutamate as substrate) were both decreased by about 25% in diabetic mitochondria. By contrast, the specific activities of NADH and succinate dehydrogenases, exclusively products of cytoplasmic translation, were not decreased in muscle mitochondria from diabetic animals; the specific muscle content of mitochondria was also unchanged in the diabetic. These results suggest that the considerable muscular atrophy observed in diabetics may involve decreases in both cytoplasmic and mitochondrial protein synthesis, the latter reflected in profound differences in the respiratory chain.

By contrast with these findings, comparison of kidney mitochondria from control and diabetic rats revealed no real differences in the rates of protein synthesis *in vitro*, cytochrome content, state 3 respiration, specific activity of succinate dehydrogenase, and the recovery of mitochondria from kidney homogenates. Kidney mitochondria are thus like liver mitochondria in being relatively unaffected by insulin deprivation. The translation product synthesized *in vitro* by mitochondria from rat kidney, liver, and skeletal muscle are very similar to each other.

The close correlation between the effects of diabetes mellitus upon the rates of total cytoplasmic protein synthesis

in vivo and mitochondrial protein synthesis *in vitro* suggested that, as in lower eukaryotes, mitochondrial protein synthesis may be governed by cytoplasmic translation products. This hypothesis was further investigated by comparing the ability of cytoplasmic post-polysomal supernates from control, diabetic, and cycloheximide treated animals to stimulate protein synthesis *in vitro* by isolated rat muscle, rat liver, and yeast mitochondria. The results of these experiments indicated some degree of support for this hypothesis. All of these findings are discussed within the context of the current picture of mitochondrial biogenesis.

DEDICATION

In memory of my beloved mother, Lillian, an accomplished woman of arts and letters.

In appreciation of my father, Robert, a great man of science and education.

To my wife, Eltha, for her patient support and encouragement.

To the future of my son, Redmond, who had the good sense to arrive after the completion of this work.

To Dr. Joseph T. Bashour, who introduced me to the world of biochemistry.

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I. INTRODUCTION

A. General Introduction to Mitochondrial Studies

> "It is a good thing for the entire enterprise [the eukaryotic cell] that mitochondria and chloroplasts have remained small, conservative, and stable, since these two organelles are, in a fundamental sense, the most important living things on earth. Between them they produce the oxygen and arrange for its use. In effect, they run the place." (1)

The foregoing quote, penned by Lewis Thomas in 1972, indicates a keen appreciation of the importance of mitochondria in eukaryotic cell function, a perspective based on more than a century of histological and biochemical observations by scores of scientists. Indeed, the history of mitochondriology is in many ways a synopsis of the development of the science of biochemistry. Accordingly, the following summary of the salient events leading to our current state of knowledge will of necessity be highly abbreviated. Kölliker (2) reported the observation of "sarcosomes" in skeletal muscle as early as 1853. Pflüger demonstrated in 1872 that oxygen was consumed by all mammalian tissues, not just the blood and lungs (3). In 1886, MacMunn (4) reported the presence of cellular pigments, which he termed "histohematins" but which are now called cytochromes. In the early 1890s, Altmann (5) developed methods for staining mitochondria; based upon his observations of size, shape, and distribution he proposed that mitochondria were bacteria-like organisms living autonomously within larger cells.

The period between 1910 and 1940 yielded tremendous advances in the understanding of cellular energy production and its relationship to respiration. Between 1912 and 1923, Wieland (6) investigated the role of dehydrogeneses in cellular metabolism. Beginning at about the same time, Warburg (7) tackled the process of oxygen "activation" by respiratory enzymes, showing that iron was involved and that respiration was inhibited by cyanide. In 1925, Keilin (8) reported the independent rediscovery of the cellular pigments which he renamed "cytochromes;" he examined their spectral changes during respiration. Several years later, Warburg (9) demonstrated the presence of heme in respiratory enzymes. In 1929, Fiske and Subbarow (10) reported the presence of ATP and phosphocreatine in skeletal muscle. In 1931, Engelhardt (11) demonstrated that phosphorylation of ADP and respiration were coupled processes. During the next nine years, Warburg (12) examined the role of flavins and pyridine nucleotides in respiration and demonstrated that ATP formation and dehydrogenation were coupled; Keilin (13) examined reconstituted electron transport in heart preparations; Dayson and Danielli advanced the lipid bilayer membrane model (14); and Krebs et al. elucidated the urea cycle (15) and the tricarboxylic acid cycle (16). Between 1939 and 1941, Lipmann (17) examined the role of ATP in cellular energy transfer.

The 1940s were a very productive decade. The discovery of cellular fractionation procedures by Claude (18) led to his isolation of liver mitochondria (19);

refinement of the isolation procedures by Hogeboom, Schneider, and Palade (20) led within a very short time to the demonstration by Kennedy and Lehninger that the mitochondrion was the intracellular locus of the Krebs cycle, the β-oxidation of fatty acids, and oxidative phosphorylation (21).

In the 1950s, Chance (22) and others, using difference spectroscopy, were able to deduce the sequence of carriers in the electron transport chain. Later, Green (23) postulated that the electron-transport chain was composed of four lipid-protein complexes; these were subsequently isolated and studied by Hatefi et al. (24). In 1961, Mitchell (25) proposed the chemiosmotic mechanism of energy coupling, a hypothesis which specifically invoked the necessary participation of the inner mitochondrial membrane.

As a result of the aforementioned reports and others too numerous for individual citation, the following summary of mitochondrial structure and functions has evolved:

1. Mitochondria from different species are morphologically and biochemically different. Even within a given individual animal, there is tissue-specific heterogeneity in mitochondrial ultrastructure and metabolic capabilities (26).
2. The organelle comprises an outer membrane, an intermembrane space, an inner membrane, and a central matrix. Each of these components has distinct structural and metabolic

features. The total number of different enzymes localized in mitochondria is over one hundred (27).

3. The mitochondrial matrix is the site of dehydrogenation of pyruvate, fatty acids, ketone bodies, most Krebs cycle intermediates, and amino acids, yielding NADH (27).
4. NADH feeds electrons into the respiratory chain, located in the inner membrane. The process of electron transport leads to the formation of an electrochemical proton gradient across the inner membrane which can be utilized to drive the phosphorylation of ADP, and the uptake of Ca^{++} and many other substances (28).
5. Mitochondria also participate in metabolic pathways which may occur partially in the cytoplasm, including fatty acid biosynthesis, ketogenesis, heme biosynthesis, and steroidogenesis (28).
6. The respiratory chain can be separated into four enzymatic complexes which transfer electrons in the following manner (28):

complex	electron donor	electron acceptor
I	NADH	Coenzyme Q (CoQ)
II	succinate	CoQ
III	$CoQH_2$	cytochrome c
IV	cytochrome c	O_2

B. The Origin of Mitochondria

The recognition of the metabolic importance of mitochondria naturally prompted inquiries into the processes involved in their formation. In 1950, Ephrussi and Sloninski (29) reported that treatment of yeast cells with acriflavin or ethidium bromide gave rise to so-called "petite" mutants (due to their slow growth, they formed small colonies) lacking cytochrome oxidase; these mutations were inherited in non-Merdelian fashion. Within two years, Potter et al. (30) and Hogeboom and Schneider (31) independently reported the presence of DNA in mitochondrial preparations; however, it was more than ten years before these observations became generally accepted. DNA had also been reported to be present in trypanosomal kinetoplasts (32) and in chloroplasts (33, 34). In the late 1950s, two groups of investigators reported that amino acids could be incorporated into proteins by isolated mitochondria (35, 36); once again, it took several years before these findings were generally accepted.

The presence of DNA in mitochondria was confirmed by electron microscopy (37) and autoradiography of cells labelled with tritiated thymidine (38, 39); even more convincing were the demonstrations that unique DNA molecules whose buoyant density differed from that of nuclear DNA from the same species could be isolated from mitochondria (40, 41); the less-dense mitochondrial DNA had a much higher A-T content than nuclear DNA. By 1970, sufficient evidence had been amassed (42) to indicate that mt DNA (from essentially all species) was small

(1-5 x 10^7 daltons; 15-75 kbp), circular, and devoid of histones.

In 1962, Bahr and Zeitler (43) observed dumbbell-shaped mitochondria in rat liver and suggested that they were undergoing division. This evidence was supported by the experiments of Luck (44) with Neurospora crassa. A choline-requiring mutant was labelled in vivo with [^{14}C]-choline, and subsequently transferred to medium containing excess unlabelled choline and grown for three division cycles. Autoradiographic examination of the cells at various times indicated a random (uniform) distribution of label throughout the mitochondrial population; the density of labelling decreased with each division cycle. These results were interpreted to indicate that new mitochondria were formed by addition of new (unlabelled) phospholipids to the old (labelled) mitochondrial membranes. Similar conclusions were reached by Parsons and Rustad (45), who labelled the mt DNA of Tetrahymena with [^{3}H]-thymidine and monitored its fate during subsequent cell division. Partitioned mitochondria were observed by Tandler et al. in mouse liver (46): feeding mice with galactoflavin for several weeks induced formation of giant mitochondria; upon administration of riboflavin to the animals, the mitochondria divided and returned to normal dimensions.

The possible informational role of mt DNA was implied by the reports of two groups in 1964 that mitochondria contained a DNA-dependent RNA polymerase (41, 47). RNA had been reported in mitochondria as early as 1948 (20), but it was

not until 1967 that the possibility of contamination with cytoplasmic RNA was convincingly excluded (48-50). Meanwhile, several groups demonstrated that the earlier observations of protein synthesis by isolated mitochondria were indeed correct (51-57). Roodyn et al. (51) examined the amino acid and energy requirements for amino acid incorporation by isolated rat liver mitochondria; Wintersberger (53) performed similar studies with yeast mitochondria; mitochondria from rat brain (54), beef heart (52), and N. crassa (56) were also shown to be capable of protein synthesis in vitro. Wheeldon and Lehninger (55) demonstrated that ATP itself, not some high-energy intermediate, provided the energy for mt protein synthesis; they performed these experiments under conditions where bacterial contamination was minimized. The possibility that the observed incorporation of labelled amino acids was due to bacterial contamination was definitively excluded by Beattie et al. (57), who also demonstrated that the inner membrane was the location of the labelled mitochondrial translation products (58).

Among the most useful tools in investigations of mitochondrial protein synthesis are antibiotics which selectively inhibit either the mitochondrial or cytoplasmic translation processes. The first report that amino acid incorporation by isolated mitochondria and chloroplasts could be inhibited by chloramphenicol (CAP) was made in 1964 (59). Vazquez (60-61) had shown that CAP binds to the large subunit of bacterial ribosomes from several species. The inhibitory effect of

CAP on mitochondrial protein synthesis *in vitro* was subsequently reconfirmed (55, 57). On the other hand, cycloheximide, an inhibitor of translation by cytoplasmic ribosomes (62-66) had no effect on protein synthesis *in vitro* by isolated mitochondria (57, 67-69). The potential use of these inhibitors as tools for investigation of mitochondrial biogenesis was developed most extensively by Linnane's group (68, 70-73). In particular, they showed that CAP could block the normal development of mature mitochondria in yeast undergoing glucose derepression (73). Furthermore, they showed (69) that lincomycin, erythromycin, spiramycin, carbomycin, and oleandomycin also inhibited mitochondrial translation; these drugs also inhibit bacterial protein synthesis in the same way that chloramphenicol does (74).

The transcription of mt DNA can also be inhibited selectively by drugs which are known to interact with prokaryotic DNA. Ethidium bromide, used by Ephrussi *et al.* to generate petite mutants of yeast (29), selectively inhibits replication and transcription of mt DNA (75, 76). Mitochondrial transfer RNAs and their activating enzymes were first reported in 1967; they were shown to be distinct from their cytoplasmic counterparts (77, 78). The other soluble factors required for mitochondrial translation were also shown to differ from their cytosolic counterparts (79). Similarly, there appeared many reports of the isolation of unique mitochondrial ribosomes from *N. crassa* (49, 56), liver (48), HeLa cells (80, 81), and *X. laevis* (82). It was further demon-

strated that, as in prokaryotes but not in eukaryotic cytosol, mitochondrial translation is initiated with N-formyl methionine (83).

The many similarities observed among plastid (mitochondrial and chloroplast) and bacterial DNA, RNAs, ribosomes, and protein synthesis led to the reactivation of the "endosymbiont hypothesis" (84-86) first implied by Altmann (5). More recently, comparisons of ferredoxin, c-type cytochromes, and ribosomal RNA sequences have been employed to construct a phylogenetic tree indicating the evolutionary interrelationships between mitochondria, chloroplasts, eubacteria, cyanophytes, and eukaryotes (87). However, it is possible to become overinfatuated with such speculations. We now know that the mitochondrial genetic code and protein-synthetic apparatus are unique; they share both similarities with and important differences from either the prokaryotic or eukaryotic nucleocytoplasmic systems (cf. Ref. 88); details of these differences will be discussed in a subsequent section.

In summary, the following points had been reasonably firmly established by the early 1970s:

1. The lipid components of the mitochondrial membrane are synthesized primarily in the cytoplasm and subsequently transferred into the cytoplasm (89).
2. Most (>90%) of the proteins (enzymes) of the mitochondria are also translated in the cytoplasm and subsequently transferred into

the mitochondria; cytoplasmic translation is inhibited by cycloheximide (90).

3. Isolated mitochondria are capable of protein synthesis *in vitro* when supplied with a source of ATP, amino acids, and suitable concentrations of inorganic ions. They contain unique DNA, ribosomes, tRNAs, and other soluble factors involved in protein synthesis (90).
4. The products of mitochondrial protein synthesis are a limited number of very hydrophobic proteins whose predominant destination is the inner mitochondrial membrane (90).

C. Mitochondrial Genes and Translation Products

The demonstration that mitochondria contain DNA and synthesize proteins does not necessarily prove that the two are related. Indications that mt DNA did indeed code for the mitochondrial translation products were reported by Mahler et al. The effects of ethidium bromide, a specific inhibitor of mitochondrial DNA replication and transcription, upon a variety of parameters of mitochondrial formation, were very similar to those of chloramphenicol, a specific inhibitor of mitochondrial translation (91). Furthermore, the formation of mitochondrial polysomes was insensitive to conditions which blocked the synthesis of cytoplasmic RNA, but sensitive to conditions which inhibited the formation of mitochondrial RNA (92). These findings were also supported by the report of Tzagoloff et al. (93) that a series of yeast mutants with specific lesions in cytochrome oxidase and cytochrome b showed non-Mendelian inheritance of the mutations, indicating that mt DNA was the site of the mutations.
The most convincing evidence indicating the mitochondrial genomic origin of mitochondrially-synthesized proteins is the demonstration that RNA isolated from mitochondria hybridizes to mitochondrial but not nuclear DNA (94-97). Isolation of mitochondrial mRNA was facilitated by the demonstration that it possessed short 3' terminal polyadenylate sequences in HeLa cells (98), Ehrlich ascites cells (99), and yeast (100), although the latter finding has been disputed (101). Translation of yeast mitochondrial poly $(A)^+$RNA in a E. coli cell-

free system yielded products which could be immunoprecipitated with antisera to cytochrome oxidase (100). Their electrophoretic mobilities corresponded to those of the three largest subunits of cytochrome oxidase, which had previously been shown to be translated on mitochondrial ribosomes (102).

Identification of mitochondrial translation products has been accomplished primarily by two methods:

1. Proteins synthesized by mitochondria are selectively labelled (i.e. *in vivo* by intact cells in the presence of cycloheximide or *in vitro* by isolated mitochondria) and their functional roles assigned by reacting with antisera raised against various purified mitochondrial enzyme complexes, either before or after electrophoretic separation.
2. Examination is made of the effects of defective mitochondrial protein synthesis upon various enzyme complexes suspected to contain mitochondrial translation products. In this case, total proteins of the mitochondria can be labelled *in vivo* in the presence of inhibitors of mitochondrial transcription or translation. Alternatively, mutations in mitochondrial proteins which display non-Mendelian inheritance patterns can be isolated and studied.

It is hardly accidental that most of our knowledge of mitochondrial genes and their translation products has been obtained from studies of a single organism, the yeast Saccharomyces cerevisiae; there are substantial advantages militating for the use of this organism. Large quantities of genetically-identical cells are easily grown and harvested. The conditions of growth can be varied appreciably, and the effects of such changes on mitochondrial functions can be examined. Growth on media containing high concentrations of fermentable substrates (glucose, galactose, mannose, etc.) leads to a decreased rate of cellular respiration; this condition is known as catabolite or glucose repression (103). Under such circumstances, ethanol is produced and excreted by the cells; as the fermentable carbon source becomes exhausted, the cells undergo a transition from anaerobic to aerobic metabolism, thus permitting utilization of the previously-formed ethanol. This transition is known as glucose (catabolite) derepression. During this process, the levels of various respiratory enzymes increase. This increase involves protein synthesis in both cytoplasm and mitochondria; however, their synthesis and subsequent assembly appear to proceed in an asynchronous manner (104, 105).

Alternatively, yeast cells can be grown anaerobically, if fermentable substrates, ergosterol, and unsaturated fatty acids are provided [the latter two components require oxygen for their synthesis (106, 107)]. Under these growth conditions, the cells are utterly devoid of respiratory activity

(108); however, they do contain mitochondria-like structures ("promitochondria") which contain mt DNA, oligomycin-sensitive ATPase, and reduced levels of various NAD^+-linked dehydrogenases (109, 110); the promitochondria are capable of protein synthesis *in vitro* (110). In these structures there is a complete lack of enzymatic activity in complexes II, III and IV of the respiratory chain (111); correspondingly, coenzyme Q and spectrally-detectable cytochromes are also totally absent (108, 110). The lattermost observation is not particularly surprising, since oxygen is necessary for the biosynthesis of protoporphyrin IX (112).

Upon aeration, anaerobically-grown yeasts rapidly acquire respiratory capacity, a process known as respiratory adaptation (108). This process involves formation of cytochromes *a*, a_3, *b*, *c*, c_1 (113), coenzyme Q (114), ergosterol, and unsaturated phospholipids (115). Respiratory adaptation is retarded if the cells are catabolite-repressed (116). Adaptation is also blocked by inhibitors of cytoplasmic translation and by inhibitors of mitochondrial transcription or translation (117), indicating the participation of both nucleocytoplasmic and mitochondrial genetic systems in this process. Further studies of respiratory adaptation in the presence of CHX or CAP by Chen and Charalampous (118, 119) and Brown and Beattie (120) indicated that the formation of the inner-membrane enzyme complexes proceeded asynchronously, with the formation of the cytoplasmic translation products seeming to precede that of the mitochondrial products. This

theme will be dealt with in greater detail in Chapter IV (Discussion).

In 1971, Tzagoloff et al. pulse-labelled yeast cells in the presence of cycloheximide and rapidly isolated the mitochondrial rutamycin-sensitive ATPase by immunoprecipitation, revealing that four of the subunits [5, 6, 7, and 9] were mitochondrial in origin (121). Similar techniques were employed by Mason and Schatz (102) and Rubin and Tzagoloff (121) to demonstrate that the three largest subunits of cytochrome oxidase [I, II, III] were also mitochondrially synthesized. Weiss et al. (122) and Lin and Beattie (123) showed that in N. crassa and S. cerevisiae, respectively, cytochrome b was also mitochondrially translated. Similarly, one protein component of the small ribosomal subunit was demonstrated to be of mitochondrial origin in N. crassa by Lambowitz et al. (124) and in S. cerevisiae by Groot et al. (125) Interestingly, in S. cerevisiae, this protein displays considerable variability in electrophoretic mobility among different strains of the organism (126, 127).

Analogous identification of mitochondrial translation products in animals has proven somewhat more difficult. The most convincing studies have generally been performed using separated cell preparations (i.e., cultured hepatocytes, HeLa cells, etc.) or isolated mitochondria in vitro Using isolated rat hepatocytes and rat hepatoma cells, Nelson et al. (128) demonstrated that the mitochondrial translation products include the three largest subunits of cytochrome oxidase,

cytochrome b, and two subunits of the proton-translocating ATPase. Cytox I, II, and III are also synthesized by isolated rat liver mitochondria (129). By contrast, Attardi et al. have detected over 20 mitochondrial translation products in He La cells (130); most of these products remain unidentified, but it is likely that they include cytox I, II, and III, cytochrome b, and at least one of the ATPase subunits (131).

In addition to their suitability for various physiological manipulations, yeasts also possess substantial advantages over animal cells for genetic studies. Consequently, detailed knowledge of the characteristics of the yeast mitochondrial genome has generally preceded that of mammals. The following summary of yeast reproduction has been adapted from Sager (132).

Haploid strains of S. cerevisiae exist in two mating types ("sexes") which are controlled by chromosomal alleles designated a and α. Upon mixing, cells of opposite mating types fuse to form diploid zygotes, which can divide mitotically to give rise to clones of diploid cells. Under starvation conditions, diploid cells can be induced to sporulate; through meiosis, a single diploid cell yields four refractile haploid spores encased within an ascus. It is possible to isolate individual ascospores; upon germination, each gives rise to a haploid clone. Crosses of haploid cell types can be subjected to genetic analysis; either the resultant diploids or the haploid ascospores may be used for this purpose. The a and α alleles, like other nuclear genes which differ

in the parents, segregate among the ascospore tetrad in a Mendelian (2:2) ratio. Deviations from this pattern are indicative of the presence of extrachromosomal genetic elements. During cell fusion but prior to nuclear fusion, there is a period where buds can be produced. Sometimes, these buds contain only one of the unfused haploid parental nuclei, but they contain cytoplasm from both parents.

Parental strains with different chromosomal mutations not directly related to mitochondrial function (e.g., amino acid auxotrophy, etc.) can be employed to facilitate subsequent selection of particular progeny. As previously discussed, Ephrussi et al. were able to generate respiration-deficient petite mutants of S. cerevisiae which displayed non-Mendelian inheritance by treatment with acriflavin or ethidium bromide (29). When crosses of petite strains with wild-type strains were analyzed by Wright and Lederberg (133) [530 mating pairs; 91 appropriate haploid clones], six clones contained wild-type nuclei but exhibited petite phenotypy. By contrast, five other clones contained nuclei from the petite parent but exhibited normal respiration. Hence, the petite property segregated independently of the nucleus (133). Earlier Chen et al. (134) reported the isolation of a series of mutants with petite phenotype, but with a Mendelian pattern of genetic segregation. Crosses of these nuclear petites with cytoplasmic petites yield two wild-type and two petite ascospores; by contrast, crosses between two cytoplasmic petites always yield petite progeny. Since nuclear and

cytoplasmic petites complement one another, the genes involved are clearly nonidentical (132). Where the location of the genetic lesion is known, nuclear petites are designated by the abbreviation pet; cytoplasmic (mitochondrial) petites are designated as ρ^- (or ρ^0, if they completely lack mt DNA). In higher eukaryotes (Chlamydomonas, plants) as well as yeast, some mechanism for preferential segregation of the cytoplasmic genes appears to exist (132); in yeast, but not in mammals, the mitochondrial genomes appear to undergo recombination prior to meiosis (135). Thus in yeast, the ascospore tetrads generally inherit these determinants in a 4:0 or 0:4 pattern (132). Hence, genetic analysis provides a powerful tool in the investigation of the mitochondrial genome.

In addition to the ρ^- mutants, several other classes of useful mutations in yeast mitochondrial DNA are also available; they include mutants resistant to compounds which specifically inhibit mitochondrial functions (protein synthesis, electron transport, oxidative phosphorylation). Such mutants can be induced or arise spontaneously. Particular inhibitors of mitochondrial translation (chloramphenicol, paromomycin) electron transport (antimycin A, Diuron), and oxidative phosphorylation (oligomycin, rutamycin) have proven particularly useful in this regard. Additionally, there are two additional classes of mutants in which mitochondrial protein synthesis is defective. In the class of mutants designated mit^-, synthesis of one or more mitochondrial translation products is defective, presumably due to a mutation in a

structural gene. In a class of temperature-sensitive (syn^-) mutants, growth at the nonpermissive temperature leads to the rapid induction of the petite phenotype; components of the mitochondrial protein-synthesizing machinery (e.g., tRNA) are presumably involved. Both of these classes are distinct from ρ^- mutants, which completely lack mitochondrial protein synthesis (135).

Once a particular phenotype has been demonstrated to display a non-Mendelian pattern of inheritance, it can be subjected to genetic analysis. Alternatively, mitochondrial DNA (from any organism) can be isolated and examined by a variety of physical and biochemical techniques. These include DNA-DNA hybridization [e.g., ρ^- vs. wild-type, known as deletion mapping]; hybridization of mtDNA with mt RNA [heteroduplex analysis]; restriction enzyme (136) analysis [physical mapping]; and DNA sequencing studies (137, 138). Among the interesting findings of such investigations is the demonstration that the ρ^- mutants retain only a small portion of the wild-type mt genome; however, through tandem reduplication, the total size of the ρ^- mt DNA approximates that of the ρ^+ wild-type genome [25μ contour length; 70^+ kbp] (135). This provides a simple explanation of the observation that ρ^- mutants are incapable of reversion (132).

Heteroduplex analysis has unequivocally demonstrated that mitochondrial DNA contains the genes for mitochondrial rRNAs and tRNAs. By use of well-defined ρ^- strains and suitable restriction fragments of ρ^+ mt DNA, the specific locations of these genes could be determined. Furthermore, the results

so obtained could be complemented by classical recombinational analysis, permitting correlation of the genetic and physical maps of the mitochondrial genome. The information so assembled for S. cerevisiae confirms that its mt DNA carries the structural genes for cytox I, II, and III, cytochrome b, two subunits of the proton-translocating ATPase, and a ribosome-associated protein, as well as for the ribosomal and transfer RNAs (135). Their relative locations are indicated in Illustration B, which also includes for comparison the recently-determined genome map for human placental mt DNA (131).

An interesting class of yeast mutations in the structural gene for cytochrome b exists in which the expression of cytox I is also affected; these are termed box or cob mutants. Genetic analysis led to the recognition of several complementation groups within the box locus; further investigation by genetic and physical techniques led to the realization that the gene for cytochrome b is split, containing five exons [coding segments] interspersed with four introns [noncoding segments]; the entire gene is transcribed and subsequently processed (139a,b). Slonimski et al. have proposed that the excised intron transcripts are also ligated and translated, yielding a "maturase" required for proper processing of the pre-mRNA for cytox I (140), which is also transcribed from a split gene.

By comparing the (partial) amino acid sequences of known mitochondrial translation products with the base sequences of ρ^- DNA retaining the corresponding genes (for

apocytochrome b, cytox II, and ATPase subunit 9), Tzagoloff's group (141) discovered that the genetic code in mitochondria does not obey the "universal" rules determined by the groups of Nirenberg (142) and Ochoa (143). Instead, the UGA "termination" codon is recognized by a mitochondrial "suppressor" $tRNA^{trp}$. Further differences between the mitochondrial and the universal genetic codes and unique features of the mechanism by which the former is decoded by the mitochondrial transfer RNAs have subsequently been reported by several groups using a number of different organisms, including animals (144-150). These results helped clear up the mystery of how the limited number [$\leq$ 22] of tRNAs could suffice for complete translation when the wobble hypothesis (151) required a minimum of 32 tRNAs.

In the 75 kbp mitochondrial genome of yeast, the tRNA genes are primarily localized in a single region, there are at least three split genes [for cytochrome b, cytox I, and the 21S rRNA], the genes are separated by A-T rich noncoding spacer segments, and unique promoter sequences and an origin of DNA replication have not been identified (135). Recently, the complete sequence of the 16,569-base-pair human placental mitochondrial genome has been determined (131). Several important differences from the yeast mt genome have been observed. The human mt genome appears to contain neither spacer segments nor split genes. Moreover, the structural genes for the ribosomal RNAs and the mitochondrially-synthesized proteins are "puntuated" by the tRNA genes which are scattered around

the genome (152-154). Mammalian mitochondrial tRNAs are not imported from the cytoplasm (155).

Despite its small size, the mammalian mitochondrial genome apparently possesses a respectable amount of genetic information. The general arrangement of genes appears to be identical in mt DNA from humans, cattle, mice and rats, although their individual base sequences vary (156). The sequence analysis of human and bovine mt DNA has revealed the presence of eight "unidentified reading frames" which begin with a methionine codon and end with a termination codon (131); these could presumably represent genes for [as yet unidentified] mitochondrial proteins, since the human sequences show a high degree of homology with analogous regions in bovine mt DNA, and it seems unlikely that non-functional genes would be retained in such a compact genome (157). It is also interesting to note that "polymorphic forms" of mt DNA have been identified in populations of horse, man, sheep, goat, and rat (158-160; cf. additional sources in Ref. 161). Several groups have employed these observations to prove the exclusively maternal inheritance pattern of mitochondrial DNA in mammals (161). There will undoubtedly be many more equally-intriguing reports in the near future.

D. The Biogenesis of Mitochondria

It is clear that the continuous, orderly production of mitochondria within any growing eukaryotic cell requires a coordinated interplay of several basic processes:

1. The synthesis of the lipid components of the outer and inner membranes. All of the enzymes involved in this phase are products of nuclear genes; most of these enzymes are located in the smooth endoplasmic reticulum, with the exception of the cardiolipin synthetase (89). Wirtz and Zilversmit demonstrated that exchange of phospholipids between microsomes and mitochondria can take place *in vitro* (162). Beattie obtained evidence suggesting that the outer mitochondrial membrane may be the first component synthesized during mitochondrial biogenesis (163). Further studies *in vivo* and in rat liver slices *in vitro* (164) indicated that the kinetics of incorporation of [^{3}H]-phenylalanine into insoluble mitochondrial proteins and the incorporation of [^{14}C]-glycerol into mitochondrial phospholipids were very similar; both differed from the time courses observed for microsomal labelling. Kadenbach (165) found that the transfer of [^{32}P]-labelled phospholipids from microsomes to unlabelled mitochondria proceeds with kinetics identical to those of the transfer of [^{14}C]-labelled proteins, implying that some of the proteins are transferred as phospholipid-protein complexes. Phosphatidyl choline appears to be transferred more rapidly than phosphatidyl ethanolamine (166).

The mechanisms by which membrane lipid synthesis and transfer to the mitochondria are coordinated with the following processes are still unclear. It is likely that the major factors governing phospholipid synthesis are the availability of suitable precursors and metabolic energy. Under conditions where parts of the respiratory chain are nonfunctional due to unavailability of flavins, giant mitochondria are observed (46); this implies that phospholipid synthesis and transfer are somewhat independent of other steps in mitochondrial biogenesis.

2. The synthesis of mitochondrial proteins in the cytoplasm directed by nuclear genes. As previously discussed, the vast majority of mitochondrial proteins are synthesized extramitochondrially in the cytoplasm; they are translated from mRNA which has been transcribed in the cell nucleus. The mechanisms governing nuclear transcription and cytoplasmic translation are extremely complex. In animal cells, protein synthesis is governed not only by the availability of amino acids and metabolic energy, but also by a large number of hormones and other factors. Transcription of genes for many enzymes is regulated by steroid hormones (167); in prokaryotes and eukaryotes, cAMP is also implicated in transcriptional control (168). Control at the level of translational initiation is also commonly observed; among the factors implicated here are cAMP (169), hemin (170-177), oxidized glutathione (176-178), GTP (179) and double-stranded RNA (177, 180-183). Moreover, cytoplasmic mRNA may exist in "masked"

(dormant) forms (176, 177). Inasmuch as hundreds of polypeptides of cytoplasmic origin are involved in the spectrum of mitochondrial enzymic activities, it is clearly impractical to attempt a comprehensive discussion of the control of their synthesis. Selected aspects of appropriate control mechanisms will be discussed in sections I.E. and IV.

3. Transfer of cytoplasmically-synthesized proteins into the mitochondrion. The experiments of Kadenbach (165) implied that cytochrome c is synthesized on the ribosomes attached to the rough endoplasmic reticulum and subsequently transferred to the mitochondrion as a phospholipid complex. Kellems and Butow (184) observed that cytoplasmic 80S ribosomes were associated with the outer membrane of yeast mitochondria; with the elucidation of the "signal hypothesis" by Blobel et al. (185), the mechanism by which cytoplasmically-synthesized proteins could be imported by mitochondria seemed quite clear. It is now known, however, that these early observations were probably incorrect in most regards.

Neupert's group conducted kinetic studies in vivo (186) and in vitro (187) on the transport of cytoplasmically-synthesized proteins into the mitochondria of N. crassa. Import of cytoplasmically-synthesized proteins into mitochondria was not prevented when cytoplasmic translation was inhibited by cycloheximide; hence, a vectorial mechanism of cotranslational protein segregation was excluded. Moreover, mitochondrial matrix proteins labelled in pulse and pulse-

chase experiments were first detected in the cytosol and subsequently in the mitochondria. More recently, the same group has demonstrated that in N. crasse and S. cerevisiae, apocytochrome c is the only form of this particular enzyme which can be taken up by the mitochondrion; the heme group is attached after the apoprotein has been imported (188,189).

There are selective mechanisms governing the mitochondrial import of cytoplasmic proteins. Marra et al. (190-192) have demonstrated that the mitochondrial isozymes of asparate aminotransferase and malate dehydrogenase are imported by rat liver mitochondria in an energy-dependent manner, while the cytoplasmic isoenzymes are not imported. Mitochondrial asparate aminotransferase appears to be made in the cytoplasm as a larger precursor (193); the protease which processes it is located in the mitochondrial matrix (194).

There does not appear to be a single common mechanism for import of cytoplasmically-synthesized proteins by mitochondria. Neupert and Schatz (195) have postulated the existence of at least two classes of "receptors" for such import on the outer mitochondrial membrane. Some, but not all, mitochondrial proteins of cytoplasmic origin are synthesized as larger precursors (195); these appear to be translated predominantly on "free" ribosomes (195,196). Research into this important aspect of mitochondrial biogenesis is continuing.

4. The replication of mitochondrial DNA. As previously indicated, the enzymes involved in this process appear to be entirely of cytoplasmic origin. By contrast with most other

aspects of mitochondrial biogenesis, somewhat more is known about DNA replication in animals than in yeast. Electron micrographic observations indicate that initiation of replication of mt DNA involves the synthesis of a 7S single-strand DNA fragment which displaces the opposite (L) strand, leading to formation of a characteristic "D-loop" (197). Shearman and Kalf (198) first demonstrated that DNA replication occurs on the inner mitochondrial membrane in rat liver; Attardi et al. showed that the point of attachment to the membrane is near the H-strand origin of replication - i.e., at the D-loop (199). The mitochondrial DNA polymerase in mammalian cells belongs to the class designated as γ-polymerases (200). Replication of the L-strand begins at a point about 1/3 of the way around the genome from the H-strand origin, and does not occur until this region has been exposed by H-strand synthesis (131).

The mechanisms controlling the initiation of mt DNA replication are not well understood. In different organisms, several different patterns of correlation of mt DNA replication with the overall cell cycle have been observed (201). One can only speculate on the role of the membrane attachment in the replication of mt DNA: perhaps changes in transmembrane electrochemical potential are involved. Alternatively, regulatory proteins of cytoplasmic origin or ion (Mn^{++}, Zn^{++}) translocation could be involved. A substantial number of questions in this area remain to be answered (202).

5. Intramitochondrial transcription and translation. In

yeast, transcription of mt DNA is partially symmetrical (203); it is initiated from at least five separate promoters (204). By contrast, in mammalian mitochondria both strands of DNA appear to be completely transcribed (119); the promoter for the H-strand is located in the vicinity of the D-loop (i.e., near the origin of replication), while the L-strand promoter is located about 1/3 of the way around the genome (131). There appears to be a transcription attenuator following the two rRNA genes which are located near the origin of replication of the H-strand, from which most, but not all, of the genetic information is transcribed (131). Although the L-strand is also completely transcribed in HeLa cell mitochondria, the resultant transcripts are much more rapidly degraded than the H-strand transcripts (205).

Transcriptional regulation in mammalian mitochondria thus appears to be a "primitive affair" in which differential gene expression is achieved by a combination of transcription-attenuation, control of the cleavages required to generate transcripts of individual genes, and stability of the final transcripts (157). The "punctuation" of the pre-mRNA transcripts at their 5' ends by various tRNAs which must be excised prior to translation (152-154) implies the possibility that most of the structural genes are coordinately expressed. Interestingly, in several transcripts, subsequent 3'-polyadenylation is necessary to generate the mitochondrial termination codons (152).

In most mammalian mt mRNAs, the initiation codon (AUA)

or AUG) is very close to the 5' terminus, unlike in prokaryotic and eukaryotic cytoplasmic mRNAs (153). Furthermore, mammalian mt mRNAs lack the 5' "cap" structure which is a distinctive feature of most of their cytoplasmic counterparts (206). Details of the regulation of the mitochondrial translation process are still poorly understood, but it is likely that, as in other systems, the primary point of control is at the level of peptide chain initiation as opposed to elongation or termination (176, 177, 207). At present it is difficult to ascertain whether transcriptional or translational control is more important in regulating the synthesis of mitochondrial proteins, since these processes appear to be closely coupled in both yeast and mammals (52, 208-210). What does seem quite clear is that the rate of synthesis of proteins within the mitochondria is primarily governed by proteins of cytoplasmic origin. In order to avoid excessive repetition, the evidence leading to this conclusion will be examined in Chapter IV (Discussion).

6. Assembly of lipids and proteins of cytoplasmic and mitochondrial origin into functional enzyme complexes of the mitochondrial inner membrane. As previously discussed, relatively little is known about the control of lipid accumulation within the inner mitochondrial membrane. On the other hand, many studies have indicated that the assembly of the various enzyme complexes of the inner membrane which contain products of both cytoplasmic and mitochondrial translation does not proceed in a synchronous manner in yeast

and other organisms (90, 104, 105, 113, 117-121, 209). If [unspecified] cytoplasmic translation products are not present, mitochondrial translation is depressed (209, 210); on the other hand, if mitochondrial translation is blocked, some of the cytoplasmically-synthesized subunits appear to be degraded rapidly within the mitochondrion (108, 110, 111, 128). Withal, it appears reasonable to conclude by restating that optimal production of mitochondria requires the coordinated interplay of all of the component processes discussed in this section. Many of the details of these processes remain to be elucidated, but it is clear that protein synthesis in both cytosol and mitochondria make essential contributions to mitochondrial biogenesis.

E. Diabetes Mellitus, Insulin, and Protein Synthesis

1. General Background (211-220)

Diabetes mellitus is one of the most prevalent of endocrine disorders in humans. It is estimated that around five million Americans may suffer from this disorder, which has been recognized for thousands of years. The Ebers Papyrus (ca. 1500 B.C.) mentioned one of the classic symptoms of the disease, excessive urination (polyuria). Polyuria was also familiar to the Roman writers of the first century A.D., Celsus and Aretaeus the Cappadocian; Aretaeus used the term "diabetes" [Gr. "flowing through"] to designate a malady characterized by polyuria, loss of weight, and unquenchable thirst (polydipsia, the second component of the classic symptomatic triad). The presence of sugar in the urine (identified by its sweet honey-like taste; hence "mellitus" [L. mel, honey]) was known to the ancient Chinese, Japanese, Hindus (Susruta, Sixth Century A.D.), and Arabs (Avicenna, 980-1037 A.D.). In 1807 Chevreul identified the sweet component of diabetic urine as "grape sugar" (glucose). The development of Fehling's test for reducing sugars in 1848 permitted subsequent investigations of the metabolic effects of diabetes. Soon, the presence of hyperglycemia as well as glycosuria was recognized as a characteristic feature of the disease, and many investigators studied the effects of dietary changes upon these symptoms.

In the 1870s, Langerhans reported the observation of

"little heaps of cells" in the pancreas; these were subsequently named "islets of Langerhans" by Laguesse. In 1889, von Mering and Minkowski demonstrated that pancreatectomy led to the development of diabetic symptoms. Several years later, Opie and Sobolev independently discovered that the pancreatic islets were the site of the pathology; ligation of the pancreatic duct led to acinar destruction, but spared the islets. These studies culminated with the discovery of insulin in 1921 by Banting and Best, who showed that it could ameliorate the disease in dogs and humans. In the 1930s and 1940s, Houssay et al. demonstrated that hypophysectomy could reduce the severity of the disease; Young showed that anterior pituitary extracts could produce a diabetic-like state; Conn demonstrated that ACTH was the factor responsible.

It is now known that diabetes mellitus is not a single disease entity, but a family of diseases with many different etiologies leading to similar pathologies and symptomatologies with wide variations in severity. Diabetes mellitus most commonly results from decreased production or release of insulin by the pancreatic β-cells. The causes of this defect can be due to pancreatic damage (chronic pancreatitis), exposure to specific toxins (alloxan, streptozotocin) or viral diseases (mumps, rubella, etc.); in many individuals, genetic factors are also clearly involved. Diabetes mellitus can also result from immunological factors: antibodies against insulin itself or cellular insulin receptors can interfere with the normal actions of the hormone.

Defects in the insulin receptors themselves may also be involved. Alternatively, many of the features of diabetes are seen in diseases in which there is overproduction of hormones which antagonize the effects of insulin, e.g., cortisol, glucagon, ACTH, or catecholamines.

2. Experimental Diabetes Mellitus

As stated previously, the most common types of diabetes result from a decreased availability of insulin. Experimental investigations of the mechanisms by which insulin exerts its effects have been greatly facilitated by the discovery of agents which damage the β-cells of the islets of Langerhans in a relatively selective manner. These include alloxan (2,4,5,6-tetraoxohexahydropyrimidine), which was first discovered in biological material by Liebig in 1862 (221). Hyperglycemia as a result of alloxan injection in rabbits was first noted by Jacobs (222). Since then, scores of studies investigating the effects of alloxan administration have been published; these have been reviewed by Rerup (223). The major disadvantage of alloxan as a diabetogenic agent is its toxic action on other organs in some species, including the kidneys, liver, and adrenals.

More recently, streptozotocin [D-glucose-2-(N'-methyl-N'-nitroso urea)] has been widely employed to induce experimental diabetes mellitus. Streptozotocin was isolated from Streptomyces achromogenes in 1960; its highly specific diabetogenic effect was first reported by Rakieten et al. in 1963 (224). Because of its very short biological half-life

the drug must be administered intravenously; in rats, doses of 100mg/kg produce diabetes in 100% of the animals (223).

The effects of alloxan and streptozotocin are very similar (223). An early, marked hyperglycemia lasting for one to four hours is followed by a more or less severe hypoglycemia lasting up to 48 hours, which can result in convulsions and death. Surviving animals then display chronic hyperglycemia, the result of decreased plasma levels of insulin. In rats, the effects of streptozotocin are somewhat less severe than in other species.

Histologically, frank necrosis of the β-cells was observed seven hours after administration of the drug; before this time, the β-cells were degranulated and some disruption of the islets was observed, although the α-cells appeared normal (223). The mechanism by which streptozotocin specifically affects the β-cells is not fully understood, but the glucose moiety is essential for its diabetogenic, but not antitumor, effects; this implies that this part of the molecule may be a "carrier". The primary effects, whatever they may be, are produced within 15 minutes of streptozotocin injection (223).

Streptozotocin in high doses can also damage other organs, including the kidneys, the liver, and the exocrine pancreas; moreover, the drug is a carcinogen. In the doses and time periods employed in the studies reported later, these effects should not have been manifest (223).

3. Metabolic Effects of Diabetes Mellitus

Diabetes mellitus is characterized in its chronic state by hyperglycemia, glycosuria, ketonemia, ketonuria, acidosis,

and increased net protein breakdown. Over prolonged periods, degenerative changes in the blood vessels, retinas, lenses, kidneys, and nervous system develop (213). These latter changes are a direct consequence of the prolonged hyperglycemia; since these tissues are freely permeable to glucose, hyperglycemia leads to elevated intracellular glucose concentrations which, in turn, lead to formation and accumulation of sorbitol within these tissues(225). Elevated concentrations of intracellular sorbitol lead to osmotic water influx, accompanied by sodium ions; these changes increase the premeability of the cell membranes, leading to losses of potassium, ATP, amino acids, and other small molecules (225). Additionally, many proteins such as hemoglobin A_1 and serum albumin, become more extensively glycosylated (28).

The hyperglycemia, ketoacidosis, and protein breakdown are consequences of both the relative deficiency of insulin and the relative excess of several hyperglycemic hormones, notably cortisol, glucagon, ACTH, and catecholamines (211-220). Lipolysis in adipose tissue is accelerated by the latter group of hormones and retarded by insulin; in diabetes mellitus, the fatty acids so released travel to the liver, where they are partially catabolized to yield "ketone bodies". Accumulation of ketone bodies produces acidosis and disturbances in fluid and electrolyte balance. The hyperglycemia is a consequence of the decreased ability of glucose to enter skeletal muscle and adipose tissue cells in the absence of insulin; inasmuch as these tissues form the greatest part of the body mass, this effect is far from trivial. Moreover, the (relatively) elevated levels of cortisol and glucagon

stimulate hepatic glycogenolysis and gluconeogenesis, thereby further elevating blood glucose levels. Additionally, cortisol induces proteolysis in skeletal muscle, providing the amino acids which are substrates of hepatic gluconeogenesis.

The effects of diabetes mellitus on protein metabolism are multiplex and profound. Insulin promotes the entry of amino acids, as well as glucose, into skeletal muscle and other tissues (219). However, the decreased rates of protein synthesis in skeletal muscle cannot simply be attributed to decreased availability of glucose and amino acids in muscle (207, 215, 226-232), although these factors also contribute to the overall decrease in protein synthesis. In a series of investigations on the effects of insulin upon protein synthesis in skeletal muscle, Wool (207, 226) discovered that the 60S ribosomal subunit in diabetic skeletal muscle has an impaired ability to participate in translation of exogenous mRNA; its ability to associate with the 40S subunit to form 80S ribosomes is decreased. Subsequently, a large number of investigators demonstrated that the state of phosphorylation of a 40S subunit protein, S6, was altered by the actions of various hormones; however, a clear correlation between the presence of phosphate groups on S6 and the rate of translation could not be established (233); perhaps more than one phosphorylation site is involved. There is evidence that insulin deficiency leads to a decreased rate of peptide chain initiation in skeletal and cardiac muscle (215, 230-232); these effects are particularly pronounced in skeletal muscles

with a high content of white fibers (230, 231), which are more dependent on glycolysis as a source of energy than are red fibers (234). The mechanism of this decrease in the rate of initiation is not well understood; formation of the ternary complex [eIF-2, GTP, met-tRNA^f] does not appear to be affected (230-233). A role of glucose-6-phosphate as an allosteric stimulator of initiation independent of its role in glycolysis or NADPH generation has been demonstrated in reticulocyte lysates (235). A similar role for this compound in skeletal and cardiac muscle has been suggested, but not definitively demonstrated (230). However, insulin is capable of stimulating peptide chain initiation in these tissues in the absence of glucose-6-phosphate (230), implying additional mechanisms of action as yet unknown. It is suspected (230-232) that, in analogy with the well-known mechanisms of translational control in reticulocyte lysates (168-183), changes in the phosphorylation state of initiation factors (or other components) of the translational system in muscle may be involved; however, decisive evidence in support of this hypothesis has yet to be obtained.

In states where there is an insulin deficiency, including diabetes mellitus and starvation (236), there is an increased rate of proteolysis in skeletal muscle as well as a depressed rate of protein synthesis (230, 231, 237-241). Lysosomal proteases are primarily involved in this process; their activities are governed by a large number of hormones (242). Insulin decreases overall rates of proteolysis in

skeletal muscle; although the mechanism of this effect is presently unknown, it is noteworthy that when insulin binds to its plasma membrane receptors, some of these complexes can be internalized by the cell and transferred to the lysosomes (243-246). The amino acids released by proteolysis in skeletal muscle are partially catabolized there; branched-chain amino acids are preferentially degraded (247). The primary amino acids released to the blood by muscle are alanine and glutamine (248); the former amino acid is a major gluconeogenic precursor for the liver, while the latter is preferentially utilized by the kidney and the intestine (229). Proteolysis in skeletal muscle can be accelerated by cortisol, glucagon, and thyroxine (242); in other tissues, response to these hormones may be more selective.

The effects of insulin deprivation on protein synthesis in other tissues are generally less dramatic than in skeletal muscle. Streptozotocin-induced diabetes in rats reduced overall protein synthesis in vivo by 70% in skeletal muscle and 44% in heart, but produced no real change in liver (249). The rate of total protein synthesis is not decreased in rat kidney (250) or Chinese hamster liver or kidney (251) from diabetic animals. However, the synthesis of specific enzymes in these tissues may be increased or decreased (232).

4. Mechanisms of Insulin Action

Insulin, like many other hormones, produces a "pleiotypic response" in its target tissues (215, 226). Clearly, several mechanisms of action must be involved; they are still rather

poorly understood. Aggregation or cross-linking of insulin receptors appears to play an important role in generation of insulin effects; (aggregation can be brought about not only by insulin, but also by antibodies against the insulin receptor and by lectins such as concanavalin A); these latter agents mimic many of the effects of insulin (252-254). Although several investigators have demonstrated the internalization of receptor-bound insulin (243-246) and suggested the existence of intracellular receptors for insulin (cf. 255) which could be involved in mediating its actions, the insulinomimetic effects of antibodies and lectins appear to render this hypothesis unlikely for most insulin effects (glucose and amino acid transport, stimulation of Na^+/K^+ and Mg^{++} ATPases, etc.). While it is possible that the transport of glucose and amino acids across the plasma membrane might be accelerated by some type of direct membrane signal, the altered intracellular functions undoubtedly require the participation of some second messenger (256, 257). As might be expected, a great deal of labor has been invested in the search for such a mediator.

Several candidates have been proposed as the intracellular second messenger of insulin. They include Na^+, Mg^{++}, Ca^{++}, cAMP, cGMP, and fragments of the insulin molecule (257). Activity of the plasma membrane Na^+/K^+ ATPase is increased by insulin. This might be due to alterations in its degree of phosphorylation, inasmuch as at least one adipocyte plasma membrane peptide displays a decreased extent of phosphorylation after insulin treatment (258). Additionally, at least one

mitochondrial protein also loses phosphate groups after insulin treatment of adipocytes (258); this has been identified as the α-subunit of pyruvate dehydrogenase (259). However, the increased activity of the Na^+/K^+ ATPase probably represents one of the many terminal cellular responses to insulin, not a primary mediating event, since there is a negative correlation between Na^+ pump activity and insulin-like metabolic effects under a variety of conditions (256). Nonetheless, alterations in intracellular $[Na^+]$ and $[K^+]$ could modulate some of these effects.

Unequivocal evidence that cAMP or cGMP are involved in mediating any of insulin's effects is lacking, but they may be involved in a limited or secondary sense (257). Spence et al. have provided a model for the regulation of glucokinase in cultured hepatocytes by insulin, glucose, and cGMP (260); in this model, changes in [cGMP] are secondary to the increased transcription of glucokinase mRNA induced by insulin and the subsequent metabolic effects. Interestingly, insulin treatment in vivo increases, by an unknown mechanism, the translational efficiency of poly(A)$^+$ mRNA, when measured in vitro (261). This may be a general phenomenon involved in other tissues as well (261).

As discussed by Czech (256,257), there is no convincing evidence that changes in intracellular $[Mg^{++}]$ are of primary importance in mediating insulin action. On the other hand, Ca^{++} has been advanced by many investigators as a possible mediator of insulin action (256, 257), the manifold

roles of Ca^{++} in cellular regulation (262, 263) make this a tempting hypothesis. However, as Czech points out, there is a wealth of contradictory data on the connection between insulin and Ca^{++}; perhaps intracellular Ca^{++} is highly compartmentalized and these compartments are specifically affected by hormones (256,257). Much further work in these areas will be required to delineate the interrelationships between insulin and Ca^{++}.

Recently, the generation of a second messenger *in vitro* has been demonstrated upon addition of insulin to adipocyte plasma membranes (253, 264); it has been shown to activate mitochondrial pyruvate dehydrogenase phosphatase (264). Larner *et al.* found a similar substance in skeletal muscle which they proposed to act as a Ca^{++}-ionophore (265). The material from adipocytes displays peptide-like properties and appears to originate by proteolysis of some plasma membrane component; it has a molecular weight of between 1000 and 4000 (266, 267). However, its actions appear to be independent of Ca^{++} or Mg^{++} (267). Recently, a component isolated from insulin-treated hepatocytes which stimulates RNA synthesis in isolated nuclei has been reported (268); it has not been characterized except to note that its molecular weight appears to be less than 1000. An insulin-dependent cytoplasmic factor with characteristics similar to those of the mediator described by Seals *et al.* (266) and Kiechle *et al.* (267) has been shown to stimulate mitochondrial Ca^{++} uptake (269). These fascinating observations will undoubtedly lead to an

improved understanding not only of the basis of insulin's actions, but also of cellular functions in general.

F. Objectives of this Thesis

The basic current of research in this laboratory over the years has been the examination of the role of mitochondrial protein synthesis in mitochondrial protein synthesis in controlled (57,58,90, 104, 120, 123,163, 164, 210,270,274,276, 285, 302, 333). The studies reported in Chapter III began as an outgrowth of the observations by Mockel and Beattie (270) that skeletal muscle mitochondria isolated from diabetic rats exhibited much lower rates of protein synthesis under optimal conditions *in vitro* compared to control muscle mitochondria. On the other hand, liver mitochondria from control and diabetic rats displayed identical rates of protein synthesis *in vitro* (270). Thus, the control of mitochondrial protein synthesis in mammals was subject to tissue-specific variations.

It thus seemed worthwhile to reexamine these findings from several aspects:

1) Did the decreased rates of *in vitro* mitochondrial protein synthesis correlate with other parameters of mitochondrial function? Were there important physiological changes associated with these observations?

2) Were these effects observable in other mammalian tissues (e.g., kidney)? Was there a coherent explanation for these tissue-specific differences?

3) Was it possible to pinpoint the reason for these changes? Could further insight into the control mechanisms of mammalian mitochondrial protein synthesis be gained?

As has been previously stated and will be subsequently discussed, there is ample evidence that mitochondrial protein

synthesis is governed by products of cytoplasmic translation. I repeat this now to emphasize that my approach to these questions has been rather biased in this direction. Investigations of these phenomena in mammals are complicated enormously by their structural and metabolic complexity. Moreover, even in inbred strains of rats and mice, there is considerable biological variation from individual to individual. Such variation is even evident in the mitochondrial genome itself (158-161).

II. EXPERIMENTAL PROCEDURES

A. Materials

Male Sprague-Dawley rats, weighing 150-200g, were obtained from Perfection Breeders. ATP, ADP, sodium phosphoenolpyruvate, pyruvate kinase, Bicine, Tricine, streptozotocin, cycloheximide, chloramphenicol, ammonium aurintricarboxylate, bovine insulin, trypsin, and unlabelled amino acids were obtained from Sigma Chemical Co. L-[4,5-^{3}H]-leucine (58.5 Ci/mMol) was from New England Nuclear, while L-[^{35}S]-methionine (800-1200 Ci/mMol) was from Amersham. Acrylamide and N,N' methylene-bis-acrylamide were from Eastman. Pactamycin was the generous gift of Dr. Joseph Wu, New York Medical College. Other reagents were of the purest grades commercially obtainable. Solutions used for mitochondrial isolation and protein synthesis *in vitro* were prepared with glass-distilled water and filtered through 0.45 μ Millipore filters before use. All glassware used was sterilized by autoclaving.

B. Treatment of Animals.

Unless otherwise indicated, rats were watered and fed *ad libitum* on a standard diet of laboratory chow. Diabetes mellitus was induced by the intravenous administration of streptozotocin (10mg/100g body weight) dissolved in citrate buffer (pH 3.8-4.2); controls received an equivalent volume of buffer only (271). The rats were diabetic after 2-3 days and were generally used 5 days after injection, when blood glucose levels of 400mg/dl or greater were present. Where the effects of insulin were to be investigated, soluble

insulin (1 unit) was injected subcutaneously at the end of the third day after streptozotocin administration, and the dose was repeated on the morning and evening of the fourth day. On the morning of the fifth day, two units of insulin were administered(249) and the animals were sacrificed two hours later. Determinations of blood glucose made prior to the first insulin injection and also at the time of sacrifice confirmed that the streptozotocin - induced hyperglycemia was reduced to control levels by insulin treatment. Fasted rats (175-200g) were caged separately and deprived of food for 48-72 hours before sacrifice. Where indicated, cycloheximide (0.5 mg/ml in 0.85% NaCl) was administered intraperitoneally from two to twenty-four hours before sacrifice.

C. Preparation of Mitochondria.

Animals were killed by decapitation. Skeletal muscles with high content of white fibers (234) (gastrocnemius, rectus femoris, vastus lateralis, longissimus dorsi) were cleaned of fat and connective tissue, quickly excised, and placed in cold medium M (0.21 M mannitol, 0.07 M sucrose, 0.01 M Tris·HCl, pH 7.4, 0.002 M EDTA). All subsequent operations were performed at 0-4°. The muscles were finely minced with scissors and processed through an Edco hand tissue press. The resultant suspension was adjusted to a concentration of 10g tissue/100ml medium M. Trypsin was added in a ratio of 2.4mg/10g tissue, and the mixture was stirred magnetically at 100 rpm for 30 minutes at 0-4°. The resultant suspension was centrifuged for 10 minutes at 650 x g

to remove debris, and the supernatant was filtered through several layers of cheesecloth or a single layer of fine silk mesh. Mitochondria were collected from the resultant filtrate by centrifuging for 10 minutes at 10,000 x g (270). The final mitochondrial pellet was resuspended in medium M at a concentration of 5-15mg/ml and kept at 0° until use.

Because of the possibility that mitochondria from trypsinized skeletal muscle might have sustained some degree of damage, skeletal muscle mitochondria were also prepared from mechanically - homogenized muscles (272). Excised muscles were finely minced with scissors, homogenized in a Dounce homogenizer with ground-glass mortar, processed through the Edco hand tissue press, and homogenized again in the Dounce homogenizer. Mitochondria were collected from the homogenate by the same procedure described above.

Rat kidney mitochondria were prepared under sterile conditions by the method of Devlin and Ch'ih (273) in medium K (0.20 M mannitol, 2 mM HEPES, 1 mM EDTA, pH 7.2) and were washed three to four times. Rat liver mitochondria were prepared by the method of Beattie (274) in medium L (0.21 M mannitol, 0.07 M sucrose, 10 mM HEPES, 2 mM EDTA, pH 7.7) and washed four times.

D. Amino Acid Incorporation by Isolated Mitochondria.

The standard medium for incorporation of amino acids by isolated rat tissue mitochondria(270) contained 50 mM Bicine, pH 7.6, 1 mM EDTA, 5 mM potassium phosphate, 90 mM KCl, 1mg/ml cycloheximide, 50µg/ml of a mixture of 19 unlabelled

amino acids (minus leucine) (51), $MgCl_2$ (6 mM for kidney, 10 mM for liver or skeletal muscle), 10μCi/ml of L-[4,5-^{3}H]-leucine, and 1mg/ml mitochondrial protein. Two alternative energizing systems were employed. Exogenously-regenerated ATP was produced using 2 mM ATP (pH adjusted to 7.6 with KOH) plus 5 mM phosphoenolpyruvate and 16.8μg/ml pyruvate kinase. Alternatively, ATP could be synthesized endogenously from added ADP (2 mM) in the presence of an oxidizable substrate (25 mM glutamate). The complete mixtures were incubated in open 25ml polycarbonate flasks at 37° in a New Brunswick metabolic shaker at 60-100 rpm. The basic incorporation medium could be modified by the addition of inhibitors of protein synthesis (chloramphenicol, ammonium aurintricarboxylate, pactamycin) or postpolysomal cytosolic supernates.

Incorporation of [^{3}H]-leucine into trichloroacetic acid-insoluble products was measured in triplicate by the filter disc method of Mans and Novelli(275); sample discs were immersed in 10ml of Betafluor and counted with a Beckman LS 9000 liquid scintillation counter. Corrections were made for zero-time incorporation. Bacterial contamination was evaluated by plating an aliquot of incubation medium at the end of an experiment onto blood-agar plates. After 72 hours of incubation at 37°, the number of colonies observed never exceeded 100/ml. Protein synthesis *in vitro* was measured in isolated yeast mitochondria incubated at 30° in a substantially similar manner as described by Everett *et al.*(276)

E. Enzyme Assays

Rates of oxygen uptake were measured polarographically with an oxygen electrode (Yellow Springs) at pH 7.4 in a medium (final volume 3.0ml) containing 15 mM KCl, 30 mM potassium phosphate, 25 mM tris·HCl, 45 mM sucrose, 10 mM mannitol, 5 mM $MgCl_2$, 7 mM EDTA, 0.2% bovine serum albumin, 20 mM glucose, 0.5 mM NAD^+, and 1-2mg mitochondrial protein. The oxidizable substrate was 10 mM glutamate. State 3 rates were measured after addition of 0.506µmol aliquots of ADP. Uncoupler - stimulated respiration was measured in the presence of 2.5 µM carbonyl-cyanide-m-chlorophenyl-hydrazone (CCCP).

Succinate dehydrogenase was assayed by the method of King(277) in both mitochondria and tissue homogenates, while NADH dehydrogenase was assayed in mitochondria only as described previously (104).The recovery of mitochondria from homogenates, expressed as a percentage, was calculated by dividing the total succinate dehydrogenase activity of the mitochondria by the total activity of the homogenate. Cytochrome content of mitochondria was determined by recording the difference spectrum of dithionite-reduced vs. ferricyanide-oxidized mitochondrial suspensions, using a Perkin-Elmer model 557 dual-wavelength double-beam spectrophotometer. The wavelength pairs and extinction coefficients used for calculations of cytochrome content were taken from Wilson (278). Protein concentrations were measured by the method of Lowry et al (279), using crystalline bovine serum albumin as standard, in the presence of 0.1% sodium deoxycholate.

F. Electrophoretic Procedures.

For labelling of mitochondrial translation products *in vitro*, the standard incorporation medium was modified by substituting 400µCi/ml of carrier-free L-[^{35}S]-methionine for [^{3}H]-leucine. An equimolar mixture of 19 amino acids (30µM each) minus methionine was used. After 60 minutes of incubation at 37° mitochondria were reisolated from the medium by centrifuging for 6-8 minutes at 4° in an Eppendorf micro-centrifuge (15,000 x g), and washed three times with medium M containing 1 mM unlabelled methionine. The final mitochondrial pellet was dissociated overnight at 25° in 50-200µl of a solution containing 5% (w/v) sodium dodecyl sulfate (SDS), 5% (w/v) 2-mercaptoethanol, 50 mM Tris·HCl, pH 6.8, 2 mM EDTA, and 10% (v/v) glycerol. (280) Often, the protease inhibitor phenylmethylsulfonyl fluoride (PMSF), dissolved in dimethyl sulfoxide (DMSO), was added to a final concentration of 1 mM. Some mitochondrial pellets were treated with 10µl of 0.1 M NaOH for 15 minutes at 25° before the dissociation solution was added(281). Total radioactivity and trichloroacetic acid-insoluble radioactivity were determined for each sample before electrophoresis. Immediately prior to electrophoresis, a small quantity of bromphenol blue in glycerol was added to the samples.

Polyacrylamide slab gels 1.2 mm thick were prepared according to Studier(282) with minor modifications (280). The discontinuous buffer system of Laemmli(283) was used for electrophoresis. The stacking gels were 5% acrylamide, while

the resolving gels contained concentrations of acrylamide ranging between 8% and 15%. Usually, the resolving gel contained 12% acrylamide. After electrophoresis, the slab gels were stained for 60-90 minutes with 1.25% (w/v) Coomassie brilliant blue R in 45% (v/v) methanol: 9.7% acetic acid and destained for 6 minutes in a Canalco electrophoretic destainer. The gels were mounted on Whatman 3 MM filter paper and dried *in vacuo* using a BioRad gel dryer. The positions of the molecular weight standards (Pharmacia "low molecular weight" kit, covering the range of 14,400-94,000 daltons) were marked using a fountain pen containing ink with added [^{14}C]-leucine. The dried, marked gels were exposed at -70° to Kodak NS-ST X-ray film for differing periods of time, depending on the number of counts/min in the samples loaded. Usually 100,000 counts/min (trichloroacetic acid-insoluble) required an exposure time of 4-5 days. Autoradiograms were scanned with a Canalco model G microdensitometer.

G. Preparation of Cytosolic Post-polysomal Supernates ("S-140")

All operations were performed at 0-4°C. Skeletal muscles were excised, cleaned of fat and connective tissue, minced finely, and homogenized with a Brinkmann Polytron homogenizer in medium S (10 mM potassium phosphate, pH 7.4, containing 1 mM EDTA, 10% (v/v) glycerol, and 1 mM each of the protease inhibitors phenylmethylsulfonyl fluoride (PMSF) and N-α-p-tosyllysine chloromethyl ketone (TLCK). PMSF and TLCK were added shortly prior to use in the form of freshly prepared stock solutions in dimethylsulfoxide (DMSO). (284)

Immediately after sacrifice, livers were subjected to retrograde perfusion via the vena cava with ice-cold pH 7.4 130 mM NaCl, 10 mM potassium phosphate, 1 mM EDTA, in order to remove erythrocytes, then excised and minced finely. The livers were homogenized using a Potter-Elvehjem Teflon/glass homogenizer in the same medium used for skeletal muscles (medium S).

The homogenates were centrifuged for 10 minutes at 650 x g to remove most tissue debris, and the resultant supernates were centrifuged twice for ten minutes at 13,000 x g to remove mitochondria and other organelles. The post-mitochondrial supernate was then centrifuged for 90 minutes at 140,000 g_{av}. The supernate was removed carefully, avoiding the lipid at the top of the tube and the pellet and supernate in the bottom of the tube. The supernate was dialyzed overnight in Spectrapore tubing (mw cutoff 3500) against 10 mM potassium phosphate (pH 7.4), 10% (v/v) glycerol, with or without 1 mM EDTA, with three to four changes of buffer. The dialyzed supernate was concentrated by immersing the dialysis bags in dry Sephadex G-25 or in polyethylene clycol (mw_{av}=20,000) to a final protein concentration of 25-40mg/ml, divided into aliquots, frozen quickly with liquid nitrogen or dry ice/acetone, and stored at -80°. This final fraction is designated S-140.

III. RESULTS

A. Optimal conditions for protein synthesis in vitro by isolated rat kidney mitochondria.

Initial studies were directed toward establishing optimal conditions for the incorporation of amino acids into protein by isolated kidney mitochondria. In these studies, which employed L-[4,5-^{3}H]-leucine as the label, the parameters examined included various concentrations of leucine, Mg^{++}, amino acids, and mitochondrial protein, as well as two alternative energy-providing systems. The concentration of leucine used for further studies (Figure 1) was selected as a compromise between the desire to maximize the absolute quality of protein synthesized (pmol leucine incorporated/mg mitochondrial protein/hr) and the need for incorporation of sufficient quality of label to permit accurate counting of samples. The final reaction mixture chosen contained a total of 15.2 µM leucine with 10 µCi of [^{3}H] leucine and resulted in 64% of the maximum incorporation achieved with 100 µM total leucine with the same amount of radioactive label.

The optimal [Mg^{++}] concentration was determined to be 6 mM (Figure 2), somewhat lower than the 10 mM optimal [Mg^{++}], reported for amino acid incorporation by mitochondria isolated from rat liver or skeletal muscle. The extent of protein synthesis was essentially proportional to the quantity of mitochondrial protein used in the mixture up to 2 mg/ml (Figure 3), but was relatively insensitive to changes in the concentrations of the 19 unlabelled amino acids used in the

range of 40 and 100 µg/ml (Figure 4). Use of a complete mixture of 19 unlabelled amino acids was far superior to use of a system employing only L-proline (data not shown), which has been reported to be capable of supporting liver mitochondrial protein synthesis in vitro (55).

The extent of protein synthesis in isolated rat kidney mitochondria is markedly influenced by the nature of the energy-providing system used. Amino acid incorporation can be supported by either exogenously-generated ATP, consisting of ATP, phosphoenolpyruvate and pyruvate kinase, or by endogenously-made ATP formed by use of excess oxidizable substrate, such as glutamate or succinate, with added ADP. In the latter case, glutamate (20-30 mM was clearly superior to succinate (20 mM) as an electron donor for supporting mitochondrial translation in vitro (Table I). The highest levels of amino acid incorporation were observed using the exogenous ATP-generating system (Tables II and III; Figures 5 and 11).

The extent of leucine incorporation into mitochondrial protein was essentially linear with time for 60 minutes (Figure 5) when the external ATP-generating system was used. Mitochondrial protein synthesis under the conditions employed was inhibited by 94% in the presence of 250 µg/ml chloramphenicol (Table III).

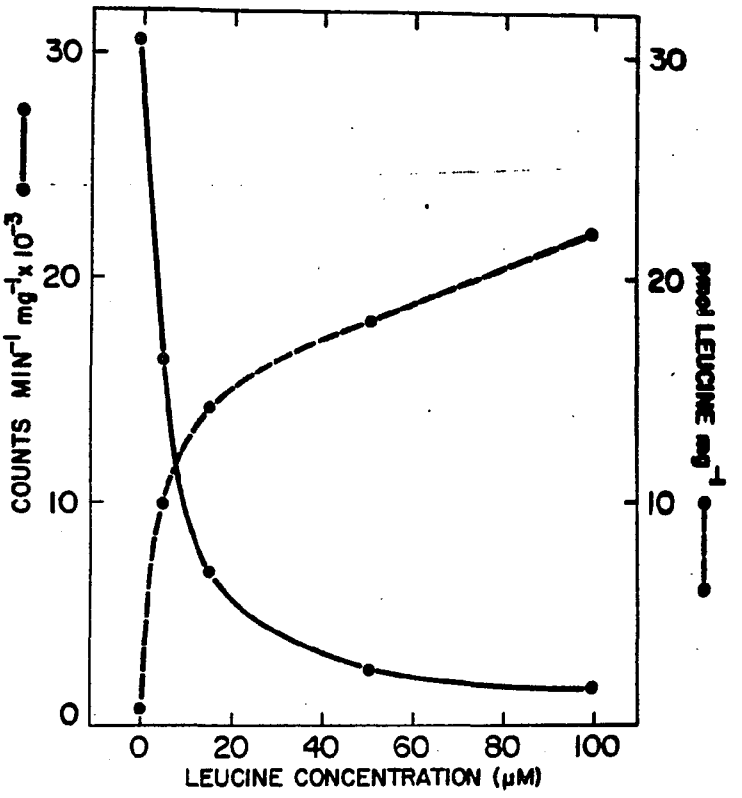

Figure 1.

Dependence of kidney mitochondrial incorporation of labelled leucine upon total leucine concentration. Isolated rat kidney mitochondria were incubated in vitro at 37° for 30 minutes in the basic medium described in "Experimental Procedures". Each 1 ml of incubation mixture contained 1 mg mitochondrial protein, 1 mg of cycloheximide and 10 μCi of L-[4,5-^{3}H]-leucine (0.8 μM), and various concentrations of unlabeled L-leucine as shown. The energy was provided by ATP-phosphoenolpyruvate-pyruvate kinase. Trichloroacetic acid-precipitable radioactivity in the resultant mixture was determined as described in "Experimental Procedures"

(●——●) counts $min^{-1}mg^{-1}$ mitochondrial protein incorporated.
(●----●) pmol leucine mg^{-1} mitochondrial protein incorporated.

Further studies employed 10 μCi of L-[4,5-^{3}H]-leucine and 15 μM added unlabelled leucine, representing a reasonable compromise between obtaining sufficient incorporation of label for accurate counting and achieving a substantial degree of actual protein synthesis. These values were used for mitochondria from kidney, liver and skeletal muscle.

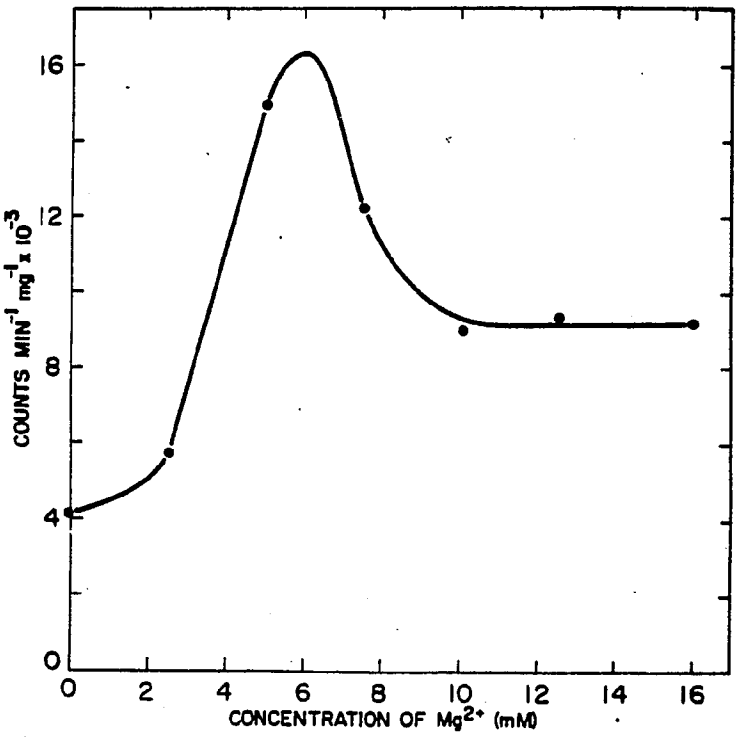

Figure 2.

Dependence of mitochondrial incorporation of labelled leucine upon magnesium concentration. Isolated rat kidney mitochondria were incubated in vitro at 37° for 30 minutes in the basic medium described previously in the legend to Figure 1, using 10 μCi of [^{3}H]-leucine and 15 μM unlabelled leucine; the concentration of $MgCl_2$ was varied. The optimal concentration of 6 mM Mg^{++} was used for all further studies; it should be noted that this figure is lower than that employed (10 mM) for similar experiments with liver or skeletal muscle mitochondria.

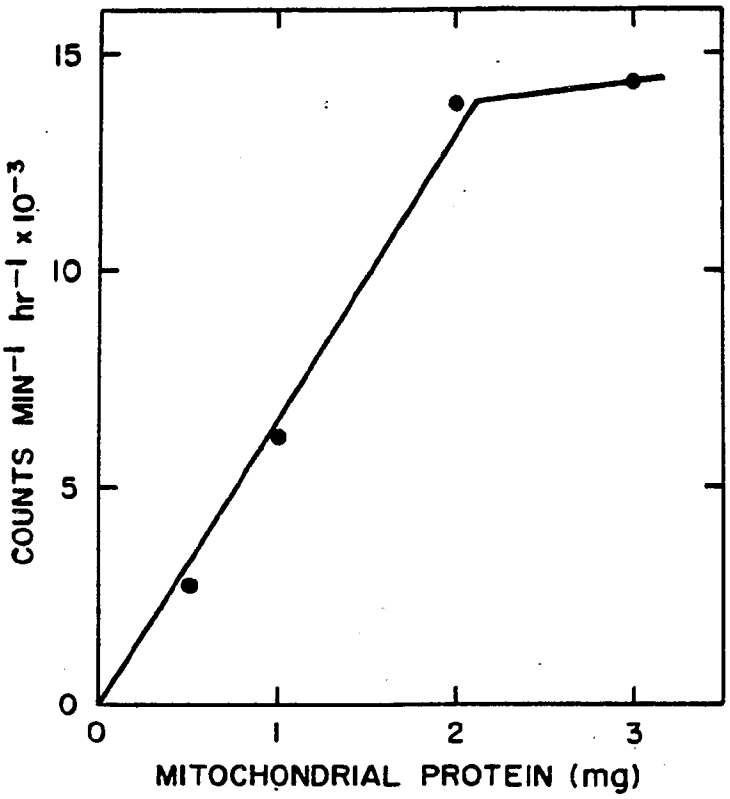

Figure 3.

Dependence of leucine incorporation by isolated kidney mitochondria upon mitochondrial protein concentration. Rat kidney mitochondria were incubated at 37° for 30 minutes in the basic incorporation medium using the optimal conditions described in Figure 1 and 2. Trichloracetic acid-precipitable radioactivity was determined as described in "Experimental Procedures".

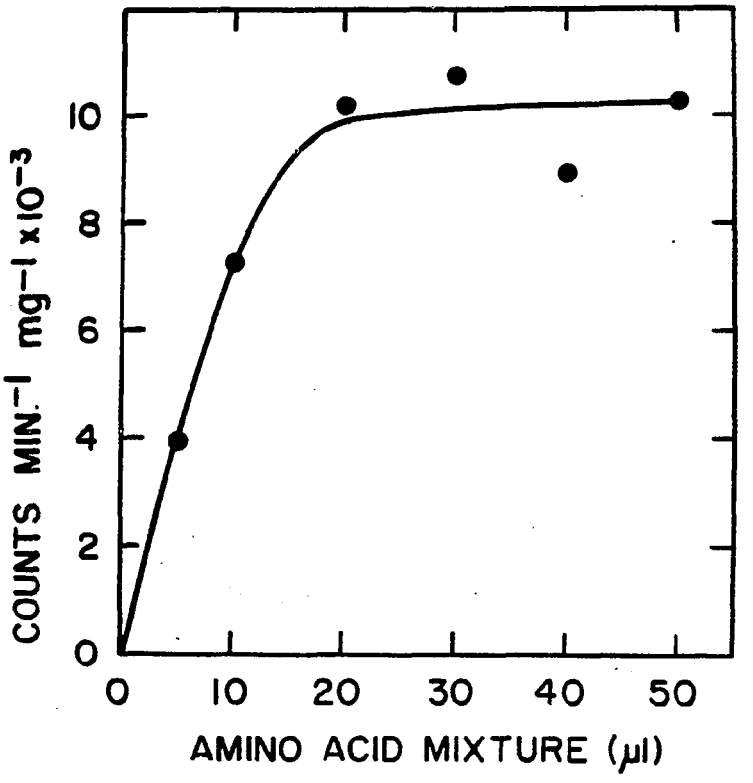

Figure 4.

Dependence of incorporation of labelled leucine by isolated kidney mitochondria upon concentration of amino acid mixture. Isolated rat kidney mitochondria at a protein concentration of 1 mg/ml were incubated as described in the legend to Figure 3 with varying amounts of the amino acid mixture previously described. All further studies using [^{3}H]-leucine employed 26 µl (50 µg) of amino acid mix per ml of incubation mixture.

TABLE I

Effect of Different Oxidizable Substrates upon Incorporation of [^{3}H]-Leucine into Protein by Isolated Rat Kidney Mitochondria.

	Counts min^{-1} mg^{-1} Mitochondrial Protein with	
Substrate (mM)	ADP-Glutamate	ADP-Succinate
10	12,800	11,200
20	18,800	12,300
30	20,400	8,950

The standard incorporation medium described in "Experimental Procedures" was used with the indicated concentration of oxidizable substrate. Determinations of incorporated radioactivity were performed in triplicate.

TABLE II

Effects of Varying the Energy Source on Amino Acid Incorporation *In Vitro* by Isolated Rat Kidney Mitochondria.

	Counts min^{-1} mg^{-1} Mitochondrial Protein	
Energy Source	Control	Diabetic
ATP-Generating System[1]	14,700±593	13,700±1,080
ADP-Glutamate	7,760±832	6,834±1,230

The standard incorporation mixture described in "Experimental Procedures" was used. Results in each case are the mean ± standard error of the mean for six animals. Determinations of incorporated radioactivity were performed in triplicate.

[1]ATP-generating system consists of ATP, phospholenol-pyruvate and pyruvate kinase.

TABLE III

Inhibition of Rat Kidney Mitochondrial Amino Acid Incorporation by Chloramphenicol.

	Counts min^{-1} mg^{-1}	% of Control
Control	23,610	100
+250 µg/ml Chloramphenicol	1,350	6

The standard incorporation medium (1.0 ml) contained either 10 µl of ethanol (control) or 10 µl of chloramphenicol (25 mg/ml in ethanol; 250 µg/ml final concentration). Radioactivity incorporated after 60 minutes at 37° was measured as described in "Experimental Procedures".

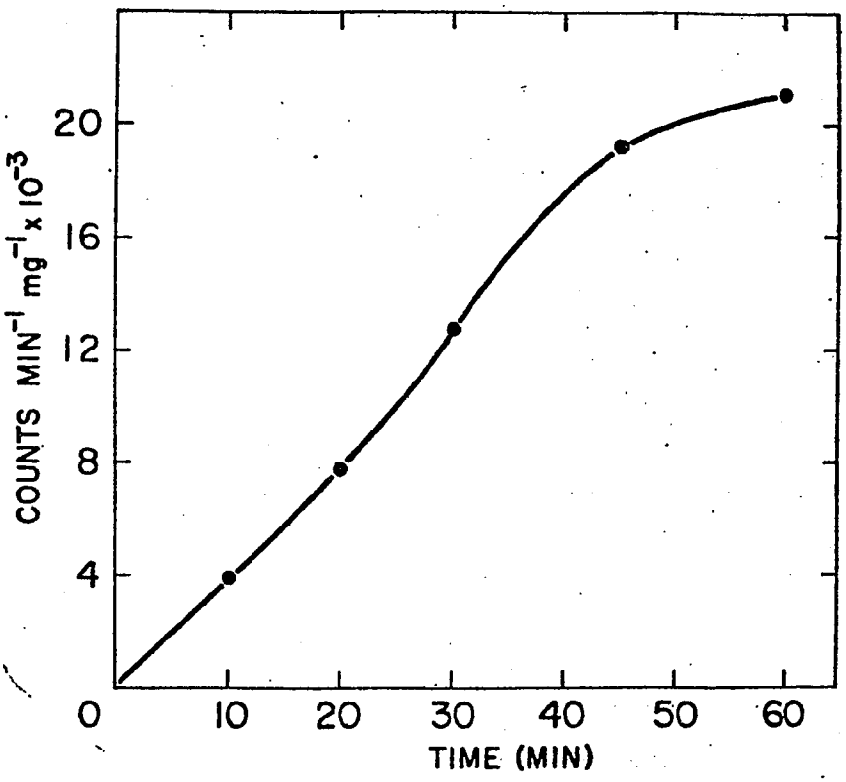

Figure 5.

Time course of leucine incorporation by isolated rat kidney mitochondria. Kidney mitochondria from a control rat were incubated using the optimal conditions described in the legend to Figure 4. The measurement of TCA-insoluble radioactivity on aliquots (100 µl) containing 0.1 mg protein was as previously described in "Experimental Procedures." A virtually identical time course was observed with diabetic kidney mitochondria.

B. Effects of Diabetes Mellitus on Protein Synthesis *in vitro* by Isolated Rat Kidney and Skeletal Muscle Mitochondria.

The optimal conditions for measuring the rate of protein synthesis *in vitro* by mitochondria isolated from skeletal muscle (270) and liver (285) were determined several years ago in this laboratory. With the exception of the optimal concentration of magnesium ion, which is 6 mM for kidney mitochondria (Figure 2), or 10 mM for liver and muscle mitochondria (270, 285), the optimal values for most other parameters (concentrations of leucine, amino acid mixture, and mitochondrial protein) are identical for all three types of mitochondria. However, the extent of protein synthesis in isolated mitochondria is markedly influenced by the nature of the energizing system used; furthermore, the relative superiority of a given energizing system varies from tissue to tissue. Amino acid incorporation by rat kidney mitochondria supported by externally-generated ATP proceeded at a rate about twice that observed when ATP was generated by intramitochondrial oxidative phosphorylation (Table II). Regardless of which energizing system was used, the rates of amino acid incorporation by kidney mitochondria isolated from diabetic animals were essentially identical to those observed in control kidney mitochondria (Table II). By contrast, amino acid incorporation by isolated skeletal muscle mitochondria (Figure 6) proceeded at a higher rate when ATP was generated by intramitochondrial oxidative phos-

phorylation than when ATP was regenerated externally. When either energizing system was employed, the rate of protein synthesis *in vitro* by skeletal muscle mitochondria isolated from diabetic animals was markedly depressed from control values. This effect was more pronounced when the energy was supplied by the respiratory chain with glutamate as substrate, which may reflect the observed decreases in cytochrome content and oxygen consumption in diabetic muscle mitochondria which are reported later.

The time course of protein synthesis *in vitro* was similar in skeletal muscle mitochondria from control and diabetic animals (Figure 6). Amino acid incorporation was linear for thirty minutes with a slightly decreased rate in the next thirty minutes. Hence, mitochondria obtained from the muscles of diabetic rats have a lowered capability of protein synthesis throughout the period of incubation. These data confirm the results of previous studies (270, 286).

In order to exclude the possibility that muscle mitochondria from diabetic animals were more sensitive than control mitochondria to trypsin-induced damage, a comparison was made of the rates of protein synthesis *in vitro* by mitochondria isolated by two different procedures: the standard method of trypsinization and mechanical homogenization. As shown in Table IV, the diabetic muscle mitochondria prepared by either procedure showed a pronounced decrease in the rate of protein synthesis relative to identically-prepared control mitochondria. Mitochondria prepared by trypsinization showed slightly higher

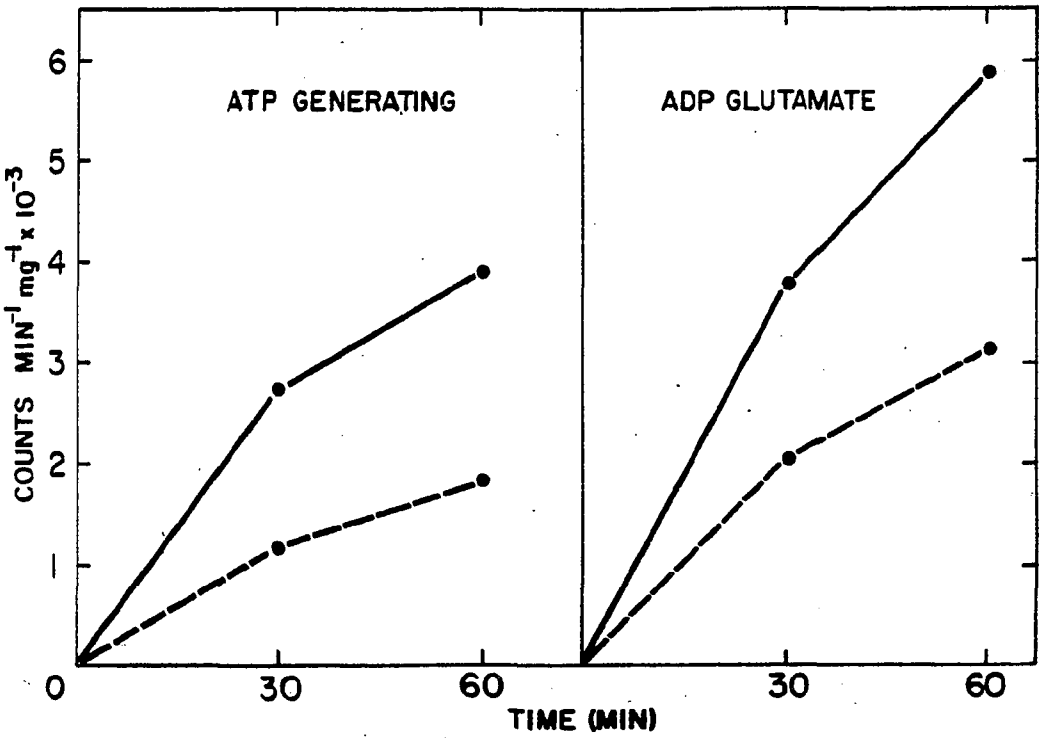

Figure 6.

Time course of leucine incorporation *in vitro* by isolated skeletal muscle mitochondria. The ATP generating system consisted of 2 mM ATP, 5 mM phosphoenolpyruvate and 16.8 µg/ml of pyruvate kinase, and the ADP-glutamate system contained 2 mM ADP and 25 mM glutamate. The incubation medium was described in "Experimental Procedures".

(●——●) control
(●------●) diabetic.

Each point is the mean of values obtained from 3 animals.

rates of protein synthesis in vitro (Table IV), as well as slightly higher yields (data not shown) and greater ease of handling larger number of animals.

The decreased rate of protein synthesis by skeletal muscle mitochondria from diabetic rats was completely reversed by administration of insulin to the animals (Figure 7). Insulin administration also lowered the blood glucose levels to those of the control rats (100-150 mg/dl). By contrast, insulin administration to control animals did not result in any change in the rate of muscle mitochondrial protein synthesis. Many of the physiological effects of diabetes mellitus are also observed during starvation, in which plasma insulin levels are lowered (236). A two-day fast resulted in a significant 23-27% decrease in the rate of amino acid incorporation in vitro by isolated skeletal muscle mitochondria whether the energy was supplied by the respiratory chain or by an external ATP regenerating systme.

The products of protein synthesis by skeletal muscle mitochondria from control and diabetic rats labelled in vitro with [^{35}S]-methionine were examined by electrophoresis on 12% polyacrylamide slab gels in the presence of 0.1% sodium dodecyl sulfate, followed by autoradiography of the dried gel. As shown in Figure 8A, isolated muscle mitochondria could incorporate L-[^{35}S]-methionine into several proteins whose apparent molecular weight ranged from under 14,000 to over 65,000 daltons. Although the extent of protein synthesis in diabetic muscle mitochondria is markedly depressed when compared to

TABLE IV

Effect of Method of Mitochondrial Preparation on Skeletal Muscle Mitochondrial Protein Synthesis *in vitro*

	Method of Mitochondrial Preparation	
	Trypsinization	Homogenization
	Counts $min^{-1}mg^{-1}$	
Control	5,640	5,120
Diabetic	2,150	1,670
% Change	-62.9	-67.4

Skeletal muscles from two control and two diabetic rats were pooled by type and processed through an Edco tissue press in medium M. The resultant suspensions were divided into two equal portions. One portion of each type was subjected to trypsinization in the normal fashion, while the other was homogenized mechanically as described in "Experimental Procedures". Incorporation of [^{3}H]-leucine into trichloroacetic acid-insoluble products after 60 min of incubation at 37° was performed as previously described. The system was energized with ADP and glutamate.

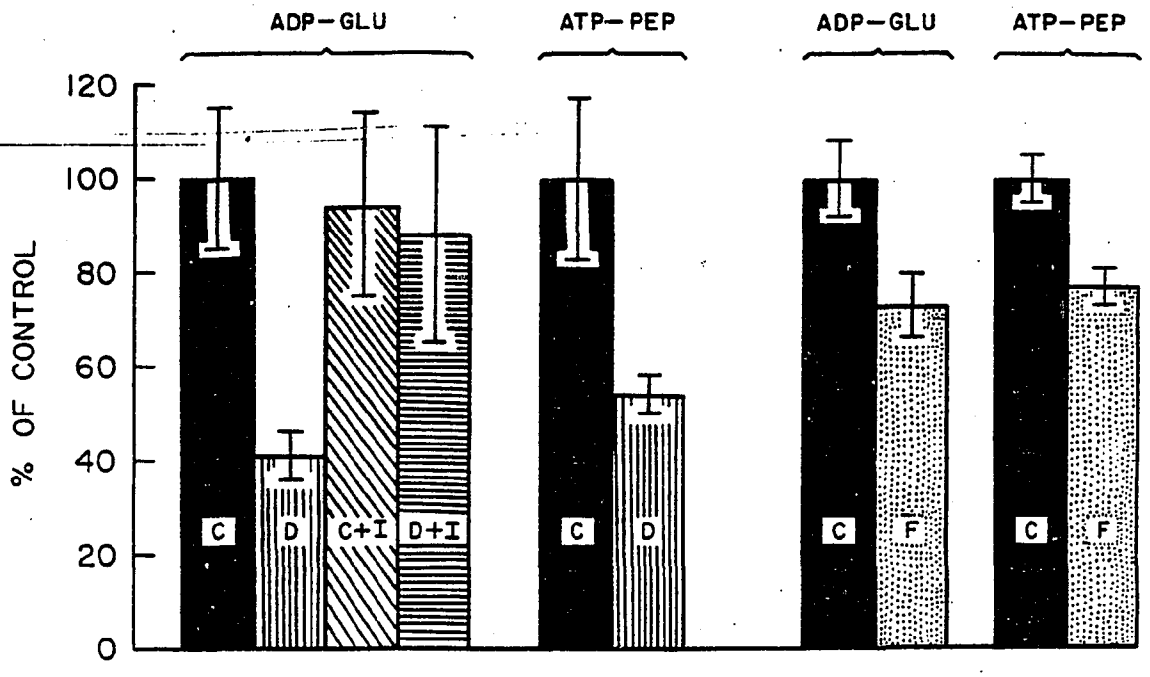

Figure 7.

Rates of protein synthesis in vitro by skeletal muscle mitochondria from control, diabetic, insulin-treated, and fasted rats.

Skeletal muscle mitochondria were prepared and incubated as described under "Experimental Procedures". Because considerable variations in the rate of protein synthesis were observed among different groups of control animals, control and treated rats from the same shipment were paired by weight and sacrificed on the same day. Each value represents the mean of 8-10 values ± the standard error of the mean.

C = Control
D = Diabetic
+I = Insulin-treated
F = Fasted 48 hrs
ADP-GLU = Energized by respiratory chain
ATP-PEP = Energized by externally generated ATP.

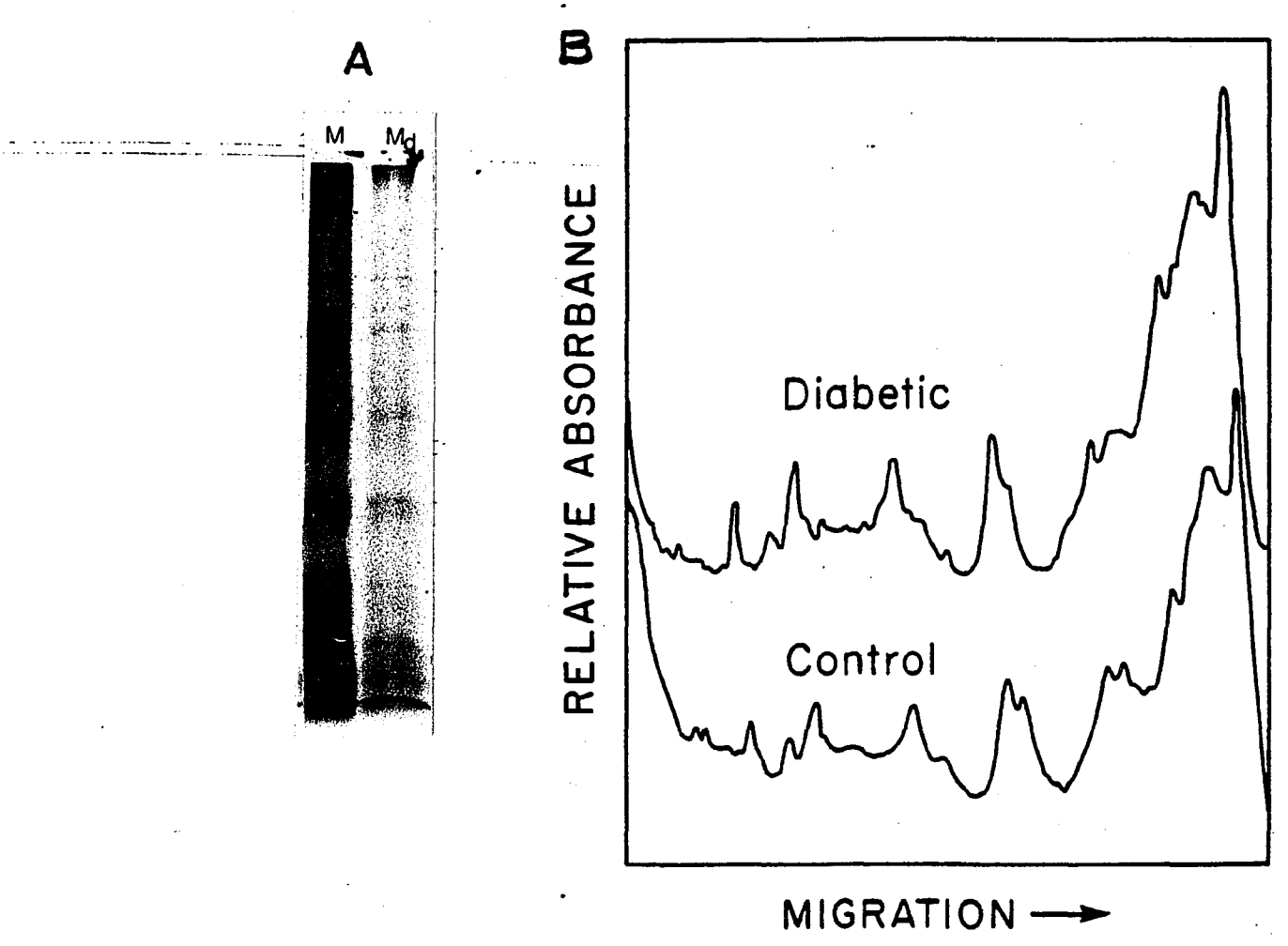

Figure 8.

Mitochondrial in vitro translation products from control and diabetic rat skeletal muscle. Skeletal muscle mitochondria were incubated with L-[^{35}S]methionine and subsequently prepared for electrophoresis as described in "Experimental Procedures". A. Samples of control and diabetic muscle mitochondria were resolved by electrophoresis on a 12% polyacrylamide slab gel with a 5% stacking gel, both containing 0.1% SDS. Arrows indicate the migration of molecular weight standards of 94,67,43,30,20 and 14 x 10^3 daltons. B. Densitometric scanning of autoradiograms of control and diabetic skeletal muscle mitochondrial translation products scanned with a Canalco microdensitometer. The autoradiogram of the diabetic was analyzed at an expanded sensitivity scale because fewer counts were applied to the gel. The zero absorbance has also been offset.

controls, the products made by both control and diabetic mitochondria appeared to be identical in number, apparent molecular weight, and relative quantity as indicated by densitometric scanning of the autoradiograms of control and diabetic muscle mitochondrial translation products (Figure 8B). It thus appears that the synthesis of all translation products in diabetic muscle mitochondria is depressed to the same extent; this might indicate that these products are expressed coordinately.

Use of a specific inhibitor of peptide chain initiation, ammonium aurintricarboxylate(287), indicates that the defect in protein synthesis in vitro in skeletal muscle mitochondria from diabetic animals exists primarily at the level of initiation (Figure 9). At a concentration of 1 mM, aurintricarboxylate inhibited protein synthesis in vitro by almost 80% in muscle mitochondria from control animals, but only by 40% in diabetic muscle mitochondria (Figure 10). The convergence of the curves in Figure 5 suggests that the rates of peptide chain elongation in skeletal muscle mitochondria from control and diabetic animals differ by at most a factor of two, while the overall rates of protein synthesis differ by a factor of close to five. Similarly, protein synthesis in vitro by kidney mitochondria is inhibited by 90% at 1 mM aurintricarboxylate (Figure 10). The time course of protein synthesis in the presence of low concentrations of aurintricarboxylate indicates that peptide chain initiation in isolated kidney mitochondria occurs throughout the period of incubation (Figure 11).

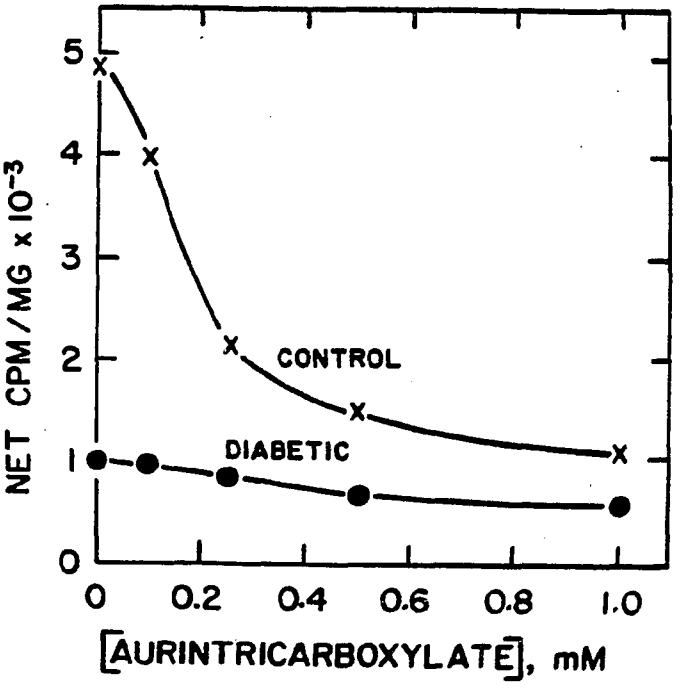

Figure 9.

Inhibition of skeletal muscle mitochondrial protein synthesis by aurintricarboxylate. Skeletal muscle mitochondria were isolated from control and diabetic rats and incubated *in vitro* as described in "Experimental Procedures" in the presence of the indicated concentrations of ammonium aurintricarboxylate.

(x——x) control
(●——●) diabetic.

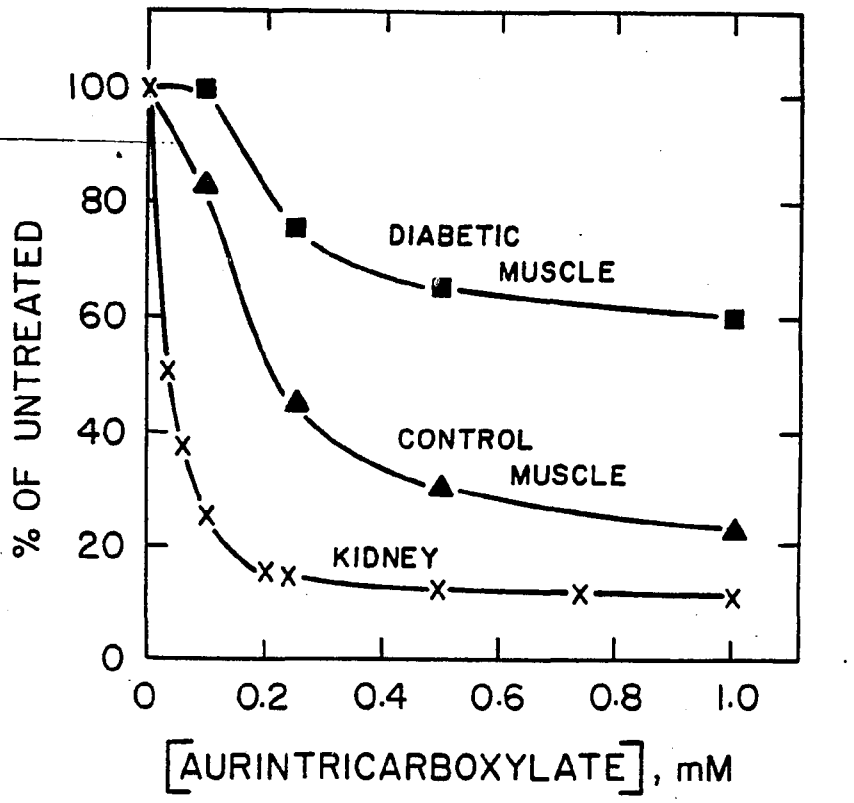

Figure 10.

Comparison of the effects of aurintricarboxylate upon protein synthesis in vitro by isolated mitochondria from control kidney and control and diabetic skeletal muscle. Kidney mitochondria were isolated and incubated in vitro as described in "Experimental Procedures". The data from Figure 5 for skeletal muscle mitochondria were normalized to 100% incorporation in the absence of ammonium aurintricarboxylate and replotted.

(x——x) kidney
(▲——▲) control muscle
(■——■) diabetic muscle.

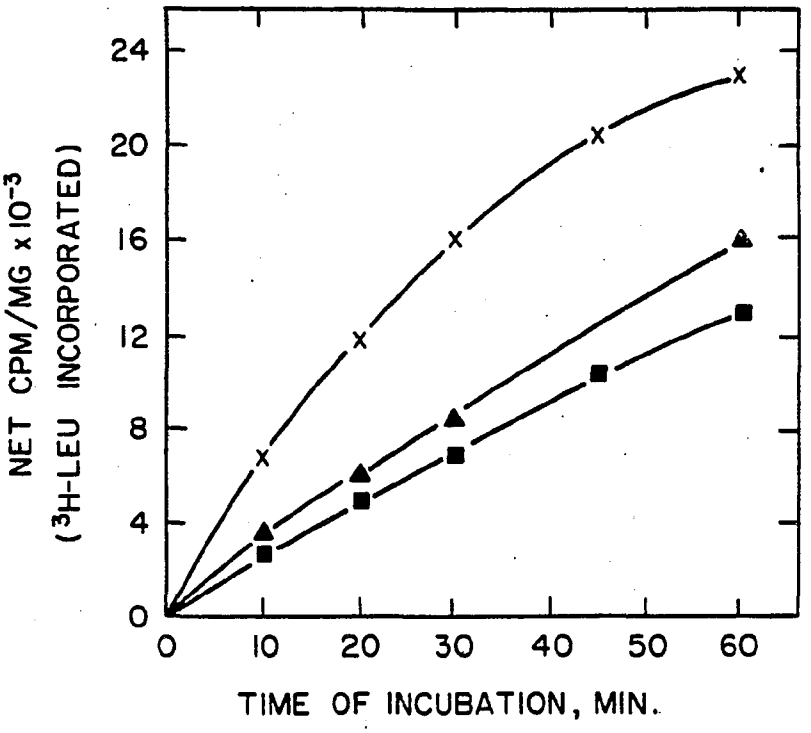

Figure 11.

Time course of kidney mitochondrial protein synthesis *in vitro* in the presence and absence of low concentrations of aurintricarboxylate. Isolated rat kidney mitochondria were incubated in the standard incorporation medium as described previously in the absence or presence of ammonium aurintricarboxylate. Aliquots were removed at the times indicated and trichloroacetic acid-insoluble radioactivity determined as described in "Experimental Procedures".

(x——x) Control (no ammonium aurintricarboxylate);
(▲——▲) +25 μM ammonium aurintricarboxylate;
(■——■) +50 μM ammonium aurintricarboxylate.

At 0.5 mM aurintricarboxylate, which inhibited protein synthesis *in vitro* by 71% in mitochondria from control skeletal muscle, addition of chloramphenicol (250 µg/ml) to the medium increased the degree of inhibition to 95% (Table II). Addition of this concentration of chloramphenicol alone inhibited protein synthesis by 88% (Table V).

Similar results were obtained when pactamycin, another specific inhibitor of peptide chain initiation (287), was employed in the concentration range of 0.1 - 1.0 µM (288) (Figure 12). A 61% maximum inhibition of protein synthesis in mitochondria from control animals was observed, while a 38% maximum inhibition was observed in the diabetic, again indicating that the rate of chain initiation is decreased in the diabetic. Again the two inhibition curves appeared to converge, suggesting that the rates of chain elongation do not differ appreciably in the control and diabetic skeletal muscle mitochondria.

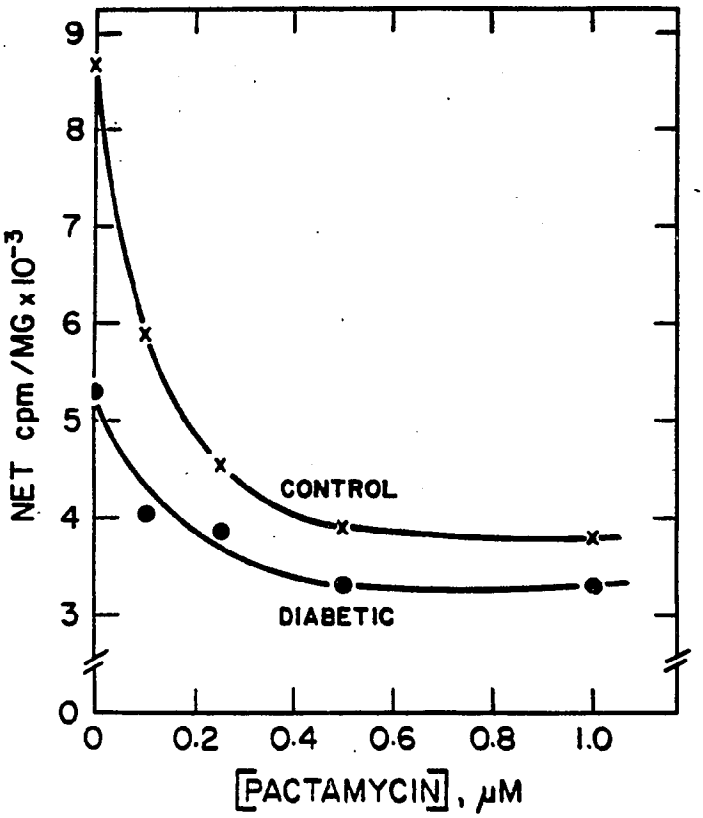

Figure 12.

Inhibition of skeletal muscle mitochondrial protein synthesis by pactamycin. Skeletal muscle mitochondria were isolated from control and diabetic rats and incubated *in vitro* as described in "Experimental Procedures" in the presence of the indicated concentrations of pactamycin.

(x——x) control
(●——●) diabetic.

TABLE V

Inhibition of Control Muscle Mitochondrial Protein Synthesis *In Vitro* by Aurintricarboxylate and/or Chloramphenicol.

Addition	Counts min^{-1} mg^{-1} Mitochondrial Protein	% of Control
None	8,320	100.0
+0.5 mM aurintricarboxylate	2,420	29.2
+250 µg/ml chloramphenicol	960	11.6
+0.5 mM aurintricarboxylate and 250 µg/ml chloramphenicol	420	5.1

The standard incorporation mixture described in "Experimental Procedures" was supplemented with 0.5 mM ammonium aurintricarboxylate and/or 250 µg/ml chloramphenicol.

The system was energized with ADP and glutamate and incubated at 37° for 60 min. Trichloroacetic acid-insoluble incorporation of [^{3}H]-leucine was determined as described in "Experimental Procedures".

C. Mitochondrial cytochrome content in control and diabetic animals.

Both cytochrome oxidase and cytochrome b have been shown to contain products of mitochondrial protein synthesis in lower eukaryotes (289), while the synthesis of cytochrome oxidase by isolated rat liver (129) and HeLa cell (130) mitochondria has been reported. Since it has been well established that blocking mitochondrial protein synthesis in lower eukaryotes results in a lowered content of cytochromes aa_3 and b, it was of some interest to determine whether the cytochrome content of skeletal muscle mitochondria from diabetic rats had decreased as a consequence of the lowered rates of muscle mitochondrial protein synthesis in these animals. The content of cytochromes aa_3, corresponding to cytochrome oxidase, was decreased by 27% in muscle mitochondria from diabetic animals (Table VI). These changes were significant at the $p=0.009$ levels. In addition, a similar decrease in the levels of cytochrome $c+c_1$ was observed, but this decrease was not as significant as that of the other cytochromes. By contrast, the levels of all classes of cytochromes were identical in kidney mitochondria from control and diabetic animals. (Table VI).

TABLE VI

Cytochrome Content of Skeletal Muscle and Kidney Mitochondria from Control and Diabetic Rats.

Cytochrome	a-a_3	b	c+c_1
Skeletal Muscle			
Control (7)	0.153±.007	0.254±.019	0.247±.028
Diabetic (7)	0.098±.015	0.185±.009	0.176±.048
% Change	-36	-27.2	-28.8
P	0.007	0.009	0.044

Cytochrome	a-a_3	b	c+c_1
Kidney			
Control (6)	0.268±.032	0.376±.014	0.626±.007
Diabetic (6)	0.264±.020	0.382±.016	0.628±.003

Cytochrome content, expressed as nmol per mg of mitochondrial protein, was determined as described in "Experimental Procedures". Values given are the mean ± standard error of the mean. Numbers in parentheses indicate the number of animals used. The p values were calculated by Student's t-test.

D. Respiratory rates of skeletal muscle mitochondria.

The rate of oxygen uptake by skeletal muscle mitochondria was measured with an oxygen electrode using glutamate as substrate. As seen in Table VII, a 27% decrease in the rate of oxygen uptake in the presence of ADP was observed with a p value of 0.006 when comparing mitochondria from diabetic and control animals. The respiratory control ratios, however, did not differ in the mitochondria from the two groups of animals. These ratios are lower than those previously reported for skeletal muscle mitochondria prepared by the same procedure as used in this study (270). Indeed, in some experiments we have observed respiratory control ratios of 5 or 6, but the overall results obtained are lower.

The rate of oxygen uptake was also measured in the presence of the uncoupler, CCCP, to determine whether the lowered rate of state 3 respiration in the mitochondria obtained from the diabetic resulted from a lowered rate of phosphorylation rather than from the decrease in respiratory chain activity (Table VII). The uncoupler-stimulated rate of oxygen uptake was identical to the state 3 rate in the mitochondria from both the control and diabetic rats. These results indicate that the maximum rate of oxygen uptake in the skeletal muscle mitochondria from the diabetic is significantly lower than that from the control.

TABLE VII

Respiratory Rates of Skeletal Muscle Mitochondria from Control and Diabetic Rats.

	Oxygen uptake (natom-g O min^{-1} mg^{-1} mitochondrial protein)		RCR
	State 3	CCCP-stimulated	
Experiment 1			
Control (8)	119.3±8.01	n.d.	2.56±0.33
Diabetic (10)	82.8±5.39	n.d.	2.12±0.16
% Change	-30	-	-17
P	0.001	-	N.S.
Experiment 2			
Control (5)	97.3±11.46	96.5±9.01	n.d.
Diabetic (6)	77.3±7.33	78.3±10.9	n.d.
% Change	-21	-19	

Rates of oxygen uptake were measured with an oxygen electrode as described in "Experimental Procedures". State 3 is defined as the rate of oxygen uptake in the presence of ADP and glutamate. The uncoupler-stimulated rate of oxygen uptake is the rate obtained with CCCP and glutamate. RCR=respiratory control ratio, defined as the rate of oxygen uptake in the presence of ADP divided by that obtained in its absence. Numbers in parentheses indicate the number of animals used in each experiment.

E. Succinate dehydrogenase, NADH dehydrogenase and mitochondrial recovery.

The activity of both primary dehydrogenases, which are not products of mitochondrial protein synthesis, was also compared in the skeletal muscle mitochondria from diabetic and control rats. No significant changes in the enzymatic rate of either enzyme complex were observed when the enzyme was assayed in either mitochondria or homogenates (Table VIII). The recovery of mitochondria, calculated by dividing the specific activity of succinate dehydrogenase in the mitochondria by the specific activity of succinate dehydrogenase in the homogenate, was also shown to be identical in the control and diabetic. Similarly, with rat kidney mitochondria there were no significant differences in the specific activity of succinate dehydrogenase or in mitochondrial recovery. (Table VIII).

TABLE VIII

NADH and Succinate Dehydrogenase Activities in Skeletal Muscle and Kidney.

	Control	Diabetic	% Change	P
Skeletal Muscle				
NADH-Dehydrogenase[1]				
Mitochondria (8)	952 ± 69	937 ± 16	-1.6	N.S.
Succinate Dehydrogenase[1]				
Mitochondria (11)	15.3± 2.7	14.7 ±3.0	-3.9	N.S.
Homogenate (11)	6.6 ±2.1	5.0 ±1.5	-24.7	N.S.
% Recovery	15.3 ±1.6	18.6 ±1.5	+21.6	N.S.
Kidney				
Succinate Dehydrogenase[1]				
Mitochondria (10)	149 ± 9	144± 2	-3.3	N.S.
Homogenate (10)	53± 4	68± 6	+28.3	N.S.
% Recovery	24.8± 1.8	21.1± 1.0	-14.9	N.S.

Enzyme activities were determined as described in "Experimental Procedures". The values reported are the mean ± the standard error of the mean. The numbers in parentheses indicate the number of animals in each group. Recovery was calculated by dividing the total enzyme activity in the mitochondrial fraction by the total activity in the tissue.

[1] nmol $min^{-1}mg^{-1}$

F. Products of protein synthesis in vitro by isolated mitochondria from rat kidney, liver, and skeletal muscle.

In order to permit better autoradiographic visualization of mitochondrial in vitro translation products separated by electrophoresis on SDS-polyacrylamide slab gels, L-[^{35}S]-methionine of high specific activity was substituted for L-[^{3}H]-leucine as the labelling agent, with appropriate changes in the composition of the mixture of 19 unlabelled amino acids. Protein synthesis in vitro by kidney and liver mitochondria was energized by externally generated ATP, while that by skeletal muscle mitochondria was energized with intramitochondrially generated ATP, with ADP and oxidizable substrate (glutamate) supplied.

As shown in Figure 13, isolated mitochondria from rat liver, kidney, and skeletal muscle are capable of incorporating labelled methionine into at least twelve strongly-labelled polypeptides, whose apparent molecular weights range from under 14,000 to over 94,000 daltons. Most of these translation products appear to be identical in mitochondria from all three tissues, but certain minor tissue-specific differences in electrophoretic patterns were noted consistently each time that these experiments were performed. The significance of this observation is not clear; it is unlikely that the rat mitochondrial genome differs from one tissue to another (161). A more likely explanation is that there are tissue-specific differences in proteolytic activity

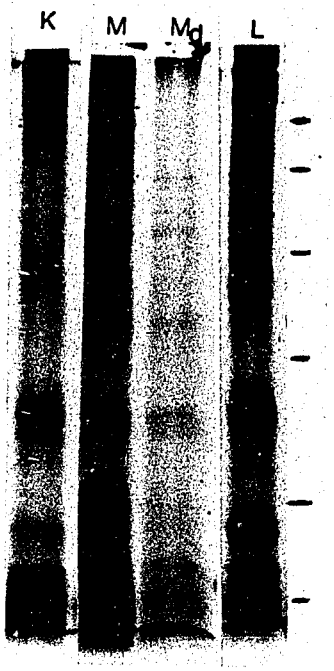

Figure 13.

Mitochondrial *in vitro* translation products. Mitochondria from rat kidney, liver, and skeletal muscle were incubated with L-[^{35}S]-methionine and subsequently prepared for electrophoresis as described in "Experimental Procedures." Samples were resolved on a 12% polyacrylamide slab gel with a 5% polyacrylamide stacking gel in the presence of 0.1% sodium dodecyl sulfate. The gel was dried using a BioRad gel dryer and exposed to Kodak NS-5T film for 28 days at -70°. The positions of the molecular weight standards are indicated at the right-hand side: phosphorylase *b* (M_r=94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400)

Lane K - rat kidney mitochondria
M - rat control skeletal muscle mitochondria
Md - rat diabetic skeletal muscle mitochondria
L - rat liver mitochondria.

present, caused by differential expressions of nuclear genes in these tissues. The substantial number of fainter bands visible in some of the autoradiograms may also represent the results of limited specific proteolysis of the actual translation products during the period of incubation. Omission of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) from the sample dissociation buffer did not appear to alter the observed electrophoretic patterns of mitochondrial translation products (data not shown), indicating that proteolysis by chymotrypsin-like enzymes did not occur during sample dissociation. Comparative interpretation of these autoradiograms is also hampered to some degree by the rather great differences in the efficiency of incorporation of [^{35}S]-methionine by mitochondria from different tissues (relative ratios: kidney, 1.0; liver 0.5; control muscle 0.1); thus, the low relative incorporation of methionine by muscle mitochondria could make some products difficult to visualize. Attempts to concentrate the labelled translation products by extracting water-soluble bulk mitochondrial proteins with low concentrations of Lubrol PX (Lubrol: mitochondrial protein = 1:5 w/w) did not produce any apparent difference in the products observed in liver or kidney mitochondria, but caused extensive proteolysis of the products obtained from skeletal muscle mitochondria (data not shown). Whether this was due to endogenous proteases or residual trypsin from the preparation of muscle mitochondria is not known.

Sevarino and Poyton observed that when yeast mito-

chondria were labelled *in vitro* with [^{35}S]-methionine in the presence of aurintricarboxylate at a concentration (500μM) which inhibited protein synthesis by about 50%, a new labelled mitochondrial translation product was formed; it was shown to be a precursor of subunit II of cytochrome oxidase with a short N-terminal extension (290). In order to ascertain whether analogous precursors might be formed in mammalian mitochondria, isolated rat kidney mitochondria were incubated *in vitro* with [^{35}S]-methionine in the absence and presence of ATA [25 and 50μM; the latter concentration of ATA inhibited protein synthesis in kidney mitochondria by about 60% (Figure 10)]. No differences whatsoever were observed in the electrophoretic mobilities of labelled products from kidney mitochondria incubated with or without ATA (data not shown). This would seem to indicate that the minor translation products observed are not precursors of the major products.

The possibility that some of the translation products observed were due to the presence of contaminating cytoribosomes was rendered highly unlikely by several observations. The standard medium for amino acid incorporation contained a concentration of cycloheximide (1.0mg/ml) sufficiently high to suppress cytoplasmic protein synthesis (57). As shown in Table IX, the addition of 250μg/ml chloramphenicol to the incubation mixture inhibited by 96% incorporation of [^{35}S]-methionine into protein by isolated kidney mitochondria in either the presence or the absence of cycloheximide. When kidney mitochondrial labelled with [^{35}S]-methionine in the

TABLE IX

Inhibition by Chloramphenicol of Incorporation of [^{35}S]-Methionine by Rat Kidney Mitochondria *in vitro* in the Presence and Absence of Cycloheximide.

Additions	[^{35}S]-methionine incorporated	
	net counts min^{-1} mg^{-1}	% of control
Cycloheximide (1.0mg/ml)	1,291,500	100.0
Chloramphenicol (250µg/ml)	52,800	4.1
Cycloheximide (1.0mg/ml) plus chloramphenicol (250µg/ml)	55,000	4.3

Isolated rat kidney mitochondria (1.0mg/ml) were incubated *in vitro* at 37° for 60 min with 400µCi of L-[^{35}S]-methionine in the standard medium for protein synthesis described in "Experimental Procedures," and subsequently reisolated and washed three times in medium K containing 1 mM unlabelled methionine. The washed labelled mitochondrial pellets were dissociated overnight in 50µl of sample dissociation buffer [50mM Tris.HCl,(pH6.8), 5% (w/w)SDS, 5%(w/v), 2-mercaptoethanol, 10% (v/v) glycerol, 2mM EDTA, 1mM PMSF]. Trichloroacetic acid-insoluble radioactivity was determined on 2µl aliquots of each sample prior to electrophoresis. Total incorporation of [^{35}S]-methionine was calculated by assuming a sample concentration of 1 mg mitochondrial protein per 50µl sample volume. Data presented are the mean of two separate experiments. The resultant pattern of electrophoretic separation of the products for one of these experiments is shown in Figure 14.

presence and absence of chloramphenicol were analyzed by electrophoresis and autoradiography, it was seen that inclusion of chloramphenicol almost totally abolished the pattern of labelled mitochondrial translation products, whether cycloheximide was present or not (Figure 14). Consequently, it appears rather certain that the translation products observed in kidney mitochondria are genuine mitochondrial translation products. The close correspondence of these products to those seen in liver and skeletal muscle mitochondria (always labelled in the presence of cycloheximide) provides strong support to the mitochondrial origin of these products. Furthermore, if contaminating cytoplasmic translational activity were present as a significant factor, one might expect that its products would represent major proteins synthesized in the cytoplasm of the tissue involved. If this were true, there should be major tissue-specific differences apparent in the observed translation products. Yet, the only differences observed in mitochondrial translation product from kidney, liver, and skeletal muscle were quite minor. Moreover, the electrophoretic pattern of labelled mitochondrial translation products bore essentially no relationship to the Coomassie blue-stained electrophoretic pattern of the mitochondria in which they were synthesized (data not shown). Since around 90% of the protein content of mitochondria is of cytoplasmic (extramitochondrial) origin (90), if cytoplasmic contamination were a significant factor, one might expect that the pattern of labelled proteins would closely resemble that of the bulk mitochondrial proteins.

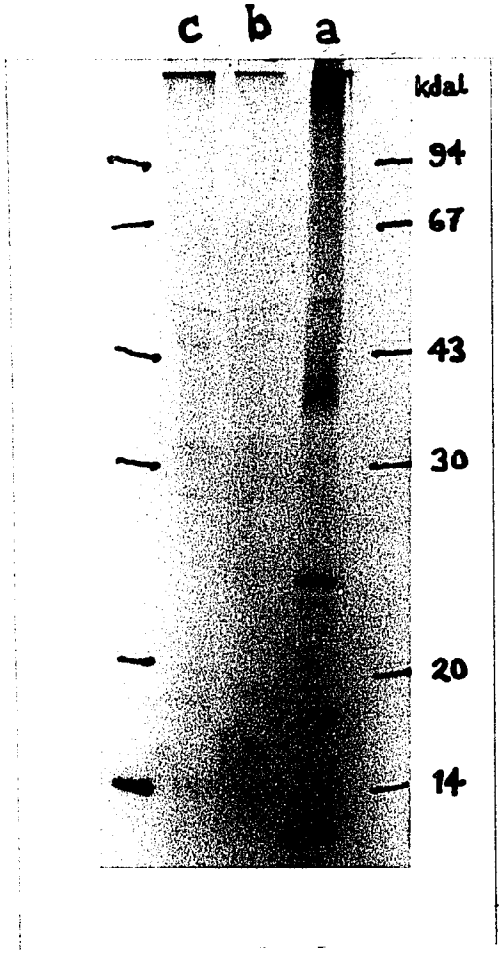

Figure 14.

Translation products of rat kidney mitochondria incubated with L-[^{35}S]-methionine in the presence and absence of chloramphenicol. Rat kidney mitochondria were incubated and prepared for electrophoresis as described in the legend to Table IX. Samples were analyzed by electrophoresis on a 12% polyacrylamide slab gel which was autoradiographed as described in "Experimental Procedures".

Lane a: + 1.0mg/ml cycloheximide (44,000 CPM in 2µl of sample)

Lane b: + 1.0mg/ml cycloheximide and 250µg/ml chloramphenicol (17,000 CPM in 30µl)

Lane c: + 250µg/ml chloramphenicol (23,000CPM in 30µl)

Without specific antisera to the electron-transport complexes of the inner mitochondrial membrane of the rat, it was not possible to identify directly any of the observed translation products. However, a few tentative assignments could be made by comparisons with results reported by other investigators. For example, Rascati and Parsons(129) showed that the three largest subunits of rat liver cytochrome oxidase are of mitochondrial origin; their reported molecular weight (66,000, 39,000, 23,000) correspond closely to labelled products observed in rat kidney, liver, and skeletal muscle reported here (Figure 13) but are markedly different from values reported by other investigators (128, 130, 131) which report molecular weights ranging from 35,000 to 42,000 for cytox I; 20,000 to 27,000 for cytox II, and 18,000 to 26,000 for cytox III from mammalian mitochondria (beef heart, human placenta, rat liver). Much of the uncertainty in the assigned molecular weight values arises from the anomalous migration properties of mitochondrial translation products in a variety of electrophoretic systems (280,291). The recent report of the complete sequence of human mitochondrial DNA (131) provides an alternative method for assigning molecular weights to mitochondrial translation products. Comparison of the DNA base sequences of human and bovine mt DNA and consideration of known (complete or N-terminal) amino acid sequences of mammalian and yeast mitochondrial translation products permitted the identification of the genes for (and estimation of the molecular weights of) subunits I, II, and III of cyto-

chrome oxidase (M_r=57,000; 25,500; and 30,000, respectively) cytochrome b (M_r=42,700) and subunit 6 of the mitochondrial ATPase (M_r=24,800). These values correspond closely to those calculated for labelled mitochondrial translation products observed in this study. (Figure 15, Lane A-C) Furthermore, Anderson et al.(131) were able to detect the presence of eight "unidentified reading frames" (URF's) which showed strong evolutionary conservation when bovine and human mt DNA sequences were compared. An unidentified reading frame begins with a mitochondrial methionine codon (ATA, ATA, ATT) and ends with a mitochondrial termination codon (TAA, AGA, TAG, AGG), and hence contains information which could potentially direct the amino acid sequence of mitochondrial translation products. Interestingly enough, as shown in Figure 15, the predicted molecular weights of the hypothetical URF gene translation products (Lane E) also correspond quite closely to those determined in this study for mitochondrial in vitro translation products. Prudence dictates that one not carry such speculation too far, since, as previously mentioned, the actual molecular weights of mitochondrial translation products may vary considerable from those calculated from electrophoretic data (291). With this caveat, the product identities were tentatively assigned as outlined in Table X. It can be seen that considerable uncertainty in these assignments is still present.

The presence of several labelled products of M_r>70,000 was unexpected, since the largest molecular weight reported

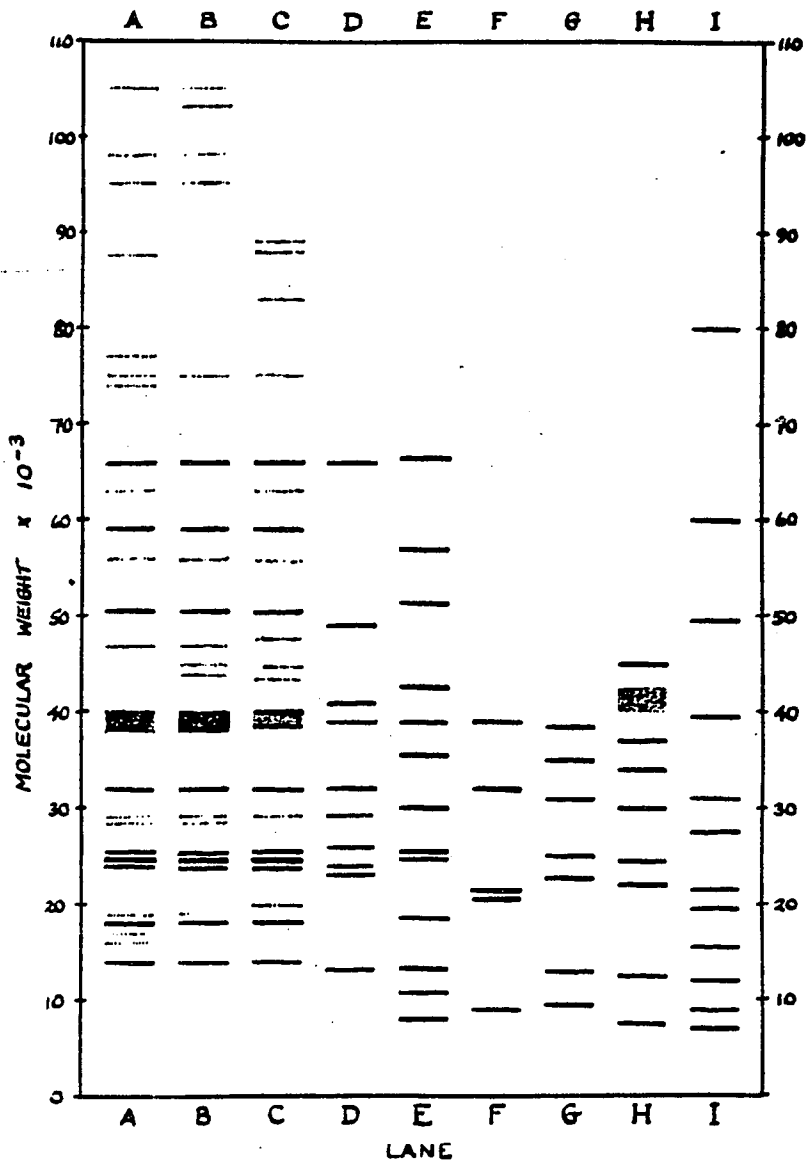

Figure 15.

Graphic comparison of molecular weights of mitochondrial translation products. This "pseudo gel" was constructed to facilitate comparison of data compiled from many sources.

Lane	Source of mitochondria	reference
A	rat kidney	this study
B	rat liver	" "
C	rat skeletal muscle	" "
D	rat liver	(129)
E	human placental cells	(131)
F	rat liver	(128)
G	chinese hamster	(292)
H	Yeast (Saccharomyces cerevisiae	(126)
I	Neurospora crassa	(293)

TABLE X

Tentative Identifications of Rat Mitochondrial *in vitro* Translation Products.

Product	M_r (approximate)	Possible Identities[1]
I	66,000	URF-5[a]; cytox I[b]
II	59,000	cytox I[a]
III	51,000	URF-4[a]
IV	47,000	?
V	43,000	cytochrome *b*[a]
VI	38,000-40,000	cytox I[c]; cytox II[b]; URF-2[a]
VII	32,000	cytochrome *b*[c]
VIII	30,000	cytox III[a]
IX-XI	23,000-26,000	ATPase 6[a]; cytox II[c]; cytox III[b]
XII	18,000	URF-6[a]
XIII	14,000	URF-3[a]

Apparent molecular weights (M_r) of the more strongly-labelled bands consistently observed in autoradiograms of rat kidney, liver and muscle mitochondrial translation products (labelled *in vitro* with [^{35}S]-methionine and separated by electrophoresis on polyacrylamide slab gels in the presence of 0.1% SDS as described previously) were calculated by comparing the relative mobilities (R_f) of the labelled products with those of the molecular weight standards.(280) These values were compared with those reported by other investigators for tentative assignment of identity.

[1]References for possible identities:
- a Anderson *et al.*(131)
- b Rascati and Parsons (129)
- c Nelson *et al.* (128)

for a mammalian mitochondrial translation product is 66,000 (129). Furthermore, the largest predicted molecular weight for a putative translation product (URF) of mammalian (human, bovine) mitochondria is about 67,000 (131). Since mammalian mitochondrial structural genes appear to lack intervening sequences(131) and are "punctuated" by flanking tRNA genes (152) this figure would appear to represent an upper limit to the size of mammalian mitochondrial translation products. Mitochondrial translation products are generally quite hydrophobic (90) and show a marked tendency to aggregate in aqueous systems(121). In order to determine whether such aggregation was responsible for the appearance of high-molecular-weight products, mitochondria labelled with [^{35}S]-methionine were reisolated from the incubation medium and treated with 0.1M NaOH before the electrophoresis sample dissociation buffer was added, following the procedure of Tzagoloff and Meagher (281). As shown in Figure 16, treatment with NaOH produced no apparent change in the electrophoretic mobility of any kidney of liver mitochondrial translation products. Thus, although the weakly-labelled chloramphenicol-sensitive, cycloheximide-resistant electrophoretic bands of $M_r \geq 70,000$ observed in this study were not dissociated by NaOH treatment, they probably represent specific aggregates of smaller mitochondrial translation products which are resistant to complete denaturation by SDS and/or NaOH under the conditions employed.

Figure 16.

Mitochondrial in vitro translation products. Mitochondria from rat kidney and liver were incubated with L-[^{35}S]-methionine and subsequently prepared for electrophoresis as described in "Experimental Procedures." An aliquot of the translation product mixture was treated with 100 mM NaOH as described by Tzagoloff and Meagher (281). Samples of untreated NaOH-treated translation products were separated on a 12% polyacrylamide slab gel with a 5% acrylamide stacking gel in the presence of 0.1% SDS, and an autoradiogram prepared as previously described.

Lane a, untreated kidney mitochondria;
Lane b, untreated liver mitochondria;
Lane c, molecular weight standards;
Lane d, NaOH-treated kidney mitochondria;
Lane e, NaOH-treated liver mitochondria.

G. Effects of cytoplasmic postpolysomal supernates (S-140) on mitochondrial protein synthesis in vitro.

As was discussed previously in the Introduction, there exists a considerable body of evidence which indicates that mitochondrial protein synthesis in lower eukaryotes is controlled in some fashion by proteins synthesized on cytoplasmic ribosomes. Consequently, it was of interest to test this hypothesis directly in mammalian mitochondria by including cytoplasmic postpolysomal supernates (S-140 extracts) from various sources in the incubation mixtures for mitochondrial protein synthesis in vitro. These incubations were performed in the presence and absence of GTP in order to rule out the possibility that any observed stimulatory capacity of such supernates was due to tightly-bound guanine nucleotides which might be present even after exhaustive dialysis of the supernates, as has been claimed by Schatz (294). As shown in Table XI, addition of 100 μM GTP to the incubation mixture for control or diabetic skeletal muscle mitochondrial protein synthesis in vitro produced an increase of about 10% in the rate of protein synthesis, whereas in yeast mitochondria a three-to-five-fold stimulation of protein synthesis has been observed at this concentration of GTP (284). It can also be seen in Table XI, that none of the S-140 extracts tested (control muscle, diabetic muscle, yeast) caused a reproducible effect upon protein synthesis in vitro by isolated muscle mitochondria. In some experiments, stimulation was observed, while in other experiments the same supernate at the same

TABLE XI

Effect of Cytoplasmic S-140 Extracts from Control and Diabetic Skeletal Muscle and Yeast upon Protein Synthesis *in vitro* by Skeletal Muscle Mitochondria.

Source of Mitochondria (Method of Preparation)	Additions	Mitochondrial Protein Synthesis (% of value without additions)		
		Experiment 1	2	Average
Control Muscle (trypsinization)	None	100	100	100
	100μM GTP	112	-	112
	Control Muscle S-140			
	0.25mg	96	-	96
	0.38mg	101	-	101
	0.50mg	64	63	64
	0.75mg	81	58	70
Diabetic Muscle (mechanical homogenization)	None	100	100	100
	100μM GTP	112	105	109
	0.25mg Control Muscle S-140	92	109	100
	0.25mg Diabetic Muscle S-140	96	165	130
	0.25mg Yeast S-140	97	-	97

Mitochondria isolated from control skeletal muscle (prepared by trypsinization) were incubated at 1.0mg/1.0ml in the standard medium for protein synthesis supplemented with the indicated quantities of GTP or S-140 extract from control muscle. Diabetic muscle mitochondria (prepared by mechanical homogenization) were incubated at 0.5mg/0.5ml in the standard medium supplemented with the indicated quantities of GTP and S-140 extracts from control muscle, diabetic muscle, or yeast. Incorporation of [^{3}H]-leucine into trichloroacetic acid-insoluble material was determined as described in "Experimental Procedures," and is expressed as a percentage of the values observed in the absence of GTP and S-140 extracts (Control: 5340-5750 counts-min^{-1}mg^{-1}; diabetic: 1820-2740 counts-min^{-1}mg^{-1}).

concentration inhibited protein synthesis by muscle mitochondria. Mitochondria prepared by mechanical homogenization instead of by trypsinization (to avoid the possibility of trypsin-induced damage to putative "receptors" (195) on the mitochondrial membranes) also failed to respond significantly to addition of cytoplasmic S-140 extracts.

Although the lack of substantive results from these studies was disappointing, an alternative method of testing the hypothesis was available, based upon observations in this laboratory that protein synthesis *in vitro* by isolated yeast mitochondria could be stimulated markedly by cytoplasmic S-140 extracts from yeast, *E. coli*, and rat liver (276). Accordingly, yeast mitochondria were utilized to test the stimulatory capabilities of various cytoplasmic S-140 extracts. As seen in Table XII, S-140 fractions prepared from skeletal muscle or liver of control and diabetic animals were capable of stimulating yeast mitochondrial protein synthesis beyond the level produced by added GTP. However, the stimulatory activities of S-140 extracts from diabetic rat skeletal muscle or liver appeared to be greater than those of the corresponding extracts from control animals.

Still another approach to test the hypothesis was suggested by investigations of Devlin *et al.* into the effects of cycloheximide administered *in vivo* upon RNA and protein synthesis *in vivo* in rat liver (295-300). These studies indicate that after administration of a nonlethal dose (2.0 mg/kg) of cycloheximide, RNA and protein synthesis showed

TABLE XII

Effects of Cytoplasmic S-140 Extracts from Control and Diabetic Rat Liver and Skeletal Muscle upon Protein Synthesis *in vitro* by Yeast Mitochondria.

S-140 added	[^{3}H]-leucine incorporated net counts $min^{-1}mg^{-1}$ Experiment 1	Experiment 2
None	51,280	61,720
Control Muscle (1.0mg)	70,690	-
Diabetic Muscle (1.0mg)	97,890	-
Control Liver (1.5mg)	-	97,860
Diabetic Liver (1.5mg)	-	118,430

Isolated yeast mitochondria (0.5mg/0.5ml) were incubated at 30° as described by Everett, *et al*. (276) with the indicated quantities of cytoplasmic S-140 extracts from control and diabetic rat liver and skeletal muscle in the presence of 100μM GTP. Incorporation of [^{3}H]-leucine into trichloroacetic acid-insoluble material was performed as described previously.

a rapid decline, evident after 2 hours, followed by a stimulatory recovery phase which lasted from 6 to over 48 hours after cycloheximide injection (295-298). Furthermore, they observed that synthesis of total mitochondrial protein (90% of which is of cytoplasmic origin) was inhibited at 2 hours and stimulated maximally at 24 hours after cycloheximide treatment (299). When the rates of liver mitochondrial protein synthesis *in vitro* were measured at several intervals after administration of cycloheximide *in vivo*, a decline of about 40% was evident by two hours, and was still evident after 16 hours (Figure 17). However, by 24 hours after cycloheximide treatment, the ability of liver mitochondria to synthesize proteins *in vitro* had increased to slightly above the control level (Figure 17). Thus, it appeared that inhibition of cytoplasmic translation led to an inhibition of mitochondrial protein synthesis; restoration of cytoplasmic translation (after hepatic metabolism and renal excretion of cycloheximide) was followed by a recovery of mitochondrial protein synthesis to control levels or slightly higher. Use of higher doses of cycloheximide killed the animals before 24 hours had passed. However, when the dose dependence of the effect of cycloheximide administration *in vivo* upon liver mitochondrial protein synthesis *in vitro* was examined two hours after injection, a clear dose-response relationship was observed (Table XIII).

These results encouraged further pursuit of this line of investigation. Liver cytoplasmic S-140 extracts were

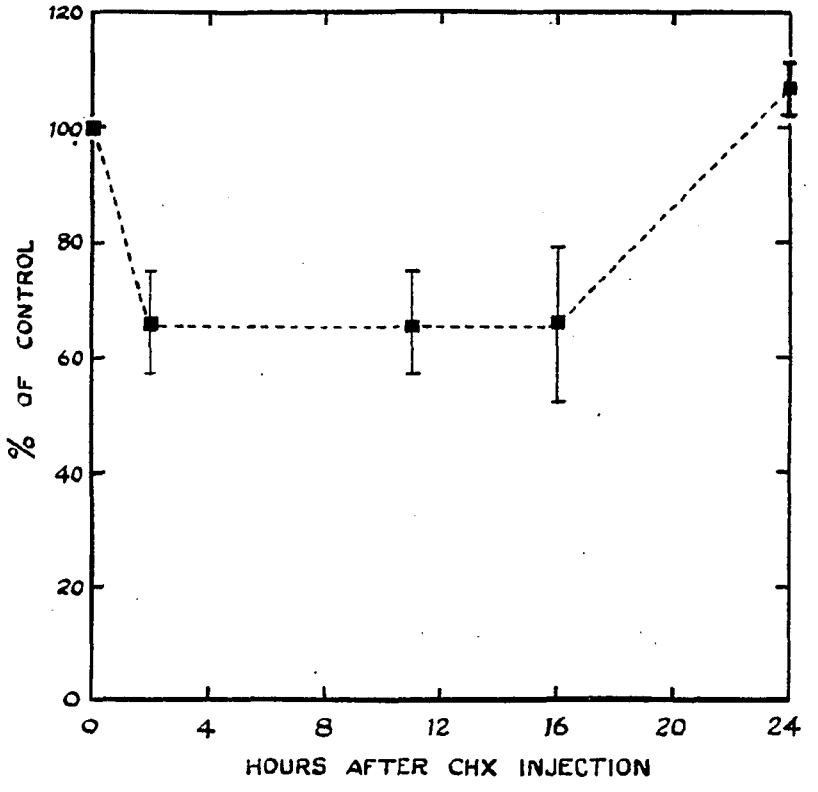

Figure 17.

Effect of time after cycloheximide administration *in vivo* upon protein synthesis *in vitro* by isolated liver mitochondria. Sublethal doses (2.5 mg/kg) of cycloheximide in physiological saline were injected intraperitoneally at the indicated times prior to sacrifice. Protein synthesis by isolated liver mitochondria was determined as described in "Experimental Procedures". The data shown represent the mean values and ranges of two separate experiments.

TABLE XIII

Effect of Dosage Level of Cycloheximide Administered *in vivo* upon Liver Mitochondrial Protein Synthesis *in vitro*.

Dosage of cycloheximide mg/kg	Hours after injection	Liver mitochondrial protein synthesis % of control
0	2	100.0
2.0	2	82.3
10.0	2	79.6
100.0	2	58.3
1.25	18	85.8
2.50	18	70.1

The indicated dose of cycloheximide dissolved in physiological saline was injected intraperitoneally at the indicated time prior to sacrifice. Protein synthesis *in vitro* by isolated liver mitochondria was measured as described in "Experimental Procedures" after 60 min of incubation at 37°.

prepared at various times after intraperitoneal injection of cycloheximide (2.5 mg/kg), and their ability to stimulate protein synthesis *in vitro* by isolated yeast mitochondria was determined. As seen in Table XIV, the liver cytoplasmic S-140 extracts were all capable of stimulating protein synthesis by yeast mitochondria well beyond the stimulation produced by GTP alone. While the stimulatory activity of the S-140 extracts from cycloheximide-treated animals did not fully reach control levels, some degree of restoration of stimulatory activity at 24 hours after cycloheximide administration was seen.

TABLE XIV

Effect of Liver Cytoplasmic S-140 Extracts from Control and Cycloheximide-treated Rats upon Protein Synthesis *in vitro* by Yeast Mitochondria.

Liver S-140 (hours post-cycloheximide)	[^{3}H]-leucine incorporated net counts $min^{-1}mg^{-1}$
None	52,490
Control	89,630
2 hours	80,700
16 hours	77,170
24 hours	80,940

Protein synthesis *in vitro* by isolated yeast mitochondria (0.5mg/0.5ml) was determined as described in "Experimental Procedures" after incubation at 30° for 30 min in the presence of 100μM GTP and rat liver cytoplasmic S-140 extracts (1.5mg) prepared from animals sacrificed at the stated time after intraperitoneal administration of cycloheximide (2.5mg/kg). Each entry is the mean of values observed with S-140 extracts from two animals. In the absence of GTP and S-140, yeast mitochondrial incorporation of [^{3}H]-leucine was 15,300 counts-min^{-1}.mg^{-1}.

IV. DISCUSSION

A. The Control of Mitochondrial Protein Synthesis in Lower Eukaryotes.

The biogenesis of mitochondria can be dissected into several component processes: 1) the synthesis of the lipid components of the outer and inner membranes; 2) the replication of mitochondrial DNA; 3) the cytoplasmic synthesis of most mitochondrial proteins; 4) their subsequent transfer into the mitochondrion; 5) the intramitochondrial synthesis of a limited number of polypeptides; and 6) their subsequent assembly with polypeptides of cytoplasmic origin into functional enzyme complexes which are primarily embedded in the inner membrane. The major focus of research in this laboratory over the years has been the contribution of mitochondrial protein synthesis to the overall process of mitochondrial biogenesis, and the mechanisms controlling mitochondrial protein synthesis.

Relatively little is known about the control of mitochondrial biogenesis in mammals, despite the crucial functions performed by these organelles in all mammalian cells except mature erythrocytes. The greatest portion of our knowledge concerning the mechanisms governing mitochondrial biogenesis is based upon studies performed with lower eukaryotes; specifically, most of these studies have employed a single organism, the yeast Saccharomyces cerevisiae. Because many of the salient properties of mitochondria appear to have been conserved quite strongly through the course of evolution, it seems appropriate to discuss some of these findings in

lower eukaryotes before progressing to an examination of the corresponding mammalian processes.

S. cerevisiae is particularly well-suited for studies of mitochondrial biogenesis. Large quantities of genetically-identical cells can be easily grown and harvested. The intracellular structure of the organism contains much less extensive endoplasmic reticulum, lysosomes, and other membranous organelles than is found in higher eukaryotes. This yeast can be grown under a wide variety of conditions: different temperatures, different carbon sources, aerobic, anaerobic (requiring fermentable substrates and supplementation of the medium with ergosterol and unsaturated fatty acids), and in the presence of inhibitors of protein synthesis such as cycloheximide or chloramphenicol. Moreover, mutants are easily generated and selected, thus greatly facilitating genetic studies of mitochondrial biogenesis.

The results of many studies strongly indicate that the biogenesis of mitochondria in yeast and other simple eukaryotes is not regulated in an autonomous manner, but is strongly controlled by proteins of cytoplasmic origin. In the mold Neurospora crassa, growth in the presence of ethidium bromide, a specific inhibitor of mitochondrial transcription, led to increased cytoplasmic synthesis of the enzymes required for transcription and translation of mt DNA (56). When S. cerevisiae is grown in medium containing a high concentration of glucose, a decrease in mitochondrial cytochrome content and respiratory activity is observed (103). As the glucose

in the medium becomes exhausted, the synthesis of cytochromes increases and the respiratory activity also increases (104). Addition of chloramphenicol to glucose-repressed cells blocked the appearance of activity of enzyme complexes containing mitochondrially-translated subunits, but addition of cycloheximide during this stage of growth did not, even though these complexes also contain cytoplasmically-synthesized subunits, implying that a pool of the latter had accumulated to a sufficient extent to permit continued production of the holoenzyme complexes (104).

Through similar techniques of growth of yeast in the presence of cycloheximide before harvesting mitochondria, Poyton and Kavanagh demonstrated that mitochondrial protein synthesis *in vitro* ceased when an endogenous pool of cytoplasmically-synthesized proteins is depleted; moreover, addition of a cytosolic post-polysomal ("S-100") supernate to mitochondria incubated *in vitro* restored their ability to synthesize proteins (301). Everett *et al.*(276) showed that this effect is not species-specific; in fact, S-100 fractions from *E. coli* or rat liver were also capable of such stimulation. While Ohashi and Schatz (294) have claimed that this stimulatory activity is due to the presence of tightly-bound guanine nucleotides contained in the S-100, Finzi *et al.*(302) shown that such supernates are capable of dramatic additional stimulation of yeast mitochondrial protein synthesis *in vitro* in the presence of maximally active concentrations of GTP. Furthermore, the major stimulatory factor present in yeast

S-100 extracts appears to be a protein of M_r < 3500 (302).

The mechanism of action of such stimulatory proteins is unclear: they could act at the transcriptional or translational level or both. While the stimulatory factor studied by Finzi *et al.* appears to cause a uniform increase in the synthesis of all mitochondrial translation products *in vitro* (302), Brown and Beattie demonstrated that the assembly of cytoplasmically- and mitochondrially-translated subunits into functional enzyme complexes does not proceed in a synchronous fashion for the *b*-c_1 complex and cytochrome oxidase (121). Furthermore, formation of an active *b*-c_1 complex in yeast mitochondria during respiratory adaption is subject to a different mode of regulation than that seen when the yeast cells are undergoing glucose derepression (121). Interpretation of these studies is complicated by the fact that the data do not permit one to decide whether the effects observed are due to alterations in mitochondrial protein synthesis or in the subsequent assembly of inner membrane complexes. Saltzgaber-Müller and Schatz have shown that heme *a* is necessary for the assembly of yeast cytochrome oxidase (303). In the absence of heme, cytox II and III, but not cytox I, were still detectable in the mitochondria; these subunits are all products of mitochondrial protein synthesis (102). Of the cytoplasmically-translated subunits, cytox VI was present, but cytox IV, V, and VII were not detected. These observations may reflect rapid degradation of the

unassembled subunits as opposed to a decrease in this synthesis, but these subunits are detectable in other yeast mutants where assembly of cytochrome oxidase is affected (304). Thus, heme may play a role in the synthesis of some mitochondrial translation products. A similar conclusion was reached by Kumar and Padmanaban for N. crassa (305). The synthesis of mitochondrial proteins was decreased in the absence of heme, and total protein synthesis in vitro by heme-deficient mitochondrial lysates was increased by addition of hemin. On the other hand, Clejan et al. found that the apoprotein of cytochrome b was still synthesized by mitochondria from yeast mutants incapable of heme synthesis, and suggested that the assembly of the b-c_1 complex was the process which required heme (280).

Certain nuclear mutations affect the synthesis of some mitochondrial translation products, but not others. Schatz's group has studied several yeast nuclear mutants which specifically lack cytochrome oxidase activity and spectrum (304, 306). In these mutants, all four cytoplasmically-translated cytox subunits were present, but one or more mitochondrially-translated subunits were missing; these results were not due to faulty assembly of the holoenzyme, nor to a defect in the synthesis of heme a (307). Cabral and Schatz concluded that the nuclear amber mutation pet 494-1 affected the cytoplasmic synthesis of a protein which regulated the mitochondrial expression of cytox III, while in the nuclear mutant pet E11-1, the synthesis of cytox I

and II was affected; both mutants possessed spectrally-identifiable cytochrome b (307). Hence, the missing cytoplasmic regulatory proteins appeared to be specifically involved in the mitochondrial synthesis of the subunits of cytochrome oxidase; their mechanisms of action thus appear different from that of the low-molecular-weight activator studied by Finzi et al. (302).

Tzagoloff observed that the synthesis of the mitochondrial subunits of the oligomycin-sensitive ATPase in S. cerevisiae was also controlled by products of cytoplasmic protein synthesis (308). Furthermore, Tzagoloff et al. found nuclear mutants in which the mitochondrial synthesis of cytochrome b and the Fo portion of the mitochondrial ATPase were specifically blocked (93). Hence, the synthesis of proteins by yeast mitochondria appears to be controlled by products of cytoplasmic protein synthesis, but in a very complex fashion. The picture is further complicated by the speculations of two groups that mitochondrial gene products may affect the expression of nuclear genes in N. crassa (55) and S. cerevisiae (309); however, no such product has been isolated or identified. Moreover, in "petite" mutants of S. cerevisiae which completely lack mt DNA, the cytoplasmic synthesis of mitochondrial proteins proceeds at near-normal levels, and the protein composition of the (respiratory-deficient) mitochondria is identical to that seen in wild-type yeast mitochondria, except for the lack of the mitochondrially-translated peptides (121, 209). The recent

discovery that the mitochondrial gene for cytochrome b contains intervening sequences which are spliced out of the primary transcript is the first demonstration of a split gene in mitochondria (139). Even more interesting, if not astounding, is the demonstration that these "introns" contain information coding for a "maturase" which is necessary for the proper posttranscriptional processing of the mitochondrial mRNA for subunit I of cytochrome oxidase (140). It is evident that the control of mitochondrial protein synthesis in yeast is quite complex; despite the tremendous amount of research into this area, many questions remain unanswered. Hence, it is not surprising that our understanding of the regulation of mitochondrial protein synthesis in mammals, which has been studied far less extensively, is still quite murky.

B. The Effects of Diabetes Mellitus upon Mitochondrial Protein Synthesis.

This phase of the studies reported here was prompted by previous observations in this laboratory (270) that isolated skeletal muscle mitochondria from diabetic rats exhibited markedly decreased rates of protein synthesis *in vitro* compared to those seen with muscle mitochondria from control rats. Similar findings had been reported previously by another laboratory (286), but this report did not come to our attention until after most of the studies reported here had already been completed. This decrease was observed whether alloxan or streptozotocin was used as the diabetogenic agent (270); moreover, addition of streptozotocin to the incubation medium had no effect on mitochondrial protein synthesis *in vitro* (286), implying that the effect observed was a consequence of the induced diabetes, not of a direct myopathic action of the drug.

The decreased rates of protein synthesis *in vitro* by diabetic muscle mitochondria were observed whether ATP was generated within the mitochondria by oxidative phosphorylation or externally from ADP and phosphoenolpyruvate (PEP) by pyruvate kinase [(270) and Figure 6]. The possibility that a decreased activity of the adenine nucleotide translocase (ATP/ADP antiporter) of the inner mitochondrial membrane was responsible for these observations was ruled out; the highest rates of mitochondrial amino acid incorporation were observed when glutamate was supplied as oxidizable

substrate and the ATP/ADP antiporter was inhibited with atractyloside, thereby preventing the exit of intramitochondrially-synthesized ATP (270). By contrast, isolated liver mitochondria from control and diabetic rats exhibited identical rates of protein synthesis in vitro (270). These observations clearly warranted further investigation.

In order to see whether these effects were limited to skeletal muscle mitochondria, the effects of experimental diabetes mellitus on protein synthesis in vitro by rat kidney mitochondria were examined. The relative paucity of previous studies of rat kidney mitochondria necessitated the determination of the optimal conditions for measuring protein synthesis in vitro by isolated kidney mitochondria; the results of these studies were presented in Figures 1-5 and Tables I-III. The most noteworthy differences observed between kidney mitochondria and liver or muscle mitochondria were the lower optimal [Mg^{++}] required (Figure 2) and the higher rate of amino acid incorporation by kidney mitochondria. As was shown previously for liver mitochondria, kidney mitochondria isolated from control and diabetic animals exhibited identical rates of protein synthesis in vitro, regardless of the method of ATP generation employed (Table II). Moreover, the content of cytochromes was the same in kidney mitochondria from control and diabetic animals (Table VI). The renal content of mitochondria (mg mt protein/mg tissue protein), as estimated by the specific activity and recovery of succinate dehydrogenase, was also identical in control

and diabetic rats (Table VIII). These results indicate that the rates of kidney mitochondrial protein synthesis *in vitro* parallel those *in vivo*. Hence, diabetes mellitus did not seem to have a major effect on the biogenesis of rat kidney mitochondria during the period of time used in these studies.

As was discussed in the previous section, our working hypothesis is that the rate of mitochondrial protein synthesis is governed by proteins of cytoplasmic origin. In this regard, the observations that diabetes mellitus does not decrease the overall rate of cytoplasmic protein synthesis in rat liver(249), rat kidney(250), or Chinese hamster liver and kidney(251) are particularly significant. On the other hand, the rate of cytoplasmic protein synthesis is depressed by 70% in skeletal muscle and by 44% in heart in diabetic rats (249). Thus, the apparently tissue-specific effects of diabetes mellitus on mitochondrial protein synthesis probably reflect the effects of this disorder on cytoplasmic translation in the particular tissue under study.

The lack of a major effect of diabetes mellitus on synthesis *in vitro* by kidney or liver mitochondria does not imply by any means that mitochondrial protein synthesis in these tissues is immune to all environmental changes. Surgical removal of one kidney leads to compensatory changes in the remaining kidney, including an increase in the rates of cytoplasmic and mitochondrial protein synthesis *in vivo* and *in vitro* (273). Kidney mitochondria from male mice are

morphologically and biochemically different from those in female mice; orchiectomy evokes the female pattern, whereas testosterone administration induces the male pattern, including increased activity of cytochrome oxidase (310). Hypophysectomy leads to decreased rates of protein synthesis by rat liver mitochondria in vitro; administration of growth hormone to the animals reverses this decrease both in vivo and in vitro (311). In thyroidectomized rats, the rates of liver mitochondrial RNA synthesis are depressed (312); administration in vivo of physiological doses of triiodothyronine causes a marked increase in the total and specific activity of mitochondrial RNA polymerase(312), which, like the other protein components of the mitochondrial transcription and translational machinery, is a product of cytoplasmic translation (289). Addition of cAMP or its dibutyryl derivative to liver mitochondria in vitro (obtained from thyroidectomized rats) had no effect on RNA polymerase activity (312). Treatment of cultured mouse adrenal tumor cells with ACTH leads to an increase in the synthesis of eight mitochondrial proteins, of which six are of cytoplasmic origin and two of mitochondrial origin (313). Treatment of these cells with cAMP mimics the effects of ACTH (313).

Inhibition of cytoplasmic protein synthesis by cycloheximide also leads to a decrease in mitochondrial protein synthesis in vivo in a variety of animal cells, including rat liver (295-299), rat kidney (295, 300), cultured Xenopus laevis oocytes(314), and HeLa cells (315).

We have observed that the administration of cycloheximide to rats *in vivo* produces a similar decrease in mitochondrial protein synthesis *in vitro* (Table XIII, Figure 17). Rat liver mitochondrial RNA and protein synthesis *in vitro* are also inhibited when anisomycin, emetine, or α-amanitin is administered *in vivo* (316). Anisomycin is an inhibitor of cytoplasmic protein synthesis(317); emetine inhibits cytoplasmic protein synthesis preferentially at low concentrations (318); and α-amanitin specifically inhibits nuclear mRNA transcription by RNA polymerase II at low concentrations (319). All of these results strongly suggest that mitochondrial protein synthesis is regulated by proteins of cytoplasmic origin.

Skeletal muscle mitochondria are also subject to important modifications induced by a wide variety of physiological variations and pathological conditions (320-326), including diabetes mellitus (226,270,286). The substantial decrease in muscle mitochondrial protein synthesis *in vitro* observed in diabetic rats (270,286) has been confirmed here (Figures 6-8). The decrease in muscle mitochondrial protein synthesis *in vitro* appears to accurately reflect a corresponding decrease *in vivo*, since muscle mitochondria from diabetic rats possessed significantly decreased specific contents of cytochromes aa_3 and *b* (Table VI), which contain mitochondrial translation products. The observed decreases in mitochondrial content of cytochromes *c* and c_1 might be due to the decrease in cytoplasmic protein synthesis in

diabetic muscle(249), or to improper assembly into the respiratory chain caused by the decrease in cytochrome b (280). The decreased mitochondrial cytochrome content in diabetic rat muscle is also reflected in decreased rates of state 3 and uncoupler-stimulated respiration (Table VII), indicating that the functional activity of these mitochondria has been adversely affected. This is also indicated by the observation that protein synthesis *in vitro* by muscle mitochondria from diabetic animals is depressed by a greater degree when ATP is generated by action of the respiratory chain than when it is generated externally (Figure 6). By contrast, Sirotzky de Favelukes *et al*. did not observe any significant difference in state 3 respiration in mitochondria from control and diabetic rat muscle (286). No explanation for this discrepancy is immediately apparent. Interestingly enough, the specific tissue content of muscle mitochondria (mg mitochondrial protein per mg of tissue homogenate protein) appears to be essentially the same in control and diabetic rats, as estimated by the specific activity and recovery of succinate dehydrogenase (Table VIII). This indicates that the decrease in various functional activities *in vitro* may have physiological significance *in vivo*, since if control and diabetic muscle have equivalent specific content of mitochondria but the diabetic mitochondria are less capable of oxidative phosphorylation, ATP production in diabetic muscle would be adversely affected; this would tend to aggravate the already serious effects of the disease

upon skeletal muscle, which include decreased cytoplasmic protein synthesis and increased protein degradation (241).

It seems quite clear that the decrease in muscle mitochondrial protein synthesis in diabetic rats is a direct consequence of insulin deprivation. Administration of insulin to diabetic animals restores the rates of muscle mitochondrial protein synthesis *in vitro* to control levels, while insulin treatment of control animals has no effect on the rates of muscle mitochondrial protein synthesis (Figure 7). Addition of insulin to isolated control or diabetic muscle mitochondria has no effect on the rates of protein synthesis *in vitro* (286). Furthermore, in fasted rats, whose plasma levels of insulin are also decreased (236), there is also a decrease in the rates of muscle mitochondrial protein synthesis *in vitro* (Figure 7).

It has been previously demonstrated that the decreased rates of protein synthesis in cardiac (227) and skeletal (226) muscle of diabetic animals cannot be simply explained by decreased transport of glucose or amino acids into these tissues. Our data indicate that changes in mitochondrial amino acid pools are probably not involved in the decreased rates of protein synthesis *in vitro* by diabetic muscle mitochondria, since the decrease is observed when either [^{3}H]-leucine or [^{35}S]-methionine is used as the labelling agent in the presence of adequate quantities of all other amino acids (Figures 6 and 8).

It is also unlikely that the observed differences are

due to an increased sensitivity of diabetic muscle mitochondria to trypsin-induced damage, since diabetic muscle mitochondria prepared by alternate means show a similar decrease in the rates of protein synthesis in vitro (Table IV). It should be noted that the data of Table IV do not exclude the possibility that skeletal muscle mitochondria from diabetic rats are somehow more fragile than those from control animals, and thus more susceptible to damage during any isolation procedure employed. However, the recoveries of mitochondria from trypsinized control and diabetic muscle are not significantly different (Table VIII), nor are their respiratory control ratios (Table VII); the latter observation indicates that mitochondria from both sources are coupled to an equivalent extent.

The decrease in diabetic muscle mitochondrial protein synthesis in vitro appears to affect the synthesis of all mitochondrial translation products to an equal extent without altering the nature of the products themselves (Figure 8), implying that all of these products are expressed coordinately. Incidentally, these results also indicate that the net decrease in protein synthesis by diabetic muscle mitochondria in vitro is not caused by an increase in mitochondrial proteolysis of these translation products. The decreased rate of protein synthesis in vitro appear to be due primarily to a lower rate of peptide chain initiation in diabetic muscle mitochondria than in controls (Figure 9,10 and 12). It should be noted that the concentrations of

aurintricarboxylate (ATA) or pactamycin (PTM) required to reach a plateau of inhibition ([ATA]=0.8mM; [PTM]=0.8μM) are higher than those which completely inhibit translational initiation in reticulocyte lysates ([ATA]=0.08mM; [PTM]=0.5μM), but lower than the concentrations of these agents required to inhibit peptide chain elongation in reticulocyte lysates ([ATA]=1.6mM; [PTM]=16μM) (288). This may indicate that the inner mitochondrial membrane is not freely permeable to these agents, as has been previously established for erythromycin and emetine (285,326), or may simply reflect a lower degree of susceptibility of mitochondrial translation to these agents. Furthermore, addition of 250μg/ml of chloramphenicol and 0.5mM ATA inhibited muscle mitochondrial protein synthesis in vitro to a greater extent than did either agent by itself (Table V). Hence, it appears that in the concentration ranges employed in these studies, ATA and PTM are inhibiting peptide chain initiation to a substantial extent without inhibiting peptide chain elongation.

As the concentrations of either ATA or PTM are increased, the rates of protein synthesis in vitro by control and diabetic muscle mitochondria appear to converge (Figure 9 and 12), indicating that the rates of peptide chain elongation in control and diabetic muscle mitochondria do not differ by a great extent. This could be interpreted to mean that the rates of mRNA transcription in muscle mitochondria from control and diabetic animals do not differ by more than a

factor or two, while the rates of peptide chain initiation may differ by a factor of about five. Since the enzymes required for both mitochondrial transcription and translation are synthesized in the cytoplasm, a decrease in the rate of either of these processes could be explained as a consequence of the lowered rates of cytoplasmic protein synthesis in diabetic muscle. The data of Figure 10 indicate that the primary reason why the rate of protein synthesis *in vitro* by rat kidney mitochondria is so much greater than that of skeletal muscle mitochondria is a much higher degree of peptide chain initiation in the former.

An intriguing additional possible mechanism of action by which insulin could cause a coordinate increase in the synthesis of all mitochondrial translation products (Figure 8) is suggested by recent reports from four groups of investigators that binding of insulin to its receptors on the plasma membranes of adipocytes or skeletal muscle cells *in vitro* leads to a generation of a second messenger which is capable of causing the activation of mitochondrial pyruvate dehydrogenase (252, 253, 258, 259). Seals and Czech (266) and Kiechle *et al.* (267) have shown that this second messenger is a peptide of low molecular weight (1000-4000) which is liberated from some plasma membrane component; the latter group has demonstrated that the second messenger activates a protein phosphatase which then activates pyruvate dehydrogenase by dephosphorylating it (267). In as much as the phosphorylation of the cytosolic translation initiation

factor eIF-2 has been shown to inhibit its activity in reticulocyte lysates, (169-183) one might speculate that activation of an appropriate mitochondrial protein phosphatase by this low-molecular-weight peptide could possibly lead to an increased activity of the corresponding mitochondrial initiation factor. Wu and Ibrahim have found a protein-like material in rat liver mitochondria which inhibits translation in reticulocyte lysates (328). Their preliminary characterization indicates that this material has properties similar to those of the hemin-regulated translational inhibitor of reticulocytes. In this connection, the demonstration by Finzi et al. (302) that in S. cerevisiae a low-molecular-weight (<3500) peptide of cytoplasmic origin causes a uniform stimulation of the synthesis of all mitochondrial translation products is particularly tantalizing.

C. Effects of Cytoplasmic Proteins on Mitochondrial Protein Synthesis in vitro.

Poyton and Kavanagh initially demonstrated that protein synthesis in vitro by isolated yeast mitochondria was highly dependent upon the availability of a pool of cytoplasmically-synthesized proteins, and that depletion of the endogenous pool of such proteins within isolated mitochondria could be ameliorated by addition of a cytoplasmic postpolysomal sypernates (301). Everett et al. demonstrated that this effect was not species-specific, inasmuch as cytoplasmic postpolysomal supernates from E. coli or rat liver worked at least as well as those from S. cerevisiae in stimulating protein synthesis in vitro by yeast mitochondria (276). While Ohashi and Schatz (294) have claimed that the observed stimulatory activity of such supernates is due to their content of tightly-bound guanine nucleotides, Finzi et al. (302) have shown that a protein of low molecular weight contained in cytoplasmic postpolysomal supernates is capable of further stimulating yeast mitochondrial protein synthesis in vitro in the presence of maximally-active concentrations of GTP. Hence, it seemed logical to test the effects of cytoplasmic postpolysomal supernates upon protein synthesis in vitro by isolated rat mitochondria.

As shown in Table XI, the results of these experiments were inconclusive; stimulation was observed in some cases, while inhibition was seen in others. The observations of Everett et al. suggested that perhaps yeast mitochondria

could be utilized to test the stimulatory activity of cytoplasmic S-140 extracts from tissues of control and diabetic rats. Suprisingly, the S-140 extracts from diabetic muscle and liver had greater stimulatory activity than did the corresponding control S-140 extracts in this system (Table XII). The reasons for this are not at all apparent.

The observations of Devlin's group that administration of cycloheximide to rats *in vivo* caused a time-dependent inhibition of cytoplasmic and mitochondrial protein synthesis *in vivo* in rat liver (295-299) and *in vivo* in rat kidney (295, 300) suggested an alternative approach to these studies. As shown in Figure 17, administration *in vivo* of sublethal doses of cycloheximide led to time-dependent alterations in the rates of rat liver mitochondrial protein synthesis *in vitro*: a period of marked inhibition which was evident by two hours after CHX administration and which presented for 18 or more hours, and a period of slight stimulation which was observed 24 hours after CHX administration. When liver cytoplasmic S-140 extracts prepared from CHX-treated rats were added to yeast mitochondria *in vitro*, their relative stimulatory activities corresponded roughly to the rates of protein synthesis *in vitro* by rat liver mitochondria prepared at corresponding intervals after CHX administration (Table XIV; Figure 17).

The mechanism by which cytoplasmic proteins from rat liver could stimulate yeast mitochondrial protein synthesis *in vitro* is not clear. It seems unlikely that the rat liver

S-140 provides initiation or elongation factors, since incubation of rat liver cytoplasm with mitochondrial ribosomes from N. crassa, Euglena, or Xenopus fails to stimulate protein synthesis (329, 330). It has been demonstrated by two groups in N. crassa (331, 332) and by Schatz et al. in S. cerevisiae (306, 307) that nuclear gene products regulate the intramitochondrial synthesis of individual subunits of cytochrome oxidase. These nuclear gene products are not subunits of cytochrome oxidase, but regulate either the transcription or translation of specific mitochondrial proteins in these organisms. By contrast, there also appears to be a mechanism by which the synthesis of all mitochondrial translation products is uniformly affected, in yeast (302) and in rat muscle (Figure 8). In yeast, this mechanism appears to involve low-molecular-weight proteins of cytoplasmic origin (302).

Wu and Ibrahim have demonstrated that a cytoplasmic S-140 fraction from rat liver is capable of stimulating protein synthesis in vitro by isolated rat liver mitochondria, but have not shown conclusively that this effect is specifically due to proteins in the S-100 extract (328). It is quite clear that there is much room for additional investigations into these aspects of mitochondrial protein synthesis.

D. Concluding Remarks on the Control of Mitochondrial Protein Synthesis.

As has been discussed in the preceding sections, there has accumulated a substantial body of evidence which indicates that mitochondrial protein synthesis in lower and higher eukaryotes is controlled by proteins which are encoded by nuclear genes and translated in the (extramitochondrial) cytoplasm. The mechanisms by which these proteins act to stimulate mitochondrial protein synthesis are not well understood, even in such extensively-studied simple eukaryotes as S. cerevisiae. In mammals, many additional complicating factors such as tissue-specific metabolic differences and endocrine involvement in regulation of metabolic processes (including protein synthesis and degradation) further hamper efforts to understand these processes. Nonetheless, the involvement of cytoplasmic proteins in the governance of mitochondrial protein synthesis seems quite clear.

Additionally, nonprotein cellular factors are also implicated in the possible regulation of mitochondrial protein synthesis. For example, guanine nucleotides are capable of stimulating protein synthesis in vitro by mitochondria isolated from yeast (294) and rat skeletal muscle (Table XI). Heme plays an important role in the synthesis and assembly in vivo of mitochondrial translation products in S. cerevisiae (280, 303), N. crassa (305), and rat liver (332,334). When included in the incubation mixture, hemin (10^{-9}M) causes a slight increase in protein synthesis in vitro by rat liver

mitochondria (335); interestingly, at higher concentrations (1-100μM), hemin inhibits protein synthesis *in vitro* by rat liver (Dolci, unpublished observations) and yeast (Finzi, unpublished observations) mitochondria. Marcus *et al.* observed that Fe^{++} was specifically required for protein synthesis *in vitro* by rat liver mitochondria; this requirement was not satisfied by hemin or by other divalent transition metal ions (335). Recently, Bandlow *et al.* have shown that the rate of RNA transcription *in vitro* by yeast mitochondria is markedly stimulated by addition of monobutyryl cAMP (336); however, Gadaleta *et al.* found that cAMP or its dibutyryl derivative had no effect on the rate of mitochondrial transcription *in vitro* by rat liver mitochondria (312).

Mitochondria from many mammalian tissues possess saturable receptors for triiodothyronine (T_3) (337); while T_3 has been shown to increase the rate of mitochondrial transcription *in vivo*, its direct effects on mitochondrial protein synthesis *in vitro* have apparently not been examined. Similarly, it has been recently demonstrated that mitochondria from cultured 3T3 cells possess binding sites for the Ca^{++}-calmodulin complex (338). Although many mitochondrial enzymes are activated by Ca^{++} *in vitro*, the effects of this ion on mitochondrial protein synthesis are not known. Interestingly, Larner *et al.* (265) have hypothesized that the second messenger for insulin may act as a Ca^{++}-ionophore, although the experiments of Kiechle *et al.* do not appear to support this theory (267).

There are many important differences between lower and higher eukaryotes in the control of mitochondrial transcription. The existence of intervening sequences in the yeast mitochondrial genes has been well-documented (140), while such structures do not appear to exist in mammalian mt DNA (131). Yeast mt genes appear to be separated by A-T rich "spacer" segments; most of the t RNA genes are found in one portion of the genome (135). By contrast, the mammalian mt genome appears to contain no spacer segments (131); instead, the rRNA and structural genes are "punctuated" by the genes for the tRNA's, which must be excised from the primary RNA transcripts before their functions can be expressed (152,154). Moreover, there appears to be no unique origin of replication (135) or single promoter region (204) in yeast mt DNA, while in mammalian mt DNA, both the H and L strands possess a single origin of replication (131), and the H-strand (which appears to contain most of the structural genes) appears to be completely transcribed starting at a point near the H-strand origin of replication (131) which appears to be attached to the mitochondrial inner membrane(199). These results indicate the possibility that, in mammals, mitochondrial DNA replication and transcription are coordinately controlled, and that the rate of transcription and the rate of translation may also be closely coupled. The explosive, almost exponential increase in the number of studies of mitochondrial biogenesis published over the past few years will obviously continue unabated for some time. The findings yet to be reported in

this area should prove to be even more fascinating and significant than those already published.

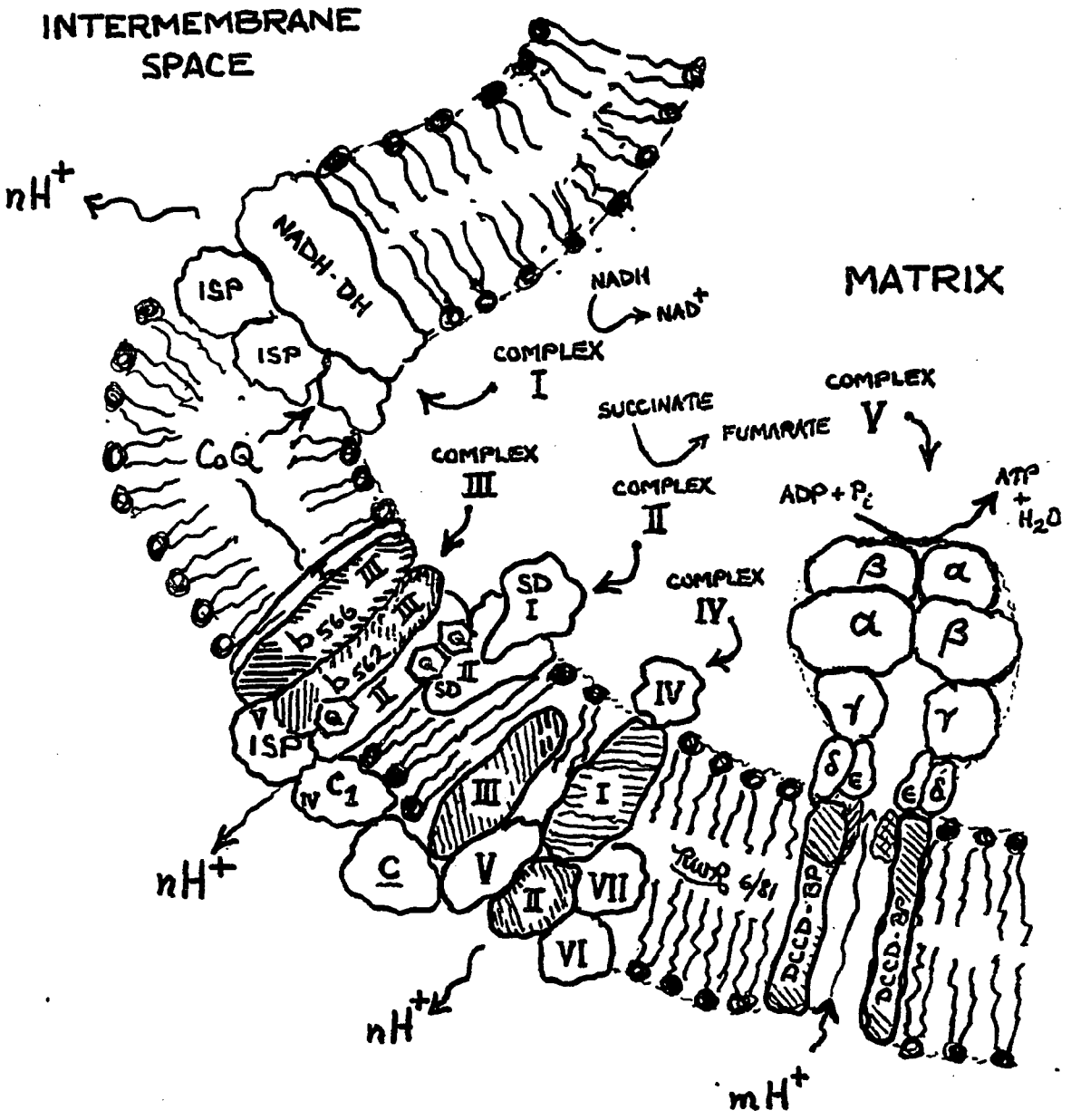

Illustration A

The electron-transport system and ATPase of the inner mitochondrial membrane according to current concepts. Complexes I-IV are defined in terms of their enzymatic activities (page 4); Complex V is the proton-translocating ATPase. Mitochondrially-translated subunits (mammalian) are shaded. The many transport systems of the inner membrane are not shown. Drawing is not to scale.

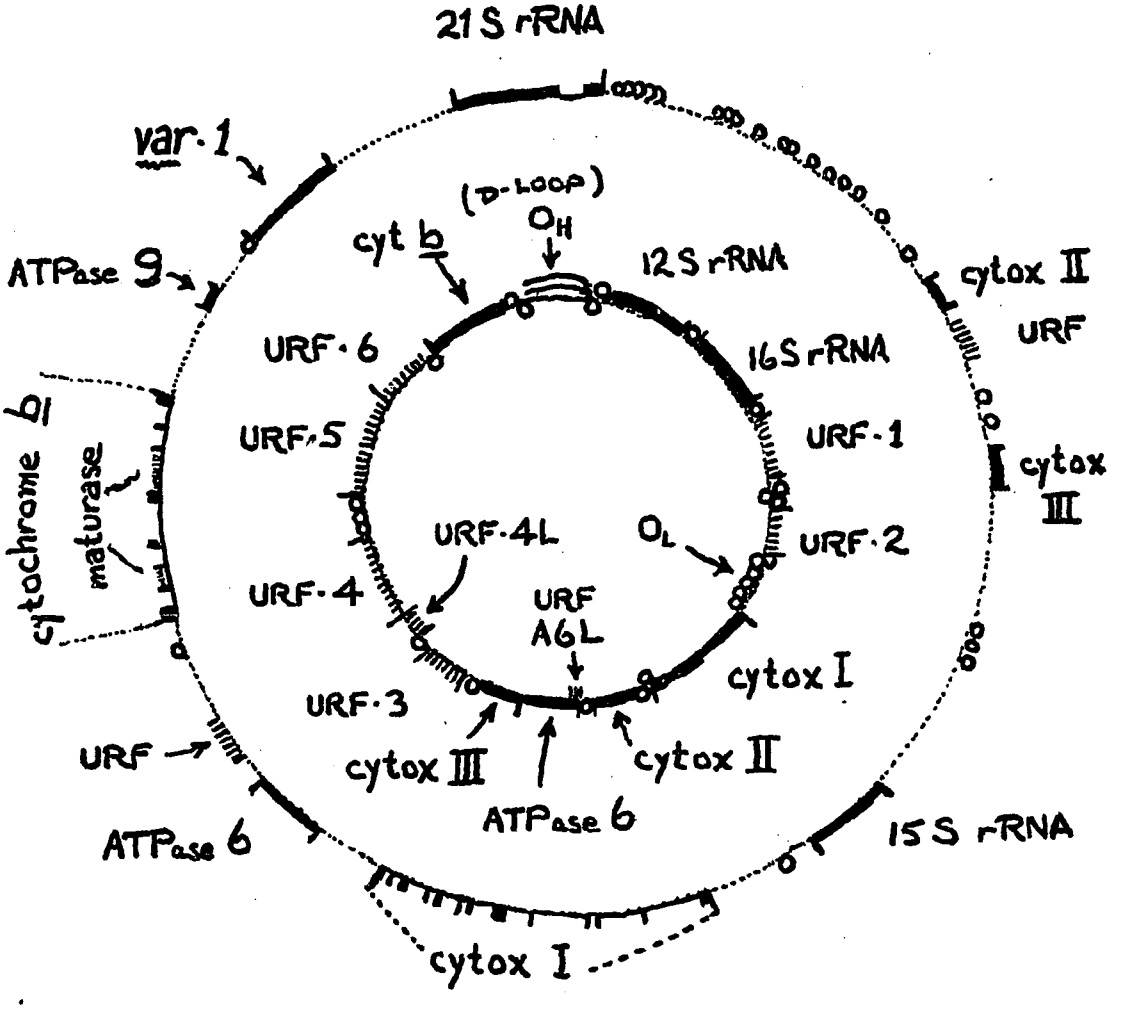

Illustration B

Organization of Yeast and Human Mitochondrial Genomes.

The outer circle represents the mitochondrial genome of S. cerevisiae strain KL 14-4A; the inner circle represents the genome of human placental cell mitochondria; drawings are not to scale. Genes named outside their respective circles are transcribed from the H-strand, while those inside are transcribed from the L-strand. Transfer RNA genes are indicated by open circles. Adapted from Borst and Grivell (157) and Anderson et al. (131). Note the split genes for the 21S rRNA, cytochrome b, and Cytox I in the yeast genome, and the tRNA "punctuation" in the mammalian genome.

ABBREVIATIONS

ACTH	adrenocorticotropic hormone
ADP	adenosine-5'-diphosphate
ATA	aurintricarboxylic acid (ammonium salt)
ATP	adenosine-5'-triphosphate
cAMP	(cyclic) adenosine-3',5'-monophosphate
CAP	chloramphenicol
CCCP	carbonyl cyanide m-chlorophenylhydrazone
CHX	cycloheximide
CPM	counts per minute
Cytox I,II,III etc.	cytochrome oxidase subunits I, II, III, etc.
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid (disodium salt)
GLU	glutamic acid
GTP	guanosine-5'-triphosphate
HEPES	N-2-hydroxyethylpeperazine-N'-2-ethanesulfonic acid
kbp	kilobase pairs
M_r	(relative) molecular weight
mt DNA, RNA	mitochondrial DNA, RNA
MW_{av}	average molecular weight
PEP	phosphoenolpyruvate
PMSF	phenylmethyl sulfonyl fluoride
PTM	pactamycin
RCR	respiratory control ratio
R_f	relative electrophoretic mobility (to bromphenol blue)
SDS	sodium dodecyl sulfate

Abbreviations contd...

S-140	cytosolic postpolysomal supernate (dialyzed and concentrated)
T_3	triiodothyronine
TLCK	Nα-p-tosyl-L-lysine chloromethyl ketone
URF	unidentified reading frames

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