Studies on the Biosynthesis of Mitochondrial Protein Components*

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ABSTRACT: Rat liver and kidney mitochondria labeled *in vivo* with radioactive leucine or valine were fractionated to provide a number of fractions that are reasonably well-recognized biochemical entities. The specific activities of the proteins of these fractions were compared to the specific activity of the unfractionated mitochondrial protein at times varying from 2 min to 8 hr after injection of the labeled amino acid. At short times the specific activities of the water-soluble proteins and the fraction containing cytochrome *c* were significantly lower than the specific activity of the unfractionated mitochondrial protein from both tissues. At 2 hr, the specific activity of the water-soluble proteins from kidney mitochondria had increased 300% over that of the unfractionated mitochondrial protein. By 8 hr, the specific activities of all fractions approximated that of the whole mitochondria. These results suggest that certain mitochondrial proteins may be synthesized outside the mitochondria and subsequently incorporated into the mitochondrial structure.

The *in vivo* incorporation of radioactive amino acids into mitochondrial protein has been shown by Hultin (1950) and by Keller *et al.* (1954). In an attempt to locate the sites of amino acid incorporation in mitochondria, Truman (1963) examined submitochondrial fragments for those with the highest protein specific activity after the *in vivo* administration of a radioactive amino acid. He fractionated liver mitochondria by three different procedures which yield submitochondrial particles with varying degrees of electron transport and oxidative phosphorylation activity: digitonin treatment, deoxycholate treatment, and ethanol treatment. He found that particles associated with the mitochondrial membrane were the site of most rapid incorporation of amino acids into proteins.

The problem of the biogenesis of the protein components of mitochondria has also been approached in this study by the fractionation of liver and kidney mitochondria labeled *in vivo* with radioactive amino acids. The method of fractionation used, however, is based on differential solubility of mitochondrial components to provide a number of fractions that are reasonably well-recognized biochemical entities. The time course of *in vivo* amino acid incorporation into mitochondrial proteins has been examined at intervals between 2 min and 8 hr. The data indicate that the specific activities of the fractions containing the water-soluble proteins and cytochrome *c* show the greatest variation when compared to the specific activity of the proteins of unfractionated mitochondria. These results have lead to some suggestions concerning the biogenesis of some of the protein components of the mitochondria.

Experimental Section

Thirty microcuries of uniformly labeled [^{14}C]L-leucine or L-valine (specific activity 200 mc/mmole) was administered intravenously to adult male rats and the animals were killed at 2, 5, and 30 min, and 2, 4, and 8 hr after injection. The mitochondria of the liver and kidney were prepared in 0.25 M sucrose by the method of Schneider and Hogeboom (1950) as modified by Weinbach (1961) and routinely washed four times with 0.25 M sucrose.

Fractionation of the Mitochondria. The washed mitochondria were extracted with water for 5 min at 30° to remove the water-soluble proteins, which contain, among other things, malic and glutamic dehydrogenases (Bendall and de Duve, 1960), and centrifuged for 10 min at 15,000 rpm in the SS 34 rotor of the Serval RC-2 centrifuge. The resulting pellet was then extracted with 0.9% KCl for 5 min at 30° to remove cytochrome *c* (Jacobs and Sanadi, 1960) and centrifuged at 12,000 rpm for 10 min. The resulting pellet was then extracted with 0.6 M KCl for 10 min at 30° to remove contractile protein (Ohnishi and Ohnishi, 1962) and some remaining cytochrome *c* and centrifuged at 15,000 rpm for 10 min.

The residue after removal of soluble protein, cytochrome *c*, and contractile protein was treated with sodium cholate, sodium deoxycholate, and sodium lauryl sulfate according to the procedure of Criddle *et al.* (1962) for the preparation of structural protein. The solubilized supernatant was treated with a few milligrams of solid $Na_2S_2O_4$ and K_2CO_3, and brought to 13% saturation with neutralized saturated ammonium sulfate. The structural protein, which was sedimented by centrifugation at 17,000 rpm (34,800*g*) for 10 min, was washed with 0.25 M sucrose, precipitated with trichloroacetic acid, and extracted as described under preparation

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TABLE I: Specific Activities of the Proteins from Rat Liver Mitochondria and Submitochondrial Fractions at Various Times after Administration of [^{14}C]L-Leucine.[a]

Fraction Corresponding to	2 Minutes cpm/mg of Protein	% Change	p	5 Minutes cpm/mg of Protein	% Change	p	30 Minutes cpm/mg of Protein	% Change	p
Whole mitochondria	88.3 ± 8.4 (6)			180 ± 22 (5)			433 ± 46 (5)		
Water-soluble proteins	33.9 ± 9.1 (5)	−62	<0.01	109 ± 22 (5)	−39	<0.1	387 ± 46 (5)	−11	Insig
Cytochrome *c*	29.9 ± 4.4 (6)	−66	<0.001	117 ± 36 (4)	−35	<0.2	404 ± 35 (5)	−7	Insig
Other cytochromes	73.4 ± 12.7 (6)	−17	Insig	131 ± 16 (5)	−27	<0.2	405 ± 47 (5)	−7	Insig
Contractile protein	125 ± 45 (6)	+41	<0.2	191 ± 59 (4)	+6	Insig	489 ± 63 (5)	+13	Insig
Structural protein	92.5 ± 17.3 (6)	+5	Insig	170 ± 23 (5)	−5	Insig	467 ± 55 (5)	+8	Insig

Fraction Corresponding to	2 Hours cpm/mg of Protein	% Change	p	4 Hours cpm/mg of Protein	% Change	p	8 Hours cpm/mg of Protein
Whole mitochondria	468 ± 59 (4)			424 ± 83 (3)			379 ± 100 (2)
Water-soluble proteins	397 ± 33 (5)	−15	Insig	335 ± 24 (3)	−21	Insig	332 ± 66 (2)
Cytochrome *c*	369 ± 36 (5)	−21	<0.2	368 ± 23 (3)	−13	Insig	362 ± 79 (2)
Other cytochromes	392 ± 80 (5)	−16	Insig	379 ± 57 (3)	−11	Insig	340 ± 154 (2)
Contractile protein	394 ± 64 (4)	−16	Insig	443 ± 43 (3)	+5	Insig	465 ± 67 (2)
Structural protein	414 ± 53 (5)	−11	Insig	352 ± 11 (3)	−17	Insig	335 ± 93 (2)

[a] The specific activities are presented as the mean ± the standard error. The figures in parentheses indicate the number of experimental animals. When only two animals were involved, *p* values were not calculated. Insig = insignificant.

of protein, rather than treatment with butanol, deoxycholate, and methanol as described by Criddle *et al.* (1962). The supernatant after sedimentation of the structural protein was increased to 50% saturation with ammonium sulfate to yield a fraction designated cytochromes.

Spectral examination of the various fractions indicated the following distribution of components (the quantities given are expressed as the total amount of the component obtained from one rat liver): water-soluble fraction, flavin, and traces of denatured cytochrome or hemoglobin; cytochrome *c* fraction, 0.064 μmole of cytochrome *c;* contractile protein fraction, 0.027 μmole of cytochrome *c;* structural protein fraction, traces of denatured cytochrome or hemoglobin; and cytochrome fraction, 0.147 μmole of cytochromes *a*, *b*, and c_1.

Preparation of Protein. The proteins of the mitochondria and all fractions were precipitated with trichloroacetic acid in a final concentration of 5% and washed by a modification of the method of McLean *et al.* (1958). This consisted of washing the protein two times with cold 5% trichloroacetic acid and once with 5% trichloroacetic acid at 90° for 15 min. The proteins were then dissolved in 2 ml of 0.4 M NaOH containing 2 mg of the appropriate unlabeled amino acid and allowed to stand at room temperature for 20 min. The proteins were reprecipitated with trichloroacetic acid in a final concentration of 10% and then extracted once with 95% ethanol containing 10% potassium acetate and two times with 3:1 ethanol–ether for 5 min at 60°. The proteins were dissolved in a known volume of 3% NaOH and an aliquot was removed for protein determination by the Lowry method (1951). The remaining protein was quantitatively reprecipitated with trichloroacetic acid, washed once with water, and dissolved in Hyamine. A small aliquot (0.1 ml) of the protein dissolved in Hyamine was counted in 10 ml of Bray's solution (1960) in a liquid scintillation counter.

Distribution of [^{14}C] Leucine in Mitochondrial Protein. Mitochondria isolated from the liver of animals injected with radioactive leucine were treated with fluorodinitrobenzene (FDNB)[1] and hydrolyzed with acid according to the method described by Fraenkel-Conrat *et al.* (1955). The counts/min were corrected for quenching due to light absorption of the dinitrophenyl (DNP) amino acids.

Results

The specific activities of the proteins from mitochondria, and submitochondrial fractions, isolated from rat liver at various time intervals after the intravenous injection of uniformly labeled [^{14}C]L-leucine are given in Table I. When the specific activities of the proteins of the submitochondrial fractions were compared to those of the proteins of unfractionated mito-

[1] Abbreviations used: FDNB, fluorodinitrobenzene.

TABLE II: Specific Activities of the Proteins from Rat Kidney Mitochondria and Submitochondrial Fractions at Various Times after Administration of [^{14}C]L-Leucine.[a]

Fraction Corresponding to	2 Minutes		5 Minutes			30 Minutes			2 Hours			4 Hours	8 Hours
	cpm/mg of Protein		cpm/mg of Protein	% Change	p	cpm/mg of Protein	% Change	p	cpm/mg of Protein	% Change	p	cpm/mg of Protein	cpm/mg of Protein
Whole Mitochondria	150 ± 30	(2)	315 ± 47 (5)			681 ± 40 (4)			547 ± 114 (5)			618 ± 49 (2)	624 ± 323 (2
Water Soluble Protein	113 ± 12	(2)	105 ± 35 (5)	−67	<0.01	800 ± 237 (4)	+17	Insig	2140 ± 298 (5)	+291	<0.01	2180 ± 990 (2)	714 ± 336 (2)
Cytochrome *c* + Contractile Protein	80.2 ± 5.8	(2)	132 ± 26 (5)	−58	<0.02	628 ± 50 (4)	−8	Insig	510 ± 115 (3)	−7	Insig	546 ± 189 (2)	...
Other Cytochromes	75.4 ± 21	(2)	202 ± 19 (5)	−36	<0.1	485 ± 72 (4)	−29	<0.05	458 ± 84 (4)	−16	Insig	364 ± 9 (2)	536 ± 151 (2)
Structural Protein	119 ± 13	(2)	478 ± 71 (4)	+52	<0.2	583 ± 39 (4)	−14	<0.1	412 ± 58 (4)	−25	Insig	507 ± 34 (2)	419 ± 183 (2)

[a] The specific activities are presented as the mean ± the standard error. The figures in parentheses indicate the number of experimental animals. When only two animals were involved, p values were not calculated. Each of the two determinations at 2 min represents the pooled kidneys from two rats. Insig = insignificant.

chondria, it may be seen that differences occur only at the shortest time intervals. At 2 min the specific activities of the protein of the water-soluble fraction and the fraction containing cytochrome *c* were significantly lower (62 and 66%) than the specific activity of the proteins of unfractionated mitochondria. This decrease in specific activity persisted, at a lower level, at 5 min, although the change in the cytochrome *c* fraction was of doubtful significance. At times greater than 5 min, no changes in specific activity of these fractions were observed. The remaining three fractions, that containing the other cytochromes and those corresponding to the contractile protein and the structural protein, showed no significant change from the unfractionated mitochondria throughout the times investigated.

With the kidney the procedure for the fractionation of the mitochondria was modified slightly. Because of the small amount of material available from this organ, the cytochrome *c* fraction and the contractile protein fraction were extracted in one step. At 5 min this fraction, as well as the water-soluble proteins and the fraction containing the other cytochromes, had specific activities significantly lower than the proteins of the unfractionated kidney mitochondria (Table II), while the specific activity of the structural protein fraction showed an increase, although of only marginal significance. It is to be noted that at 2 min all fractions had specific activities lower than that of the unfractionated mitochondria. At times greater than 5 min, the specific activity of the structural protein fraction decreased relative to that of the unfractionated mitochondria while the specific activities of fractions containing cytochrome *c* plus contractile protein and the other cytochromes increased so that at 2 hr all these fractions had essentially the same specific activity as the unfractionated mitochondria. The specific activity of the water-soluble proteins, however, increased so that at 2 hr it was 300% greater than the proteins of the unfractionated mitochondria. Not until 8 hr was the specific activity of this fraction approximately equal to that of the unfractionated mitochondria.

Similar results have also been obtained using [^{14}C]-L-valine in place of [^{14}C]L-leucine. It is of interest that the specific activity of the proteins of the unfractionated mitochondria of both the liver and the kidney reached a maximum at 30 min.

After treatment of the washed protein from unfractionated mitochondria with FDNB, only 45 cpm (0.8%) was found in the ether-soluble, DNP-amino acid fraction, and 5330 cpm (99.2%) in the water-soluble fraction. These results indicate that leucine is not incorporated preferentially into the N-termini of mitochondrial proteins.

In an attempt to check for microsomal contamination, the mitochondrial supernatant and the microsomes, obtained by centrifugation of the mitochondrial supernatant at 100,000*g* for 60 min, were isolated from a rat liver 5 min after the intravenous injection of [^{14}C]-leucine. The supernatant containing 600,000 cpm and the microsomes containing 900,000 cpm were added to a nonradioactive rat liver homogenate and the mito-

chondria were isolated and fractionated in the usual manner. The mitochondria obtained under both experimental conditions contained less than 0.1% of the added counts. Each mitochondrial subfraction had the same specific activity as the unfractionated mitochondrial protein. These results suggest that microsomal contamination is negligible and does not affect the distribution of radioactivity in the submitochondrial fractions.

Discussion

Several theories have been presented concerning the biogenesis of mitochondria (Rouiller, 1960; Novikoff, 1961; Wallace and Linnane, 1964). There is now evidence which suggests that in the liver (Bahr and Zeitler, 1962) and in *Neurospora crassa* (Luck, 1963) mitochondria can reproduce by fission and subsequent growth. Thus, it seems probable that in relatively short term experiments, after administration of radioactive substances, one would observe the addition to preexisting mitochondria of radioactive components of varying degrees of complexity. On isolation and subfractionation of these mitochondria, the specific activity of a given fraction would be a function of both the radioactive and nonradioactive parts of the mitochondria. In this view any difference in the specific activity of a submitochondrial fraction from that of the unfractionated mitochondria takes on additional significance.

The experiments reported here were designed in an attempt to clarify the problem of the biogenesis of the protein components of mitochondria by following the *in vivo* time course of incorporation of a radioactive amino acid into mitochondrial protein fractions which have been shown by others to have a reasonably well-defined biochemical function. The results obtained indicate that those protein components of the mitochondria which are most easily removed from the mitochondrial structure have lower initial specific activities than those components which require extensive treatment with detergents for their extraction, apparently a reflection of the lipoprotein nature of the substance in these fractions. Thus, 2 min after the injection of [^{14}C]-leucine the specific activities of the water-soluble proteins and of the proteins of the cytochrome *c* fraction of liver mitochondria were more than 60% lower than the specific activity of the proteins of the unfractionated mitochondria. At 5 min the specific activity of only the water-soluble proteins was significantly lower. The specific activity of the other fractions was not significantly different from that of the unfractionated mitochondria at these times. With increasing time the specific activity of all fractions tended to become equal to that of the unfractionated mitochondria.

A similar situation was obtained with kidney mitochondria. The specific activity of the proteins of the water-soluble fraction and the cytochrome *c* fraction was lowest at early times. In this organ, however, the specific activity of the proteins of the water-soluble fraction increased dramatically at 2 hr and only returned to that of the unfractionated mitochondria after 8 hr. No such increase was found for this fraction in the liver mitochondria, although additional experiments were carried out at 15 and 60 min. However, in view of the similarity in behavior of this fraction in both organs at short time intervals, it may be suspected that a similar change in this fraction may occur in liver mitochondria but has not been detected under these experimental conditions.

The lower specific activity of the water-soluble proteins and the proteins of the cytochrome *c* fraction compared to the proteins of the unfractionated mitochondria, at short time intervals, may be interpreted on the hypothesis that the proteins of these fractions are synthesized more slowly within the mitochondria than are other proteins of this organelle. An alternate explanation involves the assumption that the proteins of these fractions are synthesized outside the mitochondria and are subsequently incorporated into the mitochondrial structure. There are several observations which would favor the latter interpretation. The great increase in specific activity of the water-soluble proteins in kidney mitochondria at 2 hr is not consistent with a slow synthesis of these proteins within the mitochondria, but is consistent with synthesis outside the mitochondria and a slow transport of the proteins into the mitochondria. In this respect, it is of interest that Solomon (1959) observed that glutamic dehydrogenase activity of embryonic chick liver started to increase in the mitochondrial fraction after the twelfth day of incubation, followed by a sharp drop in the activity of this enzyme in the supernatant fraction at 15 days of incubation. He suggested that this might be due to the transfer of this enzyme from extramitochondrial sources to the mitochondria. In a similar vein, Wilgram and Kennedy (1963) have shown that the final step in the formation of lecithin takes place in the microsomes. They have pointed out that since lecithin is a major constituent of mitochondrial phospholipids that at least this portion of mitochondrial structure is not synthesized within the mitochondria. Results obtained from the labeling of mitochondrial protein *in vitro* also bear on this problem. Roodyn *et al.* (1962) observed negligible labeling of the soluble proteins, including cytochrome *c*, catalase, and malic dehydrogenase, compared to insoluble mitochondrial proteins when isolated rat liver mitochondria were incubated with radioactive amino acids. This type of result has led Truman (1964) to suggest that lipoproteins may be synthesized in the mitochondria themselves and other proteins synthesized elsewhere and then transferred to the mitochondria. The results presented in this paper lend support to such a suggestion.

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In Vitro Synthesis of Brain Protein. II. Properties of the Complete System*

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ABSTRACT: Ribosomes and microsomes prepared from rabbit brain are active in protein synthesis. The requirements for optimal incorporation are similar to those of other mammalian cell-free systems, and include adenosine 5′-triphosphate (ATP) and an ATP regenerating system, guanosine-5′-triphosphate (GTP), pH 5 fraction, potassium, and magnesium. Amino acids are required for optimal activity in the ribosomal system. Glutamic acid alone restores 90–95% of full activity in the absence of an amino acid mixture. Ribonuclease and puromycin inhibit the system, whereas deoxyribonuclease, actinomycin, and chloramphenicol do not. Although initially the postmitochondrial fractions consist almost entirely of polysomes, the final preparation used in incorporation studies consists of both single and aggregated ribosomes. The aggregates are more active than the single ribosomes in *in vitro* protein synthesis. This system provides a useful *in vitro* model for studying the products and control mechanisms of brain protein synthesis.

The role of proteins in the specific functions of nervous tissue is not known. Molecular theories of neuronal transmission and memory require that macromolecular substances subserve specific roles in either impulse propagation, synaptic transmission, or recognition of specific neuronal circuits (Schmitt, 1964). Cell-free systems derived from brain are of interest not only as models of mammalian subcellular protein synthesis, but also as possible means of investigating relationships between electrical activity in the nervous system and dynamic aspects of polypeptide synthesis. In a previous publication we reported the formation of a characteristic nervous tissue protein in cell-free systems derived from brain (Rubin and Stenzel, 1965). These systems may thus provide *in vitro* models for studying products and control mechanisms of brain protein synthesis and the relationships of these metabolic parameters to the unique properties of neurons. This report describes the characteristics of cell-free systems derived from homogenates of rabbit brain cortex.

Materials and Methods

Reagents. Inorganic reagents were commercial

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