The Effects of L-Leucine on the Synthesis of Urea, Glutamate and Glutamine by Isolated Rat Liver Cells

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With either alanine or a mixture of 15 different amino acids as nitrogen source, the addition of L-leucine inhibited the synthesis of urea by isolated rat liver cells. With alanine present leucine promoted the production of glutamate and glutamine. Comparison of effects of leucine on soluble glutamate dehydrogenase, mitochondria and isolated cells supports the postulate that leucine exerts its effect through activation of glutamate dehydrogenase. It is suggested that this latter enzyme may not be as important for the production of NH_3 for carbamoyl phosphate synthesis as has been considered hitherto.

It has been shown (McGivan *et al.*, 1973) that the addition of L-leucine to isolated rat liver mitochondria causes an inhibition of citrulline synthesis and a corresponding increase in glutamate synthesis from NH_3 as nitrogen source. These effects have been attributed to a large stimulation of glutamate dehydrogenase activity induced by leucine which is expressed only in the direction of glutamate synthesis. Stimulation of this enzyme activity in the direction of glutamate deamination is not expressed, probably because of the relatively low rate-limiting activity of the system responsible for glutamate transport (Bradford & McGivan, 1973).

It was decided to see whether this effect of leucine on soluble glutamate dehydrogenase was reflected in the nitrogen metabolism of intact cells. The advantages of using liver cells rather than slices or perfused liver have been enumerated (Krebs *et al.*, 1973; Sauer & Mahadevan, 1973). It has been reported briefly that leucine inhibits the formation of urea from endogenous amino acids and from exogenous NH_3 by perfused rat liver (Buttery & Boorman, 1971). No reason was put forward to account for these effects. In the present paper the effects of leucine on the production of several metabolites during the utilization of amino acids by isolated rat liver cells are reported.

It was shown by using L-[^{14}C]leucine that this amino acid did not, under the conditions used, contribute its carbon skeleton to any significant extent to the formation of any of the metabolites measured.

Experimental

Isolation of rat liver cells

Cells were isolated from the livers of 200–250 g male Wistar rats (starved for 48 h) by using collagenase

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and hyaluronidase, by a modification of the method of Berry & Friend (1969) as suggested by Cornell *et al.* (1973) and Krebs *et al.* (1974), except that in the present work a simplified perfusion apparatus was used. This consisted of a simple glass vessel which served as both reservoir and oxygenator. The perfusion rate was 25 ml/min. The enzyme-containing solution was used after an initial 10 min perfusion, during which the bulk of blood cells was removed and some intercellular linkages were loosened or broken because the medium had no added Ca^{2+} . After 20–30 min perfusion with the enzyme-containing medium the liver was removed, chopped thoroughly and shaken for 25 min in 50 ml of fresh enzyme-containing medium bubbled with $\text{O}_2 + \text{CO}_2$ (95:5). The cells were washed three times with enzyme-free Ca^{2+} -containing Krebs–Henseleit medium (Krebs & Henseleit, 1932) containing 2.5% (w/v) dialysed serum albumin before use. The whole procedure was carried out at 37°C, since this minimizes the loss of intracellular K^+ (Heckmann & Parsons, 1959).

The viability of the cells prepared by us was 85–95% as judged by staining with 0.02% Trypan Blue. The rate of formation of glucose from 10 mM-lactate was 1.5–3.4 nmol/min per mg of protein at 37°C. The ratio of water space to inulin space of the packed cells was 2.3–3.0. These parameters are comparable with those reported by other workers (Cornell *et al.*, 1973; Tager *et al.*, 1973; Quistorff *et al.*, 1973).

Measurement of metabolite changes

The cells (14–30 mg wet wt. of cells/ml of incubation medium) were suspended in the washing medium described above. Substrates were added as indicated and the suspension was gassed with $\text{O}_2 + \text{CO}_2$ (95:5) and shaken continuously at 37°C. At intervals samples were removed and deproteinized with HClO_4 (final concn. 1.5%, v/v). After centrifugation a sample

of the supernatant was neutralized with 1M-K₃PO₄. After cooling, the precipitate of KClO₄ was removed by centrifugation and the supernatant used for metabolite assays, as follows. Glutamate (Bernt & Bergmeyer, 1965), lactate (Hohorst, 1965), pyruvate (Bücher *et al.*, 1965), glucose (Krebs *et al.*, 1963), alanine (Williamson *et al.*, 1967b) and aspartate (Pfleiderer, 1965) were assayed enzymatically. NH₃ was assayed as described by Kirsten *et al.* (1963) and urea was determined in the same sample by measuring the release of NH₃ consequent on the addition of urease (0.1 mg/ml). Glutamate was determined as described by Bernt & Bergmeyer (1965) except that a correction was made for the slow reaction of leucine with the glutamate dehydrogenase. Protein was determined by the biuret method of Gornall *et al.* (1949).

Experiments with radioactive amino acids

Suspensions of rat liver cells were incubated under the conditions described above with either L-[U-¹⁴C]-leucine (10 μ Ci) or L-[U-¹⁴C]alanine (10 μ Ci). The radioactive amino acids as supplied were taken to dryness by storage *in vacuo* over P₂O₅ for 48 h to remove the ethanol in which they were supplied by The Radiochemical Centre, Amersham, Bucks., U.K. This is important, since ethanol has marked effects on the metabolism of alanine by liver (Krebs *et al.*, 1973). The amino acids were redissolved in water.

Samples (0.025–0.05 ml) of the deproteinized and neutralized incubation media were placed on sheets of Whatman 3MM paper (20 cm $\times$ 20 cm) and subjected to electrophoresis for 30 min in an aqueous solution containing pyridine–acetic acid (1:0.4, v/v) (pH 5.3) at 1000 V and 20°C in one direction and, after drying, to chromatography at right angles to the direction of electrophoretic migration. When labelled leucine was used, ascending chromatography was in phenol–water and for labelled alanine it was in ethanol–NH₃ as described by Smith (1969). The solvent front was allowed to reach the top of the paper (4.5 and 2 h respectively). The papers were dried and placed in contact with Kodirex X-ray film [47.5 cm (19 in) $\times$ 42.5 cm (17 in)] KD-54T for 48 h and 120 h respectively. Under these conditions of separation the amino acids of interest, that is leucine, glutamate, glutamine and alanine, and glucose and lactate (together with 3-hydroxybutyrate and acetoacetate), were well separated, and produced, when present, distinct blackening of the X-ray film. The areas of paper corresponding to the compounds of interest were excised and placed in a plastic vial with 2 ml of water and shaken for 30 min and the ¹⁴C present was determined by scintillation counting in a Nuclear–Chicago Isocap model 300 counter after addition of 10 ml of the scintillation fluid. The scintillation system contained 900 ml of dioxan and

100 ml of methanol in which was dissolved 60 g of naphthalene, 4 g of 2,5-diphenyloxazole, 0.2 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene. The values given in the Tables are those obtained after correction for quenching.

Chemicals and enzymes

Collagenase (type II) was purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Nucleotides and other enzymes were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. Other chemicals were obtained from British Drug Houses Ltd., Poole, Dorset, U.K.

Results

The addition of L-leucine to isolated mitochondria markedly inhibited citrulline synthesis from ornithine and stimulated glutamate formation from 2-oxo-glutarate when NH₃ served as the nitrogen source (McGivan *et al.*, 1973). To determine whether leucine has comparable effects on whole cells the effect of this amino acid on urea and glutamate formation with alanine as carbon and nitrogen source was determined.

In preliminary experiments it was shown by using six separate cell preparations that the addition of 5 mM-L-leucine inhibited the rate of urea synthesis from L-alanine by 69.8 $\pm$ 6.6% (S.E.M.). In the same experiments the rate of glutamate formation was enhanced by 78 $\pm$ 21%. More information was obtained by measuring simultaneously the changes in a number of metabolites (Table 1). The time-courses for the production of lactate, glutamate, glutamine, urea and glucose were approximately linear for 60 min. The results show that the amino N lost from alanine almost quantitatively appeared in glutamate, glutamine and urea, irrespective of the presence or absence of added leucine. However, when leucine was present urea synthesis was markedly decreased and that of glutamate and glutamine elevated. The overall rate of alanine utilization was not materially affected. This latter finding indicates clearly that if either the transport or transamination of alanine is rate-limiting in its metabolism, then neither of these processes is affected by the presence of leucine.

The results shown in Table 1 also show that in the presence of leucine gluconeogenesis is partially inhibited but that there is an increased flow of carbon into glutamate and glutamine synthesis.

The possibility that leucine contributed its nitrogen or carbon significantly in these experiments seems to be unlikely, since the activity of leucine–2-oxo-glutarate aminotransferase in rat liver is very low (Awapara & Seale, 1952; Ichihara & Koyama, 1966).

Table 2. *Absence of L-leucine metabolism in isolated rat liver cells*

The incubations were performed as described in the Experimental section. The reaction was started by the addition of alanine (10 mM final concn.) together with [U-¹⁴C]leucine (5 mM). Samples were taken at the intervals shown and counted for radioactivity after separation of leucine by electrophoresis and chromatography. The results shown are typical of those obtained from several batches of cells.

Incubation time (min) ...	Radioactivity (d.p.m./mg of protein)			
	0	20	40	60
Neutralized extract	200 800	193 400	185 000	198 500
Leucine spot after electrophoresis, chromatography and elution	173 100	169 900	159 100	171 100
Percentage of counts recovered after leucine separation	86.2	87.9	86.0	86.2

Table 3. *Effect of L-leucine on urea formation from a mixture of amino acids*

Isolated liver cells were incubated as described in the Experimental section. The reaction was started by addition of a mixture of amino acids such that the final concentrations were 0.6 mM-L-alanine, 0.16 mM-L-arginine, 0.015 mM-L-aspartate, 0.6 mM-L-glutamine, 0.45 mM-L-glycine, 0.1 mM-L-histidine, 0.4 mM-L-lysine, 0.1 mM-L-methionine, 0.05 mM-L-ornithine, 0.1 mM-L-phenylalanine, 0.3 mM-L-serine, 0.3 mM-L-threonine, 0.12 mM-L-tryptophan, 0.12 mM-L-tyrosine and 0.3 mM-L-valine. Progress curves were drawn for values for samples taken at 0, 20, 40 and 60 min. Similar results were obtained in experiments of this type with several batches of cells.

Further additions	Rate of urea formation (nmol/min per mg of protein)	Inhibition (%)
None	13	(0)
0.15 mM-L-Isoleucine	9	34
0.25 mM-L-Leucine	7	46
5 mM-L-Leucine	5	62

amount of any particular compound formed is taken as:

$$\frac{\text{amount of that compound formed}}{\text{amount of total metabolites assayed}} \times 100\%$$

This allows for the contribution of endogenous substrate to the changes observed, which would not be the case if the amount of alanine disappearing were used in the denominator. In the radioactivity experiment (Table 4) the relative amount of any particular compound formed was calculated on the basis:

$$\frac{\text{d.p.m. in the particular compound}}{\text{sum of d.p.m. in glutamate, glutamine and glucose}} \times 100\%$$

It was not possible to use the amount of radioactivity disappearing from the labelled alanine added as the denominator, since CO₂ liberated in decarboxylation reactions would be rapidly lost (the incubation mixture was vigorously bubbled with

Table 4. *L-Alanine utilization by isolated rat liver cells*

The incubations were performed as described in the Experimental section. The reaction was started by the addition of [U-¹⁴C]alanine (10 mM final concn.; 353.7×10^3 d.p.m./mg of protein) ('Ala') or [U-¹⁴C]alanine (10 mM; 291.2×10^3 d.p.m./mg of protein) together with leucine (5 mM) ('Ala + Leu'). Samples were taken at 0, 20, 40 and 60 min and counted for radioactivity. The results shown are those obtained from progress curves obtained by using a single batch of cells. Similar results were consistently obtained in several experiments of this type.

	Metabolite (d.p.m./mg of protein)									
	Alanine		Glucose		Glutamate		Glutamine		Lactate + ketone bodies	
	'Ala'	'Ala + Leu'	'Ala'	'Ala + Leu'	'Ala'	'Ala + Leu'	'Ala'	'Ala + Leu'	'Ala'	'Ala + Leu'
Change after 60 min	-37300	-36000	+7900	+5800	+2600	+7100	+3700	+9700	+16000	+9100

Table 5. Pattern of distribution of metabolic products resulting from the incubation of liver cells with alanine in the presence and absence of leucine.

See Tables 1 and 4 and the text. 'Sum of ^{14}C in metabolites' is the sum of d.p.m. in glucose, glutamate and glutamine; see the text.

Metabolite assays Conditions	Changes in alanine (nmol/h per mg of protein)	Change in amount of metabolite assayed (nmol of C_3 units/h per mg of protein)				Sum of C_3 units assayed (nmol/h per mg of protein)
		Glucose	Glutamate	Glutamine	Lactate	
No leucine	-738	+468	+144	+228	+86	+926
Contribution (%)	—	(51)	(16)	(25)	(9)	(100)
With leucine	-726	+322	+292	+398	+52	+1064
Contribution (%)	—	(30)	(27)	(37)	(5)	(100)

^{14}C distribution Conditions	Change in ^{14}C in alanine (d.p.m./h per mg of protein)	Change in ^{14}C in separate metabolites (d.p.m./h per mg of protein)				Sum of ^{14}C in metabolites (d.p.m./h per mg of protein)
		Glucose	Glutamate	Glutamine	Lactate	
No leucine	-37300	+7900	+2600	+3700	—	+14200
Contribution (%)	—	(56)	(18)	(26)	—	(100)
With leucine	-36000	+5800	+7100	+9700	—	+22600
Contribution (%)	—	(26)	(31)	(43)	—	(100)

non-labelled CO_2) and ^{14}C would be rapidly incorporated into lactate by enzymic exchange reactions catalysed by alanine aminotransferase and lactate dehydrogenase in reactions not involving net metabolism.

Discussion

The results presented in this paper show that the addition of leucine to a suspension of isolated rat liver cells caused an inhibition of urea formation and an increase in the formation of glutamate and glutamine, when alanine served as nitrogen source. The inhibition of urea synthesis by leucine was also observed when a mixture of 15 different amino acids at physiological concentrations replaced alanine. In this latter case the rate of urea synthesis was four times higher than with alanine alone, a finding which indicates that the site of action of leucine is not one that is peculiar to alanine metabolism.

Leucine was not degraded to any detectable extent by isolated liver cells under the conditions used in the experiments described in this paper (Table 2), a finding which is consistent with the inability of Awapara & Seale (1952) and Ichihara & Koyama (1966) to detect leucine-2-oxoglutarate aminotransferase in significant amount in rat liver extracts. It seems likely therefore that leucine exerts its effect directly and not as a result of its metabolic degradation.

An established effect of leucine is a marked activation of the glutamate dehydrogenase activity of ultrasonically disrupted mitochondria (McGivan *et al.*, 1973). The effect of leucine is to increase the apparent K_m for glutamate, 2-oxoglutarate, NAD^+ , NADH , NADP^+ , NADPH and to some extent NH_3 .

Leucine also causes a marked increase in $V_{\max}$ for the enzyme. Similar, but less marked, effects have been observed with the crystalline enzyme (Kun & Achmatowicz, 1965). Leucine also markedly activates the glutamate dehydrogenase activity of intact isolated rat liver mitochondria in the direction of glutamate synthesis, but has no significant effect on the rate of glutamate deamination. This effect has been attributed to the rate-limiting effect of the inward transport of glutamate into mitochondria (McGivan *et al.*, 1973; Bradford & McGivan, 1973). Leucine has no detectable effect on the rate of citrulline synthesis from NH_3 , CO_2 and ornithine (McGivan *et al.*, 1973).

In the present context it is pertinent to note that when isolated mitochondria are provided with a continuous rate-limiting supply of NH_3 (by addition of adenosine and its deaminase) under conditions where they are able to synthesize glutamate (from added oxoglutarate) and citrulline (from added ornithine and CO_2) the addition of leucine favours glutamate synthesis at the expense of citrulline formation. This effect parallels very closely the effects described here on isolated whole cells. It therefore appears that, in order to account for the effect of leucine on the nitrogen metabolism of isolated liver cells, no other effect than that of leucine on the glutamate dehydrogenase need be invoked (see Scheme 1). This does not imply that other effects of leucine may not occur, for example the inhibition of urea synthesis at an extra-mitochondrial site.

It is commonly held with regard to glutamate dehydrogenase that it is directly responsible for the production of NH_3 for carbamoyl phosphate synthesis and thus of one of the two nitrogen atoms of the

urea molecule (see, e.g., Chamalaun & Tager, 1970) and that the enzyme is so active *in vivo* that the reaction which it catalyses is in 'thermodynamic equilibrium' or nearly so (Williamson *et al.*, 1967a; Krebs & Veech, 1969; Brosnan *et al.*, 1970). If the latter view were correct then it follows that the glutamate dehydrogenase cannot be rate-limiting in any process, by definition. Particularly, it would be expected that increasing the activity of any enzyme which was already sufficiently active to catalyse a steady-state 'thermodynamic equilibrium' would have no effect on the rate of the overall process. However, this is clearly not the case in the experiments described here, since leucine, an activator of glutamate dehydrogenase, caused an increase in glutamate (and glutamine) synthesis and an inhibition of urea production. This implies that the glutamate dehydrogenase reaction is not at equilibrium in isolated liver cells. The question may also be posed as to whether this enzyme is as important in NH_3 production for urea synthesis as current opinion suggests.

The NH_3 for carbamoyl phosphate synthesis may be derived from some source other than the deamination of glutamate by its dehydrogenase. This NH_3 could arise from the deamination of glutamine or adenylate either in the liver or in extrahepatic tissues, e.g. muscle and kidney. Preliminary experiments by Miss K. Moss in this Laboratory have shown that the deamination of AMP produced from IMP and aspartate (Scheme 1) (Tornheim & Lowenstein, 1972) can lead to the production of 3 nmol of NH_3 /min per mg of cytosol protein at 37°C, which corresponds approximately to a rate of production of 0.35 μmol of NH_3 /min per g wet wt. of liver. By comparison rat liver mitochondria produce 4 nmol of NH_3 /min per mg of mitochondrial protein at 30°C from 20 mM-glutamate (McGivan *et al.*, 1974), which corresponds to a maximum rate of NH_3 production of approx. 0.2 μmol /min per g wet wt. of liver at 37°C. This latter rate is probably too high in that it represents the calculated rate of NH_3 production from 20 mM-glutamate. The apparent K_m for the inward transport of glutamate in mitochondria is 4 mM (Bradford & McGivan, 1973), so that at physiological concentrations of glutamate (2–3 μmol /g wet wt.) (Krebs & Veech, 1969) the rate of NH_3 production from glutamate may be calculated, by using the Michaelis–Menten relationship, to be approx. 0.08 μmol /min per g wet wt. of liver, that is the rate of production of NH_3 from glutamate is considerably less than that which would appear to be possible from the purine nucleotide cycle (Tornheim & Lowenstein, 1972). This estimate of the rate of production of NH_3 from glutamate is based on measurements with intact mitochondria and takes into account the restricted permeability of the mitochondrial membrane to glutamate, which previous estimates had not.

The observed decrease in the rate of glucose synthesis from alanine in the presence of leucine (Tables 1 and 4) may also arise from stimulation of glutamate dehydrogenase resulting in increased glutamate synthesis, which would cause the intracellular nicotinamide nucleotides to become relatively more oxidized, which would tend to inhibit gluconeogenesis and favour the formation of 2-oxoglutarate and glutamate from the carbon skeleton of alanine.

Leucine is an essential amino acid, the plasma concentration being reflected by the dietary intake (Fernstrom *et al.*, 1971). If the situation in the isolated liver cell reflects the situation *in vivo* it appears that under conditions of high-protein feeding the relative amounts of nitrogen excreted as urea or stored as glutamine may be dictated in part by the intracellular leucine concentration.

Note Added in Proof (Received 1 November 1974)

The possibility that leucine inhibits urea synthesis by isolated liver cells at a site additional to its action on the glutamate dehydrogenase was considered in the Discussion section. It is known from previous work that the enzymes involved in the conversion of ornithine into citrulline with NH_3 as nitrogen source are not affected by leucine (McGivan *et al.*, 1973). That part of the urea cycle in which citrulline is converted into arginine and then into urea and ornithine occurs in the cytosol (see Scheme 1). It has now been shown that these reactions are unaffected by the presence of leucine. Rat liver homogenates were centrifuged at 40000g for 30 min in the presence of 10 mM-MgCl₂ in 0.15 M-KCl and 10 mM-potassium *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonate buffer, pH 7.2. Endogenous substrates were removed by passing the supernatant down a column of Sephadex G-25 and elution with the preparation medium described above. Urea production was followed in a medium containing 10 mM-citrulline, 10 mM-aspartate, 10 mM-ATP and cytosol protein (10 mg/ml), in the presence and absence of 5 mM-leucine. The reaction medium was bubbled continuously with O₂+CO₂ (95:5, v/v) at 37°C. Samples were taken at 0, 5, 10, 20, 30, 40, 50 and 60 min and were assayed for urea (see the Experimental section). The rate of urea formation in one series of experiments was 13.3±2.5 (means±s.e.m., eight observations) in the absence of leucine and 13.9±0.8 (8) in its presence. In another separate set of experiments these values were 12.0±1.2 (7) and 11.2±1.0 (7) nmol/min per mg of protein respectively.

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