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In vitro ^{14}C-Leucine Incorporation into Nonsynaptic and Synaptic Rat Brain Mitochondria Isolated by Ficoll Gradients

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G. PARIS-VICENTE, M. J. LOPEZ-PEREZ, S. A. K. HARVEY and J. B. CLARK. In vitro ^{14}C-*Leucine Incorporation into Nonsynaptic and Synaptic Rat Brain Mitochondria Isolated by Ficoll Gradients.* Rev. esp., Fisiol., **39**, 399-408, 1983.

The *in vitro* incorporation of ^{14}C-leucine by nonsynaptic and synaptic rat brain mitochondria purified by means of discontinuous Ficoll gradients has been characterised. The incorporation was linear for the first 45 min for both pupulations. Synaptic mitochondria showed a higher rate of incorporation than the nonsynaptic mitochondria at high concentrations of leucine. The incorporation was more effective in the presence of Mg^{2+} and inhibited by dinitrophenol. The incorporation was sensitive to chloramphenicol and insensitive to cycloheximide. Bacterial contamination was in any case lower than 1,000 colonies per ml after the incubation period. The incorporation was carried out in the presence of either an external ATP-generating system consisting of ATP, phosphoenolpyruvate and pyruvate kinase or with mitochondria respiring with oxidisable substrates plus ADP (state III). The rates obtained for incorporation in this state III were higher for all the substrates assayed (succinate, pyruvate and glutamate) than in the presence of exogenous ATP. The highest rate obtained was found when glutamate was the respiratory substrate. No significant metabolic oxidation of leucine occurs in either synaptic or nonsynaptic mitochondria in the presence of exogenous ATP. Glutamate did not increase leucine uptake in any mitochondrial populations.

After BACHELARD (1) showed aminoacid incorporation into proteins of mitochondrial preparations from cerebral cortex and spinal cord, several authors have reported that protein syntesis in brain mitochondria was relatively insensitive to chloramphenicol (8, 10, 24). However, when purification of mitochondria was improved by use of sucrose density gradients it was shown that brain mitochondria were sensitive to chloramphenicol and that contaminating ribosomes and synaptosomes were responsible for the insensitivity to the antibiotic (5, 18, 22).

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These observations emphasize the need to use «pure» brain mitochondrial preparations to study *in vitro* amino-acid incorporation.

It is now well established that the incorporation of amino acids into isolated mitochondria has two energy requirements: a constant ATP generating system and the mitochondrion has to be maintained in an energized state. Apparently initiation of the peptide chain depends on the maintenance of the transmembrane potential (21). Therefore, experiments involving incorporation of amino acids into protein must be carried out with mitochondrial preparations which are not only metabolically competent but show good respiratory control.

Previous work on *in vitro* protein synthesis in brain mitochondria was carried out either with crude mitochondria or with mitochondria purified by sucrose gradient which showed poor respiratory control. The object of this work was the comparative study of the *in vitro* incorporation of ^{14}C-leucine into nonsynaptic and synaptic mitochondria. The preparations were obtained using discontinuous Ficoll gradients (12-14) which yield two populations of brain mitochondria (free and synaptosomal mitochondria) which are minimally contaminated and have good respiratory control.

Two methods may be used to provide the necessary source of ATP: an exogenous ATP-generating system consisting of ATP, phosphoenolpyruvate and pyruvate kinase, and an endogenous system by which mitochondria are under state III respiration with an oxidizable substrate plus ADP. Preparations of muscle mitochondria incorporate amino acids at a higher rate using an endogenous ATP-generating system than with an exogenous source of ATP, especially when glutamate is the oxidizable substrate (16). A similar effect is reported here for brain mitochondria prepared by discontinuous Ficoll gradients.

Materials and Methods

ATP, ADP, CoA, NADH, succinate, malate, pyruvate, phosphoenolpyruvate, glutamate, oxaloacetate, acetylcholine dithionitrobenzoic acid, chloramphenicol and cycloheximide were obtained from Sigma. Pyruvate kinase was purchased from Boehringer, scintillation chemicals from Packard, Ficoll 400 from Pharmacia Fine chemicals, was purified according to LAI and CLARK (12). L-(U-^{14}C) leucine of specific activity of 300 mCi/mol was purchased from the Radiochemical Centre. The rest of chemicals were obtained from Merck, and were of Analar grade.

Animals. Adult male rats (150-200 g) of Wistar strain were used in all experiments.

Isolation of Mitochondria. The technique used was a modification of that of LAI and CLARK (12), and BOOTH and CLARK (4). Eight chopped forebrains were washed in isolation buffer (0.32 M sucrose, 1mM EDTA-K^+, 10 mM Tris Cl pH 7.4) to remove contaminating blood. The material was resuspended in 60 ml of isolation buffer and homogenized in a Dounce-type homogenizer (with a glass pestle), total clearance 0.1 mm, with 12 up-and-down strokes. The homogenate was then diluted with isolation buffer to a final volume of 120 ml, and centrifuged at 1.200 × *g*, for 3 min. The supernatant was centrifuged at 17,000 × *g*, for 10 min. The pellet was resuspended in 9 ml of isolation buffer and diluted to 4 ml with 12 % (w/w) Ficoll, 0.32 M sucrose, 50 μM EDTA-K^+, 10 mM Tris HCl pH 7.4 and carefully homogenized in a Potter homogenizer, clearance 0.375 mm. 9 ml aliquots of this suspension (6 tubes total) were placed in centrifuge tubes and 5 ml of 7 % (w/w) Ficoll, 0.32 M sucrose, 50 μM EDTA-K^+, 10 mM Tris HCl pH 7.4 carefully layered on each. Finally, 3 ml of isolation buffer was layered on top of the

7 % Ficoll. All the tubes are centrifuged at 99,000 × *g*, for 30 min in a SW-271 rotor of a L 5-50, Beckman Ultracentrifuge. Free (nonsynaptic) mitochondria were pelleted at the bottom of the tube. Synaptosomes were collected from the 7 %-10 % Ficoll interphase and diluted with 60 ml of isolation buffer centrifuged at 9.000 × *g*, for 10 min and the pellet lysed by resuspension in 6 mM Tris HCl buffer pH 8.1 (2 ml/g of fresh forebrain homogenized) for 30 min, gently pipetting during this period. The lysate was layered in four aliquots on gradients comprising of 6 ml of 7 % (w/w) and 6 ml of 10 % (w/w) Ficoll medium (see before) and centrifuged as above. The pellet consisted of synaptic mitochondria. The free and synaptic mitochondria were washed with 20 ml of bovine plasma albumin (BPA) medium (10 mg BPA in 20 ml isolation buffer) centrifuged at 9,800 × *g*, for 10 min, and the final pellets were resuspended in a small (1 ml) volume of isolation buffer. All operations were carried out at 4°C.

Enzymatic and respiratory activities. Lactate dehydrogenase (EC 1.1.1.27) (6), acetylcholinesterase (EC 3.1.1.7) (9) and citrate synthase (EC 4.1.3.7) (17) were determined as previously described. Proteins were measured by the biuret method (11). Respiratory rates were measured as described by LAND *et al.* (15) in 100 mM K^+ respiration medium.

Incorporation of ^{14}C-leucine. Incubations were carried out in a final volume of 0.6 ml, containing 1 μCi/ml of the isotope with an approximate protein concentration of 1 mg/ml. Incubations were conducted in the 100 mM K^+ medium used in the respiration experiments (15). Unless stated otherwise all the experiments were carried out for 30 min in the presence of 6 mM $MgCl_2$. The exogenous ATP-generating system consisted of 5 mM phosphoenolpyruvate, 2 mM ATP and 50 μg/ml of pyruvate kinase. The endogenous ATP-generating system consisted of 10 mM succinate, 2.5 mM malate plus 10 mM pyruvate or 2.5 mM malate plus 10 mM glutamate, in the presence of 20 mM ADP. Incubations were carried out at 37°C with constant shaking in a water bath.

^{14}C-leucine uptake. Uptake of ^{14}C-leucine was based on the method of MORRE and WURTMAN (19). Incubations were carried out in the 100 mM KCl medium with or without glutamate plus malate, in a final volume of 0.6 ml, with 1 mg/ml of mitochondrial protein and 1 μCi/ml of ^{14}C-leucine and incubated for 30 min at 37°C. The blanks were prepared under the same conditions but incubated at 0°C. Samples were filtered through Millipore (GS WP 02500) filters and washed with 10 ml of isolation buffer with 10 mM cold leucine. 5 ml of scintillation liquid were added to the dried filters and counted.

CO_2 Output from ^{14}C-leucine. Incubations were carried out under the same conditions to those of ^{14}C-leucine incorporation. Determinations of $^{14}CO_2$ output was as previously described by BAXTER (2), except that the CO_2 was recovered on Millipore (GF/C) filters with 30 μl of 2 N KOH. Filters were dried overnight and counted with 5 ml of scintillation liquid.

Sterilization of the medium. In all experiments the media were previously sterilized by autoclaving for 10 min except that the incubation medium and solutions of substrates, ADP, ATP and pyruvate kinase which were filtered through 0.45 m Millipore filters. After the incubation period bacterial contamination was assessed by plating on blood agar or Tryptic soy-agar (Difco) and grown for 24 h at 37°C.

Measurement of the radioactivity. After the period of incubation of each

sample, 2 × 0.25 ml aliquots of the mixture were each added to the same volume of 20 % trichloroacetic acid and let stand for 30 min in an ice bath. The insoluble precipitate were collected on GF/C Whatman filters and washed twice with 5 ml of 5 % trichloroacetic acid. To dried filters 5 ml of scintillation liquid (5 g of 2,5-diphenyloxazole and 0.1 g of 1,4 bis (2[4-methyl-5-phenyl-oxazolyl] benzene) in 1 l of toluene and 500 ml of Triton X-100) were added and counted in a Packard Tri-Carb scintillation counter. All the samples were measured in duplicate.

Results

Mitochondrial preparations. We have slightly modified the original method of LAI and CLARK to obtain nonsynaptic and synaptic mitochondria introducing the flotation technique of BOOTH and CLARK (4). The enzymatic (Table I) and respiratory (Table II) specific activities found are in good agreement with those previously described (12,13).

Characterization of the incorporation of ^{14}C-leucine by nonsynaptic and synaptic mitochondria. The characterization of the *in vitro* incorporation of ^{14}C-leucine by nonsynaptic and synaptic mitochondria was carried out using an exogenous ATP-generating system consisting of ATP, phosphoenolpyruvate and pyruvate kinase. The incorporation was linear

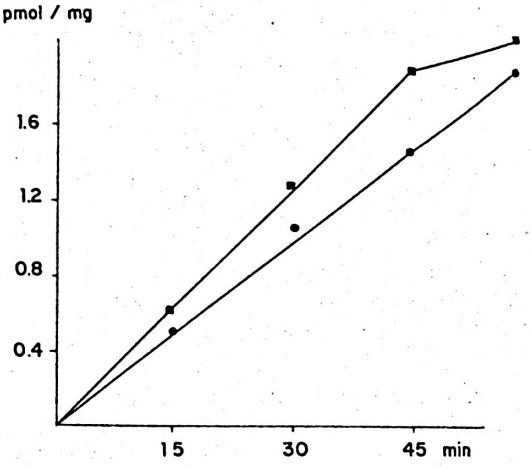

Fig. 1. *Time course of the incorporation of ^{14}C-leucine by nonsynaptic and synaptic mitochondria.* Nonsynaptic (■) and synaptic (●) mitochondria were incubated with 1/μCi/ml of ^{14}C-leucine using the exogenous ATP-generating system described in «methods». Figures are average of three experiments.

for the first 45 min for both populations of mitochondria (Fig. 1) and an incubation period of 30 min was chosen for all subsequent experiments.

When the molarity of leucine in the incubation mixture was increased, a higher ^{14}C-leucine incorporation was observed. The relationship between the increased ^{14}C-leucine incorporation and the leucine added was linear in the range tested (Fig. 2). However it can be observed that synaptic mitochondria showed a higher rate of ^{14}C-leucine incorporation than the

Table I. *Specific enzymatic activities of the fractionation.*
In brackets are expressed the percentages of enzymatic activity with respect to the total activity of the homogenate (100). All results are the mean ± S.D. of the at least six experiments.

Fraction	Enzyme activity (μmol/min^{-1} / mg^{-1} protein)		
	Lactate dehydrogenase	Acetyl cholinesterase	Citrate synthase
Homogenate	0.34 ± 0.07	0.06 ± 0.01	0.12 ± 0.03
Nonsynaptic mitochondria	0.04 ± 0.02 (0.13)	0.02 ± 0.007 (0.4)	0.86 ± 0.25 (8.1)
Synaptosomal lysate	0.35 ± 0.06 (11.2)	0.11 ± 0.03 (22.0)	0.16 ± 0.04 (15.9)
Synaptic mitochondria	0.10 ± 0.04 (0.85)	0.03 ± 0.006 (0.2)	0.65 ± 0.2 (1.7)

Table II. *Respiratory activities of nonsynaptic and synaptic mitochondria.* R.C.R. = respiratory control ratio. All results are average of three experiments ± S.D. Respiration studies were carried out in 100 mM K^+ at 25° C with approximately 1 mg of mitochondrial protein.

Incubation Conditions	Natoms O/min^{-1}/mg^{-1} protein			
	Nonsynaptic mitochondria		Synaptic mitochondria	
	State III	R.C.R.	State III	R.C.R.
5 mM Pyruvate + 2.5 mM Malate	157 ± 12	4.0	123 ±	3.9
5 mM Glutamate + 2.5 mM Malate	60 ± 2	2.6	42 ± 2	3.1
10 mM Succinate	113 ± 17	3.2	86 ± 7	2.5

nonsynaptic mitochondria at high concentrations of leucine.

Both nonsynaptic and synaptic mitochondria were highly sensitive to chloramphenicol (Table III) and insensitive to cycloheximide. In all cases, bacterial contamination was of 300 to 1000 colonies/ml after the incubation period. The incorporation was more effective in the presence of Mg^{2+} and also inhibited by dinitrophenol in both cases (Table III).

Efficacy of the exogenous and endogenous ATP-generating systems. The incorporation in the presence of external ATP-generating system compared to others in which ATP was generated internally by the mitochondria using oxidizable substrates (Table IV). Mitochondria respiring in state IV (no added ADP) with oxidizable substrate incorporate amino acids at a lower rate than those provided with an external source of ATP, except for glutamate in which the value obtained was similar to the control. However, incorporation in state III (plus ADP) increased rates for all substrates as compared to the control and particularly in the

Table III. *Effects of antibiotics, dinitrophenol and Mg^{+2} on the incorporation of ^{14}C-leucine by nonsynaptic and synaptic mitochondria.* Mitochondria are incubated for 30 min with 1/μCi/ml of ^{14}C-leucine using an exogenous ATP-generating system in the presence of 100 μg/ml chloramphenicol, 100 μg/ml cycloheximide, 0.2 mM dinitrophenol or without $MgCl_2$ in the medium. Values are the average of at least three experiments ± S.D.

Conditions	pmol incorporated/mg^{-1} protein/30 min	
	Nonsynaptic mitochondria	Synaptic mitochondria
Control (with Mg^{+2})	1.58±0.26	1.22±0.14
Chloramphenicol	0.26±0.03	0.19±0.02
Cycloheximide	1.63±0.24	1.40±0.23
Dinitrophenol	1.13±0.20	0.53±0.10
No Mg^{+2}	0.60±0.13	0.69±0.13

Table IV. *Effect of endogenous ATP-generating systems on the incorporation of ^{14}C-leucine by nonsynaptic and synaptic mitochondria.* Incubations were carried out with 1/μCi/ml of ^{14}C-leucine for 30 min in the presence and absence of ADP (State III - state IV respiratory states) (see Methods for details of respiratory substrates). Values are the average of at least three experiments ± S.D. The control was carried out in the presence of the exogenous ATP- generating system (see methods).

Conditions	pmol/mg^{-1} protein/30 min	
	Nonsynaptic mitochondria	Synaptic mitochondria
Control	1.52±0.26	1.41±0.40
Succinate	1.28±0.20	1.11±0.60
Succinate+ADP	2.53±0.50	1.66±0.51
Glutamate+Malate	1.87±0.40	1.50±0.10
Glutamate+Malate+ ADP	3.46±0.40	3.24±0.35
Glutamate+Malate+ ADP+Dinitrophenol	1.69±0.30	0.62±0.10
Pyruvate+Malate	0.47±0.20	0.67±0.20
Pyruvate+Malate+ ADP	1.91±18	1.89±0.19

case of glutamate with an incorporation rate twice that of the control.

The characteristics of glutamate as substrate are more noticeable if the rate of protein synthesis is related to a catabolic index such as the rate of oxygen consumption. With either succinate or pyruvate plus malate as substrates, the ratio of protein synthesis to oxygen uptake (nmoles ^{14}C-leucine per g-atom oxygen) is generally less than or equal to unity, irrespective of the type of mitochondria or the presence or absence of ADP. In the case of glutamate plus malate, this ratio is in the range 1.9-3.7, indicating that more protein is being synthesized for a given rate of oxidative metabolism.

Possible explanations are that glutamate may affect the rate of uptake or catabolism of leucine, and thus influence its contribution to protein synthesis indirectly. It can be seen (Table V, line 1) that differences between synaptic and nonsynaptic mitochondria with respect to leucine uptake, might be responsible for their different responses to increased leucine concentration (Fig. 2). It is evident however that the provision of glutamate plus malate has no significant effect on leucine uptake by nonsynaptic mitochondria, and actually reduces the uptake by nonsynaptic mitochondria, (Table V, line 2). Enhanced incorporation of ^{14}C-leucine cannot therefore be explained in this way.

Table V. *^{14}C-leucine uptake by nonsynaptic and synaptic mitochondria.*
Values are the average of at least three experiments ± S.D.

Incubation Conditions	c.p.m/mg^{-1} protein/30 min	
	Nonsynaptic mitochondria	Synaptic mitochondria
100 mM KCl	4,014±1,164	10,860±3,801
100 mM KCl +10 mM Glutamate +2.5 mM Malate	3,076±830	6,008±2,000

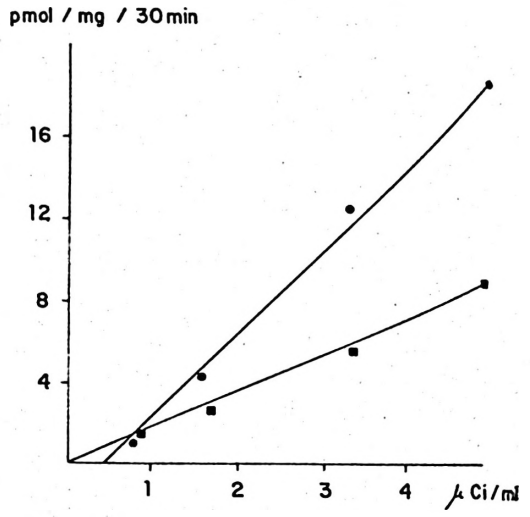

Fig. 2. *Effect of leucine concentration on ^{14}C-leucine incorporation into nonsynaptic and synaptic mitochondria.*
Incubations were carried out in the presence of 0.83; 1.66; 3.33 and 4.99 μCi/ml of ^{14}C-leucine corresponding to concentrations of 2.5; 4.9; 9.8 and 14.7 μM of leucine for 30 min using the exogenous ATP-generating system. Nonsynaptic (■) and synaptic (●) mitochondrial incorporation are expressed as pmol × mg^{-1} for 30 min. Results are the means of three experiments.

No significant oxidation of leucine occurs in either synaptic or nonsynaptic mitochondria in the presence of exogenous ATP (Table VI). In the presence of ADP, oxidation of leucine by nonsynaptic (but not synaptic) mitochondria occurs with glutamate plus malate present. Since protein synthesis is enhanced for both populations of mitochondria when glutamate plus malate are present instead of ATP no coherent argument can be made for glutamate enhancing ^{14}C-leucine incorporation into protein by suppressing its oxidation. It seems that the ability of leucine to contribute to the pool of oxidisable substrates, a process known to occur in brain slices *in vitro* (20) may be limited to the nonsynaptic compartment.

A plausible explanation for the effects of glutamate involve its central role in

Table VI. *CO_2 output from ^{14}C-leucine*
Values are expressed for periods of incubations described (0 and 30 min) and are the average of at least three experiments ± S.D.

	c.p.m/mg^{-1} protein			
	Nonsynaptic mitochondria		Synaptic mitochondria	
Incubation Conditions	0 min	30 min	0 min	30 min
Exogenous TAP-generating system	160 ± 40	163 ± 66	425 ± 178	498 ± 200
100 mM KCl medium +10 mM Glutamate +2.5 mM Malate +20 mM ADP	2,020 ± 300	5,144 ± 600	856 ± 200	101 ± 96

amino-acid/ketoacid balance. By acting as an amino group donor to endogenous ketoacids, glutamate may facilitate protein synthesis. This process may lead to decreased tricarboxylic acid cycle activity if the mitochondrial ketoacid concentrations are reduced in consequence.

Dinitrophenol strongly inhibits the incorporation of ^{14}C-leucine in the presence of glutamate plus malate plus ADP, by 51 % in the case of nonsynaptic mitochondria and by 81 % for the synaptic preparation. RABINOWITZ *et al.* (21) have shown that the effect of uncouplers on protein synthesis in mitochondria does not correlate with activation of ATP ase activity.

Discussion

The mitochondria obtained by Ficoll gradients were relatively uncontaminated as shown by the low content of lactate dehydrogenase and acetylcholinesterase in both nonsynaptic (free) and synaptic (synaptosomal) populations. The respiratory control carried out in a 100 mM K^+ medium demonstrates their metabolic integrity and intactness of the preparations in good agreement with previous reports (12, 13).

As the previous studies on *in vitro* protein synthesis of brain mitochondria have been carried out with crude preparations (8, 24) or the enzymatic characterization of the purity of the preparations have not been reported (1, 10, 18), it is not possible to easily compare the results obtained in this paper with those reported elsewhere. In any case, our mitochondrial preparations showed a complete insensitivity to cycloheximide and a high sensitivity to chloramphenicol. This chloramphenicol inhibited incorporation cannot be due to bacterial contamination, which is extremely low. Also, the complete insensitivity to cycloheximide indicates the absence of cytoplasmic ribosomes which might interfere with our results.

Although it has been described that Mg^{2+} decreases the respiratory control of brain mitochondria (17), its absence from the incubation medium inhibits the incorporation of ^{14}C-leucine. This observation probably reflects the dependence of the protein synthetic system on Mg^{2+}. Mitochondria respiring in state III incorporated ^{14}C-leucine at a higher rate than that of preparations incubated with an exogenous source of ATP. This fact has been also observed in muscle mitochondria (16) and may be explained by the different levels of intramitochondrial ATP in both cases. When an exogenous ATP-generating system is used, ADP formed by the protein synthetic system has to be exchanged with the extramito-

chondrial ATP, a process whose vectorial demands are not normally favoured by the adenine nucleotide translocase (23). However, in the state III intramitochondrial ADP is rapidly phosphorylated to ATP. On the other hand, inhibition by dinitrophenol is higher in the case of mitochondria incorporating leucine with glutamate and malate and ADP, than with an exogenous ATP-generating system. This observation may be explained by the fact that coupling of mitochondria is higher in state III than in the presence of a PEP-pyruvate kinase ATP-generating system and it is now well established that mitochondrial protein synthesis is dependent on the maintenance of the mitochondrial membrane potential (21).

The higher stimulation observed in the incorporation in the presence of glutamate and ADP has also been observed in muscle (16), but not other mamalian mitochondria (3, 7). As has been previously mentioned this may be a direct effect of glutamate on mitochondrial protein synthesis but further investigations will be necessary to pinpoint the mechanism of this effect.

Acknowledgements

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Resumen

Se caracteriza la incorporación *in vitro* de ^{14}C-leucine en mitocondrias sinápticas y no sinápticas de cerebro de rata purificadas mediante gradientes discontinuos de Ficoll. La incorporación es lineal durante los primeros 45 min. en ambas poblaciones. Las mitocondrias sinápticas muestran una incorporación mayor que las no sinápticas a concentraciones altas de leucina. La incorporación es más efectiva en presencia de Mg^{2+} y se inhibe por dinitrofenol. La incorporación se muestra sensible al cloranfenicol, pero no a la cicloheximida. La contaminación bacteriana es, en cualquier caso, inferior a 1000 colonias/ml después del período de incubación. La incorporación se lleva a cabo bien en presencia de un sistema generador de ATP que consiste en ATP, fosfoenolpiruvato y piruvato quinasa, bien con mitocondrias que respiran con un sustrato oxidable y ADP. Los valores obtenidos para la incorporación en estado III son superiores en todos los sustratos utilizados (succinato, piruvato y glutamato) que en presencia de ATP exógeno. En estas condiciones el glutamato es el más efectivo de los sustratos respiratorios utilizados. No tiene lugar una oxidación metabólica significativa de leucina en presencia de ATP exógeno ni en las mitocondrias sinápticas ni en las no sinápticas. El glutamato no aumenta la captura de leucina en ninguna población mitocondrial.

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