Effect of L-Leucine on the Nitrogen Metabolism of Isolated Rat Liver Mitochondria

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1. L-Leucine strongly activated intramitochondrial glutamate dehydrogenase in the direction of glutamate synthesis. 2. In the deamination direction, the enzyme was not stimulated by leucine. This was probably due to a rate-limiting transport of glutamate across the mitochondrial membrane. 3. The effect of leucine on the kinetic constants of glutamate dehydrogenase in a mitochondrial sonicate was studied. 4. In isolated mitochondria, leucine did not stimulate the synthesis of citrulline with glutamate as the source of NH_3 . 5. Leucine very markedly stimulated the synthesis of glutamate from added 2-oxoglutarate + NH_4Cl . 6. Under conditions where glutamate and citrulline could be synthesized simultaneously from added NH_4Cl , leucine greatly increased glutamate synthesis at the expense of citrulline synthesis. 7. It is suggested that the intramitochondrial leucine concentration may be a factor influencing the nitrogen metabolism of the liver cell.

Glutamate dehydrogenase is of central importance in the nitrogen metabolism of rat liver (Chalamaun & Tager, 1970). In the deamination direction, the enzyme is thought to provide the NH_3 required for carbamoyl phosphate synthesis in the urea cycle. In the reverse direction it may catalyse the synthesis of glutamate from NH_3 produced by various deaminases.

Glutamate dehydrogenase is located exclusively in the mitochondrial matrix, and its activity is to some extent dependent on the redox state of the intramitochondrial nicotinamide nucleotides. In isolated mitochondria added glutamate is metabolized mainly through the transaminase pathway, and relatively little is deaminated (Borst, 1962; De Haan *et al.*, 1967). It has been proposed that intramitochondrial glutamate dehydrogenase reacts preferentially with NADP^+ and its activity in the deamination direction is controlled by the high concentration of NADPH present owing to the operation of the energy-linked transhydrogenase (Papa *et al.*, 1967).

Purified glutamate dehydrogenase is inhibited by NADH, ATP and GTP, and is activated by ADP. These modifiers influence the degree of association of the enzyme subunits (Frieden, 1963, 1971). The physiological importance of these effects has not been established. Yielding & Tomkins (1961) observed that isolated glutamate dehydrogenase is activated by L-leucine and certain other amino acids, although the stimulation observed was relatively small. The effect of leucine on the isolated enzyme was further investigated by Kun & Achmatowicz (1965). Recently, evidence for the formation of a complex between leucine and glutamate dehydrogenase has been obtained (Prough & Fisher, 1972; Prough *et al.*, 1972).

In the present paper the stimulation by leucine of glutamate dehydrogenase in intact mitochondria is reported. Leucine is found to exert a profound influence on mitochondrial nitrogen metabolism.

Experimental

Rat liver mitochondria were isolated as described by Chappell & Hansford (1972). In experiments where citrulline was to be assayed, the sucrose in the isolation medium was replaced by mannitol (Charles *et al.*, 1967).

In experiments where the production of metabolites in a mitochondrial suspension was measured, mitochondria were incubated in small flasks with the appropriate medium, which was gassed with $\text{O}_2 + \text{CO}_2$ (95:5). The flasks were shaken on a metabolic shaker. At appropriate intervals, samples were withdrawn and immediately deproteinized with HClO_4 (final concn. 5%, w/v). The precipitate was removed by centrifugation, and the supernatant was neutralized in the cold with alkaline K_3PO_4 . The KClO_4 precipitate was removed by centrifugation.

The intramitochondrial volume available to externally added leucine was determined as described by Chappell (1968). Mitochondria were incubated in a medium containing 0.12M-KCl, 0.02M-Tris-HCl, 10 μM -rotenone, 10 μM -antimycin A, $^3\text{H}_2\text{O}$ and L-[1- ^{14}C]leucine at pH 7.3 and 20°C. The suspension was centrifuged after 2 min and the amounts of ^3H and ^{14}C in the pellet and supernatant were determined by dual-channel liquid-scintillation counting in a Nuclear Chicago mark I liquid-scintillation spectrometer with a scintillator containing, in 1 litre: 600 ml of toluene, 400 ml of 2-methoxyethanol, 80 g

of naphthalene and 6g of 5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole. In control experiments, the radioactive leucine was replaced by [U- 14 C]sucrose. The matrix space of the mitochondria was assumed to be that available to $^3\text{H}_2\text{O}$ but not to [14 C]sucrose.

Citrulline was assayed by the method of Archibald (1944). Aspartate (Pfleiderer, 1963) and glutamate (Bernt & Bergmeyer, 1963) were assayed enzymically. NH_3 was assayed by the method of Levitski (1970) by using glutamate dehydrogenase. Careful calibration of the assay was necessary, since the presence of leucine in the extracts activated the glutamate dehydrogenase used. Mitochondrial protein was determined by a biuret method (Gornall *et al.*, 1949).

L-[1- 14 C]Leucine, [U- 14 C]sucrose and $^3\text{H}_2\text{O}$ were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Enzymes and nicotinamide nucleotides were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

Results

Effect of L-leucine on the activity of mitochondrial glutamate dehydrogenase

Glutamate dehydrogenase was assayed in intact mitochondria by measurement of the oxidation of intramitochondrial NAD(P)H in the presence of added 2-oxoglutarate and NH_4Cl . Malate and phosphate were added to facilitate rapid penetration of 2-oxoglutarate into the matrix space, and carbonyl cyanide phenylhydrazone was present to eliminate any effect of a transmembrane pH gradient on the transport of 2-oxoglutarate or NH_3 .

Fig. 1 shows that the initial rate of NAD(P)H oxidation was stimulated between 10- and 15-fold by 10mm-leucine, and that half-maximal stimulation was obtained at 1mm-leucine; 10mm-isoleucine produced an eightfold stimulation. Control experiments showed that no oxidation of NAD(P)H occurred in the absence of 2-oxoglutarate and that leucine did not affect the transport of 2-oxoglutarate into the mitochondria. The specificity of the activation of glutamate dehydrogenase is shown in Table 1. Of the amino acids tested, only leucine, isoleucine and valine had a significant effect when added at a concentration of 10mm. Histidine and methionine, which have been reported to activate the purified enzyme (Yielding & Tomkins, 1961; Yamaguchi, 1971), did not activate the intramitochondrial enzyme.

Glutamate dehydrogenase was assayed in the opposite direction by measurement of intramitochondrial NAD(P) $^+$ reduction on the addition of glutamate to mitochondria that had been preincubated with carbonyl cyanide phenylhydrazone to cause oxidation of the internal nicotinamide nucleotides and whose respiration was then inhibited by the addition of antimycin A. Leucine at concentrations

Fig. 1. *Activation of intramitochondrial glutamate dehydrogenase by leucine and isoleucine*

Mitochondria (3.15 mg of protein) were suspended in 2ml of a medium containing 0.12M-KCl, 0.025M-Tris-HCl, 1.25mm-2-oxoglutarate, 2.5mm-potassium phosphate, 1.25mm-malate and 0.5μM-carbonyl cyanide phenylhydrazone at pH7.3 and 25°C; 10μM-rotenone was added, followed by leucine (or isoleucine) and 5mm- NH_4Cl . The initial rate of intramitochondrial NAD(P)H oxidation was followed at 340–373nm by using a double-beam spectrophotometer. ●, Leucine; ○, isoleucine.

Table 1. *Specificity of activation of glutamate dehydrogenase by amino acids in isolated mitochondria*

Conditions are those described in Fig. 1. Amino acids were added at 10mm final concentration.

Amino acid added	Rate of NAD(P)H oxidation (μmol/min)
None	0.058
L-Leucine	0.813
L-Isoleucine	0.450
L-Valine	0.150
L-Methionine	0.070
L-Serine	0.050
L-Alanine	0.063
L-Histidine	0.063

up to 20mm stimulated the enzyme by less than 20% whatever the glutamate concentration.

The characteristics of the activation of mitochondrial glutamate dehydrogenase by leucine were studied in ultrasonically disrupted mitochondria to remove permeability barriers between the enzyme and

its substrates. The effect of 5mM-leucine on the kinetic constants of the enzyme is shown in Table 2. The V_{max} of the enzyme was increased two to three times in the deamination direction and approximately ten times in the direction of glutamate synthesis. The K_m for all the substrates was also increased, that for 2-oxoglutarate about fivefold and that for the other substrates between one- and three-fold. No further increase of V_{max} was obtained on increasing the leucine concentration to 20mM.

Permeability of mitochondria to L-leucine

Activation of glutamate dehydrogenase by leucine in intact mitochondria requires penetration of leucine into the matrix space. Fig. 2 shows the equilibrium distribution of L-[1- 14 C]leucine as a function of external leucine concentration. Leucine appeared to accumulate inside the mitochondria. The penetration of leucine was very rapid, but the presence or absence of a leucine-transporting system could not be established, since the apparent rate of leucine penetration was not affected by the presence of analogues such as isoleucine and valine, and no inhibitor of leucine penetration was found. The existence of a leucine-specific transporting system has been postulated by Buchanan *et al.* (1969). However, at the very low [14 C]leucine concentration (1 μ M) used by these authors, it is not possible to discriminate between mitochondrial radioactivity caused by leucine transport and that caused by leucine binding to the membrane.

Fig. 2. Equilibrium distribution of leucine in a mitochondrial suspension

For details see the text.

Table 2. Effect of leucine on the kinetic constants of glutamate dehydrogenase in sonicated mitochondria

Ultrasonically disrupted mitochondria (2mg of protein) were suspended in 2ml of a medium containing 0.125M-KCl, 0.025M-Tris-HCl and 10 μ M-rotenone at pH7.3 and 25°C; 5mM-leucine was present in some experiments. Initial rates of oxidation and reduction of nicotinamide nucleotides were measured at 340nm. Values of K_m and V_{max} were determined from Lineweaver-Burke plots.

Concentration of substrates added (mM)				K_m for variable substrate (mM)		V_{max} (leucine present) V_{max} (leucine absent)
2-Oxoglutarate	NADH	NADPH	NH ₄ Cl	Leucine absent	Leucine present	
Variable	—	0.15	20	0.15	0.70	9.5
2.5	—	0.15	Variable	11.9	13.3	13.2
2.5	—	Variable	20	0.026	0.060	9.2
2.5	Variable	—	20	0.047	0.107	10.1
Glutamate	NAD ⁺	NADP ⁺				
Variable	—	1		3.1	5.0	2.6
Variable	1	—		5.3	10	2.1
50	—	Variable		0.067	0.185	2.7
50	Variable	—		0.135	0.417	2.4

Effect of leucine on citrulline synthesis with glutamate as the NH_3 source

When liver mitochondria are incubated with ornithine, bicarbonate, ATP and a source of NH_3 , citrulline is synthesized. The parameters determining the rate of citrulline synthesis have been extensively investigated (Charles *et al.*, 1967; Graafmans *et al.*, 1968).

Mitochondria were incubated with an excess of ornithine and bicarbonate under conditions where the rate of NH_3 production could be shown to determine the rate of citrulline synthesis. It was found that where the glutamate dehydrogenase reaction was the only source of NH_3 , citrulline synthesis was stimulated by less than 20% on the addition of leucine. In some experiments, vitamin K_3 was added to cause oxidation of mitochondrial NADPH (Charles *et al.*, 1967). The rate of citrulline synthesis in this case was rather less and was not stimulated by leucine. These results are consistent with the very small stimulation of intramitochondrial glutamate dehydrogenase in the deamination direction reported above.

Fig. 3. Formation of glutamate from NH_3 +2-oxoglutarate

The reaction was started by addition of mitochondria (5.5 mg of protein/ml) to a medium containing 0.1 M-KCl, 0.02 M-Tris-HCl, 10 mM-pyruvate, 5 mM-2-oxoglutarate, 10 mM-phosphate, 1 mM-malate and various concentrations of NH_4Cl at pH 7.3 and 25°C. The reaction was terminated after 3 min. Formation of aspartate was negligible. ●, 5 mM-Leucine; ○, no leucine.

Effect of leucine on mitochondrial reactions involving NH_3 as substrate

NH_3 can be metabolized by two different pathways in isolated liver mitochondria. In the presence of 2-oxoglutarate and a source of reducing equivalents (e.g. pyruvate), glutamate is formed by the reversal of the glutamate dehydrogenase reaction. Fig. 3 shows that in the absence of leucine very little glutamate was formed when NH_4Cl was present at low concentrations. The addition of leucine to the system stimulated glutamate dehydrogenase with the consequent rapid formation of glutamate at concentrations of NH_4Cl down to 0.2 mM.

In the presence of ornithine and bicarbonate, added NH_4Cl can be metabolized to carbamoyl phosphate and hence to citrulline. The dependence of the rate of citrulline synthesis on NH_4Cl concentration is shown in Fig. 4. In the absence of added 2-oxoglutarate, no glutamate could be formed from NH_4Cl . Leucine did not affect citrulline synthesis under these conditions.

Simultaneous synthesis of citrulline and glutamate from NH_4Cl

Mitochondria were incubated in the presence of ornithine, bicarbonate, 2-oxoglutarate and pyruvate. Under these conditions, added NH_4Cl was metabolized to both citrulline and glutamate. Table 3 shows

Fig. 4. Citrulline synthesis from added NH_4Cl

The reaction medium contained 0.08 M-KCl, 0.02 M-Tris-HCl, 5 mM-succinate, 3 mM-ATP, 0.02 M- KHCO_3 , 10 mM-phosphate, 10 mM-ornithine-HCl and various concentrations of NH_4Cl at pH 7.3 and 25°C. Mitochondria (4.5 mg/ml) were added and citrulline formation was assayed after 4, 8 and 12 min. ●, 5 mM-Leucine; ○, no leucine.

the metabolic fate of added NH_4Cl in this system. In the absence of leucine virtually equal amounts of glutamate and citrulline were formed together with some aspartate (formed from glutamate by transamination). When leucine was present the NH_3 was used faster. Glutamate (+aspartate) synthesis was greatly stimulated, whereas the synthesis of citrulline was inhibited. In both cases, NH_3 disappearance was nearly quantitatively accounted for by the production of citrulline+glutamate+aspartate.

Table 4 shows the result of a similar experiment where different concentrations of NH_4Cl were used. At all NH_4Cl concentrations the addition of leucine

markedly stimulated glutamate synthesis and inhibited citrulline synthesis. (The small amount of glutamate formed in the absence of NH_4Cl was due to transamination of 2-oxoglutarate and ornithine by intramitochondrial ornithine-2-oxoglutarate transaminase, and could be abolished by transaminase inhibitors.) The inhibition of citrulline synthesis in these experiments was caused by competition for added NH_4Cl between citrulline synthesis and glutamate synthesis, which was much increased by leucine. Leucine was shown to have no effect on citrulline synthesis under conditions where glutamate synthesis cannot occur (Fig. 4).

Table 3. *Effect of leucine on the metabolic fate of NH_4Cl*

The incubation contained 0.08M-KCl, 0.02M-Tris-HCl, 5mM-2-oxoglutarate, 10mM-phosphate, 3mM-ATP, 10mM-pyruvate, 1mM-malate, 15mM- KHCO_3 , 10mM-ornithine-HCl, 1.6mM- NH_4Cl and 5.2mg of mitochondrial protein/ml at pH 7.3 and 25°C. In some experiments 5mM-leucine was also present. The values shown are corrected for the small amounts of metabolites formed in the absence of added NH_4Cl . Assays were performed in duplicate.

Time (min)	Additions	NH_3 disappearance ($\mu\text{mol/ml}$)	Formation of metabolites ($\mu\text{mol/ml}$)		
			Glutamate	Aspartate	Citrulline
4	None	0.56	0.19	0.06	0.19
8	None	0.92	0.27	0.12	0.35
12	None	1.15	0.46	0.15	0.56
4	Leucine	0.98	0.56	0.13	0.14
8	Leucine	1.38	0.71	0.26	0.19
12	Leucine	1.59	0.81	0.47	0.20

Table 4. *Effect of leucine on NH_3 metabolism*

The reaction medium was that described in Table 3 except that various concentrations of NH_4Cl were used and the mitochondrial protein concentration was 4.1mg/ml; 5mM-leucine was present where shown. Assays were performed in duplicate.

[NH_4Cl] (mM)	Time of incubation (min)	Formation of metabolites ($\mu\text{mol/ml}$)					
		Leucine absent			Leucine present		
		Glutamate	Aspartate	Citrulline	Glutamate	Aspartate	Citrulline
0	5	0.07	0	0.04	0.07	0	0.01
0	10	0.25	0.03	0.07	0.25	0.04	0.06
0	15	0.32	0.04	0.06	0.33	0.05	0.05
0.5	5	0.10	0.02	0.11	0.31	0.02	0.06
0.5	10	0.35	0.03	0.15	0.55	0.03	0.07
0.5	15	0.57	0.04	0.16	0.73	0.05	0.07
1.0	5	0.10	0.01	0.11	0.41	0.07	0.08
1.0	10	0.32	0.03	0.18	0.64	0.09	0.10
1.0	15	0.49	0.08	0.19	0.83	0.15	0.11
1.5	5	0.11	0.07	0.33	0.50	0.14	0.30
1.5	10	0.36	0.10	0.57	0.79	0.18	0.36
1.5	15	0.62	0.14	0.75	1.01	0.25	0.37

Fig. 5. Simultaneous synthesis of glutamate (a) and citrulline (b) with adenosine as NH_3 source

The reaction medium was that described in Table 3 except that NH_4Cl was replaced by 5 mM-adenosine and the protein concentration was 3.3 mg/ml. The reaction was started by the addition of adenosine deaminase and was terminated after 10 min. ●, 5 mM-Leucine; ○, no leucine.

To obtain conditions nearer to those in the intact cell, NH_3 was produced continuously in the incubation medium by the action of added adenosine deaminase with adenosine as substrate. The stimulation of glutamate synthesis (Fig. 5a) and consequent inhibition of citrulline synthesis (Fig. 5b) in this system was very marked.

Absence of leucine metabolism in liver mitochondria

The first reaction of leucine metabolism is the transamination of leucine with 2-oxoglutarate to form α -oxo-4-methylpentanoate and glutamate, catalysed by the enzyme L-leucine-2-oxoglutarate transaminase. This enzyme has very low activity in rat liver, although it is active in other tissues (Awapa & Seale, 1952; Ichibara & Koyama, 1966). Ultrasonically disrupted mitochondria were incubated with 10 mM-leucine plus 10 mM-2-oxoglutarate (in the presence of rotenone) for periods of up to 2 h at 20°C . No glutamate was detected in the incubation mixture. It was concluded that leucine-2-oxoglutarate transaminase has negligible activity in rat liver mitochondria and that none of the effects reported above involved the metabolism of leucine.

Discussion

Leucine is a very potent activator of intramitochondrial glutamate dehydrogenase. It increases the V_{max} and also the K_m of the enzyme for all the substrates. A similar effect of leucine on crystalline

glutamate dehydrogenase has been reported (Kun & Achmatowicz, 1965), but the stimulation of V_{max} observed in this case was only threefold in the direction of glutamate synthesis, even in the presence of 50 mM-leucine. These results suggest that the mitochondrial enzyme *in situ* is far more responsive to activation by low concentrations of leucine than is the crystalline enzyme previously studied.

The apparent failure of leucine to stimulate intramitochondrial glutamate dehydrogenase in the deamination direction indicates that the enzyme may be rate-limited in this direction by the transport of glutamate into the mitochondria. Preliminary experiments (N. M. Bradford, unpublished work) have shown that the transport of glutamate is very much slower than that of other metabolites such as malate and phosphate.

The very small stimulation by leucine of citrulline synthesis with glutamate as the NH_3 source is also consistent with a rate-limiting transport of glutamate across the mitochondrial membrane. If glutamate deamination were controlled mainly by the concentration of intramitochondrial NADPH (Papa *et al.*, 1967), then the addition of vitamin K_3 , which causes NADPH oxidation (Charles *et al.*, 1967), should allow a greater stimulation of glutamate deamination and hence of citrulline synthesis by leucine. The results obtained indicate that the rate of glutamate deamination in isolated mitochondria may be controlled primarily by the rate of glutamate transport. Since glutamate synthesis is so markedly increased by leucine, it is possible that the main function of

mitochondrial glutamate dehydrogenase is glutamate synthesis rather than glutamate deamination.

The synthesis of glutamate from low concentrations of NH_3 is extremely slow in the absence of leucine (Fig. 3). Leucine at low concentrations appears to 'switch on' glutamate synthesis. The stimulation of glutamate synthesis is so marked that glutamate synthesis competes successfully with citrulline synthesis for added NH_4Cl . Under appropriate conditions, the effect of adding leucine is to switch the major part of NH_3 metabolism in mitochondria from citrulline synthesis to glutamate synthesis (Fig. 5).

In the liver cell, NH_3 is continuously produced in the cytoplasm, in particular by serine dehydratase, threonine dehydratase and adenylate deaminase. This NH_3 can be excreted in the form of urea or stored as glutamate (or glutamine) for subsequent amino acid synthesis. The results reported in the present paper suggest that the intramitochondrial concentration of leucine may be a factor determining the relative amounts of NH_3 stored and excreted by the liver cell. Small variations in the cytoplasmic leucine concentration between 0 and 1 mM would be expected to have a significant effect on the relative rate of synthesis of citrulline (and hence urea) and glutamate from cytoplasmic NH_3 .

The available evidence indicates that no large changes in the whole liver content of leucine occur under conditions of metabolic stress such as diabetes (Kirsten *et al.*, 1961), starvation or hyperglycaemia (Burns *et al.*, 1970). Whether significant changes in the intracellular distribution of leucine occur under these conditions is not known at the present time.

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