The role of leucine in ketogenesis in starved rats

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The quantitative significance of the conversion *in vivo* of L-[U-¹⁴C]leucine to ketone bodies was determined in rats starved for 3 or 48 h. In animals starved for 3 h, 4.4% of ketone-body carbon is derived from the metabolism of leucine, and in rats starved for 48 h the corresponding value is 2.3%. This conversion occurs rapidly, and the specific radioactivity of ketone bodies in blood is maximal at 2 min after the intravenous injection of labelled leucine for both periods of starvation. The flux of leucine in the blood is 1.01 and 1.04 $\mu\text{mol/min}$ per 100 g body wt. respectively for animals starved for 3 and 48 h. The specific radioactivity of blood ketone bodies was compared at 2 min after the injection of labelled leucine, lysine and phenylalanine. The specific radioactivity was 4–5-fold higher with leucine than with lysine or phenylalanine.

It is believed that ketone-body carbon atoms are derived almost entirely from the carbon atoms of fatty acids. However, studies previously reported do not permit any quantitative conclusions to be drawn about the fraction of ketone-body carbon derived from fatty acid carbon. Another potential source of ketone bodies is from the metabolism of the ketogenic amino acids leucine, isoleucine, lysine, phenylalanine, tyrosine and tryptophan. These compounds are believed to make an insignificant contribution, because of their concentrations in blood, the extent of protein degradation that might be needed during high rates of ketogenesis, the large amount of available triacylglycerol and the high flux of fatty acids in blood. However, there are no measurements available on the quantitative contribution of these amino acids to ketogenesis *in vivo*. There is clear evidence that liver can convert the 2-oxo analogue of leucine, 4-methyl-2-oxopentanoate, into ketone bodies. In fact, rat hepatocytes have the capacity to carry out this conversion at the same rate at which they can form ketone bodies from oleate or butyrate, and the conversion from 4-methyl-2-oxopentanoate appears to be relatively insensitive to the dietary state of the animals (Krebs & Lund, 1977). These results indicate there is a significant potential for ketone-body formation, at least from leucine.

The experiments described below were designed to provide a quantitative assessment *in vivo* for the contribution of leucine to ketogenesis in rats starved for a short or a long time and also to measure the flux of this amino acid in blood.

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Experimental

Animal procedures

The animal procedures used were described by Abrams & Cooper (1976). One group was deprived of food 48 h before use, and the second group had their Purina chow removed at 22:30 h and, at 06:30 h the following morning, received 2 ml of a liquid diet by stomach tube and were injected with [¹⁴C]leucine 3 h later. The weights of both groups are shown in Table 1.

Intravenous injections were made into a tail vein of unanaesthetized rats and contained 10–20 μCi of L-[U-¹⁴C]leucine (300 Ci/mol; Amersham Corp., Arlington Heights, IL, U.S.A.) in 0.5 ml of 0.15 M-NaCl/0.2 mM-phosphate, pH 7.4. Animals were killed at the appropriate time by decapitation, and the blood was collected into heparinized tubes, for 5 s from animals killed at 1 min or less after injection of [¹⁴C]leucine, for 10 s for the 2 and 5 min points and for 15 s for all other time points. A 0.6–1.0 ml sample was used for ketone-body analyses, and 1.5–2.0 ml for the leucine analysis.

Ketone-body measurements

For this 1 ml of heparinized blood was mixed with 3 ml of 10% (v/v) HClO₄ and centrifuged at 25 000 g for 10 min at 4°C. The supernatant fraction was neutralized and used for ketone-body analyses. 3-Hydroxybutyrate was determined spectrophotometrically in a solution containing 200 μl of neutralized HClO₄ extract, 800 μl of 20 mM-Tris/50 mM-hydrazine sulphate, pH 8.5, and 50 μl of 55 mM-NAD⁺. The reaction was started by adding

10 μ l of 3-hydroxybutyrate dehydrogenase (60 units/ml; H-6126, Sigma Chemical Co., St. Louis, MO, U.S.A.) that had been dialysed with 0.1 M-Tris/HCl, pH 8.5, for 90 min. Acetoacetate was determined in a solution containing 600 μ l of extract, 400 μ l of 25 mM-phosphate, pH 6.9, and 50 μ l of 17 mM-NADH. The reaction was started with 10 μ l of 3-hydroxybutyrate dehydrogenase.

The radioactivity in ketone bodies was measured by using 50 ml conical flasks fitted with standard taper tops and two internal wells. One well held a plastic tube containing a folded filter paper wetted with 70 μ l of 10 M-NaOH to trap CO₂. The second well held a tube containing 500 μ l of hydrazine lactate to trap acetone. The main compartment contained 1.0 ml of extract, 1.0 ml of 1 M-Tris/HCl, pH 9.6, 0.24 ml of 0.1 M-NAD⁺, 0.05 ml of water and 20 μ l of dialysed 3-hydroxybutyrate dehydrogenase. A 200 μ l sample was removed for radioactivity counting. The flasks were closed with spring clips and incubated for 90 min at 37°C. The flasks were then put in solid CO₂ for 10 min, 300 μ l of 5 M-H₂SO₄ was added to the main compartment, and the flasks were re-closed immediately. After 30 min at 20°C, the flasks were incubated for 18–20 h at 37°C. The hydrazine lactate tube was removed and counted for radioactivity, as was a sample from the main compartment, with Formula-963 (New England Nuclear Corp.) as scintillant. The cup containing NaOH was removed and the paper and cup were placed separately in another set of 50 ml flasks fitted with a centre well that contained a tube holding 500 μ l of Hyamine hydroxide solution (New England Nuclear Corp.). The main compartment contained 8 ml of 40% (v/v) methanol in water. The flasks were closed with serum stoppers, 500 μ l of 5 M-H₂SO₄ was added to the main compartment with a syringe, and the flasks were incubated for 90 min at 20°C. The tube containing the Hyamine hydroxide was then removed and counted for radioactivity. The recoveries were (means $\pm$ S.E.M., $n = 96$): total, 97 $\pm$ 1%; CO₂, 99 $\pm$ 1%; 3-hydroxybutyrate, 87 $\pm$ 1%. Appropriate corrections were applied to the results obtained with experimental samples.

Determination of leucine specific radioactivity

A 1.5–2.0 ml portion of heparinized blood was diluted with 3 vol. of cold water, sonicated for 10 s, and enough 15% (w/v) sulphosalicylic acid added to make the final concentration 3.8%. After 10 min at 0°C, the solution was centrifuged at 20 000 g for 10 min at 4°C. The supernatant fluid was kept, the pellet washed with 4 $\times$ 2 ml of 0.2 M-sodium citrate/2% (v/v) thiodiglycol/0.2% (v/v) Brij 35, pH 2.20, and the washings were combined with the original supernatant fluid. The combined solution was applied (with air pressure) to a jacketed column (0.9 cm $\times$ 17 cm) containing Aminex A-6 resin (Bio-

Rad Laboratories, Richmond, CA, U.S.A.) equilibrated previously with 0.2 M-sodium citrate/0.5% thiodiglycol/0.2% Brij 35 buffer, pH 3.25 $\pm$ 0.01 at 43°C. The leucine was eluted with the equilibration buffer, was separated completely from isoleucine, and appeared in a total volume of 10–15 ml after about 85 ml had passed through the column. A 100–200 μ l portion of each fraction containing leucine was counted for radioactivity. The labelled peak, and ten 1.5 ml fractions on either side, were assayed for amino acid by the ninhydrin method.

Results

The relative contributions of fatty acids and ketogenic amino acids to ketogenesis may vary considerably with the physiological state. Conditions in which there is a high rate of lipolysis, such as prolonged starvation and diabetes, may represent one end of a spectrum, whereas a high-protein diet or brief deprivation of food may be the other end.

Fig. 1. Specific-radioactivity/time curves for blood leucine and blood ketone bodies in rats starved for 48 h. Each point represents the mean $\pm$ S.E.M. obtained with four to eight rats. The points for times less than 1 min were at 15, 25 and 40 s for both curves and, in addition, an 8 s point was determined for the leucine curve.

We therefore decided to determine the conversion of leucine into ketone bodies under two sets of conditions, and we selected prolonged and brief starvation.

Conversion of leucine into ketone bodies in rats starved for 48 h

The specific-radioactivity/time relationship for blood leucine was obtained with animals starved for 48 h (Fig. 1). The area under the curve from zero to infinity was 16.4 when units of specific radioactivity were expressed as per cent of injected dose of tracer/ μmol of leucine carbon corrected to a rat weight of 100 g. The area from 0 to 60 min was estimated by the trapezoid rule and that from 60 min to infinity by an exponential extrapolation (Shipley & Clark, 1972). The irreversible disposal rate of leucine from blood was calculated by dividing the dose of tracer by this area (Shipley & Clark, 1972). Therefore, for leucine carbon:

Irreversible Disposal Rate = administered dose (100%)/area under disposal curve (16.4)
 = $6.27 \mu\text{mol}$ of leucine carbon/min per 100 g body wt.
 = $1.04 \mu\text{mol}$ of leucine/min per 100 g body wt.

The rate constant for the flux of blood leucine, expressed as the fraction of the blood leucine pool disposed of per min, was determined by dividing the Irreversible Disposal Rate by the pool size of blood leucine ($\mu\text{mol}/100 \text{ g body wt.}$). The latter term was calculated from the relationship:

Concn. of leucine ($\mu\text{mol}/\text{ml}$ blood) $\times$ serum volume per 100 g body wt. (Abrams & Cooper, 1976)/
 (1 - haematocrit).

The rate constant in this instance was $1.04/0.923 = 1.13 \text{ min}^{-1}$. The value for the concentration of leucine in blood is in good agreement with the values reported by Vina & Williamson (1981).

The area under the specific-radioactivity/time curve for the total ketone-body pool in blood was also determined (Fig. 1). By dividing the area under the ketone-body curve by that under the leucine curve, the fraction of ketone-body carbon derived from leucine carbon was calculated as described by Shipley & Clarke (1972) and was $(0.372/15.96) \times 100 = 2.33\%$.

During the brief overnight fast in humans there is mobilization of amino acids from muscle and enhancement of gluconeogenesis in liver, and lipolysis is relatively low. It is possible that during a brief fast the fraction of ketone-body carbon derived from ketogenic amino acids may be greater than during prolonged starvation. To find the best starvation time to test this possibility, the specific radioactivity of blood ketone bodies was measured at 1 and 2 min after injection of labelled leucine in

animals deprived of food for 3–10 h. The specific radioactivity was 5–10-fold higher after starvation for 3–4 h than it was at 5, 7 or 10 h. The concentration of plasma ketone bodies and the rate of ketogenesis do not rise in rats until the starvation period exceeds 5–6 h (McGarry *et al.*, 1973). In addition, the concentration of fatty acids in plasma does not reach a plateau until about 10 h of starvation, and is elevated only moderately (about 1.5-fold) after 3 h of starvation (McGarry *et al.*, 1973). Starvation for 3 h was therefore chosen for the second group of animals.

Conversion of leucine into ketone bodies in rats starved for 3 h

The specific-radioactivity/time curve of blood leucine in rats starved for 3 h (Fig. 2) was used to calculate the Irreversible Disposal Rate under these circumstances. It was $1.01 \mu\text{mol}$ of leucine/min per 100 g body wt., a value that cannot be distinguished from the rate of 1.04 obtained with rats starved for 48 h. In addition, the concentration of blood leucine

Fig. 2. Specific-radioactivity/time curves for blood leucine and blood ketone bodies in rats starved for 3 h. The numbers of animals per time point and the time points used were the same as described in Fig. 1.

Table 1. *Summary of parameters measured*All values are expressed as means $\pm$ S.E.M.

Parameter measured	Time of starvation (h) ...	3	48
Body wt. (g)		238 $\pm$ 2 (n = 72)	200 $\pm$ 4 (n = 55)
Blood leucine			
(μ mol/ml)		0.128 $\pm$ 0.003 (n = 72)	0.124 $\pm$ 0.005 (n = 55)
(μ mol/100 g body wt.)		1.005	0.923
Blood ketone bodies (μ mol/ml)		n = 55	n = 55
Total		0.433 $\pm$ 0.031	1.805 $\pm$ 0.136
3-Hydroxybutyrate		0.287 $\pm$ 0.022	1.409 $\pm$ 0.118
Acetoacetate		0.146 $\pm$ 0.010	0.396 $\pm$ 0.023
Ratio 3-hydroxybutyrate/acetoacetate		1.97	3.56
Irreversible disposal rate for blood leucine (μ mol/min per 100 g body wt.)		1.01	1.04
Irreversible disposal rate coefficient (min ⁻¹)		1.00	1.13
Ketone-body carbon derived from leucine carbon (%)		4.44	2.33

Table 2. *Comparison of specific radioactivity of blood ketone bodies at 2 min after injection of lysine, phenylalanine and leucine*All values are expressed as means $\pm$ S.E.M.

Parameter measured	Lysine (n = 3)	Phenylalanine (n = 3)	Leucine (n = 5)
Label injected (μ Ci/100 g body wt.)	13.2	5.4	4.7
Amino acid concn.			
(μ mol/ml of blood)	0.396 $\pm$ 0.015	0.065 $\pm$ 0.008	0.122 $\pm$ 0.011
(μ mol/100 g body wt.)	3.147 $\pm$ 0.219	0.556 $\pm$ 0.063	1.001 $\pm$ 0.092
Estimated specific radioactivity at zero time (Ci/mol of amino acid)	4.19	9.71	4.70
Ketone-body specific radioactivity at 2 min (% of injected dose/ μ mol of ketone-body carbon per 100 g body wt.)	0.00453 $\pm$ 0.00157	0.00409 $\pm$ 0.00129	0.01997 $\pm$ 0.00162

is also the same within experimental error (Table 1). The comparable curve for blood ketone bodies was also determined (Fig. 2), and from the areas under these curves it was calculated that $(0.732/16.49) \times 100 = 4.44\%$ of ketone-body carbon is derived from blood leucine in rats starved for 3 h. Table 1 summarizes all measurements made.

Possible role of lysine and phenylalanine as ketone-body precursors

The results presented above show that leucine is not of much importance quantitatively in rats as a precursor for ketone-body formation during starvation for 3 or 48 h. It is conceivable, however, that it may assume greater importance in this regard during a high-protein diet. It is difficult to extrapolate from the contribution of leucine to ketogenesis to that of all ketogenic amino acids. We carried out experiments to predict, on the basis of early specific-

radioactivity values, the probable importance of ketogenesis from lysine and phenylalanine compared with leucine. Rats starved for 48 h were injected with L-[U-¹⁴C]lysine (Amersham), L-[U-¹⁴C]-phenylalanine (New England Nuclear Corp.) or L-[U-¹⁴C]leucine. They were killed 2 min later, the blood was collected, and the specific radioactivity in blood of ketone bodies and of the injected amino acid determined. The values were much greater with leucine as the precursor than they were with the other two ketogenic amino acids tested. This was the case even though the estimated initial specific radioactivity in blood of leucine (4.70 Ci/mol) was considerably less than that with lysine (9.71 Ci/mol) and was comparable with that of phenylalanine (4.19 Ci/mol) (Table 2). Even though these data provide only a crude estimate of the true conversion into ketone bodies, it is likely that these other amino acids are less important than leucine as precursors for ketone bodies in rats starved for 48 h.

Discussion

There has been considerable interest in the metabolism of branched-chain amino acids *in vivo*. The development of the concept of a glucose–alanine cycle has raised questions regarding the ultimate disposition of the carbon chains of this class of amino acids. In rats, the enzymes responsible for the metabolism of 4-methyl-2-oxopentanoate, the 2-oxo analogue of leucine, are believed to be located primarily in liver, whereas the transaminase is mainly in skeletal muscle (Shinnick & Harper, 1976). However, this view is not accepted universally (Odessey & Goldberg, 1979). If the premise is correct, the amino nitrogen of leucine mobilized from muscle protein would be available for transamination to form alanine, while the carbon skeleton would move to liver for possible conversion into acetoacetate and acetyl-CoA. The potential for ketone-body formation from leucine would then depend on the rate of turnover of muscle protein and the fraction of the flux of leucine and/or 4-methyl-2-oxopentanoate in blood that was accounted for by hepatic uptake. It has been shown that virtually all of the 2-oxo analogues of branched-chain amino acids put out by muscle *in vivo* are taken up by liver (Livesey & Lund, 1980), and hepatocytes are known to convert 4-methyl-2-oxopentanoate into ketone bodies at a high rate (Krebs & Lund, 1977). Early in starvation, before protein turnover has been suppressed, leucine could provide a significant fraction of ketone-body carbon, whereas during prolonged starvation, where fatty acid is a predominant fuel and nitrogen is being conserved, it would probably be an insignificant source. Our results showed that leucine itself is of minor importance under both circumstances even though its flux in blood is fairly high. The value that we obtained for the flux of leucine in blood of 1.01–1.04 $\mu\text{mol}/\text{min}$ per 100g body wt., can be contrasted with values of 4.2–4.3 for palmitate (Abrams & Cooper, 1976), 3.1–3.8 for glucose (Shipley *et al.*, 1967; Heath & Corney, 1973) and 9.5 for ketone bodies (Barton, 1976). These values

for palmitate, glucose and ketone bodies are for rats starved for 48, 20, 24 and 30h respectively. Clearly the flux of leucine in blood of rats is significant and is of the same order of magnitude as that of the other major fuels. However, the fraction of this flux going to liver remains unknown, as does the fraction of the hepatic uptake used for ketogenesis. It appears from our data that one or both of these latter parameters must be low to account for the relatively small contribution that leucine appears to make toward ketogenesis.

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