MITOCHONDRIAL PROTEIN SYNTHESIS *IN VITRO* IS *NOT* AN ARTIFACT

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1. Introduction

The first report that mitochondria, *in vitro*, can incorporate amino acids into protein was published in 1958 [1]. Since that time, numerous studies have detailed this process in mitochondria isolated from mammalian and insect tissues, as well as from cultured mammalian cells, yeast, fungi, and plants (cf. [2, 3]). In these studies, it was demonstrated that amino acid incorporation by isolated mitochondria was not due to either microsomal [4] or bacterial contamination [5, 6]. In recent publications, Hochberg et al. [7, 8] have suggested that mitochondrial protein synthesis *in vitro* may be an artifact resulting from specific binding of radioactive amino acids to mitochondrial protein–lipid structures. We have carefully reinvestigated this problem and observed that three different concentrations of leucine are not incorporated into heat or acid-denatured mitochondria significantly above the zero time values. Under the conditions used in our laboratory, leucine is incorporated into proteins of intact mitochondria at a rate nearly 20-fold greater than that reported by Hochberg et al. [7]. Possible reasons for these differences will be discussed as well as other evidence which demonstrates that protein synthesis by isolated mitochondria *in vitro* is not an artifact.

2. Materials and methods

Liver mitochondria were prepared under sterile conditions from 150 g male Sprague-Dawley rats. The livers were homogenized in 20 volumes of a medium consisting of 0.25 M sucrose, 10 mM Tris-HCl, pH 7.8, and 1.0 mM EDTA (sodium salt). The mitochondria were prepared by previously described methods [9] which yield a mitochondrial fraction which is 3% contaminated with microsomal protein. Inner membrane-matrix fractions were prepared by the digitonin method [10]. Boiled and TCA-precipitated mitochondria were prepared exactly as described by Hochberg et al. [11].

Amino acid incorporation was determined in a medium containing 50 mM Bicine buffer, pH 7.6, 10 mM $MgCl_2$, 1 mM EDTA, 5 mM potassium phosphate, pH 7.6, 90 mM KCl, 2.0 mM ATP, 5 mM PEP, 10 μg/ml of pyruvic kinase, 22.5 μg of an amino acid mixture minus leucine [4], and 2.0–3.0 mg of mitochondrial protein in a final volume of 2.0 ml in a metabolic shaker at 30°. Radioactive amino acids were added in the concentrations described in the legends of the tables. Proteins were prepared for counting as described previously [12] and counted in a scintillation counter with an efficiency for ^{14}C of 90% and ^{3}H of 50%.

Polyacrylamide gel electrophoresis was performed in 7 cm gels as described by Tzagoloff [13].

ATP, PEP, pyruvic kinase, chloramphenicol, erythromycin, and cycloheximide were obtained from Sigma, emetine-HCl from Eli Lilly and carbomycin from Pfizer. Sparsomycin was a gift of Dr. A.C. Trakatellis. [UL-^{14}C]L-leucine (342 mCi/mmole) was obtained from Amersham-Searle and [G-^{3}H]L-tryptophan (4 Ci/mmole) from New England Nuclear.

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Table 1
The incorporation of leucine into intact and denatured mitochondria.

Time (min)	Specific activity (cpm/mg protein)			
	Intact mitochondria	0° Incubation	Hdn. mitochondria	TCA mitochondria
0	7	7	4	8
15	2,100	–	10	7
30	4,100	8	22	11
60	4,300	11	23	18

Radioactive precursor was 0.25 μCi/ml of uniformly labelled [^{14}C]L-leucine.

3. Results

Rat liver mitochondria were incubated at 30° in the presence of the optimal concentrations of KCl, ATP, $MgCl_2$ and phosphate recently determined in our laboratory [14]. The rate was linear for the first 30 min (table 1); however, little increase in radioactivity was observed during the next 30 min of incubation. In contrast, no significant incorporation above the zero time occurred when the incubation was performed at 0° or when the mitochondria had been denatured by boiling or treatment with acid.

In these experiments, only a trace amount of leucine was added. Several groups [15, 16] have reported that maximum incorporation in terms of pmoles/mg required a final conc. of leucine of 40–60 μM. We have confirmed this observation and demonstrated that the incorporation under these conditions procedes with identical kinetics with a final incorporation of 47 pmoles/mg/30 min. No incorporation into boiled mitochondria was observed with 30 μM leucine as substrate (table 2).

Hochberg et al. [7] have recently reported that leucine (1 μCi/0.3 μmole/tube) was incorporated to a greater extent into heat denatured than into intact mitochondria. Incubations of this concentration of leucine under *our* experimental conditions resulted in an incorporation of 70 pmoles/mg/30 min in intact mitochondria and essentially no incorporation into boiled mitochondria (table 2). Chloramphenicol caused a 75% inhibition of incorporation of leucine at this concentration. However, incubations under these conditions with the same concentration of tryptophan (300 μM), resulted in very high zero time values and significant incorporation into boiled mitochondria.

The rate of amino acid incorporation measured with 40 μM leucine as substrate was depressed severely by several inhibitors of protein synthesis (table 3). Both emetine and erythromycin only inhibited the incorporation rate in the inner membrane-

Table 2
Incorporation of high concentrations of leucine and tryptophan into mitochondrial protein.

Conditions	Specific activity (cpm/mg)					
	Leucine (30 μM)[a]		Leucine (300 μM)[b]		Tryptophan (300 μM)[a]	
	Intact	Hdn.	Intact	Hdn.	Intact	Hdn.
zero	7	28	10	25	1,750	1,260
30 min	782	39	235	42	3,210	2,080
+chloramphenicol	172	13	63	52	2,650	2,240

a) 30 μM leucine, 0.25 μCi/ml; b) 300 μM leucine, 0.50 μCi/ml; c) 300 μM tryptophan, 1.0 μCi/ml; d) 50 μg/ml + chloramphenicol.

Table 3
Effect of inhibitors of protein synthesis on leucine incorporation.

Inhibitors			% of control	
			Intact mitochondria	Inner membrane-matrix fraction
Chloramphenicol	50	μg/ml	23	20
Erythromycin	50	μg/ml	100	58
	100	μg/ml	100	33
Carbomycin	2	μg/ml	53	58
	10	μg/ml	22	27
Emetine	100	μg/ml	100	49
	300	μg/ml	71	25
Sparsomycin	0.1	μg/ml	65	–
	1	μg/ml	10	8
Puromycin	100	μg/ml	5	7
Cycloheximide	100	μg/ml	95	99

Radioactive precursor 40 μM leucine (0.25 μCi/ml); control specific activity 64 pmoles/mg/30 min.

matrix fraction; however, carbomycin, chloramphenicol, sparsomycin and puromycin inhibited the incorporation to the same extent in intact mitochondria as in the inner membrane fraction. Cycloheximide had no significant effect on the incorporation rate.

The proteins labeled after an *in vitro* incubation were compared to the products of mitochondrial protein synthesis *in vivo*. Liver mitochondria were obtained from rats injected with [^{3}H]leucine after treatment with sufficient cycloheximide to block microsomal protein synthesis more than 97% [17]. Mitochondria were labeled *in vitro* in the usual incorporation medium except that the [^{14}C]leucine concentration was raised to 2 μCi/ml; both mitochondrial preparations were fractionated with acetic acid and then Lubrol. This procedure resulted in a mitochondrial pellet with a specific activity 7-fold greater than that of the intact mitochondria. In addition, the pellet retained 70–80% of the total radioactivity and less than 10% of the total protein. The pellets obtained from the *in vivo* incubation (with [^{3}H]leucine) and the *in vitro* incubation (with [^{14}C]leucine) were thoroughly mixed. Approx. 100

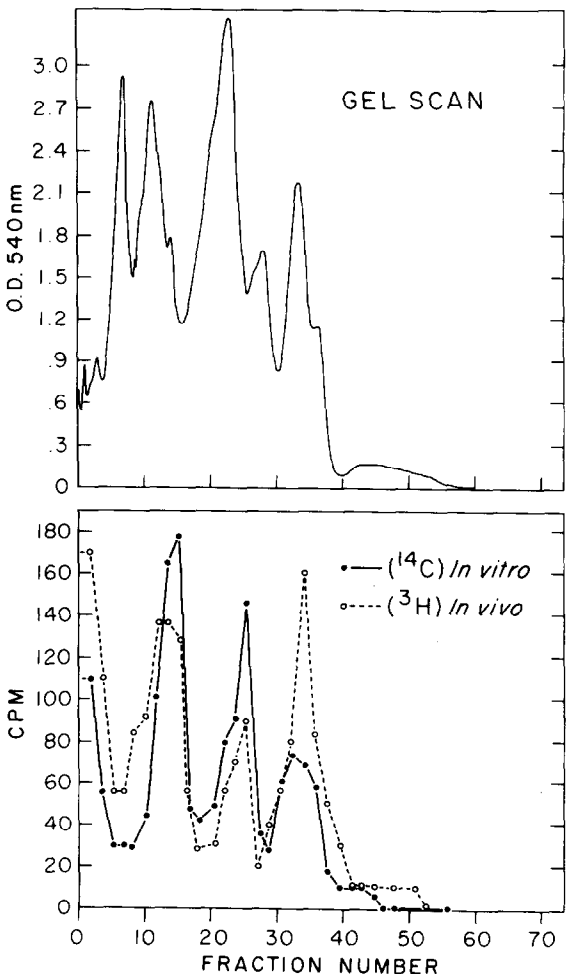

Fig. 1. Gel electrophoresis of mitochondria labeled *in vitro* with [^{14}C]leucine (●—●—●) and *in vivo* with [^{3}H]leucine (○—○—○). The mitochondria were extracted with acetic acid and Lubrol, the pellets thoroughly mixed, and 150 μg of the mixture added to the gel. The upper box is an optical density scan of the gel and the lower box is a profile of the radioactivity in each slice.

μg of this mixture were analyzed by SDS-gel electrophoresis. The proteins labeled with [^{3}H]leucine migrated with the same relative mobilities as those labeled with [^{14}C]leucine (fig. 1).

4. Discussion

The data presented in this paper indicate that radio-

active leucine is incorporated into protein at a significant rate by isolated rat liver mitochondria. This incorporation is almost completely repressed by addition of 6 different inhibitors of protein synthesis which are known to act at different and specific sites on the ribosome [18]. Leucine, at any of the concentrations tested, is *not* incorporated into mitochondria previously denatured either by acid precipitation or by boiling.

These results differ from those of Hochberg et al. [7], who recently reported that 300 μM leucine was incorporated into protein to a greater extent by heat-denatured than by intact mitochondria. Furthermore, addition of chloramphenicol caused little inhibition of the incorporation. One reason for this discrepancy may be the very low incorporation, calculated to be 1–4 pmoles/mg/hr, compared to those reported here, 50–70 pmoles/mg/30 min. It should be emphasized that our incorporation values are comparable to or even greater than the 33 pmoles/mg/hr reported by Kroon and DeVries [15]. Contrary to the claim of Hochberg et al. [7], the incorporation medium used in our laboratory is significantly different than the one they used.

It is evident that the amino acid used for incorporation studies must be chosen with care. Leucine, the most commonly used amino acid for these studies, is acceptable; however, tryptophan is unacceptable as appreciable incorporation of this amino acid into zero time controls and into heat-denatured mitochondria was observed. Hence, prior to studies of amino acid incorporation it should be clearly demonstrated that the chosen amino acid is not incorporated into denatured mitochondria.

Further evidence that mitochondrial protein synthesis *in vitro* is not an artifact was provided by the gel electrophoresis studies. The proteins labeled after an incubation *in vitro* migrated to approximately the same positions on the gel as those labeled *in vivo*. Several groups [19–21] have previously demonstrated that the proteins synthesized by mitochondria *in vitro* were identical to those synthesized *in vivo*.

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