Energy-Linked Synthesis and Decay of Membrane Proteins in Isolated Rat Liver Mitochondria*

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ABSTRACT: Incorporation of radioactive amino acids into acid-insoluble membrane proteins of isolated rat liver mitochondria proceeds maximally in a medium containing 2.0 mM adenosine triphosphate (ATP), an ATP-regenerating system such as phosphoenolpyruvate plus pyruvate phosphokinase, $MgCl_2$, KCl, and L-proline at pH 7.5. Adenosine diphosphate (ADP) cannot replace ATP, but guanosine triphosphate (GTP) can, whereas adenosine monophosphate (AMP) is inhibitory. Incorporation supported by an ATP-regenerating system is far more rapid than that supported by electron transport. Furthermore, it is not inhibited by oligomycin, showing that high-energy intermediates of oxidative phosphorylation generated by electron transport or ATP hydrolysis are not obligatory in providing energy for mitochondrial protein synthesis.

Amino acid incorporation is preceded by a rapid, energy-dependent transport of external amino acids into the intramitochondrial pool. The amino acids are then largely incorporated into insoluble membrane proteins, particularly the structural protein fraction. The incorporation is sensitive to actinomycin D and to chloramphenicol and is not caused by contaminating extramitochondrial ribosomes. The newly synthesized membrane protein undergoes rapid enzymatic decay to an acid-soluble neutral peptide. The decay, which can be stimulated by puromycin, probably consists of discharge of incomplete or "nascent" polypeptide chains from the biosynthetic surface, followed by action of endopeptidases. The decay reaction is inhibited by antimycin A but greatly stimulated by GTP. The complexity of the effects of added nucleotides, respiratory substrates, and inhibitors on mitochondrial protein synthesis is due to the fact that there are many energy-linked reactions in the biosynthetic chain, and that both biosynthesis and decay of mitochondrial membrane protein are stimulated by ATP and/or GTP. None of the findings made is inconsistent with the view that mitochondrial protein synthesis proceeds by the same enzymatic pattern as that in extramitochondrial ribosomes.

The ability of isolated mitochondria to carry out energy-dependent incorporation of labeled amino acids into protein was first observed by Siekevitz (1952). Later studies by Simpson (1962), Roodyn *et al.* (1961, 1965), Truman and Korner (1962a), Mager (1960), and Kroon (1963a–c, 1964, 1965), among others, have delineated some of the major features of this process. The recent observations that mitochondria contain deoxyribonucleic acid (DNA) differing from nuclear DNA (*cf.* Luck and Reich, 1964; Rabinowitz *et al.*, 1965; Neubert, 1964; and Wintersberger, 1965) and that mitochondrial protein synthesis is inhibited by actinomycin D under some circumstances (*cf.* Kroon, 1964; Wintersberger, 1965) have generated greatly increasing interest in the biological significance of mitochondrial protein synthesis. However, very little is known regarding the biochemical details of this process and a number of conflicting experimental findings have been reported.

Some of these problems may be mentioned. It is not yet completely clear to what extent contaminating bacteria are responsible for the incorporation of radioactive amino acids in isolated mitochondria, and at least one group (Sandell *et al.*, 1966) has concluded that bacteria are responsible for all the protein synthesis they observed. Secondly, there are many conflicting observations on the energy source and on the requirement of adenine nucleotides, respiratory substrates, and the effect of respiratory inhibitors (Simpson, 1962; Roodyn *et al.*, 1961, 1965; Truman and Korner, 1962a, Kroon, 1963a–c, 1964, 1965). Some studies have suggested that high-energy intermediates generated during electron transport are required to support amino acid incorporation, without the intervention of ATP[1] (Bronk, 1963; Kroon, 1964). This suggestion raises the question as to whether mitochondrial protein synthesis proceeds by the same enzymatic processes as "classical" protein synthesis in cytoplasmic ribosome preparations. Although ribosomes have been reported to be present in mitochondria (Elyaev, 1965; Howell

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[1] Abbreviations used: ATP, adenosine triphosphate; TCA, trichloroacetic acid; AMP, adenosine monophosphate; ADP, adenosine diphosphate; GTP, guanosine triphosphate; NAD, nicotinamide–adenine dinucleotide.

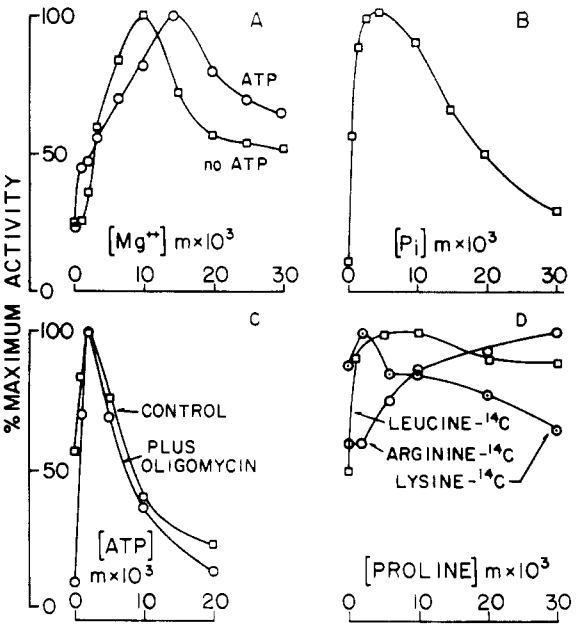

FIGURE 1: Cofactor requirements for amino acid incorporation by mitochondria. In all experiments mitochondria (6.0 mg of protein) were incubated for 60 min at 37° in 3.0 ml of medium containing sucrose (100 mM), KCl (100 mM), succinate (10 mM), Tris chloride (33 mM), and DL-[1-^{14}C]leucine (0.166 μc/ml). $MgCl_2$ and KH_2PO_4 were each 10 mM, except when these components were varied. Final pH was always adjusted to 7.5 (25°) with Tris base. (A) Mg^{2+} requirement in the absence and in the presence of 2.0 mM ATP. (B) Requirement for phosphate in the absence of ATP. (C) Optimal concentration of ATP, determined in the presence of phosphoenolpyruvate (5 mM) and pyruvate kinase (0.1 mg/ml), with and without oligomycin (4 μg/ml). (D) Effect of L-proline on the incorporation of DL-[1-^{14}C]leucine, L-[U-^{14}C]arginine, and L-[U-^{14}C]-lysine. Conditions of incubation included 2.0 mM ATP and 15 mM $MgCl_2$.

et al., 1964; Rabinowitz *et al.*, 1966), their role in protein synthesis in mitochondria is not yet clear. There is also considerable uncertainty with regard to the identity of the proteins synthesized by mitochondria and whether complete protein chains are synthesized under *in vitro* conditions.

The investigation described in this report, the first of a series of mitochondrial protein synthesis, was concerned with the development of a more reproducible assay system appropriate for obtaining further biochemical information on this process. It deals primarily with the role of adenine nucleotides, respiratory substrates, and the effect of respiratory inhibitors on the incorporation of labeled amino acids into the proteins of intact rat liver mitochondria. In the course of this work, a number of findings were made that are revelant to some of the other areas of uncertainty listed above. In a separate report (Wheeldon, 1966), the problem of bacterial contamination as an element in mitochondrial protein synthesis is considered.

Experimental Details

Methods. Mitochondria were prepared from livers of male rats of the Wistar strain by the method of Schneider (1948) and washed three times in cold 0.25 M sucrose. Microsomes were prepared from a 20% (w/v) homogenate of rat liver in 0.25 M sucrose, after removal of mitochondria (15 min at 12,000*g*), by centrifugation at 105,000*g* for 45 min. Rat liver ribosomes were prepared by the method of Korner (1964). The protein content of mitochondria, microsomes, and ribosomes was determined by the biuret method (Gornall *et al.*, 1949).

Incubations were carried out in open beakers in a Dubnoff metabolic incubator. Reactions were stopped by addition of an equal volume of 10% TCA containing nonradioactive amino acid (10 mM). The precipitates were washed twice by resuspension in TCA, then suspended in 3.0 ml of 1.0 N NaOH containing 10 mM amino acid, and allowed to stand for at least 1 hr. Acid-insoluble protein was then recovered by addition of 3.0 ml of 6 N HCl, followed by centrifugation. The suspension of the acid-insoluble protein fraction in 1.0 N NaOH and precipitation with 6 N HCl were carried out once more. The protein was then suspended in 10 ml of 0.1 N perchloric acid and placed in a boiling water bath for 5 min, and the insoluble portion recovered by centrifugation. After a final washing with 10 ml of ethanol–ether (v/v) (or 10 ml of acetone), the residue was dissolved in formic acid transferred to a tared aluminum planchet, and dried, and the weight determined on a microbalance. Samples were counted in a Nuclear-Chicago, low-background, automatic gas-flow counter with an efficiency of approximately 30%. Correction was made for self-absorption from a curve constructed with serum albumin. No correction for counting efficiency was made.

Peptide maps were made of the labeled TCA-soluble peptides formed in the presence of puromycin (*cf.* Figure 7) by the following procedure. Mitochondria (2.0 mg of protein/ml) were incubated for 60 min at 37° in a final volume of 30 ml in a medium containing the basic components listed in Figure 1, plus ATP (2.0 mM) and 50 μc of L-[U-^{14}C]leucine (sp act., 240 mc/mM). The incubation mixture was then rapidly cooled to 0° and the mitochondria were harvested by centrifuging for 10 min at 10,000*g*. The mitochondrial pellet was carefully resuspended in 0.25 M sucrose and again recovered by centrifugation. After resuspending in sucrose, the mitochondria were incubated for 30 min in a medium identical with the above but in which ATP was omitted and puromycin (40 μg/ml) was added. The mitochondria were then recovered as before and suspended in 2.0 ml of water, to which 0.2 ml of 50% TCA was added. The precipitated material was extracted with 1.0 ml of 5% TCA and the TCA extracts were combined. The combined TCA extracts were

shaken ten times with equal volumes of ether in order to remove TCA; the extracted aqueous residue was then evaporated to dryness under nitrogen at 50°. The residue was dissolved in a minimum volume of water and then applied to a sheet of Whatman 3MM paper (13 × 15 in.), previously wetted in pH 6.4 buffer composed of water–pyridine–acetic acid in the volume proportions 450:50:2. Electrophoresis for 1 hr at 1000 v was carried out using a cold-plate apparatus of conventional design. After drying, chromatography in the solvent system 1-butanol–pyridine–water–acetic acid (30:20:24:6, v/v) was carried out for 20 hr in a direction at right angles to the direction of electrophoresis. The chromatogram was then thoroughly dried. An autoradiogram was prepared by exposure of Kodak medical X-ray "no screen" film to the chromatogram for a period of 2 weeks.

Materials. All nucleotides were obtained from Calbiochem D.C., Bethesda, Md., who also supplied the Boehringer product, phosphoenolpyruvate, which was obtained as the silver–barium salt and converted to the Tris salt for use. Bovine pancreatic ribonuclease (five times recrystallized) was also a Boehringer product. Pyruvate kinase, Type II from Sigma Chemical Co., St. Louis, Mo., was recovered from ammonium sulfate suspension before use. DL-[1-^{14}C]Leucine (sp act., 20–30 mc/mM), L-[U-^{14}C]lysine and L-[U-^{14}C]arginine (both 222 mc/mM) were obtained from New England Nuclear Corp., Boston, Mass. Antibiotics were obtained from the following sources: puromycin, Nutritional Biochemicals, Cleveland, Ohio; chloramphenicol, Parke Davis, Detroit, Mich.; oligomycin, Wisconsin Alumni Research Foundation, Madison, Wis. Actinomycin D was a gift from Merck Sharp and Dohme, Philadelphia, Pa.

Results

Cofactor Requirements. Most of the experiments on leucine incorporation to be described in this section were carried out in a basic medium consisting of 100 mM sucrose, 100 mM KCl, 10 mM Mg^{2+}, and 10 mM P_i, sometimes supplemented with 5 mM proline, which was found to stimulate amino acid incorporation (see below). Either adenine nucleotides or respiratory substrates (or both) were added as energy source. The presence of KCl stimulated the incorporation of amino acid more than NaCl, in agreement with an earlier report (Truman and Korner, 1962a). Amino acid incorporation into liver mitochondria showed an absolute requirement for both Mg^{2+} and phosphate (*cf.* Roodyn *et al.*, 1961). In the basic system incorporation of amino acid was approximately linear with time for about 1 hr, but then usually declined in rate; a 30–60-min incubation period was used in most experiments. It will be shown below that the net amino acid incorporation observed, especially in longer incubations, is the resultant of the synthesis of acid-insoluble protein and its decay to acid-soluble labeled peptides. Both synthesis and decay of acid-insoluble protein were found to be influenced by most of the variables studied.

Adenine Nucleotide Requirement. Existing data on the requirement for added nucleotides are somewhat conflicting. Some authors have reported that AMP or ADP stimulates amino acid incorporation in the presence of added respiratory substrates (Roodyn *et al.*, 1961; Truman and Korner, 1962a; Kalf, 1963; Truman and Löw, 1963), whereas others have reported no stimulation by added adenine nucleotides in the absence of a substrate (Kroon, 1963c; Truman and Löw, 1963; Neubert, 1964) or in its presence (Bronk, 1963). There is one report of complete dependence on both ATP and the phosphoenolpyruvate–pyruvic kinase system (Singh *et al.*, 1964). It has been postulated by Bronk (1963) and Kroon (1964) that amino acid incorporation proceeds, possibly obligatorily, at the expense of hypothetical high-energy intermediates of oxidative phosphorylation generated during electron transport. Neubert (1964), on the other hand, has concluded that protein synthesis may be supported by ATP generated by reactions other than oxidative phosphorylation. Experiments in this and the follow-

TABLE I: Adenine Nucleotide Requirement for Amino Acid Incorporation.[a]

Expt	Additions (mM)	Leucine Incorp (cpm/mg of protein) 30 Min	120 Min
1	None	12.4	22.7
	ATP (2.0)	28.5	46.2
	ADP (2.0)	28.9	52.0
	ADP (2.0) + glucose–hexokinase	9.4	8.4
	ATP (2.0) + PEP + PK	59.0	68.0
2	None	13.0	
	AMP (2.0)	7.6	
	AMP (5.0)	4.9	
3	None		33.1
	GTP (0.2)		55.0
	GTP (0.5)		66.0
	ATP (2.0)		65.0
	ATP (2.0) + GTP (0.2)		66.0
	Pyrophosphate (10)		36.5

[a] Mitochondria (6.0 mg of protein) were incubated at 37° in 3.0 ml of a medium containing sucrose (100 mM), KCl (100 mM), KH_2PO_4 (10 mM), succinate (10 mM), $MgCl_2$ (10 mM), proline (5 mM), Tris (33 mM), and DL-[1-^{14}C]leucine (0.166 μc/ml). Final pH was 7.5 (25°). Glucose and hexokinase were added at 20 mM and 100 units/ml, and phosphoenolpyruvate (PEP) and pyruvate kinase (PK) at 5 mM and 0.1 mg/ml, respectively. Each experiment was carried out with a different preparation of mitochondria; there was some variation in their activity in the absence of added ATP.

ing sections provide some clarification of this problem.

The effects of added ATP, ADP, and AMP, as well as other energy sources, on the incorporation of leucine into mitochondrial protein are shown in Table I; succinate was present in these experiments. From expt 1 it is seen that addition of 2.0 mM ATP to the test system greatly stimulated leucine incorporation. Additions of ADP and ATP were equally effective under these conditions, presumably because ADP can be phosphorylated to ATP at the expense of electron transport. That ATP is the immediately required nucleotide is shown by the almost complete inhibition of the leucine incorporation reaction by addition of the glucose–hexokinase trap.

The rate of the ATP-supported reaction was very greatly increased when an ATP-regenerating system, composed of phosphoenolpyruvate and pyruvate kinase, was added, as shown in Table I. The effect of varying the ATP concentration in the presence of the regenerating system is shown in Figure 1C. The rate of incorporation is sharply optimal at 2.0 mM ATP, higher concentrations of ATP causing a marked inhibition of incorporation. It is clear that the concentration of ATP is very critical; if excessive concentrations are used there is no stimulation and inhibition may also result. In Figure 1C it is demonstrated that the high rate of leucine incorporation supported by the ATP-generating system is essentially insensitive to oligomycin. This finding clearly established that under these circumstances, *i.e.*, in the presence of a large excess of the phosphopyruvate-linked ATP-regenerating system, oxidative phosphorylation of ADP to ATP plays no significant role in supporting amino acid incorporation. It also demonstrates that ATP-supported amino acid incorporation does not require intervention of the oligomycin-sensitive high-energy intermediates of oxidative phosphorylation as an obligatory step. Our findings therefore do not support the suggestions by Bronk (1963) and Kroon (1964) that amino acid incorporation is dependent on high-energy intermediates generated during electron transport or ATP hydrolysis.

Data in Table I show that AMP cannot replace ATP or ADP, despite the presence of succinate and other components and conditions that are known to support oxidative phosphorylation of AMP to ATP *via* the adenylate kinase reaction. In fact, AMP was strongly inhibitory to amino acid incorporation during the first 30 min of incubation, but less inhibitory over the longer incubation period of 120 min; this finding indicates that the inhibition is reversed as AMP is converted to ATP. Inhibition of leucine incorporation by AMP is in all probability caused by a mass-action effect of AMP in preventing the formation of aminoacyl soluble ribonucleic acid (s-RNA) (*cf.* Allen *et al.*, 1960). Such an inhibition would be relieved when AMP is phosphorylated to ADP and ATP.

Replacement of added ATP by GTP also stimulated leucine incorporation, measured after 120-min incubation (Table I), but GTP did not yield a higher level of incorporation if ATP was already present, in confirmation of Neubert's (1964) observations. Considerable leucine incorporation took place in the absence of added ATP. Under these circumstances, where incorporation of amino acid was evidently supported by intramitochondrial ATP, the incorporation reaction appeared to be subject to a greater number of variables and was less reproducible.

Respiratory Substrates. The requirement for respiratory substrates to support amino acid incorporation into mitochondrial protein has also been reexamined, because previous reports on such a requirement have been quite conflicting (Roodyn *et al.*, 1961; Kalf, 1963; Truman and Löw, 1963; Kroon, 1964; Neubert, 1964; Roodyn, 1965). In view of the data on the ATP requirement reported above, a relationship between the requirements for adenine nucleotide and respiratory substrate can be expected. Data in Table II show that neither succinate nor isocitrate in the absence of ATP stimulates incorporation of leucine in fresh mitochondria; in fact, they were somewhat inhibitory. Presumably endogenous substrates suffice to support leucine incorporation, since addition of amytal suppressed endog-

TABLE II: Effect of Respiratory Substrates on Protein Synthesis.

Conditions[a]	Leucine Incorporated (cpm/mg of protein) No Substrate	Succinate	Isocitrate
Fresh mitochondria			
(1) ATP absent	57	42	20
ATP absent + amytal	24	41	15
(2) ATP added	75	72	42
ATP added + PEP + PK	153	99	111
Aged mitochondria			
ATP absent	36	80	45

[a] Fresh mitochrondria were prepared as described in the Methods section. "Aged mitochondria" were prepared by suspending in 125 mM KCl–20 mM Tris chloride, pH 7.3, at 4 mg of protein/ml and incubating for 30 min at room temperature in air. They were recovered by contrifugation and resuspended in 0.25 M sucrose. The medium contained, in a final volume of 3.0 ml, mitochondria (12.0 mg of protein), sucrose (135 mM), KCl (50 mM), nicotinamide (10 mM), $MgCl_2$ (10 mM), KH_2PO_4 (10 mM), Tris chloride (50 mM), and DL-[1-^{14}C]leucine, 0.25 μc/ml; final pH, 7.5 (25°). Succinate (Tris salt) was added at 5.0 mM and DL-isocitrate (sodium salt) at 10 mM. Incubation was for 60 min. Where added, ATP was 2.0 mM, amytal, 1.0 mM, PEP, 5.0 mM, and pyruvate phosphokinase, 0.1 mg/ml.

enous leucine incorporation and evoked stimulation by added succinate (but not by isocitrate). When ATP was regenerated independently of oxidative phosphorylation by addition of phosphoenolpyruvate and pyruvate kinase, succinate was inhibitory; it is probable that mitochondrial pyridine nucleotides are more highly reduced under these circumstances. Only in previously aged mitochondria, which were depleted of substrates, was stimulation by either succinate or isocitrate observed.

These data clarify in part the conflicting findings cited above. Although an absolute dependence for added respiratory substrate on amino acid incorporation was observed by Roodyn *et al.* (1961), their test system contained AMP, which was shown above to inhibit amino acid incorporation. The requirement for added respiratory substrate in their case may be a reflection of the removal of AMP by its oxidative phosphorylation to ATP.

The inhibition of amino acid incorporation by addition of respiratory substrates to fresh mitochondria (Table II) has also been observed by Kroon (1963a, 1964), who attributed the effect to the maintenance of mitochondrial pyridine nucleotides in a more reduced state, a condition which he postulated was unfavorable for amino acid incorporation. However, an alternative explanation is that the addition of the respiratory substrate may cause diversion of one or another endogenous amino acid into another metabolic pathway, such as transamination, removing it from participation in protein synthesis. As will be developed below, amino acid incorporation into mitochondrial protein appears to use an intramitochondrial pool of precursor amino acids; this pool of amino acids may also be involved in competing reactions such as oxidation or transamination. Although respiration at the expense of endogenous substrates can support submaximal levels of amino acid incorporation into the protein of freshly prepared rat liver mitochondria, an external ATP-regenerating system is far more effective and requires no addition of substrates.

Mg^{2+} and Phosphate. Maximum incorporation required the presence of both Mg^{2+} and phosphate in the medium, in confirmation of Roodyn *et al.* (1961). However, both requirements were profoundly influenced by the addition of ATP to the system. Figure 1A shows that the optimal concentration of Mg^{2+} was 10 mM in the absence of ATP and 15 mM in the presence of 2.0 mM ATP. The relatively high concentrations of Mg^{2+} required are reminiscent of the dependence of ribosomal protein synthesis on high Mg^{2+} (Campbell, 1965). Data in Figure 1B show that the optimal concentration of phosphate was 5.0 mM. However, phosphate was required only when ATP was absent. Phosphate may be required to sustain oxidative phosphorylation of internal adenine nucleotides in the absence of added ATP; however, mitochondria already contain significant endogenous phosphate, which could be expected to recycle during oxidative phosphorylation and protein synthesis. Phosphate is known to cause mitochondrial swelling (*cf.* Lehninger, 1962) and can also shift the equilibrium of hypothetical high-energy intermediates in oxidative phosphorylation by interacting at the oligomycin-sensitive site in the coupling mechanism (*cf.* Lehninger and Wadkins, 1962).

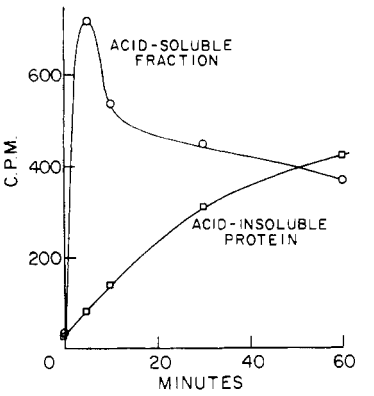

FIGURE 2: Uptake of leucine into acid-soluble and acid-insoluble fractions. Mitochondria were incubated at 37° at 2.0 mg of protein/ml in a final volume of 9.0 ml, which contained the basic components listed in Table I, plus ATP (2.0 mM) and DL-[1-^{14}C]leucine (0.166 μc/ml). At the time intervals shown, the incubation mixtures were rapidly chilled, and the mitochondria harvested by centrifugation and washed once with cold 0.25 M sucrose. The pellets were then suspended in cold 5% (w/v) TCA. Radioactivity was measured in the 5% TCA extract and in acid-insoluble protein recovered from the TCA precipitate.

Amino Acid Requirement. When ^{14}C-labeled leucine, lysine, and arginine were compared, all three were found to be incorporated into mitochondrial protein when no other amino acids were added to the system (see data at zero proline concentration in Figure 1D). Leucine and arginine were incorporated at about the same rate, whereas lysine incorporation was substantially lower. Labeled leucine was used in most experiments described in this paper; however, the rate of incorporation of different amino acids into mitochondrial protein is affected differently by the presence of other amino acids in the medium.

Addition of a complete mixture of amino acids as described by Truman and Korner (1962a) (excluding leucine) at a total concentration of 1.0 mg/ml was found to inhibit the incorporation of [^{14}C]leucine by 25%. Of twelve amino acids tested singly at 10 mM (Cys, Val, Ser, Arg, Gly, Lys, Ala, Ile, Pro, His, and Met), only proline and arginine stimulated leucine incorporation significantly, each to the extent of about 100%. Incorporation of leucine was inhibited 50% by 10 mM cysteine, 63% by methionine, and 100% by isoleucine added singly. Since proline is readily oxidized by mitochondria (Taggart and Krakaur, 1948), it was anticipated that it might stimulate leucine incorporation by serving as a respiratory substrate. However, this does not appear to be the case, since added proline stimulated leucine incorporation to the same extent,

whether or not succinate and/or ATP were added. Proline has different effects on the incorporation of lysine, arginine, and leucine. Data in Figure 1D show that very low concentrations of proline stimulated leucine incorporation, but did not affect incorporation of lysine or arginine. Higher concentrations of proline stimulated arginine incorporation, but inhibited lysine incorporation. The stimulatory effects of proline and arginine on leucine incorporation were not additive.

These findings are not easily explained on the basis of a direct action of the amino acids on the known enzymatic steps in protein synthesis *per se;* however, experiments below suggest that an amino acid transport process precedes incorporation into protein. Added amino acids may facilitate (or inhibit) the uptake of labeled leucine into an amino acid pool within the mitochondria.

Mitochondrial Uptake of Amino Acid in an Acid-Soluble Form. In Figure 2 is shown the time course of the uptake of labeled leucine by the mitochondria into a form soluble in cold 5% trichloroacetic acid. It is seen that there was an extremely rapid uptake of leucine by the mitochondria, that reached a peak in only minutes. Subsequently the amount of radioactivity in this fraction declined. The radioactivity appearing in the acid-soluble fraction at the early peak was entirely in the form of a substance having the chromatographic behavior of free leucine.

In another investigation, to be reported elsewhere, it was found that rapid accumulation of certain amino acids in acid-soluble form is stimulated by Mg^{2+} and ATP; presumably amino acid accumulation by mitochondria is the result of an energy-linked transport process. After the first minutes it can be assumed that the amino acids accumulated in the mitochondria may undergo a variety of enzymatic transformations, such as transamination, oxidation, conjugation, and incorporation into protein.

Compartmentation of amino acids in mitochondria has already been demonstrated for hippurate synthesis from [^{14}C]glycine (Garfinkel, 1963). The existence of such an intramitochondrial compartment of metabolically active amino acids, analogous to that described in yeast cells (Halvorson and Cowie, 1960) and animal tissues (Rosenberg *et al.*, 1963), would serve to enhance competition among alternative metabolic pathways of the amino acids. Roodyn (1965) has invoked a similar cause to account for anomalous incorporation of different amino acids by isolated mitochondria.

Rate of Incorporation of Leucine into Protein. Figure 2 also shows the time course of leucine incorporation into acid-insoluble protein in an ATP-supported system. Incorporation proceeded linearly for about 30 min, and thereafter decreased in rate. The linearity of this curve, as well as the specific cofactor requirements reported above, strongly suggest that the bulk of the amino acid incorporation observed is carried out by mitochondria *per se*, and not by contaminating bacteria, a matter to be discussed further below. A logarithmically increasing rate of incorporation and nondependence on ATP could be expected if bacteria were solely responsible. Although all reasonable precautions were taken to minimize bacterial contamination of media and reagents, preparations of rat liver mitochondria are known to contain bacteria (Roodyn *et al.*, 1961). Some labeled amino acid is in fact incorporated by bacteria associated with rat liver mitochondria, but this does not make a significant contribution to the total incorporation under the conditions used in this paper (Wheeldon, 1966).

Other Properties. Incorporation of leucine into mitochondrial protein was unaffected by addition of rat liver supernatant fraction (0.3 ml/ml of incubation mixture), suggesting that exogenous amino acid activating enzymes and s-RNA's are not required or that they are ineffective in reaching the sites of mitochondrial protein synthesis. Reduced glutathione (5 mM) had no effect, but oxidized glutathione (5 mM) inhibited incorporation completely; this inhibition was overcome by addition of an equal concentration of reduced glutathione, indicating a dependence of amino acid incorporation on mitochondrial SH groups.

Effect of Inhibitors of Electron Transport and Oxidative Phosphorylation. Data collected in Table III show that the incorporation of leucine in the presence of succinate (but without added adenine nucleotides) was almost completely inhibited by cyanide, antimycin

TABLE III: Effect of Inhibitors on Leucine Incorporation in Respiration- and ATP-Supported Systems.[a]

Inhibitor	% Inhib	% Stimulation
Respiration-supported systems		
KCN (5.0 mM)	95	
Antimycin A (0.6 μg/ml)	88	
Amytal (2.0 mM)		58
2,4-Dinitrophenol (0.2 mM)	88	
Oligomycin (4 μg/ml)	85	
ATP-supported systems		
Oligomycin (4 μg/ml)	8	
KCN (5.0 mM)	34	
Antimycin A (0.6 μg/ml)	64	
Amytal (2.0 mM)	0	
2,4-Dinitrophenol (0.2 mM)	81	
Oligomycin + 2,4-dinitrophenol	42	
Oligomycin + KCN	0	

[a] Mitochondria (6.0 mg of protein) were incubated for 1 hr at 37° in 3.0 ml of a medium containing sucrose (100 mM), KCl (100 mM), KH_2PO_4 (5 mM), $MgCl_2$ (10 mM), Tris chloride (33 mM), proline (5 mM), and DL-[1-^{14}C]leucine, 0.166 μc/ml. Final pH, 7.5 (25°). The respiration-supported systems also contained succinate (5.0 mM) and the ATP-supported systems contained ATP (2.0 mM), phosphoenolpyruvate (5.0 mM), and pyruvate kinase (0.1 mg/ml). Data were collected from several experiments.

A, oligomycin, and 2,4-dinitrophenol, showing dependence of the incorporation on electron transport. However, the large *stimulatory* effect of amytal (Table III) is anomalous. Amytal does not inhibit succinate oxidation, but it could be expected to inhibit endogenous NAD-linked respiration. The stimulatory effect of amytal will be considered below.

Incorporation of leucine supported by an ATP-regenerating system was not inhibited by oligomycin (Table III), showing that ATP alone can provide the energy for leucine incorporation without the participation of phosphorylating electron transport. On the other hand, 2,4-dinitrophenol (DNP) did inhibit substantially; presumably the stimulation of ATPase activity by dinitrophenol reduced the steady-state level of ATP below that required for maximum activity, as might be expected from data in Figure 1C. Oligomycin diminished the inhibitory effect of dinitrophenol on leucine incorporation, as expected, since it inhibits DNP-stimulated ATPase (Huijing and Slater, 1961). However, it was unexpected to find that the ATP-supported incorporation, presumably independent of electron transport because it is insensitive to oligomycin, was inhibited significantly by cyanide and antimycin A, but not by amytal (Table III).

Interpretation of these anomalous findings is difficult because of the fact that there are several points in mitochondrial protein synthesis that are dependent on ATP, including the energy-dependent accumulation of amino acids from the medium. Moreover, ATP may be broken down by competing reactions, such as amino acid and fatty acid activation reactions. Another factor is the rate of removal of inhibitory products such as AMP and pyrophosphate by respiration and hydrolysis, respectively.

The respiratory inhibitors cyanide, amytal, and antimycin A require special comment, since they are known to have characteristic effects on mitochondrial ATPase activity; cyanide and antimycin A strongly inhibit ATPase activity, particularly if succinate is also present, whereas amytal stimulates activity (Wadkins and Lehninger, 1959; Chefurka, 1960). Wadkins and Lehninger (1959) have attributed these effects to the oxidation–reduction state of the respiratory carriers. Kroon (1964) has found that amino acid incorporation is maximal when the endogenous pyridine nucleotides are in the oxidized state and lowered when pyridine nucleotides are reduced. This finding might explain the stimulation by amytal that occurs in the absence of ATP, provided succinate is present (Tables II and III), since amytal inhibits the succinate-supported reduction of pyridine nucleotides (Chance and Hollunger, 1961). However, in view of the fact (Table III) that leucine incorporation can proceed at a maximal rate in an ATP-supported system in the presence of succinate, oligomycin, or cyanide, conditions in which the pyridine nucleotides are probably also fully reduced, it is concluded that the oxidized state of pyridine nucleotides cannot be an absolute requirement for protein synthesis in mitochondria. As will be seen below, an additional complication is introduced by the finding that the respiratory inhibitors influence the discharge of incomplete polypeptide chains from the site of protein synthesis in mitochondria and their degradation to acid-soluble peptides.

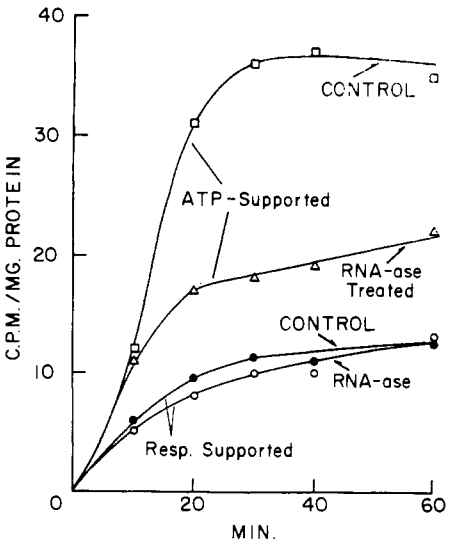

FIGURE 3: Sensitivity of ATP-supported incorporation to ribonuclease. A suspension of mitochondria in 125 mM KCl–20 mM Tris–HCl, pH 7.3 (2.5 mg of protein/ml), was divided into two equal portions, one of which served as control. To the other portion crystalline pancreatic ribonuclease was added at 0.2 mg/ml. After 20 min at room temperature, the mitochondria were centrifuged, resuspended in 0.25 M sucrose, and incubated under the conditions given in Figure 1. The time course of incorporation of DL-[1-^{14}C]leucine into acid-insoluble protein was determined for control and ribonuclease-treated mitochondria, both in the presence and absence of ATP + ATP-regenerating systems.

Effect of Inhibitors of the DNA–RNA–Protein Sequence in Intact Mitochondria. Actinomycin D was found not to inhibit amino acid incorporation in fresh intact liver mitochondria, although Kroon (1964) has found an inhibition with RNAase-treated liver mitochondria. However, we have found in other experiments not reported here that amino acid incorporation is inhibited by actinomycin D in rat liver digitonin or sonic fragments. This is consistent with the finding of Neubert and Helge (1965) that RNA polymerase activity is sensitive to actinomycin D only in damaged mitochondria. In agreement with the findings of Simpson (1962), puromycin, which interrupts peptide chain formation (*cf.* Brockman and Anderson, 1963), was found to inhibit completely amino acid incorporation in intact mitochondria.

Preincubation of the mitochondria with crystalline ribonuclease had no effect on leucine incorporation in fresh, intact rat liver mitochondria when it was supported by respiration in the absence of added ATP. However, when leucine incorporation was supported

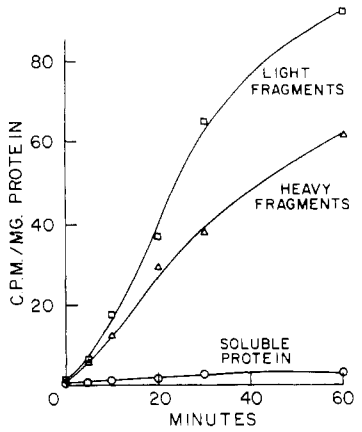

FIGURE 4: Incorporation of leucine into membrane fragments. At the time intervals shown, incubation mixtures having the composition described in Figure 2 were rapidly chilled to 0° and the mitochondria harvested by centrifugation. The mitochondria (30 mg of protein) were then suspended in 1.0 ml of 0.25 M sucrose containing 15 mM $MgCl_2$ and 1 mM ATP and then frozen and thawed with the addition of 2.0 ml of the same suspension mixture. The lumpy suspension was then sonicated for 1.5 min, using the small probe of the M.S.E. ultrasonicator at maximum output. *Heavy fragments* were recovered by centrifugation at 15,000 rpm for 5 min (Spinco rotor no. 40). *Light fragments* were obtained by centrifuging for 45 min at 40,000 rpm leaving the supernatant *soluble protein fraction.*

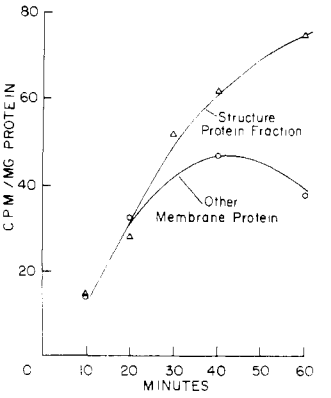

FIGURE 5: Incorporation into structure protein and other membrane proteins. Mitochondria were harvested at various intervals during incubation and sonicated as described in Figure 4. The light and heavy membrane fragments were combined and briefly sonicated in 0.45% deoxycholate, pH 7.6, to give a clear solution, and ammonium sulfate was added to 11% saturation. The precipitate was collected immediately by centrifugation (*structural protein fraction*). An approximately equal amount of protein was recovered from the supernatant solution by addition of TCA to 5% (*ammonium sulfate soluble fraction*).

by an ATP-regenerating system, then ribonuclease treatment inhibited leucine incorporation substantially (Figure 3). The simplest explanation for inhibition by ribonuclease in the presence of external ATP is the fact that it strongly stimulates ATPase activity of rat liver mitochondria (Kroon, 1964), thus lowering the steady-state level of ATP. Since ribonuclease did not inhibit respiration-supported incorporation it is unlikely that it is inhibiting protein synthesis *via* hydrolysis of mitochondrial messenger ribonucleic acid (m-RNA) or s-RNA. Elsewhere it is reported that respiration-supported ribonuclease-insensitive incorporation may be caused in part by bacterial contamination (Wheeldon, 1966).

Data in Table IV show that chloramphenicol completely inhibited leucine incorporation, confirming the early observation of Mager (1960) made on mitochondria from *Tetrahymena pyriformis.* Table IV also shows a comparison of the effects of chloramphenicol on leucine incorporation into rat liver mitochondria, rat liver microsomes supplemented with rat liver supernatant fraction, and a mixture of mitochondria and microsomes. Chloramphenicol failed to inhibit incorporation into microsomes (*cf.* Cooper, 1964). The characteristic inhibition of mitochondrial protein synthesis by chloramphenicol was not altered in a mixture of microsomes and mitochondria. It may be concluded that exogenous contaminating ribosomes do not contribute significantly to the incorporation of amino acids observed in rat liver mitochondria preparations.

TABLE IV: Effect of Chloroamphenicol on Protein Synthesis by Mitochondria and Microsomes.[a]

Mg of Protein	Chloramphenicol −	Chloramphenicol +	Chloroamphenicol-Sensitive Incorp (cpm)
Mitochondria (6.0)	220	36	184
Mitochondria + microsomes (0.5)	328	108	220
Mitochondria + microsomes (1.5)	465	282	183
Mitochondria + microsomes (3.0)	680	510	170
Microsomes alone (3.0)	410	380	30

[a] Mitochondria and microsomes were incubated for 30 min at 37° in 3.0 ml of a medium having the composition described in Table I, supplemented with rat liver supernatant fraction at 0.1 ml/ml of incubation medium. ATP was added at 2.0 mM. Values shown represent total cpm radioactivity recovered in protein. Chloramphenicol was added at 33 μg/ml as shown.

Identity of Newly Labeled Protein Fractions. In

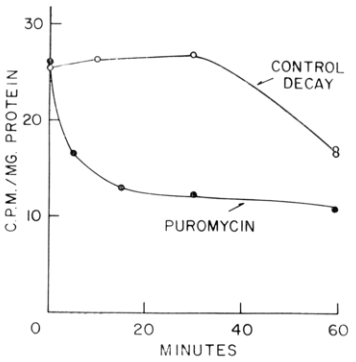

FIGURE 6: Decay of newly labeled protein and its stimulation by puromycin. Incorporation of [^{14}C]-leucine into mitochondrial protein was allowed to take place for 30 min under the conditions outlined in Table I; ATP was present at 2.0 mM. At 30 min the extent of incorporation was determined (initial point in graph) and a "chaser" of unlabeled leucine (1.33 mM) was added. The loss of radioactivity in the mitochondrial protein was followed in the ensuing 60 min. Puromycin was added at 50 μg/ml.

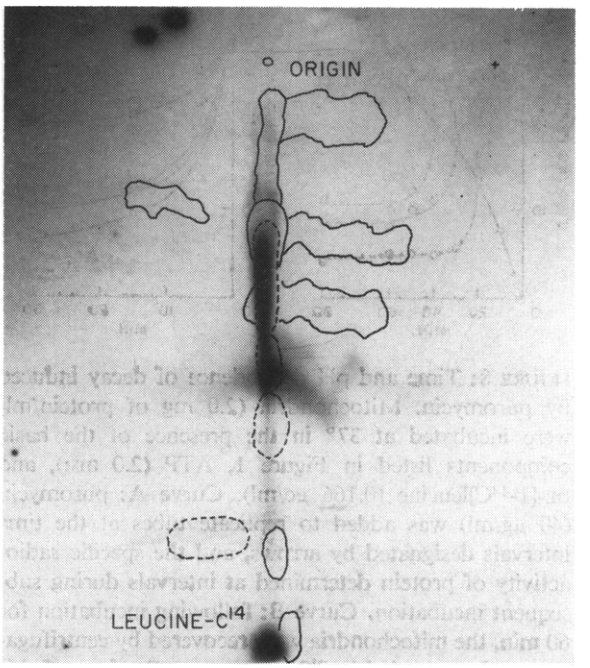

FIGURE 7: Peptide map of TCA-soluble products from puromycin-induced decay of labeled protein. The dark areas are those showing radioactivity. Areas which stained blue with ninhydrin are circled with continuous lines; those which stained yellow are circled with broken lines.

confirmation of Roodyn (1962), the radioactivity incorporated into acid-insoluble protein of mitochondria was found to be almost entirely confined to the insoluble membrane fraction. Mitochondria were harvested at successive time intervals and sonicated as described in Methods. The sonicated mitochondria were separated into three protein fractions by differential centrifugation: a "light" membrane fraction, a "heavy" membrane fraction, and a soluble protein fraction. The results are shown in Figure 4, which demonstrates that both the membrane fractions became labeled, whereas little or no radioactivity was incorporated into the soluble protein fraction, in agreement with Roodyn *et al.* (1962).

The radioactivity incorporated into the membrane fractions was found to be present largely in that protein fraction designated as "structural protein" (Criddle *et al.*, 1962), in confirmation of Roodyn (1962). The structural protein can be separated from other membrane proteins by precipitation from deoxycholate solutions with ammonium sulfate at 11–12% saturation (Criddle *et al.*, 1962). This procedure was applied to the combined "heavy" and "light" membrane fractions isolated from rat liver mitochondria incubated for various times with [^{14}C]leucine, with the results shown in Figure 5. At the beginning of the incorporation, the radioactivity was equally distributed between the structure protein and ammonium sulfate soluble fractions of the total membrane protein, but after 20 min, there was progressively higher incorporation into the structural protein fraction.

Decay of Newly Labeled Mitochondrial Protein. Newly labeled structural protein was found to undergo decay to an acid-soluble form. This was demonstrated by adding a "chaser" of excess unlabeled leucine to the system 30 min after incubation with labeled leucine had begun. The data collected in Figure 6 show that after a 30-min lag period, one-half of the newly incorporated radioactivity was lost from the acid-insoluble membrane protein fraction in the next 30 min. The rate of decay is thus very large compared to the rate of incorporation. The lag period in the decay of the labeled acid-insoluble protein was abolished when puromycin was present, as is seen in Figure 6.

The radioactivity lost from the acid-insoluble membrane protein fraction during such decay experiments could be recovered in the form of a fraction soluble in cold 5% trichloroacetic acid. This fraction was subjected to two-dimensional paper chromatography and electrophoresis to produce a peptide map developed with ninhydrin and by radioautography (Figure 7). Although it shows the presence of a number of ninhydrin-staining peptides, only one or a very few closely grouped peptides were radioactive. The radioactive material exhibited the chromatographic and electrophoretic mobility of a reasonably large, neutral peptide. The characteristics of this labeled peptide were essentially identical in acid-soluble preparations isolated from mitochondria incubated in the presence or absence of puromycin during the decay period.

Experiments in Figure 8A show that addition of puromycin caused immediate decay of labeled acid-

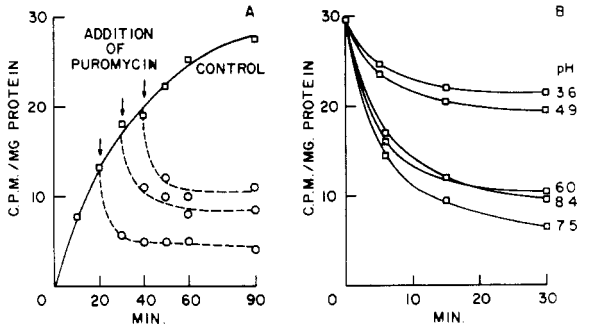

FIGURE 8: Time and pH dependence of decay induced by puromycin. Mitochondria (2.0 mg of protein/ml) were incubated at 37° in the presence of the basic components listed in Figure 1, ATP (2.0 mM), and DL-[1-^{14}C]leucine (0.166 μc/ml). Curve A: puromycin (40 μg/ml) was added to replicate tubes at the time intervals designated by arrows, and the specific radioactivity of protein determined at intervals during subsequent incubation. Curve B: following incubation for 60 min, the mitochondria were recovered by centrifugation and suspended in 0.25 M sucrose. Portions of this suspension were added to media containing puromycin (40 μg/ml) which had been adjusted to various pH values by addition of HCl or Tris base, and samples withdrawn for determination of specific radioactivity at the intervals shown. pH values were measured after a 30-min incubation.

TABLE V: Decay of Protein Synthesized by Mitochondria.[a]

Expt	Addition	Incorp of Leucine (cpm/mg of protein) 30 min	60 min	Loss of Incorp Leucine between 30 and 60 min (%)
1	None	41.6		
	Puromycin	41.6	22.1	47
	Puromycin (0°)	41.6	38.8	7
2	None	20.8	27.0	
	Chloramphenicol	20.8	20.3	2
	Chloramphenicol + puromycin	20.8	8.1	61
3	None	23.7	26.5	
	Puromycin	23.7	15.2	36
4	None	47.7	71.7	
	Puromycin	47.7	21.2	55

[a] Mitochondria (6.0 mg of protein) were incubated at 37° in a 3.0-ml medium containing sucrose (100 mM), KCl (100 mM), KH_2PO_4 (5 mM), $MgSO_4$ (10 mM), Tris chloride (33 mM, proline (5 mM)), and [1-^{14}C]leucine (0.166 μc/ml); final pH, 7.5 (25°). ATP (2.0 mM), phosphoenolpyruvate (5.0 mM), and pyruvate kinase (0.1 mg/ml) were added at zero time. Puromycin and chloramphenicol were added after 30-min incubation and the system incubated another 30 min. Chloramphenicol was added at 50 μg/ml, and puromycin at 40 μg/ml in expt 1, 50 μg/ml in expt 2, and 80 μg/ml in expt 3 and 4.

insoluble protein when added at different times during the course of leucine incorporation into mitochondrial protein. However, the fraction of total labeled protein that underwent decay decreased somewhat with time. In Figure 8B it is seen that the rate of puromycin-induced decay of acid-insoluble labeled protein was maximal at pH 7.5 and decreased greatly at pH 3.6 and at pH 4.0. This fact, together with the finding that essentially no puromycin-induced decay occurred at 0° (Table V), indicated that the decay is in all probability an enzyme-catalyzed reaction. The addition of chloramphenicol, which inhibits leucine incorporation, did not accelerate decay of the label (Table V). It is therefore probable that the decay process accelerated by puromycin is due to the release of so-called "nascent" polypeptide chains from the site of synthesis, as has been observed in ribosomal systems (Allen and Zamecnik, 1962; Morris *et al.*, 1963). To account for the solubility of the released chains in 5% trichloroacetic acid, it is necessary to assume that, once released from the template, the partially completed chains are rapidly broken down by mitochondrial endopeptidases (Umana and Dounce, 1964). That portion of the labeled acid-insoluble protein that is not discharged by puromycin may be composed of completed chains, since this fraction increased with time in proportion to the total incorporation (Figure 8B).

Effect of Respiratory Inhibitors on Puromycin-Induced Decay. Cyanide and antimycin A were found to inhibit both the puromycin-induced and the spontaneous decay of newly labeled membrane protein into an acid-soluble form (Table VI). Since ATP was present in excess, the decay is evidently dependent on respiration *per se*, rather than on the supply of ATP. This was confirmed by the finding that 2,4-dinitrophenol did not inhibit the decay. However GTP greatly accelerated puromycin-induced decay in the presence of antimycin A (Table VII). Such promotion of puromycin-induced decay by GTP is similar to that observed by Traut and Monro (1964) in a ribosomal system. It was also found that puromycin-induced decay of newly synthesized protein was completely prevented by 0.33 mM *p*-mercuribenzoate, in agreement with the findings of Traut and Monro (1964) for the ribosomal system.

It is evident from these experiments that inhibitors of energy-yielding processes in mitochondria may have striking effects on the rate of decay of the newly labeled acid-insoluble protein into an acid-soluble form, in addition to their effects on energy-linked

TABLE VI: Effect of Respiratory Inhibitors on Puromycin-Induced Decay Reaction.[a]

Expt	Addition	Incorp (cpm/mg) 30 min	60 min	Loss of Incorporated Radioactivity (%)
1	None	33.6	34.6	
	Puromycin	33.6	11.7	65
	Puromycin + antimycin A	33.6	18.9	44
	Puromycin + KCN	33.6	21.4	36
		30 min	90 min	
	None	30.4		
	Unlabeled leucine	30.4	20.6	34
	Unlabeled leucine + antimycin A	30.4	30.6	0
	Unlabeled leucine + DNP	30.4	17.1	39
	Unlabeled leucine + both	30.4	17.0	39

[a] Mitochondria (6.0 mg of protein) were incubated under the conditions described in Table V. Puromycin (50 μg/ml) and other inhibitors indicated were added after 30-min incubation; the decay in specific radioactivity is expressed as per cent of total incorporated radioactivity that was lost in the second incubation period. Antimycin A was added at 0.6 μg/ml, KCN at 3.33 mM, 2,4-dinitrophenol (DNP) at 0.33 mM, and unlabeled leucine at 1.33 mM.

TABLE VII: GTP Requirement for Puromycin-Dependent Decay Reaction in the Presence of Antimycin A.[a]

Addition	Incorporated [1-^{14}C]-Leucine (cpm/mg of protein) 30 min	60 min	Loss of Incorp Radioactivity (%)
None	256		
Puromycin		28	89
Puromycin + antimycin		262	2
Puromycin + antimycin + GTP		23	90

[a] Mitochondria (6.0 mg of protein) were incubated under the conditions described in Table V except that a higher specific activity of [1-^{14}C]leucine (0.66 μc/ml) was added; ATP was omitted. Puromycin and other additions listed were made after 30-min incubation and the change in specific radioactivity of mitochondrial protein was measured after a further period of incubation of 30 min. The change in specific radioactivity is expressed as per cent of specific radioactivity at time of addition (30 min). Puromycin was added at 40 μg/ml, antimycin A at 0.6 μg/ml, and GTP at 0.5 mM.

transport of amino acids into mitochondria and on incorporation of amino acids into protein.

Discussion

The data and considerations described in this paper have provided reasonably satisfactory rationalization in principle, if not in detail, for many of the anomalous and sometimes conflicting reports on the effects of added respiratory substrates, adenine nucleotides, and inhibitors of respiration and phosphorylation on the rate of amino acid incorporation into the proteins of isolated mitochondria. One of the reasons for this complexity of behavior is the fact that there are many energy-dependent steps in the complete process by which external amino acid is incorporated into newly formed membrane protein. The various steps and potential side reactions that can be expected to be dependent on or to be influenced by ATP energy and/or respiration may be listed: (1) energy-dependent transport of amino acids across the mitochondrial membrane(s); (2) intramitochondrial oxidation of amino acids; (3) intramitochondrial transamination reactions; (4) ATP-dependent activation of amino acids; (5) phosphorylation of various nucleoside 5′-mono- and diphosphates as precursors for m-RNA synthesis; (6) biosynthesis of m-RNA by DNA-directed RNA polymerase; (7) biosynthesis of s-RNA's, or maintenance of their terminal C-C-A nucleotide sequences; (8) formation of peptide linkages, presumably by GTP-linked reactions; (9) synthesis or maintenance of ribosomal RNA of the mitochondria.

Superimposed on these considerations is the now large body of evidence that both the structure (permeability of membranes, manner of folding of cristae, the mitochondrial volume, etc.) and many functions (electron transport, respiratory control, reverse electron flow, ion transport, swelling–contraction) are profoundly influenced by both external and internal ATP and ADP (*cf.* Lehninger, 1964). Furthermore, exchanges of adenine nucleotides across the mitochondrial membrane are themselves under control *via* an atractyloside-sensitive exchange diffusion mechanism (*cf.* Heldt *et al.*, 1965). It is therefore not unexpected that protein synthesis in intact isolated mitochondria is a more complex process than protein synthesis in isolated ribosomes. Furthermore, it can be expected that conditions influencing mitochondrial respiration and ATP formation should produce complex variations in the interrelationships among the many energy-dependent reaction steps involved in mitochondrial protein synthesis and in other relevant energy-dependent mitochondrial reactions.

A second point developed in this paper is that hypothetical high-energy intermediates of oxidative phos-

phorylation, generated either by electron transport or ATP hydrolysis, are not obligatory energy sources for mitochondrial protein synthesis, since mitochondrial protein synthesis can be supported at *maximal* rates by external ATP and an ATP-regenerating system without inhibition by oligomycin. Under no circumstances was respiration-linked protein synthesis as high in rate as the ATP-supported reaction. Our findings therefore do not support the suggestion (Kroon, 1964) that high-energy intermediates generated in electron transport are necessary to energize protein synthesis directly, without the intermediate formation of ATP. This point is of considerable significance in the experimental analysis of protein synthesis in mitochondrial extracts or other types of submitochondrial preparation, since the whole complex enzymatic apparatus of respiration and phosphorylation may now be excluded as a required component of mitochondrial protein synthesis.

A third major point developed in this paper is that the newly labeled "structural protein" fraction, as defined by Criddle *et al.* (1962), undergoes a substantially high rate of decay into a labeled acid-soluble neutral peptide. The removal of the newly synthesized peptide chain from the biosynthetic surface is greatly stimulated by addition of GTP. The mechanism of the discharge process is not clear, but presumably it is caused by some dysfunction of the GTP-dependent peptide-forming reaction similar to that produced by puromycin. Degradation of such discharged chains into acid-soluble peptides probably occurs by action of endopeptidases known to be present in mitochondrial fractions (*cf.* Umana and Dounce, 1962). Alberti and Bartley (1965) have reported a rather large rate of formation of free amino acids during incubation of isolated mitochondria, a process that may be related to the formation of the neutral, acid-soluble labeled polypeptide described here.

While the newly labeled membrane protein is recovered in the structural protein fraction of Criddle *et al.* (1962), this fraction may contain not only intact structural protein molecules but also other proteins, possible even some that are normally soluble, that have been newly discharged from the biosynthetic surface in an unfolded or insoluble state. Actually, in some experiments, up to 90% of the label incorporated into protein could be released by the action of puromycin (Table VII). Further work is in progress on the isolation of ^{14}C-labeled structural protein fraction and its analysis by means of peptide maps of tryptic digests.

None of the findings made in this study conflict with the view that protein specification and synthesis in mitochondria occur *via* the "classical" mechanisms such as have been observed in extramitochondrial ribosomes. Mitochondria are now known to contain a specific DNA (Luck and Reich, 1964; Rabinowitz *et al.*, 1965) and a DNA-directed RNA polymerase presumably able to cause synthesis of m-RNA (Luck and Reich, 1964; Neubert and Helge, 1965; Wintersberger and Tuppy, 1965). They have also been reported to contain at least some activating enzymes (Craddock and Simpson, 1961; Truman and Korner, 1962b) and s-RNA's in low amounts, although much more work is required to establish these points satisfactorily. There have also been reports that mitochondrial preparations contain ribosomes or polyribosomes (Elyaev, 1965; Howell *et al.*, 1964; Rabinowitz *et al.*, 1965).

Finally it appears significant that chloramphenicol blocks mitochondrial protein synthesis but not ribosomal protein synthesis in the rat liver preparations used. This finding makes it possible to conclude that contaminating extramitochondrial ribosomes are not responsible for protein synthesis observed in preparations of isolated mitochondria. Chloramphenicol sensitivity has been found to be characteristic of protein synthesis in preparations of isolated mitochondria and chloroplasts (Eisenstadt and Brawerman, 1964), and in bacterial systems. The mode of action of chloramphenicol in these systems is still not known (*cf.* Wolfe and Weissberger, 1965). Recently Rabinowitz *et al.* (1966) reported that protein synthesis in ribosomes isolated from heart mitochondria is insensitive to chloramphenicol, whereas protein synthesis in the intact mitochondria was inhibited by this agent. It has also been demonstrated (Linnane *et al.*, 1966) that chloramphenicol inhibits the normal development of mature mitochondria when glucose-repressed yeast cells are derepressed.

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