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Leucine and tryptophan metabolism in rats

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1. The rate of tryptophan metabolism in isolated liver cells from animals fed on a high-leucine diet was greater than for cells from control animals. 2. Leucine inhibited tryptophan metabolism and tryptophan uptake in isolated liver cells, probably by competing for membrane transport. Leucine had no effect on tryptophan 2,3-dioxygenase *in vitro*. 3. 4-Methyl-2-oxovalerate increased tryptophan oxidation in incubations containing albumin, by displacing bound tryptophan and increasing the availability of the amino acid to the cell. 4. The results suggest that, under extreme conditions, when the availability of tryptophan is low, leucine may be pellagragenic.

It has been suggested that the incidence of pellagra among people whose dietary staple is jower (*Sorghum vulgare*) may be attributable to the relative excess of leucine over tryptophan, rather than to deficiency of the latter amino acid itself (Gopalan & Srikantia, 1960). The literature contains conflicting reports to show that a dietary excess of leucine may (Gopalan & Srikantia, 1960; Ghafoorunissa & Narasinga Rao, 1973; Krishnaswany & Bapu Rao, 1978; Yamada *et al.*, 1979; Bender, 1983; Magboul & Bender, 1983) or may not (Nakagawa *et al.*, 1975; Manson & Carpenter, 1978*a*,*b*) be pellagragenic. If leucine is indeed pellagragenic, a number of mechanisms may be involved: (1) alteration of tryptophan metabolism, mediated by changes in amounts of activities of enzymes as a result of chronic exposure to the high-leucine diet; (2) alteration of tryptophan metabolism mediated by direct interaction of leucine or a metabolite with one or more enzymes; it has been suggested that kynureninase (EC 3.7.1.3) (Bender, 1983; Magboul & Bender, 1983) and picolinate carboxylase (EC 4.1.1.45) (Bender, 1983) may be potential sites sensitive to leucine; (3) decreased tryptophan metabolism as a result of decreased availability of tryptophan either intracellularly, owing to decreased tissue uptake, or in the whole body; (4) increased catabolism of nitocinamide nucleotides.

The present paper describes experiments designed to investigate the interactions between leucine and tryptophan metabolism. The results suggest that, in situations where leucine may be pellagragenic, this effect is unlikely to be mediated by either short-term or long-term changes in the activities of the enzymes of tryptophan catabolism [i.e. by mechanisms (1) and (2) above], but that decreased tissue uptake of tryptophan may result in decreased synthesis of nicotinamide nucleotides [mechanism (3) above].

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Materials and methods

Animals

Male Sprague–Dawley rats were obtained from the University of Manchester animal house. For investigation of the effects of dietary leucine, animals were weaned at 3 weeks on the maize/gelatin/sucrose diet described previously (Magboul & Bender, 1983), providing 900mg of tryptophan/kg and essentially no available niacin. The high-leucine diet provided an additional 15g of L-leucine/kg.

In other experiments, rats weighing 180–220g received standard CRM diet (Labsure, Poole, Dorset, U.K.).

All animals were given water *ad libitum*.

Chemicals

4-Methyl-2-oxovalerate (ketoleucine) and L-leucine were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Other chemicals were from sources given previously (Smith *et al.*, 1980).

Procedures

Cells were isolated from the livers of fed rats and incubated as described previously (Elliott *et al.*, 1976; Smith & Pogson, 1980; Smith *et al.*, 1980) with the following amendments. (*a*) The perfusion medium contained 20mM-D-glucose. (*b*) The washing solutions and incubation medium contained 5mM-D-glucose, 5mM-L-glutamine, 4.5mM-L-lactate and 0.5mM-pyruvate; this medium was used to maintain glycogen; unless otherwise stated, the incubation medium also contained 2% (w/v) bovine serum albumin. (*c*) Cells were preincubated for 25–30min before addition of [^{14}C]tryptophan (final concentration 0.1mM); reactions were terminated after a further 90min.

The methods for assessment of metabolic flux through the tryptophan catabolic pathway have been described elsewhere (Smith & Pogson, 1980; Smith *et al.*, 1980). The results are expressed as the means ± S.E.M. for independent observations on hepatocytes from different rats. Each observation represents the mean of three replicate determinations.

The uptake of tryptophan into cells was measured under steady-state conditions. Cells were incubated (6mg dry wt./ml) at 37°C in medium containing 5mM-D-glucose, 5mM-L-glutamine, 4.5mM-L-lactate and 0.5mM-pyruvate (but not albumin) for 25min before addition of unlabelled tryptophan (to a final concentration of 50μM). L-[*ring*-2-^{14}C]Tryptophan (final specific radioactivity 10^4d.p.m./nmol) was added after a further 5min. Cells from 1ml samples, removed after 5s, 15s and 30s of incubation, were centrifuged (12000*g*, 30s, room temperature) through 250μl portions of dinonyl phthalate/Dow–Corning 550 silicone oil (1:2, v/v) into 100μl portions of 0.92M-$HClO_4$. Contamination of cells with extracellular medium was measured simultaneously with [^{14}C]-sucrose; appropriate corrections for this contamination were made. Rates of uptake were linear for approx. 30s in all cases. Results are based on triplicate determinations at each time point.

Tryptophan 2,3-dioxygenase (EC 1.13.11.11) activity was assayed essentially as described by Metzler *et al.* (1982) in extracts of cells sonicated (three 5s bursts) in assay medium (10mg of cells in 0.6ml of medium).

Results and discussion

Animals fed on the control and high-leucine diets gained weight at similar rates. Control animals grew from 42±2 to 90±5g; those receiving the high-leucine diet grew from 44±2 to 88±3g in the same 30-day period (mean±S.D.; $n = 6$).

Liver cells from rats given the high-leucine diet exhibited significantly higher rates of tryptophan metabolism than did cells from control animals (Table 1). The activity of tryptophan 2,3-dioxygenase was also increased in proportion (as shown by the ratio of enzyme activity to flux). The high-leucine diet had no effect on the ratio of DNA to dry weight of cells (M. Salter & C. I. Pogson, unpublished work), and so results expressed per unit dry weight (as in Table 1) or per mg of DNA are comparable. The relative rates of flux through tryptophan dioxygenase (I), kynureninase (II) and to acetyl-Co (III) (see Table 1) were similar in both cell populations, suggesting that enzymes subsequent to the dioxygenase respond to an increase in substrate supply. Previous studies in this laboratory (Smith *et al.*, 1980) have shown that, in rats, the difference between the fluxes through kynureninase and to acetyl-CoA is quantitatively accounted for by the formation of the nicotinamide nucleotide precursor, quinolinate. The implication of the

Table 1. *Effect of a high-leucine diet on tryptophan metabolism in isolated liver cells from fed rats*

Metabolic fluxes were calculated from the formation of $^{14}CO_2$ and ^{14}C-labelled products not adsorbed on Norit GSX from [*ring*-2-^{14}C]tryptophan, [1-*carboxy*-^{14}C]tryptophan and [*benzene-ring*-U-^{14}C]tryptophan as described by Smith *et al.* (1980). Other procedures were as described in the text. Results are means ± S.E.M. for three independent observations. The significance of differences between means was assessed with Student's *t* test; *P* (versus corresponding controls): * < 0.05; ** < 0.005. Other differences are not significant.

		Rate (nmol/h per mg dry wt.) or ratio	
		Control diet	High-leucine diet
I.	Flux through tryptophan 2,3-dioxygenase	2.57±0.25	3.77±0.03*
II.	Flux through kynureninase	2.56±0.17	3.66±0.23*
III.	Flux through acetyl-CoA	0.38±0.04	0.59±0.05*
IV.	Assayable activity of tryptophan 2,3-dioxygenase	15.94±0.74	23.00±1.18**
	Ratio I/IV	0.16±0.01	0.16±0.01
	Ratio III/I	0.15±0.05	0.15±0.05

present results, shown in Table 1, is that the absolute rate of formation of nicotinamide nucleotides and of their precursor, quinolinate, is increased. It follows that it is unlikely that chronic changes in the enzymes of tryptophan catabolism could explain the pellagragenic action of leucine [see mechanism (1), in the introduction].

Preincubation of cells, isolated from adult rats (180–220g) fed on standard CRM diet, with 2mM-leucine led to approximately a 40% inhibition throughout the pathway of tryptophan oxidation (Table 2), and therefore decreased formation of the nicotinamide nucleotide precursor, quinolinate. The inhibition in the three segments of the pathway (fluxes I, II and III) was similar, consistent with the postulate that leucine affects either the initial step of catabolism, catalysed by tryptophan dioxygenase, or the uptake of tryptophan into the cell.

Concentrations of leucine in the physiological range, added to cells isolated from adult rats fed on the CRM diet, inhibited the initial step of tryptophan oxidation, with at most about 50% inhibition in the presence of 2–5mM-leucine (Fig. 1). The total concentration of tryptophan in the incubation medium was 0.1mM, but binding of the amino acid to the albumin added to the medium resulted in a concentration of 'free' tryptophan of approx. 0.05mM (Cook, 1983), so that the ratio leucine/'free' tryptophan was considerably higher than the ratio leucine/total tryptophan. Experiments with 10μM-tryptophan, in the absence of albumin, showed that leucine produced a very similar pattern of inhibition at this physiological concentration (results not shown). Leucine itself did not inhibit tryptophan dioxygenase in cell extracts under a number of possible conditions of haem and tryptophan availability (results not shown).

The results are most easily explained as being the outcome of a competition between leucine and tryptophan for transport across the liver cell membrane. Both amino acids are believed to be carried out by the L1 and L2 systems (Weissbach *et al.*, 1982; Kilberg *et al.*, 1983). Under steady-state conditions, leucine in the range up to 5mM inhibited the uptake of tryptophan into cells isolated from rats fed on the standard CRM diet (Fig. 2). This pattern of inhibition closely resembles the effect of comparable concentrations of leucine on the catabolic flux, with, again, a maximal inhibition of around 50%. We have recently found (M. Salter & C. I. Pogson, unpublished work) that at steady state the rate of uptake of the amino acid is similar to the rate of its metabolism (although the maximum capacity of the transport system may be greater). Mechanism (3) (see the introduction) therefore remains plausible as an explanation of leucine action *in vivo*. The possibility that tryptophan availability in the blood

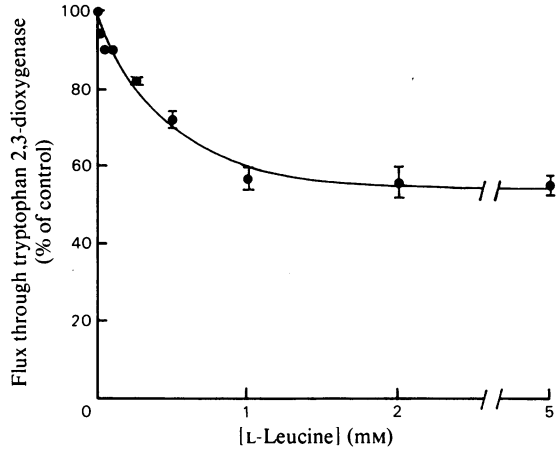

Fig. 1. *Effect of leucine on metabolic flux through tryptophan 2,3-dioxygenase in liver cells from normal fed rats*

Procedures were as described in Table 1 legend and in the text; all incubations contained 0.1mM-tryptophan and 2% albumin. Results are means ±S.E.M. for three independent observations or means for two independent observations. The control rate was 2.62±0.20nmol/h per mg dry wt.

Table 2. *Effect of 2mM-L-leucine on tryptophan metabolism in liver cells from fed rats receiving stock diet*

Procedures were as described in Table 1 legend and in the text; L-leucine was added at the beginning of the precinbuation period. Results are means ±S.E.M. for three independent observations. The significance of differences between means was assessed with Student's *t* test (*P* versus corresponding control): *<0.01, **<0.005.

		Rate (nmol/h per mg dry wt.)	
	2mM-L-Leucine ...	−	+
I.	Flux through tryptophan 2,3-dioxygenase	2.37±0.15	1.35±0.13**
II.	Flux through kynureninase	2.37±0.20	1.40±0.14*
III.	Flux through acetyl-CoA	0.36±0.03	0.20±0.02*

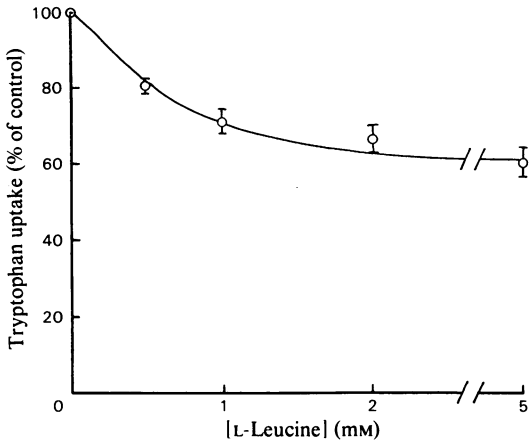

Fig. 2. *Effect of leucine on the uptake of tryptophan into liver cells isolated from normal fed rats*

Procedures were as described in the text; all incubations contained 0.05mM-tryptophan, but no albumin. Initial rates are means ± S.E.M. for three independent observations. The control rate was 3.05 ± 0.20nmol/h per mg dry wt.

may be important has been raised by Fujii (1979), who reported a decreased absorption of tryptophan from the small intestine in animals receiving a high-leucine diet. The finding that the blood concentrations of the amino acid were virtually unaltered under these conditions, however, makes this supposition less likely (Magboul & Bender, 1983). The presence of sufficiently high concentrations of competing amino acids might therefore lead to a situation whereby tryptophan disposal is limited by tissue uptake. This is particularly likely with a high concentration of circulating blood leucine, as leucine is taken up by the L system into freshly prepared hepatocytes, with a K_m approximately half that for tryptophan uptake and the other L-system substrates (Handlogten *et al.*, 1982; Kilberg *et al.*, 1983). Tannous *et al.* (1966) have, in fact, reported that a high-leucine diet, similar to the one employed in this laboratory, raises the blood leucine concentration to 1mM, a value just above the K_m for leucine uptake into isolated hepatocytes. This decrease in tryptophan uptake would result in a diminished supply of precursors for nicotinamide nucleotide synthesis. If prolonged, such an effect of a high intake of leucine might precipitate pellagra if the dietary intake of tryptophan (and niacin) were already low.

It is conceivable that a hepatic metabolite of leucine could be an effector of the enzyme, but this would have to be formed rapidly because all time courses of the flux through the dioxygenase in whole cells were linear with time and showed no sign of progressive inhibition; such a mechanism is also theoretically unlikely because leucine is metabolized only very slowly by rat liver (Crabb & Harris, 1978).

Leucine is metabolized extrahepatically in the rat by transamination to 4-methyl-2-oxovalerate (Lund, 1981); about 25% of this oxo acid is transported in the blood and oxidized in the liver (Williamson *et al.*, 1979; Livesey & Lund, 1980). It is therefore possible that leucine could exert an effect on tryptophan metabolism *in vivo* through its conversion into 4-methyl-2-oxovalerate; the concentration of the oxo acid in blood is doubled in animals fed on high-leucine diets (Yamada *et al.*, 1983*b*).

No inhibitory effect on flux through tryptophan 2,3-dioxygenase was seen when concentrations of 4-methyl-2-oxovalerate in the physiological range were incubated with cells isolated from rats fed on the standard CRM diet, in the presence of 2% albumin (results not shown). However, at super-physiological concentrations of the oxo acid, increases in flux through tryptophan 2,3-dioxygenase were seen (25% with 2mM-4-methyl-2-oxovalerate). These increases were most probably due to the binding of 4-methyl-2-oxovalerate to albumin (Livesey & Lund, 1982) in a similar manner to that of long-chain fatty acids, and displacing the albumin-bound tryptophan, thus making the displaced amino acid available for uptake and then oxidation by tryptophan 2,3-dioxygenase.

It therefore seems improbable that 4-methyl-2-oxovalerate is responsible for any decrease in nicotinamide nucleotide synthesis in the rat, although very high concentrations *in vitro* may not be without effect (Yamada *et al.*, 1983*a*).

The metabolism of leucine in man differs from that in the rat. Although little or no oxo acid is released from human muscle supplied with branched-chain amino acid (Elia & Livesey, 1983) and the oxo acid dehydrogenase activity in liver is very low (Khatra *et al.*, 1977), the presence of the low-K_m isoenzyme of the branched-chain amino acid aminotransferase (Goto *et al.*, 1977) suggests that leucine may be transaminated, but not further metabolized, in human liver. Leucine metabolites are therefore no more likely, in man than in the rat, to be involved in the inhibition of tryptophan metabolism.

The lack of effect of leucine on the relative rates of flux through the three segments of the tryptophan catabolic pathway suggests that the inhibition of kynureninase that has been observed *in vitro* (Magboul & Bender, 1983) may not be of physiological significance. The present results did not show any diminution in the production of [^{14}C]-alanine or alanine-derived $^{14}CO_2$ from [1-^{14}C]-tryptophan (representative of the kynureninase flux) in liver cells from animals fed on the high-

leucine diet, although such inhibition has been observed in whole-animal studies (Bender, 1983), when the release of [^{14}C]alanine-derived $^{14}CO_2$ (from [*methylene*-^{14}C]tryptophan) was used as an indication of the flux through kynureninase. Yet it is possible that the oxidation of [^{14}C]alanine (the alanine formed by the action of kynureninase) is decreased *in vivo* by the high-leucine diet as a result of inhibition of pyruvate dehydrogenase (Roche & Cate, 1977; Cate & Roche, 1978).

Bender (1983) also observed increased production of $^{14}CO_2$ from [*benzene-ring*-U-^{14}C]-tryptophan *in vivo*, but it is possible that the results in the whole animal reflect effects of leucine on extrahepatic picolinate carboxylase; the activity of this enzyme in kidney is considerably greater than in liver (D. A. Bender, unpublished work).

We put forward, then, that the competitive inhibition of tryptophan uptake on the L-system transporter, and the subsequent decrease in the hepatic flux to the nicotinamide nucleotides, may be a possible explanation for the precipitation of pellegra by leucine. The possible role of mechanism (4), increased catabolism of nicotinamide nucleotides, remains uninvestigated. Magboul & Bender (1983) reported statistically significant increases in the excretion of *N*-methylnicotinamide in rats on a diet similar to that employed for the present work, and Kubota *et al.* (1982) have shown that leucine activates NAD glycohydrolase *in vitro*.

We would suggest that the relative importance of these two potential mechanisms may perhaps be assessed by experiments with amino acids, other than leucine, which may inhibit tryptophan transport without affecting NAD turnover.

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