The Inner Membrane as the Site of the *in Vitro* Incorporation of L-[^{14}C]Leucine into Mitochondrial Protein*

Diana S. Beattie, R. E. Basford, and Seymour B. Koritz

ABSTRACT: Radioactive amino acids incorporated *in vitro* into mitochondrial proteins are present predominately in the inner membrane fraction prepared by two different procedures. The "structural" proteins and other membranous proteins account for essentially all of the radioactivity of the inner membrane. The structural proteins of the outer membrane appear to be poorly labeled. Inner membranes isolated by a digitonin procedure can incorporate amino acids into their proteins. The requirements for this system parallel those for intact mitochondria and include a need for adenosine triphosphate, Mg^{2+}, a complete amino acid mixture, and EDTA. The mitochondria appear to contain adequate amounts of all the amino acids except for serine, methionine, and proline. Most of the ribonucleic acid of the mitochondria is present in the inner membrane. Bicine or Tricine buffer is superior to Tris in both systems.

The incorporation process is inhibited by puromycin and chloramphenicol but not by cycloheximide. Experiments based on exposure of mitochondria to different osmolarities of sucrose suggest that a minimal structural organization is required for the incorporation process.

It is now reasonably well established (Wheeldon and Lehninger, 1966; Beattie *et al.*, 1967) that mitochondria *in vitro* have the ability to incorporate amino acids into protein. Some disagreement existed in earlier work (Roodyn *et al.*, 1961; Truman and Korner, 1962; Kroon, 1963, 1964) concerning the requirements for the incorporation process. A contributing factor to these differences was probably the presence of varying amounts of bacterial contamination in the mitochondrial preparations of these workers. The report by Wheeldon and Lehninger (1966) in which bacterial contamination was minimized and that by Beattie *et al.* (1967) in which bacterial contamination had been largely eliminated appear to have established the requirements for amino acid incorporation on a sound basis. These results made possible a consideration of the location of the labeled proteins in the mitochondria. The approach to this problem was placed on firm morphological grounds by the development of methods to separate mitochondria into inner and outer membranes (Bachmann *et al.*, 1966; Schnaitman *et al.*, 1967). The present report describes experiments in which it has been demonstrated that the intramitochondrial location of the radioactive protein is associated with the fraction corresponding to the inner membrane and predominately into the "structural" proteins and other membranous proteins of this fraction. In addition, inner membranes prepared by the digitonin method (Schnaitman *et al.*, 1967) were also capable of incorporating amino acids into their proteins *in vitro*. Evidence has also been obtained which suggests that the maintenance of some structural feature of the inner membrane is necessary for this process to take place.

Experimental Section

Preparation and Fractionation of Mitochondria. Rat liver mitochondria were prepared under sterile conditions as previously described (Beattie *et al.*, 1967). The isolated mitochondria were fractionated by several procedures. The method of Beattie *et al.* (1966) was modified such that a single extraction with KCl (0.6 N) was used to remove cytochrome *c* and contractile protein in one fraction. The inner and outer membranes of the mitochondria were separated by the following modification of the phospholipase digestion method of Allmann *et al.* (1966). Mitochondria (30 mg/ml) in sucrose (0.25 M)–Tris (0.01 M, pH 7.6) were digested for 30 min at 37° with 100 μg/ml of phospholipase prepared from snake venom according to Bachmann *et al.* (1966). A similar separation of inner and outer membranes was achieved by the Ficoll method of Schnaitman *et al.* (1967) in which 60 mg of mitochondrial protein remaining after two extractions with water were suspended in 50 ml of 0.25 M sucrose and layered over 20.0 ml of 7.5% Ficoll in sucrose prior to density gradient centrifugation. Mitochondria were also fractionated with digitonin and separated into the inner membrane (9500*g* pellet), P_2 (40,000*g* pellet), outer membrane (140,000*g* pellet), and soluble fractions (140,000*g* supernatant) as described by Schnaitman *et al.* (1967). Protein in the whole mitochondria and in the various submitochondrial fractions

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was determined by the biuret method (Gornall *et al.*, 1949).

Amino Acid Incorporation into Mitochondrial Protein. Incubations were performed in duplicate as described in the legends to the tables. To terminate the reaction, the beakers were placed in ice, 2 mg of unlabeled L-leucine was added to each beaker, the contents were mixed, and 2 ml of 30% trichloroacetic acid was added to precipitate the protein. The values reported are corrected for a small zero-time control in ice.

Preparation of Protein. Proteins were prepared for counting as described previously (Beattie *et al.*, 1966) with a slight modification. After extraction with organic solvents, the proteins were dissolved in a known volume of 0.4 N NaOH. Aliquots (0.1 and 0.2 ml) were removed for counting in 10 ml of the solution described by Bray (1960) in a liquid scintillation counter (85% counting efficiency) and for protein determination by the method of Lowry *et al.* (1951).

Preparation of Structural Protein and Gel Electrophoresis. Structural proteins were prepared by the method of Criddle *et al.* (1962) and washed with acetone as described by Richardson *et al.* (1964). The protein was solubilized with 0.4 N NaOH, 2 M urea, and 0.1% sodium lauryl sulfate (200–500 μg), placed on polyacrylamide gel (Takayama *et al.*, 1966), and subjected to electrophoresis at 5 ma for 2 hr. The protein bands were visualized with 0.1% Amido Black stain for 1 hr, then destained electrophoretically at 10 ma until the gel was clear. The bands were sectioned and homogenized in Bray's (1960) solution containing 3% Cabosil for counting.

Enzymic Determinations. Isocitric dehydrogenase, succinic dehydrogenase, and α-ketoglutaric dehydrogenase were assayed as described by Bachmann *et al.* (1966) and monoamine oxidase as described by Schnaitman *et al.* (1967).

RNA was extracted from mitochondria and submitochondrial fractions according to the Schmidt–Thannhauser–Schneider procedure as described by Volkin and Cohn (1954). The orcinol reaction (Kerr and Seraidarian, 1945) was used for quantitation and the values were checked by phosphorus determination (Gomori, 1942) after RNA hydrolysis according to LePage (1957). Yeast RNA (Worthington) was used as a standard.

Chemicals. ATP,[1] PEP, TPN, cycloheximide, and lyophilized *Crotalus atrox* snake venom were obtained from Sigma; crystalline pyruvic kinase from Boehringer Mannheim; puromycin and puromycin aminonucleoside from Nutritional Biochemicals; actinomycin D from Merck Sharp and Dohme; Ficoll from Pharmacia; Tricine and Bicine buffers from Calbiochem; and uniformly labeled L-[^{14}C]leucine (250 μc/μmole) from New England Nuclear. Digitonin was recrystallized from hot ethanol prior to use.

[1] Abbreviations used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; PEP, phosphoenolpyruvate; TPN, triphosphopyridine nucleotide; GTP, guanosine triphosphate; UTP, uridine triphosphate; CTP, cytidine triphosphate.

Results

Mitochondria prepared under sterile conditions (*i.e.*, less than 100 bacteria/ml of incubation mixture) incorporate amino acids into protein in a linear manner for 40 min in the presence of adenine nucleotides, Mg^{2+}, EDTA, phosphate, a complete amino acid mixture minus leucine, and either succinate or an ATP-generating system consisting of PEP and pyruvic kinase (Beattie *et al.*, 1967). Such mitochondria when reisolated after incubation and fractionated according to the method of Beattie *et al.* (1966) contained 80% of the radioactivity of the intact mitochondria in the fractions corresponding to structural proteins and other insoluble membranous proteins, designated cytochromes, which contains, among other components, cytochromes *a*, *b*, and c_1 (Table I). These results are in agreement with those of Roodyn (1962), Wheeldon and Lehninger (1966), and Kadenbach (1967).

TABLE I: Distribution of Radioactivity in Mitochondrial Subfractions.[a]

Fraction	Cpm/mg	Protein (mg)	Total (cpm)
Mitochondria	1,370	164	224,000
Water soluble	214	66.8	
KCl soluble	338		
Structural proteins	2,500	45.5	117,000
Cytochromes	1,270	50.7	64,500

[a] Intact mitochondria were incubated 40 min in air in a metabolic shaker. The medium contained 50 mM Tris (pH 7.6), 10 mM $MgCl_2$, 5 mM sodium phosphate (pH 7.6), 5 mM PEP, 20 μg of pyruvic kinase, 2 mM ATP, 2 mM EDTA, 22.5 μg/ml of a complete amino acid mixture minus leucine (as described by Roodyn *et al.* (1961)), 0.5 μc of uniformly labeled L-[^{14}C]leucine, 2–3 mg of mitochondrial protein, and 0.154 M KCl to a final volume of 2.0 ml. After the addition of 10 mM unlabeled L-leucine, the mitochondria were reisolated at 12,000*g*, washed once with 0.25 M sucrose, and then fractionated according to the method of Beattie *et al.* (1966).

The structural proteins which had a specific activity two times that of the intact mitochondria were washed with acetone according to the method of Richardson *et al.* (1964) and subjected to disc electrophoresis on polyacrylamide gel. In accord with the results of Haldar *et al.* (1966), the structural protein fraction prepared in this manner from rat liver mitochondria was heterogeneous and at pH 8.0 separated into twelve bands of which six had significant radioactivity.

Previous methods to determine the distribution of radioactivity in mitochondrial proteins labeled with amino acids *in vitro* had been based on solubility

TABLE II: Distribution of Radioactivity between Inner and Outer Membrane Prepared by Phospholipase Method.[a]

Fraction	Cpm/mg of Protein	Protein (mg)	Total (cpm)	Succinic Dehydrogenase Total Units
Mitochondria	5,100	56.2	287,000	644
Inner membrane fraction	13,600	19.3	264,000	487
Structural proteins	19,200			
Cytochromes	10,800			
Outer membrane fraction	682	42.5	28,900	50

[a] Intact mitochondria were incubated with [^{14}C]leucine and reisolated as described in the legend to Table I prior to fractionation according to the phospholipase method of Allmann *et al.* (1966). The succinic dehydrogenase activity is expressed as millimicromoles of indophenol reduced per minute.

TABLE III: Distribution of Radioactivity between Inner and Outer Membrane Prepared by Ficoll Method.[a]

Fraction	Cpm/mg	Protein (mg)	Total (cpm)	Succinic Dehydrogenase		Monoamine Oxidase	
				Total Units	Sp Act.	Total Units	Sp Act.
Mitochondria	3,740	102	375,000	10,900	107	596	4.9
Water-soluble I	189	43.8	8,280				
Water-soluble II	509	3.5	1,780				
Outer membrane fraction	3,110	10.8	33,500	1690	15.6	122	11.3
Inner membrane fraction	6,040	43.7	264,000	7600	173	167	3.8
Structural proteins	10,700	18.6	199,000				

[a] Intact mitochondria were incubated with [^{14}C]leucine, and reisolated as described in the legend to Table I prior to fractionation by the Ficoll method of Schnaitman *et al.* (1967). Succinic dehydrogenase activity is expressed as millimicromoles of indophenol reduced per minute (total), or per milligram of protein in the fraction (specific activity). Monoamine oxidase is expressed as millimicromoles of benzaldehyde formed per minute (total) or per milligram of protein (specific activity).

properties of these proteins. These methods afforded a distribution which could be followed in terms of enzymic activities; however, these procedures provided little information as to the morphological correlations of these activities. The development of methods to separate mitochondria into morphologically definable entities made it reasonable to apply these methods to mitochondria labeled with [^{14}C]leucine and thus determine the distribution of radioactivity in the proteins of these morphological categories.

The phospholipase digestion method of Allmann *et al.* (1966) permits the separation of the inner and outer membranes of the mitochondrion. The results presented in Table II indicate that 90% of the total radioactivity was present in the inner membrane fraction. This correlates well with retention of 91% of the total succinic dehydrogenase in this fraction. The isocitric dehydrogenase and α-ketoglutarate dehydrogenase used by Bachmann *et al.* (1966) as markers for the outer membrane were absent from the inner membrane fraction and were present in the outer membrane fraction. In contrast to the results of Allmann *et al.* (1966) who found 60% of the total beef heart mitochondrial protein in this fraction, only 31% of the original protein was found in the inner membrane fraction of incubated rat liver mitochondria. The specific activity of the inner membrane was 2.7 times that of the intact mitochondria, while structural proteins prepared from the purified inner membrane had a specific activity nearly four times that of the intact mitochondria. In one experiment, it was calculated that 80% of the total radioactivity was present in the structural proteins fraction from the inner membrane prepared by the phospholipase digestion method.

In similar fractionation experiments carried out by the Ficoll method of Schnaitman *et al.* (1967), similar results were obtained (Table III). Over 70% of the radioactivity was associated with the inner membrane

TABLE IV: Distribution of Protein, RNA, and Enzymic Activities in Liver Mitochondria Fractionated by the Digitonin Method.[a]

Fraction	Protein (mg)	RNA[b] Total (μg)	RNA[b] μg/mg	Isocitric Dehydrogenase Total	Isocitric Dehydrogenase Sp Act.	Succinic Dehydrogenase Total	Succinic Dehydrogenase Sp Act.	Monoamine Oxidase Total	Monoamine Oxidase Sp Act.
Mitochondria	127	1150	9.1			5710	45	630	4.96
Inner membrane	74.6	470	6.30	9640	129	3850	52	25.4	0.34
P_2	15.4	363	23.6			732	47	51.5	3.34
Outer membrane	5.6	76.9	13.7			61	11	219	39.5
Soluble	24.7	101	4.09	2940	119	61	2.5	490	19.8

[a] Liver mitochondria were fractionated with 1.1 mg of digitonin/10 mg of mitochondrial protein according to the method of Schnaitman *et al.* (1967). Enzymic activities expressed as described in legend to Table III, with isocitric dehydrogenase expressed as millimicromoles of TPN reduced per minute. [b] Values for mitochondria are based on phosphorus determination, all other values are based on ribose (orcinol). Ribose × (2/P) for mitochondria = 0.57; for all submitochondrial fractions = 1.

fraction and of this 75% was in the structural proteins fraction prepared from the inner membrane. Less than 10% of the total radioactivity was in the outer membrane fraction. This distribution was reflected in the specific activities of these fractions. The adequacy of the separation was confirmed by the distribution of succinic dehydrogenase activity, a marker for the inner membrane (Bachmann *et al.*, 1966) and monoamine oxidase shown by Schnaitman *et al.* (1967) to be associated with the outer membrane.

This separation is in essential agreement with that obtained by Schnaitman *et al.* (1967). The inner membrane obtained in this study appears to be cleaner than that of these workers as evidenced by a 60% increase in the specific activity of succinic dehydrogenase in this fraction as compared to a 25% increase in cytochrome oxidase, the marker used by Schnaitman *et al.* The separation of the outer membrane, however, was not as adequate, since the specific activity of monoamine oxidase increased 2.4-fold as compared to a 3.9-fold increase reported by Schnaitman *et al.*

Mitochondria were also fractionated by the digitonin method of Schnaitman *et al.* (1967); however, this method could not be applied to previously incubated mitochondria, since the enzymic activities (*i.e.*, succinic dehydrogenase and monoamine oxidase) were not distributed among the inner membrane, broken inner membrane (P_2), and outer membrane fractions in a clear-cut manner. This method was successfully applied to nonincubated mitochondria as evidenced by the data of Table IV. Approximately 60% of the total mitochondrial protein was associated with the inner membrane fraction as well as 77% of the isocitric dehydrogenase and 82% of the succinic dehydrogenase. Monoamine oxidase activity was essentially absent from the inner membrane fraction and concentrated in the outer membrane and soluble fractions. These results are in agreement with Schnaitman *et al.* Of the total RNA, 41% was in the inner membrane fraction, and 72% in the combined inner membrane and P_2 fractions. The great advantage of the digitonin method for fractionating mitochondria was the isolation of inner membrane fractions which possessed the ability to incorporate amino acids. The purified inner membranes obtained by the two previous methods were inactive in this respect.

The properties of the digitonin inner membrane particles are described and compared to those of intact mitochondria in the data presented in Table V. It may be seen that these properties are essentially identical with the exception that the ATP-generating system could not be replaced by succinate in the digitonin particles as it could be with intact mitochondria. The requirement for an amino acid mixture is apparent, but this could be partially replaced by the addition of the three amino acids, serine, methionine, and proline. The omission of any one of the amino acids of the complete mixture resulted in small decreases in the incorporation except for serine, methionine, and proline where decreases of 50, 35, and 75%, respectively, were obtained. These data would suggest that all the amino acids are necessary for the incorporation process, but that the amounts of these three amino acids are limiting in the mitochondrion.

It is noteworthy that Tricine or Bicine buffer (Good *et al.*, 1966) were superior to Tris in both systems. The deleterious effect of Tris is of interest in view of the report of Heredia and Halvorson (1966) that Tris accelerates the spontaneous hydrolysis of phenylalanyl-tRNA. We have observed that the substitution of Bicine for Tris in studies of oxidative phosphorylation measured by the oxygen electrode resulted in no significant differences.

Puromycin caused a complete inhibition of amino acid incorporation, while actinomycin D was slightly inhibitory in digitonin particles but without effect in

TABLE V: Properties of Leucine Incorporation into Digitonin Particles Compared to Intact Mitochondria.[a]

	Values Expressed as % of Complete System	
	Intact Mitochondria	Digitonin Particles
Complete	100	100
Minus amino acid mixture	23	20
Serine, methionine, and proline in place of amino acid mixture	80	79
Minus EDTA	53	26
KCl replaced by sucrose	7	8
Low Mg^{2+} (mM)	31 (5)	2 (2)
Low ATP (0.4 mM)	25	36
Succinate (10 mM) in place of PEP	135	27
Bicine buffer in place of Tris	220	173
Tricine buffer in place of Tris	195	158
Plus chloramphenicol (50 μg/ml)	15	21
Plus cycloheximide (500 μg/ml)	100	100
Plus puromycin (150 μg/ml)	5	1
Plus puromycin aminonucleoside (225 μg/ml)	100	100
Plus actinomycin D[b] (40 μg/ml)	100	70

[a] Complete system as described in legend to Table I. Digitonin particles prepared as described by Schnaitman *et al.* (1967). [b] Beakers preincubated 15 min in complete system plus inhibitor prior to addition of [^{14}C]-leucine.

intact mitochondria. A surprising observation was the finding that cycloheximide did not inhibit the incorporation process, while chloramphenicol, as previously reported by Kroon (1963) and Wheeldon and Lehninger (1966), was very inhibitory. Although very high concentrations of cycloheximide were used in these experiments, the possibility existed that this inhibitor did not enter the mitochondrion or the digitonin inner membrane. This possibility was approached experimentally by subjecting the mitochondria to a number of conditions which are known to alter mitochondrial structure. The data of Table VI indicate that freezing and water treatment of mitochondria decreased the incorporation ability; however, under all these conditions cycloheximide was still without any significant effect while chloramphenicol caused a large inhibition.

The inhibition of amino acid incorporation by water treatment or freezing of the mitochondria is subject to several interpretations. One possibility is that the inhibition is due to the dilution of small molecules necessary for the incorporation process; however, supplementation of the incubation mixture with GTP, UTP, and CTP as well as the concentrated supernatant from the water-treated mitochondria and the high-speed supernatant from the whole homogenate was without effect. A second possible explanation is that the inhibition is caused by a loss of essential enzymes, since Caplan and Greenawalt (1966) have shown that water extracts some mitochondrial proteins. A third explanation is the activation of degradative enzymes which might destroy components of the incorporation process. Still another possibility is that osmotic shock may modify an intramitochondrial structural feature indispensable for amino acid incorporation.

An experimental approach to differentiate among these last three possibilities is presented in the experiment described by Figure 1. Mitochondria, after four washes, were suspended in various molarities of sucrose ranging from 0.25 M to water for 5 min in an ice bath. The mitochondria were reisolated and suspended in 0.25 M sucrose at a final concentration of 6 mg/ml of protein and incubated under the indicated conditions. In addition, the protein content and isocitric dehydrogenase activity of the supernatant from the sucrose treatments were also measured. The data of Figure 1 indicate that essentially no protein or isocitric dehydrogenase were released until the mitochondria were exposed to concentrations of sucrose less than 0.06 M; however, at this same concentration of sucrose, amino acid incorporation under all conditions studied had decreased to 30% of the maximum values obtained. In addition, it may be noted that regardless of incubation conditions, parallel inhibition curves resulted. This would indicate that pretreatment of the mitochondria with different osmolarities of sucrose has the same effect on the incorporation process whether ATP was generated *via* succinate or the PEP system (curves 1 and 2). Thus, a relationship may exist between the structural elements responsible for ATP generation from succinate and those involved in amino acid incorporation. This conclusion was further substantiated by studies of oxidative phosphorylation on mitochondria exposed to different osmolarities of sucrose in this same manner. Respiratory control and ADP:O ratios decreased at the same osmolarity as amino acid incorporation. The possibility that degradative enzymes may be involved was made unlikely by the finding that preincubation of the treated mitochondria for 30 min prior to addition of radioactive leucine resulted in an incorporation curve which decreased in a parallel manner as that observed for nonpreincubated mitochondria (curve 3).

Discussion

Little evidence is available concerning the intramitochondrial location of the proteins into which amino acids are incorporated *in vitro*. Information on this subject is of potential significance in the eventual

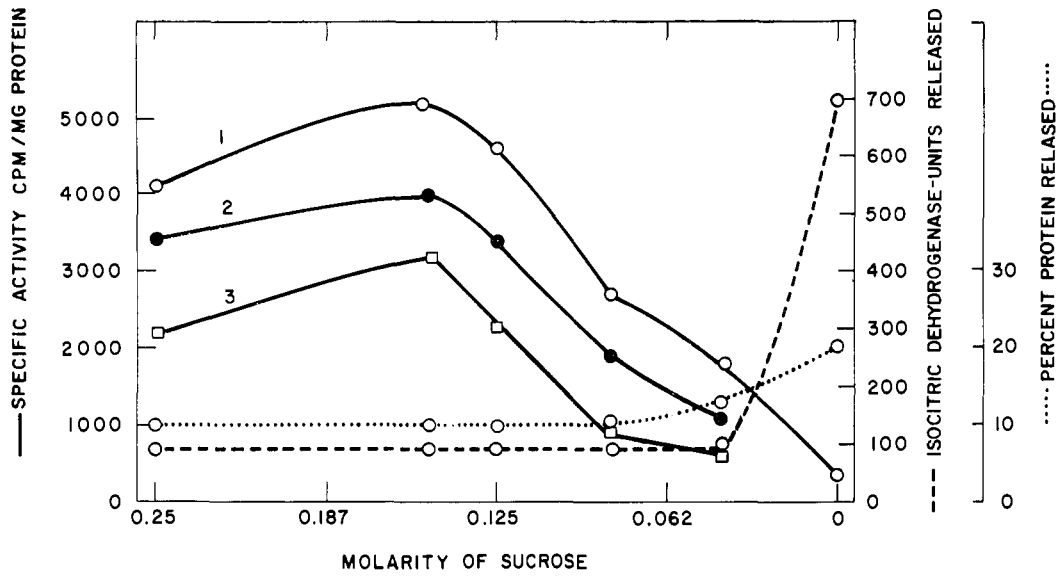

FIGURE 1: The effect of pretreating mitochondria with sucrose of different osmolarities on the [^{14}C]leucine incorporation into mitochondrial protein. The counts per minute per milligram of protein obtained with: (○—○) external ATP-generating system, (●—●) succinate, and (□—□) after preincubation of mitochondria for 30 min in complete system prior to addition of [^{14}C]leucine. (○ · · · ○) Per cent of protein released into the supernatant. (○ - - - ○) Isocitric dehydrogenase activity expressed as millimicromoles of TPN reduced per minute.

understanding of mitochondrial biogenesis. The availability of methods to fractionate mitochondria into morphologically definable subfractions has provided a first approach to this problem. The data presented in Tables II and III indicate that eight to nine times as much radioactivity appeared in the inner membrane compared to the outer membrane. When the inner membranes prepared either by the phospholipase digestion method of Allmann *et al.* (1966) or the Ficoll method of Schnaitman *et al.* (1967) were further subfractionated, the vast majority of counts was present in structural proteins (Table III). It is of interest that the specific activity of structural proteins isolated from inner membranes prepared by either method was three to four times that of intact mitochondria, while the specific activity of structural proteins prepared from mitochondria in which inner and outer membranes were not separated increased only twofold (Table I). These differences may be explained by the presence in the latter case of poorly labeled structural proteins which is present also in the outer membrane (Lenaz *et al.*, 1967).

It has been reported by Haldar *et al.* (1966) that structural protein isolated from liver mitochondria by the method of Richardson *et al.* (1964) was heterogeneous and on disc electrophoresis separated into four major bands plus some minor bands. We have confirmed these results and demonstrated that structural protein as we prepare it is a multicomponent system. Thus, it is apparent that amino acids are incorporated into a number of insoluble proteins associated with the inner membrane of the mitochondrion. This conclusion was further emphasized by the incorporation of considerable radioactivity into another insoluble membrane fraction designated cytochromes (see Table I), since in previous work this fraction had been found to contain among other things cytochromes *a*, *b*, and c_1 (Beattie *et al.*, 1966). The proteins labeled in this fraction are not known; however, Kadenbach (1967) has reported that under *in vitro* conditions succinic dehydrogenase and cytochrome *a* are not labeled. In accord with numerous reports (Roodyn *et al.*, 1962: Kadenbach, 1967) little incorporation was found in the soluble proteins.

It is to be noted that the phospholipase method of fractionation yields 70% of the mitochondrial protein in the outer membrane fraction (Table II) while in the Ficoll method, only 10% appears in this fraction (Table III). This distribution of protein is in accord with the conclusions of Parsons *et al.* (1967) that the outer membrane fraction obtained by the phospholipase method is contaminated with soluble enzymes of the tricarboxylic acid cycle which are associated with the inner membrane or the matrix. As pointed out above, such proteins are not labeled under *in vitro* conditions. Hence, their presence in the outer membrane fraction obtained by the phospholipase method would not only account for the increased protein present, but would also account for the considerably larger ratio of the specific activities of the inner to outer membrane fractions obtained using this procedure (Table II) to that obtained using the Ficoll method (Table III). In agreement with the above, is the observation that in the Ficoll procedure, 46% of the total mitochondrial

protein appears in the water-soluble fractions (Table III), and these proteins have both low specific and low total radioactivities.

The location of the vast majority of radioactivity in the inner membrane fraction after incubation of whole mitochondria suggested the possibility that purified inner membranes might have the ability to incorporate amino acids. The inability of inner membranes prepared by the Ficoll method of Schnaitman *et al.* (1967) to incorporate amino acids was not surprising, since this method involves water treatment which we have shown has a deleterious effect on the incorporating ability of intact mitochondria (Table VI and Figure 1). Inner membranes prepared by the phospholipase method of Allmann *et al.* (1966) were also inactive. This may reflect an enzymatic modification of lipoidal elements of the mitochondrial structure needed for the incorporation process. Attempts to prepare active submitochondrial particles by sonication in the medium of Hansen and Smith (1964) or in 0.25 M sucrose were unsuccessful. Inner membranes prepared by the digitonin method yielded active particles which had properties similar to those of the intact mitochondria (Table V).

TABLE VI: The Effect of Chloramphenicol and Cycloheximide on Leucine Incorporation into Disrupted Mitochondria.[a]

	Cpm/mg of Protein		
Treatment	Control	+CAP (50 μg/ml)	+Cycloheximide (500 μg/ml)
Intact mitochondria	5880	790	6260
Frozen 1 time	3610	606	3320
Frozen 2 times	1460	250	1180
Mitochondria suspended in H_2O	687	141	771
Mitochondria suspended in 0.154 M KCl	5210	998	5210

[a] Complete system as described in the legend to Table I except Tris buffer replaced with Bicine. After four washes in 0.25 M sucrose, the mitochondria were suspended in 0.25 M sucrose, water, or 0.154 M KCl such that the final protein concentration in each case was approximately 6 mg/ml.

The inhibition by chloramphenicol and the lack of inhibition by cycloheximide strongly suggest the possibility that the mitochondrial incorporation system is qualitatively different from the process occurring in microsomes. There is evidence that these inhibitors interact with ribosomes and that the differential effect of these inhibitors on mammalian and bacterial systems can be accounted for on this basis. Thus, Vazquez (1966) has demonstrated that chloramphenicol interacts specifically with the 50S subunit of 70S bacterial ribosomes and not with 80S ribosomes from a number of organisms. Siegel and Sisler (1965) have suggested that cycloheximide interacts with mammalian ribosomes. This would suggest that if the mitochondrial incorporation system proceeds by a ribosomal mechanism, which seems likely in view of the observations that tRNAs (Barnett and Brown, 1967; Barnett *et al.*, 1967) and mRNAs (Wintersberger, 1966; Luck and Reich, 1964; Neubert and Helge, 1965) have been detected and that ribosomelike particles have been observed under the electron microscope (Kislev *et al.*, 1965), then these ribosomes are different from extramitochondrial ribosomes. Evidence to this effect has been presented by O'Brien and Kalf (1967).

Determination of the RNA content of various submitochondrial fractions indicated that 72% of the total mitochondrial RNA was present in the inner membrane or derivatives thereof, although the inner membrane fraction (9500*g* pellet) used for incorporation studies contained 41% of the total RNA. The outer membrane appeared to contain RNA which could not be accounted for solely on the basis of inner membrane contamination. If the content of succinic dehydrogenase in the outer membrane is taken as a measure of contamination by broken inner membranes, the more probable contaminating fraction, a contamination of 8.4% can be calculated. On this basis the RNA content of the outer membrane would be 31 μg instead of the observed 77 μg. The outer membranes, however, are sedimented at 140,000*g* for 1 hr, conditions under which any contaminating microsomes would also be sedimented. Hence, the presence of RNA in the outer membrane of the mitochondrion is still open to question. The location of the vast majority of RNA in the inner membrane is consistent with the ability of this fraction to incorporate amino acids into proteins.

The decrease of amino acid incorporating ability in mitochondria exposed to hypotonic solutions or freezing permits a number of explanations as previously described. A possible contributing factor is the loss of essential small molecules. This does not appear to be of consequence, since reactivation could not be obtained by supplementation with concentrates of the supernatant of the water-treated mitochondria or by the addition of other substances. In support of this conclusion, is the finding that preincubation of mitochondria which had been treated with 0.15 M sucrose did not result in lower incorporation than those treated with 0.25 M sucrose; one would expect an enhanced loss of small molecules from mitochondria treated with a hypotonic solution. Neither does the decrease in the incorporating activity appear to result from the loss of an enzyme or protein, since no detectable loss of protein or isocitric dehydrogenase activity was observed until the incorporating activity had decreased by 70%. This does not obviate the possibility that an extremely small amount of an

essential protein was lost under these conditions, but this process would have to be a highly selective one.

The possibility that activation of various degradative enzymes was involved as a consequence of exposing mitochondria to hypotonic conditions suggests that preincubation of the treated mitochondria would result in an increased loss of incorporating activity. As seen from Figure 1 (curve 3), this was not found to be the case.

The hypothesis that osmotic shock destroys structural elements of the mitochondria essential for the incorporation process is consistent with the observations. The close correlation between the onset of loss of incorporation supported by either succinate or an external ATP-generating system and the decrease in respiratory control and ADP:O ratios suggests that the structural elements required for the incorporation process are sensitive to similar conditions as are those involved in oxidative phosphorylation. This does not necessarily imply that these two structural entities are identical, although they may very well have certain features in common. The necessity for structural integrity is also in accord with the observation that inner membranes prepared by phospholipase digestion are inactive. A possibility to be considered is that the translation process of the mitochondrial mRNA takes place upon an element of the inner membrane.

Added in Proof

While the manuscript was in press, Neupert *et al.* (1967) reported that mitochondria *in vitro* incorporate amino acids predominately into the proteins of the inner membrane.

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