BBA 75247

LEUCINE TRANSPORT BY RAT LIVER MITOCHONDRIA *IN VITRO*

JOHN BUCHANAN, JOAN ROSS POPOVITCH AND DONALD F. TAPLEY

Department of Medicine, College of Physicians and Surgeons Columbia University, New York, N. Y. 10032 (U.S.A.)

(Received October 24th, 1968)

SUMMARY

Leucine accumulation by rat liver mitochondria *in vitro* has been studied. Uptake was stimulated by ATP and inorganic phosphate and was inhibited by *N*-ethylmaleimide. With ATP mitochondrial leucine concentration exceeded that of the medium 2–3 fold. Leucine uptake was consistent with saturation kinetics. Certain other amino acids (isoleucine, methionine, valine, and cycloleucine) both significantly inhibited leucine uptake and effected a rapid discharge of leucine from mitochondria pre-loaded with the amino acid. The evidence indicates that rat liver mitochondria contain a leucine transport mechanism, for which several other amino acids are competitive.

INTRODUCTION

While studying the incorporation of leucine into the protein of rat liver mitochondria *in vitro*[1], we found that the addition of certain amino acids reduced leucine incorporation. Investigation revealed that these amino acids inhibited mitochondrial uptake of leucine. This suggested that a transport mechanism mediates the uptake of leucine. The present studies partially characterize leucine accumulation by rat liver mitochondria *in vitro*.

MATERIALS AND METHODS

Materials

Uniformly labeled L-[^{14}C]leucine (specific activity 200–275 mC/mmole) and other radioactive amino acids were purchased from the New England Nuclear Corporation. Nonradioactive amino acids and *N*-ethylmaleimide were obtained from Mann Research Laboratories. Phosphoenolpyruvate, crystalline pyruvate kinase (Type II), oligomycin, the disodium salt of adenosine 5′-triphosphate (ATP), and other nucleotides were procured from the Sigma Chemical Co. Chloramphenicol was supplied by Parke, Davids and Co. Other chemicals were purchased from the Fisher Scientific Co.

Preparation of mitochondria

Rat liver mitochondria were prepared in 0.25 M sucrose as previously described[1]. Final suspensions, containing 6–9 mg protein per ml, were used immediately.

Conditions of incubation

The medium contained in 5 ml: L-[^{14}C]leucine, $1 \cdot 10^{-6}$ M; potassium phosphate buffer, pH 7.4, 10 mM; Tris–HCl buffer, pH 7.4, 50 mM; $MgCl_2$, 10 mM; KCl, 50 mM; sucrose, 100 mM; chloramphenicol, 64 μg per ml, and 1.2–1.8 mg/ml of mitochondrial protein[2].

Reactions proceeded in triplicate under air for 10 min at 20° with shaking at 60 cycles/min unless stated otherwise.

Chloramphenicol minimized leucine incorporation into protein without depressing the uptake of free leucine. With chloramphenicol, incorporation into protein accounted for 1–3 % of total leucine uptake.

Sampling and counting

After incubation, aliquots were withdrawn for determination of the total uptake of leucine and its incorporation into protein. The latter was determined after precipitation of the sample with an equal volume of 10 % trichloroacetic acid followed by 2 washes with 4 ml of 5 % trichloroacetic acid. After heating to 90° for 15 min in 4 ml of 10 % trichloroacetic acid, the final precipitate was dissolved in 0.3 ml of formic acid and counted in 10 ml of BRAY's[3] solution in a Packard Liquid Scintillation Counter.

Uptake of leucine was determined as follows. Aliquots of the medium were centrifuged at 10000 $\times$ *g* for 10 min at 20° in weighed polypropylene tubes. Aliquots of the supernatants were counted in 10 ml of BRAY's solution *plus* 0.3 ml of formic acid. The supernatants were discarded and the tubes and surfaces of the pellets rinsed with 0 25 M sucrose. The tubes were dried with paper tissues and reweighed. The pellets were dissolved in 0.3 ml of formic acid and counted in 10 ml of BRAY's solution.

Identification of free leucine

Radioactive material in the pellets, excluding that portion in protein, was identified as free leucine after dispersion of pellets (identical to those dissolved in formic acid) in 5 % trichloroacetic acid. After centrifugation the acid-soluble material was desalted, and ascending chromatography was performed on Whatman No. 1 filter paper in methanol–pyridine–water (80:4:20, by vol.) and butanol–acetic acid–water (50:25:25, by vol.). Autoradiograms were made. The chromatograms were then stained with ninhydrin. All detectable radioactivity migrated as leucine. Similar procedures were performed on cellulose acetate plates. The leucine spots were counted in BRAY's solution. They contained 90–95 % of the radioactivity in the original pellets.

Inulin space

Extra-mitochondrial medium in the pellets was determined by measurement of the [^{14}C]inulin space[4]. The inulin space accounted for 29–33 % of the weights of the pellets. The size of the inulin space was not affected by concentration of mitochondria, length of incubation, or the addition of ATP, P_i, amino acids, and *N*-ethylmaleimide.

The dry weights of the pellets were measured after heating to 100° for 24 h and averaged 20 to 30 % of the pellet weights.

Results are expressed as moles of leucine taken up per g of mitochondrial water. Uptake was corrected for radioactivity in protein and the inulin space. Mitochondrial water was determined from pellet weight *minus* inulin space and dry weight.

P_i was determined by the method of FISKE AND SUBBAROW[5].

RESULTS

In preliminary experiments the effects of temperature, time, and alterations in sodium and potassium concentrations were studied.

Raising the temperature from 20 to 37° had little effect on total leucine uptake at 2, 10, and 45 min, as shown in Table I. At these temperatures mitochondrial leucine concentration exceeded that of the medium by a factor of 2.0 to 2.5. At 0 to 4° mitochondrial leucine concentration did not exceed that of the medium after 10 min. After 45 min at 0 to 4° mitochondrial leucine concentration exceeded that of the medium though it was less than that at higher temperatures (Table I).

TABLE I

THE EFFECT OF CHANGES IN TEMPERATURE ON MITOCHONDRIAL UPTAKE OF LEUCINE

The standard medium with $1 \cdot 10^{-6}$ M L-[^{14}C]leucine was employed. Samples were taken after 2, 10 and 45 min of incubation. Results are expressed as moles $\times 10^{-9}$ leucine taken up per g of mitochondrial water ± S.E. with 95% confidence limits.

Temp.	*Uptake after*			*Number of determinations in triplicate*
	2 min	*10 min*	*45 min*	
0°	0.96 ± 0.10	1.08 ± 0.09	1.90 ± 0.21	4
20°	1.99 ± 0.15	2.24 ± 0.16	2.32 ± 0.10	6
30°	2.08 ± 0.11	2.27 ± 0.16	2.41 ± 0.16	4
37°	2.15 ± 0.10	2 36 ± 0.16	2.49 ± 0.21	4

The rate of leucine uptake at 20° is shown in Fig. 1. The initial rate was rapid, and mitochondrial leucine concentration exceeded that of the medium ($1 \cdot 10^{-6}$ M) at the earliest time point. Mitochondrial leucine concentration was relatively constant after 5 to 10 min. Under different conditions, WHEELDON AND LEHNINGER[6] measured the uptake of DL-[^{14}C]leucine by rat liver mitochondria. They observed a decline in mitochondrial leucine after the first min. This was not observed in these studies.

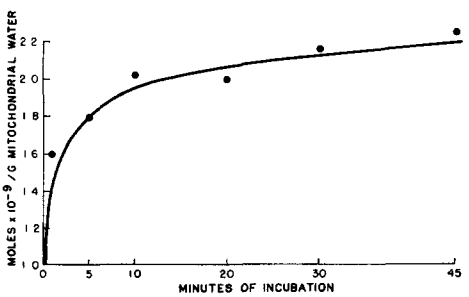

Fig. 1. Time course of leucine uptake by rat liver mitochondria *in vitro* The medium is described in the text. The initial time point, obtained by centrifugation immediately after addition of leucine, was considered to be 1 min.

Concentrations of added K^+ and Na^+ (as the chlorides) were varied from 0 to 100 mM. Added K^+ (40–100 mM) was required for maximal uptake. Added Na^+ was not required. Concentrations over 50 mM were moderately inhibitory.

The effect of phosphate

P_i (5–10 mM) stimulated leucine uptake without causing mitochondrial swelling, as determined by pellet weights (Table II).

TABLE II

THE EFFECT OF PHOSPHATE AND ATP ON MITOCHONDRIAL LEUCINE UPTAKE

Phosphate, ATP, phosphoenolpyruvate, pyruvate kinase, and L-[^{14}C]leucine (final concn., $1 \cdot 10^{-6}$ M) were added after 15 min of incubation at 20°. Samples were taken after 10 min of further incubation Results are given as moles $\times 10^{-9}$ of leucine taken up per g of mitochondrial water ± S.E. with 95% confidence limits.

Additions	*Uptake*	*Number of determinations in triplicate*
None	1.01 ± 0.08	12
P_i (1 mM)	1.19 ± 0.15	4
P_i (10 mM)	1.96 ± 0 09	4
ATP (5 mM)	2.17 ± 0.19	6
ATP (5 mM), oligomycin (5 μg/ml)	2.09 ± 0.23	4
ATP (5 mM), P_i (10 mM)	2.46 ± 0 16	4
ATP (5 mM), phosphoenolpyruvate (3.6 mM), pyruvate kinase (1.67 μg/ml)	2.70 ± 0.15	4

The effect of ATP

ATP ($5 \cdot 10^{-4}$ to $5 \cdot 10^{-3}$ M) increased mitochondrial leucine. The effect was maximal with 2 to 5 mM ATP. With 5 mM ATP the concentration of leucine, determined in triplicate in 20 experiments, varied from 1.7 to 3.5 times the concentration of leucine in the medium. The average gradient was 2.4. When mitochondria were incubated for 15 min in the absence of P_i, ATP, and leucine prior to the addition of leucine, subsequent mitochondrial leucine concentration approximated that of the medium, as shown in Table II, suggesting the depletion of endogenous ATP. The addition of ATP with leucine doubled leucine uptake. When phosphoenolpyruvate and pyruvate kinase were added as well, the uptake of leucine was further stimulated (Table II).

Oligomycin (2,5 and 10 μg per ml) was included during preincubation in similar experiments with ATP (1 to 5 mM). The concentration of P_i at the end of incubation was reduced 50% by oligocymin. P_i concentration fell from $6 \cdot 10^{-4}$–$8 \cdot 10^{-4}$ M to $3 \cdot 10^{-4}$–$4 \cdot 10^{-4}$ M with oligomycin, 5 μg/ml, and ATP, 5 mM. In spite of its inhibition of ATPase activity, oligomycin did not significantly reduce ATP-stimulated leucine accumulation (Table II).

The effects of other nucleotides were investigated. ADP *plus* either small concentrations of P_i or phosphoenolpyruvate and pyruvate kinase supported leucine uptake. GTP also appeared to stimulate uptake. Other nucleotides (AMP, ITP, UTP, CTP) did not increase mitochondrial leucine concentration.

The effect of N-ethylmaleimide

N-Ethylmaleimide inhibits transport processes by interacting with sulfhydryl groups in carrier proteins[7]. *N*-Ethylmaleimide inhibited the uptake of leucine, as shown in Table III.

TABLE III

THE EFFECT OF *N*-ETHYLMALEIMIDE ON MITOCHONDRIAL UPTAKE OF LEUCINE

The standard medium was employed. Samples were taken after 10 min of incubation. Results are expressed as moles $\times 10^{-9}$ of leucine taken up per g of mitochondrial water $\pm$ S.E. with 95 % confidence limits.

Conditions	*Uptake*	*Number of determinations in triplicate*
Control	2.30 $\pm$ 0.21	4
Plus N-ethylmaleimide ($5 \cdot 10^{-4}$ M)	1.53 $\pm$ 0.23	4

The effect of added amino acids

A barrier to diffusion of leucine was demonstrated by the addition of high concentrations of [^{12}C]leucine or other amino acids with [^{14}C]leucine. Depression of $1 \cdot 10^{-6}$ M [^{14}C]leucine uptake occurred with 3 to 5-fold excesses of [^{12}C]leucine. Inhibition was maximal 1 to 10 mM [^{12}C]leucine, suggesting saturation of a mechanism mediating leucine uptake. Leucine uptake appeared to fit saturation kinetics, as indicated in Fig. 2. The "K_m" for leucine was $2.5 \cdot 10^{-4}$ M and the "v_{max}" was $5.7 \cdot 10^{-7}$ moles/g of mitochondrial water per 10 min.

Isoleucine, valine, methionine, and cycloleucine inhibited the uptake of leucine (Table IV). Isoleucine was a competitive inhibitor (Fig. 2). Valine, methionine, and

TABLE IV

THE EFFECT OF AMINO ACIDS UPON MITOCHONDRIAL UPTAKE OF LEUCINE

The concentration of L-[^{14}C]leucine was $1 \cdot 10^{-4}$ M. Numbers of determinations, each in triplicate, are given in parentheses. Results are expressed as moles $\times 10^{-7}$ of L-[^{14}C]leucine taken up per g of mitochondrial water.

Added amino acid	*Control uptake*	*Uptake with $1 \cdot 10^{-3}$ M amino acid*	*Inhibition (%)*	*Uptake with $1 \cdot 10^{-2}$ M amino acid*	*Inhibition (%)*
None	1.50 (16)				
Alanine		1.50 (2)	0	1.43 (4)	3
α-Aminoisobutyric acid		1.51 (1)	0	1.45 (3)	1
Arginine		1.55 (2)	0	1.39 (3)	7
Aspartic acid		1.50 (1)	0	1.53 (2)	0
Cycloleucine		1.40 (4)	7	1.14 (6)	24
Glutamic acid		1.47 (4)	2	1.45 (6)	3
Glycine		1.48 (2)	1	1.44 (4)	4
Histidine		1.49 (3)	1	1.45 (3)	3
Isoleucine		1.36 (6)	9	1.01 (6)	33
D-Leucine		1.40 (3)	7	1.34 (4)	10
L-[^{12}C]Leucine		0.92 (12)	39	0.77 (16)	49
Lysine		1.42 (3)	5	1.30 (5)	13
Methionine		1.34 (5)	11	1.04 (5)	31
Phenylalanine		1.52 (3)	0	1.40 (5)	7
Proline		1.46 (2)	3	1.36 (4)	9
Threonine		1.49 (1)	1	1.50 (2)	0
Tryptophan		1.49 (4)	1	1.40 (5)	7
Valine		1.42 (5)	5	1.20 (6)	20

cycloleucine also appeared to be competitive inhibitors. Alanine, glycine, and the acidic and basic amino acids had little effect.

The uptakes of [^{14}C]isoleucine and [^{14}C]valine were studied. Leucine, methionine, valine, and cycloleucine and [^{12}C]isoleucine inhibited the uptake of [^{14}C]isoleucine. The uptake of [^{14}C]valine was inhibited by [^{12}C]valine, leucine, isoleucine, methionine, and cycloleucine.

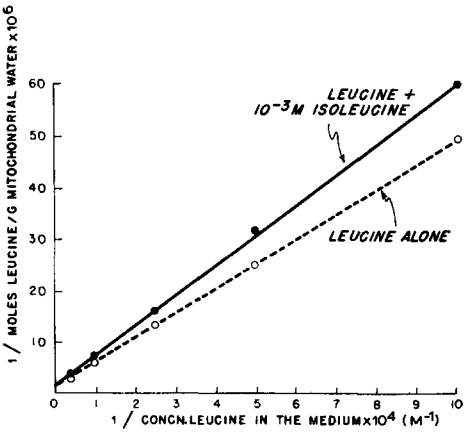

Fig. 2. Inhibition of leucine uptake by isoleucine. Apart from the addition of isoleucine and alterations in leucine concentration, the conditions are those described in the text. Uptake of leucine was determined after 10 min of incubation.

Although the uptakes of leucine, isoleucine, valine, methionine, and cycloleucine appeared to be competitive, the data did not exclude the possibility that "restricted diffusion" accounted for the observations. Since differences in the uptakes of D- and L-leucine would not occur if the process of accumulation were "restricted diffusion", the uptake of D-[^{14}C]leucine was studied. Increasing concentrations of D-[^{12}C]leucine, L-leucine, methionine, valine, and cycloleucine did not inhibit the uptake of $1 \cdot 10^{-6}$ M, $1 \cdot 10^{-5}$ M, or $1 \cdot 10^{-4}$ M D-[^{14}C]leucine. The concentration of D-leucine in mitochondria never exceeded the concentration in the medium even with raising the temperature (20 to 37°) and lengthening the incubation (to 60 min).

Release of [^{14}C]leucine by the addition of amino acids

In carrier-mediated transport, the addition of a compound capable of reacting with transport sites may effect release of molecules already bound to these sites. Underlying mechanisms have been described elsewhere[8,9]. Findings with mitochondria are described below.

Mitochondria were incubated with $1 \cdot 10^{-6}$ M [^{14}C]leucine in the standard medium in polypropylene centrifuge tubes for 5 min at 20°. Single amino acids in small volumes of water were then added, yielding final concentrations of 1 mM or 10 mM. Equal volumes of water were added to controls. The tubes were inverted once and centrifuged. Results are given in Table V as final mitochondrial [^{14}C]leucine concentration after addition of water or amino acid. Findings paralleled the inhibition experiments (Table IV). Thus, [^{12}C]leucine, isoleucine, valine, and cycloleucine significantly released [^{14}C]leucine. These studies were repeated with [^{14}C]isoleucine,

TABLE V

RELEASE OF LEUCINE FROM MITOCHONDRIA BY ADDITION OF UNLABELED AMINO ACIDS

Experimental procedure is described in the text. Concentration of L-[^{14}C]leucine in the standard medium was $1 \cdot 10^{-6}$ M Numbers of determinations, each in duplicate, are given in parentheses. The quantity of L-[^{14}C]leucine remaining in the mitochondria after addition of amino acids is expressed as moles $\times 10^{-9}$ per g of mitochondrial water.

Added amino acid	*Control*	*Plus amino acid* ($1 \cdot 10^{-3}$ *M*)	*Release* (%)	*Plus amino acid* ($1 \cdot 10^{-2}$ *M*)	*Release* (%)
None (water)	2.07 (9)				
Alanine		2.04 (3)	1	2.01 (2)	3
α-Aminoisobutyric acid				2.04 (2)	1
Arginine		1.86 (2)	10	1.88 (3)	9
Aspartic acid				2.21 (2)	0
Cycloleucine		1.91 (4)	8	1.44 (3)	30
Glutamic acid		1.86 (3)	10	1.88 (3)	10
Glycine		1.88 (1)	9	1.96 (3)	5
Histidine				2.20 (2)	0
Isoleucine		1.69 (4)	18	0 96 (4)	54
D-Leucine		1 81 (2)	12.5	1.63 (2)	21
L-Leucine		0 97 (6)	53	0 76 (6)	63
Lysine		1 81 (1)	12 5	1 94 (2)	6
Methionine		1 81 (4)	12.5	1.21 (4)	42
Phenylalanine		2.04 (1)	1	1 74 (1)	16
Proline		1 92 (3)	7	1.62 (2)	22
Threonine				2.05 (2)	1
Tryptophan		2.0 (3)	3	1.82 (2)	12
Valine		1.93 (4)	7	1.37 (4)	34

[^{14}C]valine, and D-[^{14}C]leucine as the "preloaded" amino acid. While [^{14}C]isoleucine was discharged by [^{12}C]isoleucine, leucine, valine, methionine, and cycloleucine, and [^{14}C]valine by [^{12}C]valine, leucine, isoleucine, methionine, and cycloleucine, D-[^{14}C]-leucine was not released by D-[^{12}C]leucine, L-leucine, or other amino acids.

DISCUSSION

These studies suggest that uptake of leucine by mitochondria *in vitro* is carrier mediated. Leucine uptake appears to fit saturation kinetics and consistent "K_m" and "v_{max}" values were repeatedly obtained. Further evidence supporting the existence of a transport mechanism was provided by the finding that certain amino acids inhibited leucine uptake, indicating that transport sites do not have specific affinity for leucine. Rat liver mitochondria appear to have a transport mechanism for leucine and certain other amino acids resembling that in Ehrlich cells[10].

It is unlikely that leucine uptake occurs by restricted diffusion. First, the uptake of D-leucine is dissimilar to the uptake of L-leucine. Second, discharge of leucine from mitochondria preincubated with the amino acid is effected by those amino acids which interfere with leucine uptake. This is not consistent with "restricted diffusion"; however, it is characteristic of certain carrier-mediated transport processes[8,9]. Last, the effect of *N*-ethylmaleimide favors the existence of a transport process.

Though evidence for a leucine transport mechanism in rat liver mitochondria is strong, it is not clear whether the process is active transport or "facilitated diffusion"[11].

ATP increased mitochondrial leucine 2- to 3-fold. This action was not due to P_i released from ATP as such phosphate levels were too low to stimulate uptake. Though oligomycin inhibited ATPase activity, it did not significantly depress leucine accumulation. Thus the mechanism of the effect of ATP remains to be explained.

The failure of alanine, glycine, and α-aminoisobutyric acid to inhibit leucine uptake is consistent with data suggesting that these amino acids are transported by a different mechanism in other systems[10]. An "alanine-preferring" transport site was sought in mitochondria with α-amino[^{14}C]isobutyric acid. Neither inhibition of α-amino[^{14}C]isobutyric acid uptake nor its release from preloaded mitochondria was effected by high concentrations of α-amino[^{12}C]isobutyric acid, alanine, or other amino acids. Furthermore, the mitochondrial concentration of α-amino[^{14}C]isobutyric acid did not exceed its concentration in the medium even at higher temperatures (30 and 37°) and with longer incubations (60 min). Therefore, no transport mechanism was demonstrated. The existence of other transport mechanisms (such as a dicarboxylic acid transport process) has not been investigated.

Whether or not amino acid transport mechanisms have a role in mitochondrial function *in vivo* is speculative. It would appear to be consistent with the capacity of mitochondria to synthesize protein. Since mitochondria must share the cellular amino acid pool with other highly active sites of cytoplasmic protein syntheses, it seems reasonable to hypothesize that mitochondria actively accumulate amino acids from the cytoplasmic pool.

It is important to note that incorporation of certain amino acids into mitochondrial protein *in vitro* may be inhibited by competition for transport from relatively high concentrations of other added amino acids.

ACKNOWLEDGEMENTS

The authors are most grateful to Mr. James Clohessy, who provided invaluable technical assistance.

These studies were supported by Grant No. AM-01 506 and Grant No. AM-07 970 of the National Institutes of Health, U. S. Public Health Service.

REFERENCES

1 J. BUCHANAN AND D. F. TAPLEY, *Endocrinology*, 79 (1966) 81.
2 A. G. GORNALL, C. S. BARDAWILL AND M. M. DAVID, *J. Biol. Chem.*, 177 (1949) 751.
3 G. A. BRAY, *Anal. Biochem.*, 1 (1960) 279.
4 J. B. CHAPPELL, *Brit. Med Bull.*, 24 (1968) 150.
5 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
6 L. W. WHEELDON AND A. L. LEHNINGER, *Biochemistry*, 2 (1966) 3533.
7 C. F. FOX AND E. P. KENNEDY, *Proc. Natl. Acad. Sci. U.S.*, 54 (1965) 891.
8 C. R. PARK, R. L. POST, C. F. KALMAN, J. H. WRIGHT, JR., L. H. JOHNSON AND H. E. MORGAN, *Ciba Found. Colloq. Endocrinol.*, 9 (1956) 240.
9 E. HEINZ AND P. O. WALSH, *J. Biol. Chem.*, 233 (1958) 1488.
10 D. E. OXENDER AND H. N. CHRISTENSEN, *J. Biol. Chem.*, 238 (1963) 3686.
11 W. F. WIDDAS, *J. Physiol. London*, 125 (1954) 163.