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Short Term *in vivo* Incorporation of ^{3}H-Leucine into Brain Mitochondria

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The time course of the *in vivo* incorporation of ^{3}H-leucine into synaptic (mitochondria which may be isolated enclosed within the synaptosomes) or free mitochondria separated by means of a discontinuous sucrose gradient, has been studied. Free mitochondria showed maximum incorporation 13 h after the intracerebroventricular administration of the tritiated leucine. However, in the synaptic mitochondria two peaks at 3 and 22 h were observed. In pulse-chase experiments, while cold leucine, administered intraperitoneally some hours after the isotope administration, induced 22 h later a decrease in ^{3}H-leucine incorporation into free but not into synaptic mitochondria. This finding suggests that the second peak of ^{3}H-leucine incorporation in the synaptic mitochondria population comes from the shift towards the synapse of labelled material through the slow axoplasmic flow. The *in vivo* incorporation of the isotope appears, on the other hand, to be sensitive to cycloheximide or chloramphenicol administered either intraperitoneal or intracerebroventricularly.

Several reports have been published on the synthesis of proteins by rat brain mitochondria (1, 11, 14, 16, 19, 24) and efforts have been made to find out differences between free and synaptic mitochondria with respect to their protein synthesis ability (2, 15, 16, 22). The most studied aspect has been the kinetic pattern of *in vivo* incorporation of radioactive leucine using long-time course. In these studies radioactivity incorporated into the mitochondria was determined along several weeks after the administration of the isotope in order to calculate the half-life of these mitochondria (2, 15, 22), but no significant differences have been found between both mitochondrial populations. However, it remains to be stablished whether such differences occur during short-time pulses in which a possible effect of the axoplasmic flow might perhaps be observed.

The present work was therefore undertaken to study the kinetic pattern of *in*

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vivo incorporation of ^{3}H-leucine into protein of free or synaptic mitochondria during short-time pulses. Pulse-chase experimens were also carried out to provide evidence of a possible interconversion of radioactive material between both populations. The inhibition caused by cycloheximide or chloramphenicol on the *in vivo* incorporation was also studied in order to determine the participation of the cytoplasmic (cycloheximide-sensitive) or the mitochondrial (chloramphenicol-sensitive) protein synthesis system on the incorporation of the precursor in the rat brain mitochondria.

Materials and Methods

Animals and chemicals. The experiments have been carried out with female Wistar rats weighing 250-300 g. L-4,5-^{3}H-leucine (40-60 Ci/mol) was obtained from Radiochemical Center (Amersham), chloramphenicol succinate from Parke Davis and cycloheximide from Sigma. All drugs were dissolved in saline. Intracerebroventricular (i.c.v.) injections into conscious animals were carried out as previously described (5). Constant volumes of 20 μl or 0.6 ml were used for i.c.v. or intraperitoneal (i.p.) injections respectively. ^{3}H-leucine was administered by the i.c.v. route in doses of 20 μCi/rat. Chloramphenicol succinate was administered either by the i.p. or i.c.v. route in doses of 600 mg/kg (active component) or 0.65 mg/rat respectively. Cycloheximide doses were in turn 4 mg/kg i.p. or 0.7 mg/rat i.c.v. The doses used had been previously found to inhibit the *in vivo* incorporation of radiolabelled leucine into liver mitochondria when administered i.p. (6, 7) or into brain mitochondria when given i.c.v. (20). In pulse-chase experiments the dose of cold leucine injected i.p. was 0.6 ml/rat of 0.15 M leucine in saline, which approximately represents the limit of solubility of the amino acid.

Isolation of mitochondria. Two forebrains were used in each experiment. The method used was that of GRAY and WHITTAKER (12), the only modification being the isolation buffer: sucrose, 0.32 M; EDTA, 1 mM; Tris HCl, 1 mM; pH 7.4. The 17,000 $\times$ *g* pellet was washed with isolation buffer and recentrifuged at 17.000 $\times$ *g* for 20 min. This process was repeated twice to eliminate microsomal contamination. The material sedimented in the 0.8 M and 1.2 M sucrose interphase («Synaptosomes») was diluted in three volumes of Tris-HCl 1 mM, pH 7.4 containing EDTA 1 mM and centrifuged at 17,000 $\times$ *g* for 20 min. The pellet was resuspended in 2 ml in the last buffer, carefully pipetted for 30 min and layered on the top of a sucrose gradient (12).

Citrate synthetase (E.C. 4.1.3.7.) (8) was used as enzymatic marker of mitochondria, lactate dehydrogenase (E.C. 1.1.1.27.) (8) as marker for cytoplasm either free or enclosed into particles, acetylcholinesterase (E.C. 3.1.1.7.) (10) as marker for synaptic membranes, and NADPH citochrome c oxidoreductase (E.C. 1.6.2.4.) as marker for microsomes (4). Protein was determined by the method of LOWRY (18).

Radioactive assay. We have used a similar technique to that proposed by BEATTIE *et al.* (3) for small sample preparations. Mitochondria were resuspended in isolation buffer to give an approximate final concentration of 2 mg of protein/ml; 0.5 ml of this preparation were added to 0.5 ml of 20 % trichloroacetic acid allowed to stand for 30 min at 4° C. Precipitates were filtered on Whatman GC filters, and washed twice with 5 % trichloroacetic acid. After drying the filters, 5 ml of scintillation liquid (5 g PPO, 0.1 g POPOP, 0.5 l of Triton X-100 and 1 l of toluene) were added and radioactivity measured in a Packard Tri-Card scintillation counter. In all experiments samples were assayed in duplicate.

Results

Purification of free and synaptic mitochondria. The specific activity of citrate synthetase of free and synaptic mitochondria is 2.5 and 3.5 times higher than the activity of crude mitochondria (17,000 × *g* pellet) and synaptosomes respectively. Acetylcholinesterase specific activity is 4 times lower in free mitochondria than in the crude preparation (17,000 × *g* pellet) and one half lower in synaptic mitochondria than in synaptosomes. Lactate dehydrogenase is proportionally higher than acetylcholinesterase in mitochondrial preparations (table I). No NADPH cytochrome c oxidoreductase activity is observed in any of the mitochondrial preparations.

Total radioactivity collected in free and synaptic mitochondria represents about 1 % of the total amount found in homogenate. It should also be noted the progressive decrease of radioactivity eliminated in the successive washings with isolation buffer of the 17,000 × *g* pellet (table II).

Pulse experiments. Figure 1 shows the time course incorporation of ^{3}H-leucine into both mitochondrial preparations. A single maximun of incorporation can be observed at 13 h for free mitochondria whereas two peaks at 3 and 22 h in the synaptic mitochondria.

Table II. *Total and specific radioactivity of the fractions obtained in the isolation of free and synaptic mitochondria.*
Radioactivity was determined in a fractionation carried out from two rat forebrains after a pulse of 3 h. Total radioactivity = c.p.m. of the fraction. Specific radioactivity = c.p.m. × mg^{-1}.

	Total	Specific
Homogenate	177,920	556
1,000 × *g* pellet	60,610	319
1st 17,000 × *g* supernatant	25,760	594
2nd 17,000 × *g* supernatant	31,200	780
3rd 17,000 × *g* supernatant	8,880	222
4th 17,000 × *g* supernatant	5,120	128
17,000 × *g* pellet	24,000	300
Synaptosomes	6,230	623
Free mitochondria	1,480	478
Synaptic mitochondria	1,607	480

The results obtained in pulse experiments with ^{3}H-leucine of two different specific activities are represented in table III. In both cases 20 μCi of each isotope were injected, so different concentration of leucine were used. It can be observed a clear proportionality between the amount of ^{3}H-leucine incorporated into the organelles, and the concentration of the tritiated amino acid injected.

Previous intraperitoneal administration

Table I. *Total and specific enzymatic activities of the fractions obtained in the isolation of free and synaptic mitochondria.*
These data are the means of five experiments. The S.E.M. are within 5 % of the mean in all cases. Total activity = μmol × min of the total volume of the fraction. Specific activity = μmol × min^{-1} × mg^{-1}.

	Lactate dehydrogenase		Acetyl cholinesterase		Citrate synthetase	
	Total	Specific	Total	Specific	Total	Specific
Homogenate	536.0	0.605	55.00	0.061	164	0.185
1,000 × *g* pellet	121.0	0.258	20.00	0.042	50	0.106
1st 17,000 × *g* supernatant	242.0	1.865	16.00	0.122	12	0.096
17,000 × *g* pellet	168.0	0.944	18.00	0.138	68	0.542
Synaptosomes	70.0	0.596	9.00	0.075	45	0.388
Free mitochondria	2.0	0.340	0.20	0.032	9	1.361
Synaptic mitochondria	0.3	0.201	0.07	0.038	3	1.319

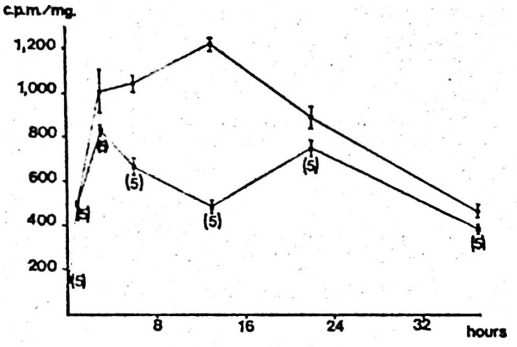

Fig. 1. *Time course of ^{3}H-leucine incorporation into free and synaptic mitochondria.* Bars shown are means ± S.E.M., within parentheses = number of experiments. (●—●) Free mitochondria. (■—■) Synaptic mitochondria.

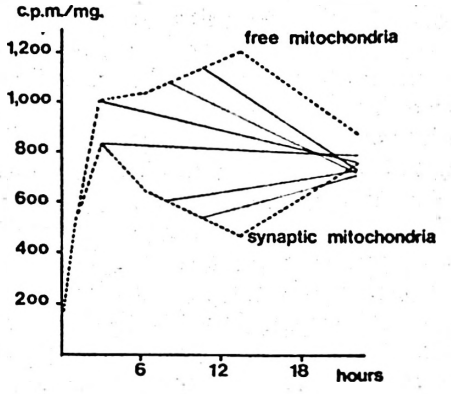

Fig. 2. *Pulse-chase experiments of ^{3}H-leucine incorporation into free or synaptic mitochondria.* Dashed lines represent values from fig. 1. Cold leucine was intraperitoneally injected after 3, 8 or 10.5 h later of isotope administration. In all cases, the rats were decapitated at 22 h and radioactivity measured in the mitochondrial preparations. The uninterrupted lines are drawn from the time of the cold leucine administration till the final time of the chase (22 h). Each point of these pulse-chase experiments is the average of at least three experiments. The S.E.M. are within 10 % of the mean in all cases.

of cold leucine resulted in a high inhibition of ^{3}H-leucine incorporation into both mitochondrial preparations (table IV). Therefore, a series of pulse-chase experiments were carried out. In these experiments, cold leucine was intraperitoneally administered to rats which had been previously injected intracerebroventricularly with ^{3}H-leucine. The radioactivity found in free mitochondria showed a general tendency to decrease. However the values obtained in the synaptic ones remained constant (fig. 2).

Table III. *Effect of ^{3}H-leucine concentration on the incorporation of the amino acid into mitochondria.*
A constant volume (20 µl) containing 20 µCi of the isotope was intracerebroventricularly injected in both cases. These data are the means of five experiments. The S.E.M. are within 10 % of the mean in all cases.

	Incorporation in pmol/mg protein	
	Free mitochondria	Synaptic mitochondria
Leucine 40-60 Ci/mol (0.4 nmol injected)	0.0616	0.0464
Leucine 135 Ci/mol (0.14 nmol injected)	0.0255	0.0160

Inhibition of in vivo *incorporation of ^{3}H-leucine by antibiotics.* The intraperitoneal administration of the antibiotics were carried out 1 h before the ^{3}H-leucine injection to avoid the effect of rebound after long-time treatments, as previously described (23). The inhibition caused by chloramphenicol on the ^{3}H-leucine incorporation into both populations is clearly less marked than that caused by cycloheximide (table IV). The inhibition caused by cycloheximide administered i.c.v. is approximately of the same order of magnitude than that observed when injected i.p. However, chloramphenicol produced a higher inhibition of the incorporation of ^{3}H-leucine when administered i.c.v. (table IV).

Table IV. *Inhibition (%) of unlabelled leucine and antibiotics on the incorporation of ^{3}H-leucine into free and synaptic mitochondria.*

Cold leucine was intraperitoneally injected 1 h before the injection of ^{3}H-leucine and the rats were decapitated 22 h later. Chloramphenicol succinate or cycloheximide were injected intraperitoneally 1 h before the injection of ^{3}H-leucine or intracerebroventricularly 15 min before the injection of ^{3}H-leucine. Rats were decapitated 50 min later. These data are the means of at least three experiments. The S.E.M. are within 10 % of the mean in all cases.

	Mitochondria	
	Free	Synaptic
Unlabelled leucine	68.6	58.5
Intraperitoneal Cycloheximide	75.0	70.6
Intraperitoneal Chloramphenicol	14.6	11.3
Intracerebroventricular Cycloheximide	62.2	77.6
Intracerebroventricular Chloramphenicol	74.0	92.5

Discussion

Purification of mitochondria. The free and synaptic mitochondria show a degree of purification like that of other preparations previously described (8, 16, 19). The absence of NADPH cytochrome c oxidoreductase activity in the mitochondrial preparations and the low specific radioactivity of the washings of the 17,000 × *g* pellet, clearly suggests the absence of microsomal contamination (table II).

Pulse labelling experiments. The i.c.v. injection of leucine present the great advantage of minimizing the effects of reutilization of the amino acid that take place after intravenous or oral administration (15). And futhermore ROBERTIS and MORELOS (21), have found that metabolism of labelled leucine is negligible in the brain, at least during the first 30 min. The results of specific activities of leucine labelled after 3 h pulse in the initial homogenate and in the free mitochondrial preparations are in excellent agreement with those described by HUNGEN *et al.* (15) when used a similar period of pulse and the same radioactive amount injected of an isotope of similar specific activity.

Although some pulse labelling experiments of brain mitochondria had been previously described, no significant differences in the kinetic pattern of free or synaptic mitochondria were observed (2, 15, 22). The present detailed study of short-time pulses show that the incorporation is clearly different for free and synaptic mitochondria. Similar results in synaptosomes (particles enclosing synaptic mitochondria) have been observed by GURD (13), with a broad peak of incorporation at 3-5 h and a second peak at 16 h. These results can be explained by the displacement of unlabelled proteins or whole organelles, moving along the axon at the time of the radioisotopic pulse by the slow axoplasmic flow, resulting in the isotopic dilution of the synaptic mitochondria and in the decay of radioactivity in this fraction at 6 and 13 h. The shift of new labelled material should produce the new increase of radioactivity in the synaptic mitochondria found at 22 h. The possibility that this second peak of incorporation in this fraction comes from a *de novo* leucine incorporation seems unlikely, since leucine concentration is not under saturation as shown in table II suggesting a rapid utilization of the isotope administered.

An alternative interpretation of these results might be that ^{3}H-leucine was first incorporated into synaptic mitochondria at 3 h and then relocated by a reverse axoplasmic transport in the cell body giving the peak of free mitochondria at 13 h. However, there is, to our knowledge, no evidence of reverse movement of mitochondria by axoplasmic transport

and therefore this possibility looks unlikely.

The inhibition of ^{3}H-leucine incorporation produced by i.p. administration of cold leucine, suggests, in accordance with CHRISTENSEN (9), the rapid diffusion of the amino acid to the brain with the subsequent isotopic dilution of the radiolabelled leucine. Pulse-chase experiments confirm the interpretation of the pulse experiments in the sense that after the isotopic dilution caused by cold leucine, a decrease of incorporation into free mitochondria occurred. However, the maintainance into the standard deviations of the values observed in synaptic mitochondria at 22 h suggests that this material was labelled before the administration of cold leucine (3, 8 and 10.5 h after ^{3}H-leucine administration) and probably moved to the synapse by the axoplasmic flow.

Inhibition of the in vivo *incorporation by antibiotics.* The inhibition on ^{3}H-leucine incorporation into rat brain mitochondria produced by cycloheximide is similar to that found by CH'IH *et al.* (7), in liver mitochondria. The results suggest that, after 3 h pulse the labelled material that appears in whole mitochondria mostly comes from the cycloheximide-sensitive cytoplasmic protein synthesis. This effect can not be explained by the toxicity of the antibiotic since no toxic effects have been found with the dose used in the present work (6, 7).

In the other hand, JONES *et al.* (16), also found a 10 and 21 per cent inhibition into cell and synaptic mitochondria respectively after incubation of brain slices with chloramphenicol for 60 min. The similar inhibitory effect obtained *in vivo* by intraperitoneal injection or by incubation of the tissue slices with the antibiotic, suggests a satisfactory penetration of the drug from plasma into brain tissue. In fact, the concentration of the drug achieved in the brain may be higher than that of the plasma one as it has been described by KATZMAN (17).

The inhibition observed after cerebroventricular administration of cycloheximide corroborates the data obtained after the intraperitoneal injection. In keeping with previous data (20), the higher inhibition produced by chloramphenicol might be due in turn to an indirect effect on the synaptic fraction rather than to an effect on the mitochondrial protein synthesis. In any case, the inhibitory effects produced by chloramphenicol given by the i.p. or i.c.v. route cannot be strictly compared since there is no direct indication in each case of the chloramphenicol levels reached in the forebrain.

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Resumen

Se estudia la incorporación *in vivo* de H^3-leucina en función del tiempo en mitocondrias libres o sinápticas separadas mediante gradientes discontinuos de sacarosa. Las mitocondrias libres muestran un máximo de incorporación de 13 h después de la administración intracerebroventricular de leucina tritiada. Sin embargo, en las mitocondrias sinápticas se observan dos máximos a las 3 y 22 h. En experimentos de pulso-caza, la administración i.p. de leucina fría posterior a la del isótopo, induce un descenso en la incorporación de H^3-leucina en las mitocondrias libres, pero no en las sinápticas. Estos resultados sugieren que el segundo máximo de H^3-leucina incorporada en la población de mitocondrias sinápticas, proviene del material marcado que es transportado a la sinapsis a través del flujo axoplásmico lento. Asimismo, la incorporación *in vivo* del isótopo se inhibe por cicloheximida o cloranfenicol cuando se administran por vía intraperitoneal o intracerebroventricular.

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