Biochem. J. (1982) **204**, 831–838
Printed in Great Britain

Failure of leucine to stimulate protein synthesis *in vivo*

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(*Received 22 February 1982/Accepted 26 March 1982*)

The effect of 100 μmol of leucine on protein synthesis in several tissues was assessed in the intact rat. Leucine had no immediate effect on protein synthesis in gastrocnemius muscle, heart, jejunal serosa, jejunal mucosa or liver in rats which were fed, starved for 2 days or deprived of dietary protein for 9 days. Leucine treatment for 1h also failed to stimulate protein synthesis in tissues of 2-day-starved animals.

Several studies have indicated that branched-chain amino acids, particularly leucine, are involved in the regulation of protein synthesis. Direct stimulation of protein synthesis in muscle tissue has been demonstrated by Buse & Reid (1975) and Fulks *et al.* (1975) with incubations of rat diaphragm *in vitro*. Together these studies have provided evidence for a role of leucine in regulating both the synthesis and the degradation of protein, which was not shared with other branched-chain amino acids and which was not related only to provision of energy for the tissue through leucine oxidation. Similar direct and rapid changes in protein synthesis in muscle tissue in response to leucine have been shown in perfused hemicorpus (Li & Jefferson, 1978) and perfused heart (Chua *et al.*, 1979).

The role of branched-chain amino acids in regulating protein metabolism has also been investigated in intact animals and man. Although this evidence is less direct, an ability of branched-chain amino acids to enhance protein synthesis has been inferred. Buse *et al.* (1979) suggested that an increase in the proportion of aggregated polyribosomes isolated from muscle tissue of starved rats (glucose and insulin were also provided) indicated a stimulation of protein synthesis. In addition, improved nitrogen balance in response to branched-chain amino acid administration has been cited as evidence of the ability of these amino acids to regulate protein metabolism. For example, Freund *et al.* (1980) induced a loss of whole-body nitrogen in rats through the trauma associated with laparotomy. Nitrogen balance was improved by infusion of a mixture of branched-chain amino acids compared with dextrose infusion, and infusion of alanine did not fully reproduce this effect. The ability of branched-chain amino acids, or their oxo acid analogues, to improve nitrogen balance has also been demonstrated in man. Although the studies in man have not investigated the specificity of the effect of branched-chain amino acids as exhaustively as those in animals, a beneficial role of these amino acids and their oxo acid analogues in starvation (Sapir & Walser, 1977; Sherwin, 1978) and after surgery (Freund *et al.*, 1979) has been demonstrated. These changes in nitrogen balance could result from changes in either the synthesis or the degradation of protein, but Sherwin (1978) has suggested that leucine acts through changes in synthesis, since 3-methylhistidine excretion, and by inference the degradation of muscle protein, remained unchanged with and without leucine.

However, the conditions that favour the sparing of body nitrogen by leucine and whether or not this effect is mediated by a direct action of leucine in stimulating muscle protein synthesis *in vivo* remains unclear. We have therefore measured the effect of leucine *in vivo* on protein synthesis in a number of tissues of rats that were either fed or losing body nitrogen as a result of starvation or lack of dietary protein.

Methods

Male Wistar rats were purchased from Charles River (Margate, Kent, U.K.) at a weight of 50g and maintained on a pelleted diet containing 23% (w/w) crude protein (Oxoid, Basingstoke, Hants., U.K.) until they weighed approx. 135g. At that weight the fed group was killed between 10:00 and 12:00h. The starved group of animals was maintained for an additional 48h without food and weighed 102g at death. A separate group of rats was maintained on the pelleted diet to 113g body wt. and then transferred to a powdered diet containing no protein

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0306-3283/82/060831-08$01.50/1

(formulated as 0% Protein Diet; ICN Biochemicals, Cleveland, OH, U.S.A.) for 9 days. The final weight in this group was 90g. All animals were individually caged and had free access to water.

Protein synthesis was measured by injection of a large amount of labelled phenylalanine as described previously (Garlick *et al.*, 1980). Briefly, unanaesthetized animals were killed at 2 and 10min after the intravenous injection of 1ml per 100g body wt. containing either 150μmol and 25–50μCi of L-[4-^{3}H]phenylalanine (10–25Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) or 150μmol and 25–50μCi of L-[4-^{3}H]phenylalanine plus 100μmol of unlabelled leucine. Liver, small intestine, gastrocnemius muscle and heart were quickly removed and chilled in ice/water. The small intestine was transferred to a chilled plate, slit longitudinally and scraped with a microscope slide to separate the mucosal and the serosal components. All tissues were weighed and frozen in liquid N_2. Frozen tissue was pulverized between two aluminium blocks (cooled on solid CO_2), precipitated with cold 2% (w/v) $HClO_4$ and centrifuged at 3000*g* for 15min. The supernatant was adjusted to pH6.3 with saturated potassium citrate and centrifuged at 3000*g* for 15min to remove $KClO_4$ before being used to determine the specific radioactivity of free phenylalanine. After thorough washing the pellet was used to determine RNA (by u.v. absorption at 260 and 232nm; Munro & Fleck, 1969), protein (by the method of Lowry *et al.*, 1951, as described by Munro & Fleck, 1969) and the specific radioactivity of protein-bound phenylalanine after hydrolysis in 6M-HCl at 110°C for 18–20h with subsequent evaporation and resuspension in 0.5M-sodium citrate, pH6.3.

To determine the specific radioactivity of phenylalanine, the amino acid was converted into β-phenylethylamine with L-phenylalanine decarboxylase (obtained as L-tyrosine decarboxylase, acetone-dried powder from *Streptococcus faecalis*, type 1, Sigma). Samples were incubated with 0.4–0.7 unit (where 1 unit corresponds to 1μmol of CO_2/min, pH5.5, 37°C) of enzyme in 1.5ml of 0.5M-sodium citrate, pH6.3, containing 0.2mg of pyridoxal phosphate, at 50°C overnight. After the addition of 1ml of 3M-NaOH, β-phenylethylamine was extracted into 10ml of n-heptane/chloroform (3:1, v/v). The organic layer was removed and to it was added 5ml of chloroform and 4ml of 0.01M-H_2SO_4. A sample of the H_2SO_4 layer was used to determine radioactivity in a xylene-based scintillant (Fricke, 1975) with a Nuclear–Chicago Delta 3000 liquid-scintillation counter (counting error <1.5%). Another sample was used to determine the amount of β-phenylethylamine fluorimetrically by a modification (Garlick *et al.*, 1980) of the method of Suzuki & Yagi (1976).

The fractional rate of protein synthesis (i.e. the proportion of the protein pool that is synthesized each day) was calculated from the equation $k_s = 100 \times S_B/\bar{S}_A t$, where k_s is the fractional synthesis rate (in %/day) and S_B is the specific radioactivity of phenylalanine in protein at 10min. $\bar{S}_A$ is the mean specific radioactivity of free phenylalanine over the time interval 0–10min, which was calculated from the rate of change in specific radioactivity assessed from animals killed at 2min (four rats) and 10min (six rats). t is the actual time of incorporation, including the time taken to remove the tissues, expressed in days.

Concentrations of leucine, isoleucine and valine in plasma were determined with a Locarte amino acid analyser from 0.25ml of plasma precipitated with an equal volume of 5% (w/v) sulphosalicylic acid, with norleucine added as an internal standard. Samples of plasma were also analysed for insulin by radioimmunoassay (Herbert *et al.*, 1965).

Results

Rates of protein synthesis in muscle tissue (gastrocnemius, heart, jejunal serosa) and visceral tissue (jejunal mucosa and liver) of fed rats receiving either 150μmol of [^{3}H]phenylalanine (control group) or 150μmol of [^{3}H]phenylalanine plus 100μmol of leucine (leucine group) are compared in Table 1. Protein synthesis has been calculated both as a fractional rate (% of the protein pool synthesized per day) and as the amount of protein synthesized per mg of RNA per day. Because the majority of RNA in the tissue is rRNA (Henshaw *et al.*, 1971), the latter calculation makes an adjustment for differences in the amount of protein-synthetic apparatus between different groups. Neither method of expressing protein synthesis reveals any significant alteration in rate in response to leucine administration.

Table 1 also shows the specific radioactivity of free phenylalanine in the tissues ($\bar{S}_A$) after injection of the radioisotope. In all tissues the specific radioactivities of the two groups were within 6% of each other, thereby ensuring that, although leucine and phenylalanine share a common system for transport (Christensen, 1969), the large dose of leucine did not alter the equilibration of labelled phenylalanine within the animal.

Table 2 shows the rates of protein synthesis in tissues from rats starved for 2 days. Fractional rates of synthesis were decreased in all tissues relative to the rates obtained in tissues from fed rats, with synthesis in muscle tissues depressed by 40–65% and in visceral tissues by 17–25%. In all tissues except the jejunal serosa there was also a lower rate of synthesis per mg of RNA. Despite depressed protein synthesis, however, leucine administration

Table 1. *Effect of 100 μmol of leucine on protein synthesis in various tissues from fed rats*
The fractional rate of protein synthesis was determined from the incorporation of 150 μmol of [^{3}H]phenylalanine (approx. 0.2 μCi/μmol)/100 g body wt. in fed animals with and without concomitant intravenous administration of 100 μmol of L-leucine. The fractional synthesis rate was divided by the ratio of RNA to protein in the tissue to give an estimate of the mg of protein synthesized per mg of RNA. Free phenylalanine specific radioactivity (S_A) was determined from acid-soluble amino acid within the tissue. Details appear in the Methods section. Results are expressed as means ± S.E.M. ($n = 6$). Statistical significance was assessed by Student's t test: $^*P < 0.05$. None of the differences in synthesis rates between leucine and control is significant ($P > 0.05$).

	Fractional synthesis rate (%/day)		Protein synthesized (mg/mg of RNA)		Sp. radioactivity of free phenylalanine (d.p.m./nmol)	
	Control	Leucine	Control	Leucine	Control	Leucine
Gastrocnemius muscle	16.9 ± 0.6	18.6 ± 0.9	16.7 ± 0.8	18.5 ± 1.2	398 ± 1	388 ± 4*
Heart muscle	19.6 ± 0.8	20.1 ± 0.7	12.5 ± 0.6	12.5 ± 0.7	368 ± 4	380 ± 2*
Jejunal serosa	52.1 ± 2.0	56.5 ± 2.8	18.2 ± 0.6	21.0 ± 1.5	361 ± 5	361 ± 4
Jejunal mucosa	123 ± 4	117 ± 3	17.9 ± 0.7	16.8 ± 0.5	321 ± 3	340 ± 4*
Liver	86.3 ± 5.6	82.1 ± 2.6	17.5 ± 0.7	15.6 ± 0.6	381 ± 8	388 ± 3

Table 2. *Effect of 100 μmol of leucine on protein synthesis in various tissues from 2-day-starved rats*
Details are the same as Table 1, except that the animals had been starved for 48 h before the measurement of protein synthesis.

	Fractional synthesis rate (%/day)		Protein synthesized (mg/mg of RNA)		Sp. radioactivity of free phenylalanine (d.p.m./nmol)	
	Control	Leucine	Control	Leucine	Control	Leucine
Gastrocnemius muscle	5.83 ± 0.33	5.76 ± 0.63	8.45 ± 0.55	8.57 ± 1.11	363 ± 5	365 ± 3
Heart muscle	11.9 ± 0.7	10.7 ± 0.9	9.10 ± 0.42	8.91 ± 0.88	321 ± 5	335 ± 6
Jejunal serosa	31.4 ± 1.2	31.2 ± 1.2	17.8 ± 0.5	17.6 ± 0.5	365 ± 3	363 ± 1
Jejunal mucosa	92.0 ± 3.2	101 ± 4	14.1 ± 0.6	16.0 ± 0.8	361 ± 1	359 ± 1
Liver	71.8 ± 2.9	73.6 ± 2.1	14.5 ± 0.7	15.9 ± 0.7	378 ± 4	384 ± 4

Table 3. *Effect of 100 μmol of leucine on protein synthesis in various tissues from protein-deprived rats*
Details are the same as Table 1, except the animals were maintained on a diet devoid of protein for 9 days before the measurement of protein synthesis, and the specific radioactivity of [^{3}H]phenylalanine injected was approx. 0.4 μCi/μmol. $^*P < 0.001$.

	Fractional synthesis rate (%/day)		Protein synthesized (mg/mg of RNA)		Sp. radioactivity of free phenylalanine (d.p.m./nmol)	
	Control	Leucine	Control	Leucine	Control	Leucine
Gastrocnemius muscle	4.05 ± 0.65	3.51 ± 0.25	10.3 ± 1.4	8.45 ± 0.47	764 ± 4	800 ± 3*
Heart muscle	10.3 ± 0.2	10.6 ± 0.4	9.26 ± 0.22	9.02 ± 0.38	738 ± 5	689 ± 7*
Jejunal mucosa	94.9 ± 6.0	99.7 ± 1.6	12.1 ± 0.8	12.4 ± 0.3	736 ± 3	727 ± 7
Liver	69.4 ± 4.3	68.6 ± 1.1	12.2 ± 0.4	12.1 ± 0.3	750 ± 1	769 ± 4*

had no significant effect on either the rates of synthesis or the specific radioactivity of free phenylalanine.

The rates of protein synthesis in tissues from protein-deprived animals (Table 3) were similar to those from 2-day-starved animals, and again leucine did not significantly affect these rates. In those tissues where leucine altered the specific radioactivity of free phenylalanine the effect was very small (<5%).

Although this study was primarily concerned with an immediate effect of leucine on protein synthesis, which would be observable within 10 min, one group of animals that had been treated with leucine for a longer time was also examined. Table 4 shows the rates of protein synthesis from another group of

Table 4. *Effect of pretreatment for 1 h with 100 μmol of leucine on protein synthesis in several tissues of 48 h-starved rats*
Details are the same as in Table 1, except that 100 μmol of leucine was injected intraperitoneally 1 h before the intravenous injection of [^{3}H]phenylalanine. *$P < 0.05$.

	Fractional synthesis rate (%/day)		Protein synthesized (mg/mg of RNA)		Sp. radioactivity of free phenylalanine (d.p.m./nmol)	
	Control	Leucine	Control	Leucine	Control	Leucine
Gastrocnemius muscle	4.48 ± 0.42	5.00 ± 0.31	8.28 ± 1.04	8.25 ± 0.57	372 ± 3	376 ± 2
Heart muscle	12.2 ± 2.0	10.1 ± 1.3	11.1 ± 2.1	8.71 ± 0.93	325 ± 1	343 ± 4*
Jejunal serosa	34.6 ± 1.8	35.5 ± 2.1	19.2 ± 0.8	17.7 ± 0.9	298 ± 5	319 ± 6*
Jejunal mucosa	93.9 ± 2.1	88.8 ± 4.1	13.0 ± 0.2	13.3 ± 0.5	334 ± 2	345 ± 3*
Liver	66.0 ± 2.5	67.0 ± 2.3	13.7 ± 0.5	14.2 ± 0.5	361 ± 3	361 ± 3

Table 5. *Concentrations of branched-chain amino acids in plasma from fed, starved and protein-deprived rats 10 min after the injection of 150 μmol of phenylalanine with or without 100 μmol of leucine*
Results are expressed in μM, as means ± S.E.M. ($n = 4$). Statistical significance between control and leucine treatment was assessed with Student's t test; *$P < 0.05$; **$P < 0.01$.

	Leucine		Valine		Isoleucine	
	Control	Leucine	Control	Leucine	Control	Leucine
Fed	126 ± 4	850 ± 27	187 ± 6	140 ± 17*	96 ± 4	66 ± 6**
Starved	132 ± 7	985 ± 39	151 ± 12	195 ± 19	81 ± 8	88 ± 9
Protein-deprived	81 ± 6	1040 ± 54	118 ± 21	96 ± 14	44 ± 4	36 ± 9

2-day-starved animals. In this case, the 'leucine group' received 100 μmol of leucine per 100 g body wt. by intraperitoneal injection 1 h before the injection of labelled phenylalanine. Rates of protein synthesis were obtained that were very similar to those reported in Table 2, and treatment with leucine for 1 h had no effect on protein synthesis or on the specific radioactivity of free phenylalanine.

The concentrations of branched-chain amino acids in the plasma 10 min after injection of 150 μmol of [^{3}H]phenylalanine or 150 μmol of [^{3}H]phenylalanine plus 100 μmol of leucine are shown in Table 5. In those animals that did not receive leucine, the concentrations of all three branched-chain amino acids were similar, in fed and starved animals, but lower in protein-deprived animals. At 10 min after the injection of 100 μmol of leucine the plasma concentrations of leucine were approx. 1 mM in all groups. In addition, leucine administration significantly lowered the plasma concentrations of valine and isoleucine in fed animals. In starved and protein-deprived animals leucine did not significantly alter valine or isoleucine concentrations.

The concentration of insulin in plasma, measured at 2 and 10 min after the injection of 150 μmol of [^{3}H]phenylalanine ('Control' columns, Table 6), was elevated in both fed and starved animals 2 min after phenylalanine injection, but had fallen by 10 min. This transient increase in insulin was not observed in animals deprived of dietary protein. Injection of 100 μmol of leucine with the phenylalanine ('Leucine' columns, Table 6) caused an even greater stimulation of insulin in fed and starved rats, and a rise was also apparent in the protein-deprived group. In the fed group the insulin concentration 10 min after injection was still significantly higher than in rats receiving only phenylalanine ($P < 0.05$).

Table 6. *Concentrations of insulin (μunits/ml, ±S.E.M.) in plasma from fed, starved and protein-deprived rats at 2 and 10 min after the injection of 150 μmol of phenylalanine (control) or 150 μmol of phenylalanine plus 100 μmol of leucine*
Insulin concentrations were determined by radioimmunoassay in plasma from fed, starved (2 days) and protein-deprived (9 days) rats. Three rats were measured at 2 min and four rats at 10 min after injection of phenylalanine or phenylalanine plus leucine.

	Control		Leucine	
	2 min	10 min	2 min	10 min
Fed	84 ± 9	36 ± 4	>150	55 ± 6
Starved	20 ± 5	7 ± 1	46 ± 6	10 ± 2
Protein-deprived	11 ± 2	13 ± 3	21 ± 11	10 ± 4

Discussion

In this study we have been unable to detect any effect of 100 μmol of leucine on the rate of protein

synthesis *in vivo* in muscle or in visceral tissues of fed, starved (2 days) or protein-deprived (9 days) rats. The results in fed rats are perhaps not surprising. Concentrations of insulin and free amino acids were both high, and it is probable that synthesis was already maximal. High concentrations of insulin in perfused muscle have been associated with the failure of branched-chain amino acids to stimulate protein synthesis (Li & Jefferson, 1978). The positive reports of an effect of leucine have been observed in tissues in catabolic states. Conditions *in vitro* are, in general, catabolic, and studies *in vivo* have mostly been made on starved and/or stressed animals or man. Therefore we have also investigated the effect of leucine on protein synthesis in starvation and protein depletion, both of which are characterized by loss of body nitrogen and low rates of tissue protein synthesis. The results in Tables 1–3 confirm previous reports (Garlick *et al.*, 1975; McNurlan *et al.*, 1979; McNurlan & Garlick, 1981) that protein synthesis under these conditions is depressed in all tissues, with skeletal muscle the most seriously affected. In both starvation and protein deprivation depressed rates of protein synthesis were accompanied by low concentrations of plasma insulin (Table 6), conditions that in the study of Li & Jefferson (1978) allowed leucine to stimulate protein synthesis in perfused muscle. The possibility that an effect of leucine on protein synthesis *in vivo* was masked by the transient increase in insulin in animals receiving [^{3}H]phenylalanine alone (i.e. control animals, Table 6) is ruled out by the results for the protein-deprived group, which showed no increase in insulin and no effect of leucine on protein synthesis. In all groups insulin concentrations were higher in animals receiving leucine, but despite this protein synthesis was not increased.

In the starved and protein-deprived animals, contrasting concentrations of branched-chain amino acids were observed (Table 5). In starvation, concentrations were little different from those in fed rats, whereas in protein deprivation they were low. There was thus no correlation between rates of protein synthesis in any tissue and the concentration of the branched-chain amino acids in plasma, confirming the observation of Millward *et al.* (1976) that there was no relationship between concentrations of branched-chain amino acids in muscle and the rate of protein synthesis. Injection of leucine caused an increase in its concentration in plasma to about 1mM (Table 5). This concentration is comparable with that which stimulated protein synthesis in incubated muscle (Buse & Reid, 1975; Fulks *et al.*, 1975), perfused hemicorpus (Li & Jefferson, 1978) and in perfused heart (Rannels *et al.*, 1974; Chua *et al.*, 1979).

In the present study, protein synthesis was measured from the incorporation of a large amount of labelled phenylalanine over a short period of time. Rather than measuring the specific radioactivity of the immediate precursor for protein synthesis (i.e. aminoacyl-tRNA), this procedure was designed to flood all free amino acid pools to nearly the same specific radioactivity (Henshaw *et al.*, 1971). Since synthesis rates calculated from the specific radioactivity in either the plasma or the tissue are nearly the same, an error is not introduced through choosing an inappropriate value. Although we had originally used a flooding amount of leucine (McNurlan *et al.*, 1979), the method was subsequently modified to use labelled phenylalanine as the flooding amino acid (Garlick *et al.*, 1980) because of reports that leucine might alter the rate of protein synthesis. Phenylalanine has not been implicated in the control of protein synthesis in muscle (Buse & Reid, 1975) or liver (Woodside & Mortimore, 1972). Indeed, high concentrations of labelled phenylalanine have been used to measure the rate of protein synthesis in perfused hemicorpus and perfused heart, in which an effect of leucine has been observed (Rannels *et al.*, 1974; Li & Jefferson, 1978). The use of large amounts of labelled amino acid to measure protein synthesis *in vivo* is of particular value in rapidly turning-over tissues such as liver and intestine. If tracer amounts of labelled amino acids are used to measure synthesis in these tissues, the specific radioactivity of the free amino acid within the tissue is quite different from that in the plasma, which introduces considerable potential for error into the estimate of synthesis (McNurlan *et al.*, 1979). Even with a flooding amount of phenylalanine it is theoretically possible for leucine to alter the specific radioactivity of the precursor for protein synthesis, since leucine and phenylalanine share a system of transport (Christensen, 1969). The observation that free phenylalanine in all the tissues had nearly the same specific radioactivity in the presence and absence of leucine (Tables 1, 2 and 3) suggests that penetration of label into cells was not altered.

Studies by Freund *et al.* (1981) and Moldawer *et al.* (1981) have reported increases in protein synthesis in the whole animal and in individual tissues in response to leucine administration, when measurements were made by continuous infusion of [^{14}C]tyrosine (Garlick *et al.*, 1973). With this technique the problem of defining the specific radioactivity at the site of protein synthesis is more serious than with the flooding dose. Indeed, in re-examining the effect of dietary protein deprivation on protein synthesis in liver with a flooding amount of [^{14}C]leucine we observed a decrease in protein synthesis (similar to that obtained in the present study with [^{3}H]phenylalanine; cf. Tables 1 and 3). This was the opposite effect to that observed previously (Garlick *et al.*, 1975) when constant

infusion of [^{14}C]tyrosine was used, and the discrepancy was attributed to an error in defining the precursor specific radioactivity with the infusion technique (McNurlan & Garlick, 1981).

In the study of Freund *et al.* (1981) on rats after laparotomy, the fractional rate of synthesis in the liver was increased from 8.2%/day with infusion of glucose to 12%/day with the infusion of a 1.5% (w/v) solution of leucine, isoleucine or valine. These values for fractional synthesis rates are quite unlike any others obtained with constant infusion (normal values are approx. 50%/day; Waterlow *et al.*, 1978), and no attempt was made to measure the specific radioactivity of the tyrosine used for protein synthesis. Instead, the specific radioactivity of tyrosine in plasma was multiplied by arbitrary numbers (0.7 for muscle and 0.5 for liver). Muscle protein synthesis was reported to be 1.2%/day in the glucose group and 1.1%/day in the group receiving leucine. Only valine appeared to stimulate muscle protein synthesis. Reported values for muscle protein synthesis are rarely this low (for a 300g rat, 5–8%/day; Waterlow *et al.*, 1978), and stimulation of protein synthesis by valine has been ruled out in a number of systems *in vitro* (e.g. Buse & Reid, 1975; Chua *et al.*, 1979).

Moldawer *et al.* (1981) also measured protein synthesis in injured rats by constant infusion of labelled amino acids. In liver the rate of synthesis appeared to be stimulated in rats receiving either branched-chain amino acids or a complete amino acid mixture, compared with that when glucose was given. With the same technique, Sakamoto *et al.* (1980) showed a stimulation in liver synthesis by leucine administration in rats with experimental pancreatitis compared with starved controls. By comparison, leucine failed to stimulate protein synthesis in perfused liver (Woodside & Mortimore, 1972), and it is perhaps more likely that with the constant-infusion technique the leucine affected the specific radioactivity of the free amino acid at the site of protein synthesis. Moldawer *et al.* (1981) also measured rates of protein synthesis in muscle, which is less likely to suffer from problems of precursor compartmentation, since the specific radioactivity of the amino acid within the tissue is closer to that in plasma (Waterlow *et al.*, 1978). Whereas administration of the complete amino acid mixture elevated muscle synthesis relative to that with glucose, branched-chain amino acids had little effect (5%), similar to the results in the present study.

The data on polyribosome aggregation from Buse *et al.* (1979), although not a direct measure of protein synthesis *in vivo*, would suggest that in the psoas muscle of starved rats there was a stimulation 1h after the injection of leucine. In the study of Buse *et al.* (1979), starved rats were also injected with glucose and insulin in addition to leucine, and it is possible that this difference has led to the observation of an effect of leucine not observed in the present study. Moreover, although a small effect of leucine was observed in rats starved for 2 days, the effect was more pronounced after 4 days of starvation. In the present studies we attempted to make measurements in rats starved for longer than 2 days. After 3 days of starvation young rats were close to death, and rates of protein synthesis in all tissues were very variable. No effect of leucine could, however, be detected.

A number of studies *in vivo*, in both man and animals, have shown that administration of leucine, branched-chain amino acids or branched-chain oxo acids caused an improvement in nitrogen balance (Sapir & Walser, 1977; Sherwin, 1978; Freund *et al.*, 1979, 1980; Blackburn *et al.*, 1979; Sakamoto *et al.*, 1980). Although this effect has almost always been attributed to the action of leucine on protein synthesis observed *in vitro* (Buse & Reid, 1975; Fulks *et al.*, 1975), it could be reconciled with our own finding of a lack of effect of leucine on muscle if synthesis in non-muscle tissues was increased. However, the data on liver and intestine suggest that this was not the case. Assuming that the rate of protein synthesis in gastrocnemius muscle is representative of all skeletal muscle, the five tissues measured account for at least 70% of the total protein synthesis in the whole animal (McNurlan *et al.*, 1980); therefore it is unlikely that leucine stimulated protein synthesis in some other tissue that was not measured, sufficiently to account for changes in nitrogen balance.

Improved nitrogen balance in response to leucine could, however, arise by an inhibition of protein degradation rather than by an increase in synthesis. In man, measurements of 3-methylhistidine excretion would suggest that this did not occur (Sherwin, 1978), but direct measurements of the degradation of total muscle protein would be necessary to substantiate this, since 3-methylhistidine may originate from protein degradation in other tissues (Millward *et al.*, 1980). Experiments with incubated muscles reported by Goldberg & Tischler (1981) have suggested that protein synthesis is not stimulated by leucine itself, but instead by its oxo acid analogue, 4-methyl-2-oxopentanoate, and protein degradation is inhibited by leucine. However, this does not completely explain our results, or the observation by Mitch *et al.* (1981) that in fasting man nitrogen was spared by infusion of 4-methyl-2-oxopentanoate but not by leucine, since leucine administration *in vivo* would give rise to the oxo acid, and vice versa.

In general, studies of nitrogen balance in man have not compared the effect of leucine with that of an equivalent source of nitrogen, but in rats with pancreatitis Sakamoto *et al.* (1980) obtained a

similar nitrogen-sparing effect with alanine as with leucine. Freund *et al.* (1981), with post-operative rats, showed similar effects with leucine, valine, isoleucine and alanine. If improvement in nitrogen balance can be brought about by amino acids such as alanine, then clearly this effect is different from the action of leucine in incubated muscle. However, the effects of leucine or branched-chain amino acids on nitrogen balance are not completely clear. Previously Freund *et al.* (1980) reported that, although alanine could spare nitrogen better than glucose, it was not as effective as a mixture of branched-chain amino acids. In addition, not all studies have demonstrated an improvement in nitrogen balance with branched-chain amino acids. Wolfe *et al.* (1981) have reported that infusion of branched-chain amino acids or their α-oxo acid analogues into 6-day-starved dogs had no effect on nitrogen balance.

Those studies that have demonstrated an effect of leucine on nitrogen balance have, of necessity, involved exposure to leucine of 12h (e.g. Sherwin, 1978) or longer (e.g. Blackburn *et al.*, 1979; Freund *et al.*, 1980; Sakamoto *et al.*, 1980). By contrast, we sought to investigate *in vivo* the effect that had been observed *in vitro* in response to incubation with leucine (Buse & Reid, 1975; Fulks *et al.*, 1975). This direct effect was observed after 1h preincubation with leucine and did not appear to require synthesis of RNA or protein (Buse & Reid, 1975). We therefore chose to use short exposure to leucine to detect direct stimulation of protein synthesis. It is possible that 10min was not long enough for such an effect to be observable, although in cultured Ehrlich ascites cells stimulation of protein synthesis can be observed within 2min of providing an amino acid that had been omitted from the medium (Austin & Clemens, 1981). However, it is unlikely that direct stimulation like that in incubated muscle could occur but not be observable at 1h. Studying the effects of leucine over longer periods of time, such as those used in studies of nitrogen balance, increases the likelihood that the effect of leucine is secondary to some other change (e.g. hormonal).

We conclude that the ability of leucine to stimulate protein synthesis does not appear to be a general phenomenon and may only occur under rather special conditions. Incubated or perfused muscle tissues are generally more catabolic than tissues *in vivo*, and it may be that such conditions are necessary for leucine to stimulate protein synthesis. This seems to be the case for perfused heart, where altering the perfusion system so that the heart was pumping against a resistance both improved amino acid balance and diminished the stimulatory effect of leucine on protein synthesis (Chua *et al.*, 1980). Starvation for 2 days and dietary protein deprivation for 9 days may not have been severe enough to induce conditions which were sufficiently catabolic, since we have been unable to demonstrate in any tissue *in vivo* the same kind of direct and immediate effect of leucine that has been observed in muscle *in vitro*. It would appear that, in rats which are fed, starved or protein-deprived, leucine does not play an important part in the regulation of tissue protein synthesis.

We thank Miss S. Dalal for measuring insulin, Mr. P. Broadbent for the analysis of plasma amino acids and Mr. P. DeSouza for help in measuring phenylalanine specific radioactivities. Generous financial support was provided by the Wellcome Trust and the Medical Research Council.

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