Valine oxidation: the synthesis and evaluation of L-[3-^{3}H]valine as a tracer in vivo

BY P. R. BECKETT*, A. CADENHEAD AND M. F. FULLER

Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB

(*Received 20 November 1990 – Accepted 1 August 1991*)

The suitability of L-[3-^{3}H]valine for measuring valine oxidation was studied by comparing its oxidation rate with that of L-[1-^{14}C]valine in rats and pigs. L-[3-^{3}H]valine was synthesized by removal of the tritium on carbon-2 of L-[2,3-^{3}H]valine by acetylation. The acetyl group was removed enzymatically using pig renal acylase 1 (*EC* 3.5.1.14) and the product was purified by ion-exchange and paper chromatography. For the first rat experiment L-[3-^{3}H]valine was synthesized in our laboratory; for the subsequent experiments it was produced by Amersham International plc. In the first experiment in rats the two tracers were given by injection and $^{14}CO_2$ was collected for 2 h. The oxidation of tritiated valine was significantly higher than that of L-[1-^{14}C]valine. In a second experiment there was no difference. This was probably due to the higher purity of the labelled valine which, for the second experiment, was shown by nuclear magnetic resonance to contain only one tritium atom. In a study with pigs in which the two tracers were given by continuous infusion there was no significant difference between them in flux or oxidation. The results of this experiment were used to evaluate a model to estimate amino acid requirements. With pigs given a methionine-limiting diet a reduction in methionine intake, by reducing protein accretion, increased valine oxidation by the same proportion.

Tritiated valine: Methionine/valine requirement: Pig

Animals have evolved mechanisms to regulate the catabolism of individual essential amino acids depending on whether they are deficient or in excess. Measurements of amino acid oxidation have been made to estimate amino acid requirements (Brookes *et al.* 1972; Bergner & Simon, 1976; Newport *et al.* 1976; Bergner *et al.* 1978; Kim *et al.* 1983; Ball & Bayley, 1986) and to study the effects of amino acids (Harper *et al.* 1984; Fuller *et al.* 1987), protein (Soliman & Harper, 1971; Kang-Lee & Harper, 1978; Fuller *et al.* 1987) or energy (Reeds *et al.* 1981) on amino acid catabolism. These measurements have been based on quantifying the release of $^{14}CO_2$ following administration of tracers labelled either generally or at specific carbon atoms. Errors may arise because of label sequestration, especially from labelled C atoms which enter the Krebs cycle. Tomera *et al.* (1983) demonstrated, for example, that the recovery of the [2-^{14}C] of ketoisocaproate, which enters the Krebs cycle, was only 25% of that of the [1-^{14}C] label which is lost directly as $^{14}CO_2$. Further restrictions in the use of this method include limited availability of suitably labelled tracers for the essential amino acids and the need for access to a respiration chamber to measure total CO_2 output. Tritiated amino acids provide an alternative tracer but have been used more rarely. Potentially they offer a comparatively simple method for measuring oxidation without the need for animal confinement. The present study was undertaken to evaluate the measurement of amino acid oxidation using tritium-labelled amino acids.

Use of a tritiated tracer requires the label to be positioned such that when the amino acid is committed to catabolism the tritium is oxidized to water in the electron transport chain

* Present address: USDA/ARS, Children's Nutrition Research Center, Baylor College of Medicine, Houston, Texas 77030, USA.

and then labels the body water pool. In the present study, L-[3-^{3}H]valine was synthesized and its oxidation by growing rats was compared with that of L-[1-^{14}C]valine. In the second part of the study a comparison was made of the oxidation rates of L-[3-^{3}H]valine and L-[1-^{14}C]valine by growing pigs, using a variation of the indicator amino acid method of Kim *et al.* (1983).

MATERIALS AND METHODS

Synthesis of L-[3-^{3}H]valine

L-[2,3-^{3}H]valine (47 Ci/mmol) was purchased from Amersham International plc, Amersham, Bucks. All other chemicals and enzymes were purchased from Sigma Chemical Co. Ltd, Dorset.

The L-[2,3-^{3}H]valine was freeze-dried without carrier, dissolved in 0·5 ml glacial acetic acid plus 0·01 ml acetic anhydride and then heated at 140° in a sealed hydrolysis tube for 2 h (Crawhall & Smyth, 1958; Adriaens *et al.* 1975). Completion of the reaction was determined by thin-layer chromatography against a valine standard, the acetyl derivative being ninhydrin negative. Excess acetic acid was removed by evaporation under reduced pressure at 37° and replaced with 0·5 ml water. Any unreacted L-[2,3-^{3}H]valine was removed from the acetylated product by passage through 1 ml cation-exchange resin (Dowex 50W, 8% crosslinking, 200–400 mesh, H^+ form) in a Poly-Prep chromatography column (Bio-Rad Laboratories, California), *N*-acetyl-[3-^{3}H]valine was eluted with 12 ml water and hydrolysed using the stereospecific enzyme acylase I (*EC* 3.5.1.14; 2000 units) in 0·5 ml 0·1 M-potassium dihydrogen phosphate, pH 7·0 (Mitz & Schlueter, 1958). Phenol red indicator was added before the enzyme and the pH adjusted if necessary during a 5 h incubation at 37°. The products, *N*-acetyl-D-[3-^{3}H]valine and L-[3-^{3}H]valine, were separated on 1 ml cation-exchange resin (details given previously) by thorough washing with water to elute the acetyl derivative, followed by elution of the L-[3-^{3}H]valine with 8 ml 2 M-ammonium hydroxide solution. After evaporation of the ammonia the L-[3-^{3}H]valine was further purified using paper chromatography with a solvent of butanol–acetic acid–water (65:15:25, by vol.) (Pichat *et al.* 1965). The final purity was 99·4% as determined by radiochemical yield as valine on preparative amino acid analysis, and the overall reaction yield was 11%.

Because contamination with D-[3-^{3}H]valine or L-[2,3-^{3}H]valine would affect the measured oxidation rate of the tracer in vivo the degree of contamination was assessed using the D- and L-amino acid oxidases (*EC* 1.4.3.3, 1.4.3.2) respectively (Greenstein & Winitz, 1961). Reaction of the valine with D-amino acid oxidase, which would produce ketoisovalerate (KIV) from any D-valine, did not produce any radioactivity in the water wash when the products were loaded onto cation-exchange resin. Since any KIV would appear in this fraction it was concluded that there was no contamination with D-valine. In the reaction of L-valine with L-amino acid oxidase to produce KIV, tritium on C-3 remains on the KIV whereas tritium on C-2 is oxidized to water. It was, therefore, necessary to freeze-dry the product to separate labelled water from labelled KIV. There was a 4% loss of radioactivity on lyophilization and a further 4% loss when the lyophilization was repeated. Use of an anion-exchange column to separate the products led to even greater breakdown of KIV. We concluded that both losses were due to radiolysis and not to contamination with tritium on C-2.

Tracers

L-[1-^{14}C]valine purchased from Amersham International plc was 100% pure as defined by the co-elution of radioactivity with carrier valine after preparative amino acid analysis. The

Table 1. *Composition of the diets (g/kg) used for the rat experiments*

Diet	Lab (Labsure CRM)	V+ (+20% valine)*	V− (−20% valine)*	V
Ingredients				
Maize starch		490·0	490·0	490·0
Glucose		150·0	150·0	150·0
Maize oil		150·0	150·0	150·0
Minerals		50·0	50·0	50·0
Vitamins		35·0	35·0	35·0
Albumin		125·0	100·9	125·0
Valine		1·1		
Synthetic amino acids excluding valine			24·1	
Estimated composition				
Digestible energy (MJ/kg)	13·3	13·2	13·2	13·2
Crude protein (nitrogen ×6·25)	179·0	99·1	99·5	100·0
Total valine	9·0	7·3	5·1	6·2

* Diets containing 20% excess valine relative to egg albumin or 20% deficiency.

tracer contained 0·9% D-[1-^{14}C]valine as determined by enantiomeric separation of leucyl-valine synthesized by the method of Mitchell *et al.* (1978). The sensitivity of the method was found to be greatly improved by first oxidizing the L-valine with the L-amino acid oxidase.

For the first experiment L-[3-^{3}H]valine was prepared as described previously. For the second experiment L-[3-^{3}H]valine was synthesized by Amersham International plc using the same procedures. For this commercial product the structure was shown by tritium nuclear magnetic resonance (NMR) to contain only one tritium atom per molecule. Radiochemical purity, by amino acid analysis, was greater than 99%. There was 1·3% D-[3-^{3}H]valine contamination determined by the dipeptide method of Mitchell *et al.* (1978).

Rat experiments

Animals and diets. Male weanling Hooded Lister rats (Rowett strain) were used. They were fed either Labsure CRM (Lab) diet (Lavender Mill, Cambridgeshire) or one of two semi-synthetic diets. Of these, one (V+) had 20% excess valine relative to egg albumin, the other (V−) had 20% deficiency (Table 1). The rats were caged in a temperature-controlled room (21°) with a 12 h light–12 h dark cycle (lights on at 06.00 hours). For the first experiment eighteen animals were allocated to six groups of three with equal mean body-weight and each diet was offered *ad lib.* to two groups.

For the second experiment eight animals were used. They were all offered *ad lib.* one semi-synthetic diet (V) which is also described in Table 1. In both experiments animals had free access to water at all times except when enclosed in the respiration chamber.

Procedures. In Expt 1 the rats were adapted to their diet for 5 d. Nine were then injected via a tail vein with $NaH^{14}CO_3$ (25 nCi) and tritiated water (50 nCi; approximately 300 μl) for measurement of bicarbonate recovery and tritium dilution volume. The other nine rats were injected similarly with L-[1-^{14}C]valine (60 nCi) and L-[3-^{3}H]valine (150 nCi; approximately 300 μl). In Expt 2 all eight rats were injected with L-[1-^{14}C]valine (1 μCi) and L-[3-^{3}H]valine (4 μCi; approximately 300 μl). After injection each animal was placed immediately into a Perspex respiration chamber of volume 1·3 l through which air was drawn at a rate of 500 ml/min. The air then passed through two sodium hydroxide traps

(50 ml 2 M-NaOH in 500 ml sinter 1 gas wash bottles) to trap CO_2 (Haggarty *et al.* 1986) and a water trap to prevent NaOH entering the pump. The system was under negative pressure to avoid loss of $^{14}CO_2$ and had been previously calibrated by liberating a known amount of $^{14}CO_2$ into the chamber; recovery of radioactivity from the chamber was 100·4 (SE 2·77) % (*n* 7); recovery from the rat was 83·3 (SE 3·12) %. After 2 h the rat was removed, killed by cervical dislocation followed by decapitation and the blood was collected by drainage from the neck vessels. To avoid microbial contamination the gastrointestinal tract was removed and the rest of the carcass was then minced.

Analytical methods. Total $^{14}CO_2$ production was determined by counting, in a Packard Tri-Carb 460CD liquid-scintillation spectrometer (Packard Instrument Co., Illinois, USA), 1 ml from each of the traps weighed into 10 ml NE260 (Nuclear Enterprises Ltd, Edinburgh). Fractional oxidation of L-[1-^{14}C]valine was calculated from the recovery of $^{14}CO_2$ over 2 h after injection and corrected for bicarbonate recovery. In the second experiment $^{14}CO_2$ in the minced carcass sample was also determined. Weighed carcass samples, approximately 1 g, were placed in 20 ml polyethylene vials containing 0·5 ml hyamine in a glass well and sealed with a Suba Seal stopper. Sulphuric acid (2 ml, 2 M) was injected to liberate the CO_2 and the vials were incubated for 6 h at 37°. The hyamine was then counted in 10 ml NE265 (Nuclear Enterprises Ltd). The remainder of each minced carcass was freeze-dried for 72 h and ground in a domestic coffee grinder. Four weighed samples of each carcass were rehydrated with 200 μl water and digested in 1 ml Soluene-100 (Packard Instrument Co, Illinois, USA) for 24 h at 37° in sealed 20 ml glass scintillation vials. Colour quench was removed by the addition of 1·7 mg benzoyl peroxide in 1 ml toluene and the vial was placed under an infrared lamp for 3 h. Hionicfluor (Packard Instrument Co, Illinois, USA; 10 ml) was added and the vials were left to stand for 1 h at room temperature to allow chemiluminescence to diminish before counting. Counting efficiency, determined by an internal standard, was 60% for ^{14}C and 19% for ^{3}H.

Eight carcass samples and the blood from each animal were vacuum distilled at 37° and the water from each sample was trapped in a liquid nitrogen cold finger (−196°). The water from each sample was weighed into plastic vials and counted in 10 ml NE265 to determine its specific activity (SA).

Tritium dilution volume was measured from the radioactivity (disintegrations/min; dpm) of tritiated water injected divided by the SA of body water 2 h after injection (dpm/g) and expressed as a proportion of body-weight (0·72 (SE 0·093)). Fractional oxidation of L-[3-^{3}H]valine, measured in separate animals, was calculated from the SA of body water 2 h after injection multiplied by the calculated tritium dilution volume, i.e. body-weight × 0·72 and divided by the amount (dpm) of L-[3-^{3}H]valine injected. The method assumes that there was no loss of tritium from the body.

Pig experiments

Animals and diets. Four Large White × (Large White × Landrace) female pigs, average weight 28 kg, were surgically prepared with two aortal PVC (NT2; Portex Ltd, Hyde, Kent) catheters introduced under Fluothane anaesthesia as described by Fuller *et al.* (1987). Catheter tip positions were confirmed by radiographic examination.

Two semi-synthetic pelleted diets were formulated, based on starch, casein and amino acids. The amino acid pattern was taken from the ideal protein pattern of Wang & Fuller (1989). Methionine was set at either 70 or 80% (diets M70 and M80 respectively) of the ideal concentration (i.e. so that it was the first-limiting amino acid) with an equal supply of cystine. Valine was set at 90% of the ideal concentration so that it was the second-limiting amino acid (Table 2). Both diets supplied 2·0 g N/kg body-weight$^{0.75}$ per d and 1·23 MJ

Table 2. *Composition of the diets (g/kg) used for the pig experiments*

	M80	M70
Ingredients		
Glucose	179	176
Maize starch	330	330
Sucrose	148	148
Cellulose	61	61
Oil	41	41
Vitamin–mineral mix	58	58
Casein	96	84
Synthetic amino acids	87	102
Calculated composition		
DE* (MJ/kg)	14·2	14·2
Nitrogen	21·9	21·9
Amino acids		
Thr	6·42	6·42
Val	6·03	6·03
Cys	2·19	1·92
Met	2·19	1·92
Ile	5·33	5·33
Leu	9·84	9·84
Tyr	5·33	5·33
Phe	5·33	5·33
Lys	8·89	8·89
His	3·01	3·01
Trp	1·64	1·64

* DE, digestible energy.

digestible energy/kg body-weight$^{0.75}$ per d. The diets were offered in equal hourly meals by continuous automatic feeder. Water was available at all times.

Procedures. The tracers, L-[3-^{3}H]valine and L-[1-^{14}C]valine, were the same as those used for the second rat experiment.

To calculate the flux of the amino acids, the stochastic model of Waterlow *et al.* (1978) was used; this assumes only one pool of free amino acids into which amino acids enter from both the diet and from protein breakdown and exit for protein synthesis and amino acid oxidation. It was further assumed that, during an experiment, this pool is of constant size and that the flux of amino acids entering and leaving the pool is continuous and steady.

Each pig received both diets, two pigs in the order M70 then M80 and two pigs in the reverse order. After surgery the animals were kept in individual pens for 1 week during which time they were offered their first experimental diet. On the third day thereafter the animal was transferred to an open-circuit respiration chamber (2300 l volume) and allowed a further 3 d acclimatization. At 15.00 hours on day 6 the catheters were connected to extension tubes which led out of the chamber which was then sealed. Between 16.00 and 16.40 hours background samples were taken of expired CO_2, plasma amino acids and body water (plasma). A solution of 100 μCi L-[3-^{3}H]valine and 25 μCi L-[1-^{14}C]valine in 18 ml physiological saline (9 g sodium chloride/l) was infused at a rate of 1·9 ml/h from 6.00 to 14.00 hours on day 7. The use of a time clock to initiate the infusion ensured that the animal was undisturbed and, therefore, more likely to be in a steady state than if the catheters were connected immediately before the infusion. Samples of expired CO_2 were collected for 40 min during each of the last 4 h of the infusion. Blood samples, 10 ml for tritiated water and 20 ml for amino acid analysis, were collected at 30 min intervals during the last 2 h.

Gaseous exchange was measured as described by Reeds *et al.* (1980). After the first infusion the diet was changed but the animal remained in the chamber for one week until the second infusion.

Analytical methods. After the addition of a norleucine standard, blood samples were deproteinized with cold sulphosalicylic acid (120 g/l; SSA). The free amino acids were desalted on a cation-exchange column (H^+ form Zeo-carb 225, mesh 50–100, dimensions 20 × 200 mm) and eluted with 100 ml 2 M-ammonium hydroxide. The free amino acids were dissolved in 2 ml 0·1 M-hydrochloric acid and frozen until analysed on an amino acid analyser (200 × 9 mm, 10 μm resin; The Locarte Co., London) in the preparative or analytical mode using 0·2 M-sodium citrate, pH 3·5, at a flow rate of 30 ml/h and a temperature of 50°. From the 2 ml sample, 1·8 ml was chromatographed preparatively to determine valine SA. The rest of the sample was analysed for valine concentration, corrected for recovery of the norleucine standard. Constant amino acid SA (plateau) was considered to be attained in the sample for which the subsequent sample had a lower SA. The SA of this and of all following samples were included in the calculation of plateau SA. This was usually the sample taken 6 h after the start of infusion. Plateau amino acid SA was also calculated from a regression equation to determine the sequential values which had a gradient not significantly different from zero. This calculation did not produce estimates significantly different from those derived by the simpler method described previously.

Expired CO_2 SA was determined by two different methods. In the first, a sample of gas was drawn through a stainless-steel Cary-Tolbert ionization chamber. The output from the ionization chamber was amplified using a Cary Model 401 vibrating-reed electrometer (Varian Associates, Palo Alto, USA). For the second method, CO_2 samples were collected by bubbling chamber gas at a rate of 4 l/min through 200 ml 0·7 M-NaOH in two 500 ml sinter-bottom gas wash bottles (Gallenkamp, Leicestershire) in parallel. The carbonate was then precipitated with excess 0·1 M-barium chloride, collected by filtration, washed with 1 l distilled water and dried overnight at 105°. A blank procedure was performed to account for barium carbonate formed in fresh NaOH. This was usually about 10% of the total collection. The SA of the carbonate was determined using a Packard 306 Oxidizer (Packard Instrument Co., Illinois, USA) using the method described by Lobley *et al.* (1985).

The recoveries of $^{14}CO_2$ by the ionization chamber and by absorption in NaOH were determined by releasing a known amount of $^{14}CO_2$ into the air leaving the chamber. The coefficient of variation of the calibration factor was 4% for the ionization chamber and 12% for the NaOH method.

All the blood samples for tritiated water analysis were centrifuged; the plasma was vacuum distilled and a weighed amount of plasma water (approximately 2 g) was counted in 10 ml NE265 to determine its SA. The increase in body water SA during the infusion was calculated from a linear regression equation using the values for the SA of body water samples before the infusion and at 6, 6·5, 7, 7·5 and 8 h. The correlation coefficient (r^2) values were greater than 0·98 except for pig 4 receiving diet M70 for which r^2 was 0·93.

Calculations

Amino acid flux (nmol/h) was calculated as the rate of infusion (dpm/h) divided by amino acid SA at plateau (dpm/nmol). The flux values were calculated taking into account the amount of infused labelled D-valine, assuming that none of it was oxidized and that it accumulated in total body water.

Total $^{14}CO_2$ production was calculated from carbonate SA, using the mean SA of precipitated barium carbonate and from the results of the ionization chamber, and from total CO_2 production corrected for 99·1% bicarbonate recovery, determined from continuous infusion of $NaH^{14}CO_3$ (Beckett, 1989). The plateau values, defined as for valine

SA, were divided by the infusion rate to give the proportion oxidized. For [^{3}H]valine total tritiated water production was calculated from the change in body water SA between the beginning and end of the infusion multiplied by the tritium dilution volume. This was determined in a previous experiment (Beckett, 1989) from measurements of body water SA in 40–50 kg pigs 1 h after injection of a known amount of tritiated water into the aorta via a catheter. The dilution volume was calculated from the radioactivity (dpm) injected divided by the radioactivity (dpm)/g body water and expressed as a proportion of body-weight (0·722 (SE 0·0149), n 9). The proportion oxidized was calculated as total tritiated water production divided by total tritium infused. Total valine oxidation rate was calculated as the valine flux multiplied by the proportion oxidized.

Statistical analysis

Statistical analysis of the rat data was performed using two-way analysis of variance (GENSTAT). For the pig data Student's t test was used to test the significance of treatment effects. Differences between the two tracers, $^3H - ^{14}C$, were analysed using a paired t test.

RESULTS

Rats

In the first experiment (Table 3) the mean oxidation rate of L-[3-^{3}H]valine was 16·4% (SE 1·1) of the dose injected, significantly higher ($P < 0·05$) than that of L-[1-^{14}C]valine, 14·8 (SE 1·0)%. There was no significant effect of diet on the proportion of valine oxidized or on the difference between the two tracers.

In the second experiment the fractional oxidation of L-[1-^{14}C]valine was 21·1 (SE 1·5)% and that of L-[3-^{3}H]valine 23·1 (SE 1·5)%; these were not significantly different (Table 3). Only 0·21 (SE 0·03)% of the injected ^{14}C was liberated from the carcass by acid. The rest, 83·8 and 83·7% for ^{3}H and ^{14}C respectively, was recovered in the carcass. The coefficient of variation of the SA of four replicate carcass samples was 9% for tritium and 6% for ^{14}C.

Pigs

All the pigs consumed their food hourly; there were no refusals.

The flux of valine calculated from ^{3}H-labelled valine was not significantly different from that calculated from the ^{14}C-labelled valine (1·03 (SE 0·92) mmol/h). The estimates of valine flux were corrected for contamination with labelled D-valine. The amount (dpm) of labelled D-valine infused was calculated from the total infusion rate (dpm) multiplied by the measured proportions of D-amino acid in the tracer, 0·013 for [^{3}H]valine and 0·010 for [^{14}C]valine. D-valine is diluted in the total body water space (Rosenhagen & Segal, 1974) and, because it co-chromatographs with L-valine, increases the measured SA of valine. From calculations of tritium-dilution volumes and plasma valine concentration the contribution of labelled D-valine to valine SA was calculated and the true L-valine SA estimated. Including this correction increased the absolute flux measurements by about 17%. After correction the difference between the two tracers (0·73 (SE 1·29) mmol/h) was not significant (Table 4). During the time it was given diet M80, pig 4 became unwell; it had a general infection which was associated with an increase in the rate of valine oxidation. Estimates made at this time were excluded from the statistical analysis when examining the effect of diet but were included in the comparison between the tracers. Excluding pig 4 on diet M80, the flux measurements with [^{3}H]valine were 2·51 (SE 0·42) and 2·54 (SE 0·22) g/kg body-weight$^{0·75}$ per d for diets M70 and M80 respectively. The same treatments with [^{14}C]valine gave flux rates of 2·84 (SE 0·36) and 2·32 (SE 0·33) g/kg body-weight$^{0·75}$ per d. There was no significant effect of diet on valine flux.

Table 3. *L-[3-^{3}H] valine and L-[1-^{14}C] valine oxidized by rats in 2 h after injection**

(L-[3-^{3}H] valine oxidation was calculated using a tritium space of 717 g/kg body-weight. L-[1-^{14}C]valine oxidation rates have been corrected for 83·3% recovery of bicarbonate)

Diet*	Body-wt (g)	^{3}H oxidized (% dose)	^{14}C oxidized (% dose)	%^{3}H dose in carcass	%^{14}C dose in carcass
Lab	63	13·0	13·7		
Lab	70	17·8	17·9		
Lab	63	10·2	9·5		
V+	61	16·3	17·0		
V+	61	20·2	18·1		
V+	96	21·1	17·5		
V−	90	16·2	13·4		
V−	85	15·5	12·1		
V−	90	17·2	14·4		
Mean		16·4	14·8		
SE		1·12	1·00		
V	113	20·8	22·2	92·5	100·3
V	114	22·8	21·8	86·3	92·6
V	140	26·7	25·5	77·4	73·2
V	157	27·5	23·9	79·9	78·7
V	165	25·9	21·3	82·9	80·0
V	190	17·2	13·0	85·7	79·9
V	180	19·1	20·2	82·1	81·5
Mean		23·0	21·1	83·8	83·7
SE		1·5	1·5	1·9	4·4

Lab, Labsure CRM diet (Lavender Mill, Cambridgeshire); V+, semi-synthetic diet containing 20% excess valine relative to egg albumin; V−, semi-synthetic diet with 20% valine deficiency; V, semi-synthetic diet.

* For details of diets and procedures, see p. 141 and Table 1.

Table 4. *Whole-body valine flux rates in pigs measured from the continuous infusion of L-[3-^{3}H]valine and L-[1-^{14}C]valine and corrected for infused labelled D-valine**

Pig no.	Diet*	[^{3}H]valine flux		[^{14}C]valine flux	
		mmol/h	g/kg$^{0·75}$ per d	mmol/h	g/kg$^{0·75}$ per d
1	M70	18·3	3·33	14·0	2·54
	M80	11·6	2·31	13·8	2·75
2	M70	6·7	1·34	9·7	1·96
	M80	12·8	2·34	9·2	1·68
3	M70	14·9	2·84	17·7	3·38
	M80	17·2	2·99	14·5	2·52
4	M70	13·5	2·53	18·5	3·46
	M80	13·0	2·65	16·4	3·34
	M70:Mean		2·51		2·84
	SE		0·42		0·36
	M80†:Mean		2·54		2·32
	SE		0·22		0·33

W, body-weight.

* For details of diets and procedures, see pp. 142–144 and Table 2.

† Excluding pig 4.

Table 5. *Whole-body valine oxidation measured by the recovery of ^3H_2O and $^{14}CO_2$ during the continuous infusion of L-[3-^{3}H]valine and L-[1-^{14}C]valine in pigs**

Valine oxidation (g/kgW$^{0.75}$ per d) was calculated from the percentage of the infused dose oxidized multiplied by valine flux)

Pig	Diet*	[^{3}H]valine oxidized		[^{14}C]valine oxidized	
		% dose	mg/kgW$^{0.75}$ per d	% dose	mg/kgW$^{0.75}$ per d
1	M70	4·28	142·7	4·56	115·6
	M80	4·73	109·2	2·08	57·2
2	M70	10·37	139·2	5·99	117·4
	M80	3·30	77·3	3·55	59·8
3	M70	13·65	388·3	10·97	370·8
	M80	6·54	192·7	9·48	239·0
4	M70	7·70	194·5	10·00	345·5
	M80	34·03	901·0	28·09	939·7
	M70: Mean	9·0	216·2	7·88	237·3
	SE	1·98	58·8	1·54	70·0
	M80†: Mean	4·8	126·4	5·0	118·7
	SE	0·91	34·4	2·26	60·2

* For details of diets and procedures, see pp. 142–144 and Table 2.
† Excluding pig 4.

The measured rates of valine oxidation are shown in Table 5. For all results the mean difference between L-[3-^{3}H]valine and L-[1-^{14}C]valine in the percentage of dose oxidized was 1·23% of the dose (SE of difference (SED) 1·13) which was not significant. Excluding pig 4 on diet M80, the effect of increasing dietary methionine from 70 to 80% of the estimated requirement was to reduce the rate of [^{3}H]valine oxidation from 9·0 (SE 1·99) to 4·8 (SE 0·91)% of the dose. The corresponding estimates with L-[1-^{14}C]valine were 7·9 (SE 1·54) and 5·0 (SE 2·26)% of the dose.

DISCUSSION

Choice of L-[3-^{3}H]valine as a metabolic tracer

In its oxidation valine is first transaminated to KIV, losing the proton from C-2. Since this reaction is reversible an H label in this position would be unsuitable for measuring oxidation. KIV is decarboxylated to isobutyryl-CoA with the irreversible loss of C-1 as CO_2. This is followed by desaturation to methacrylyl-CoA with the transfer of the proton on C-3 and one from C-4 to FAD. These protons are then oxidized to water via the electron transport chain. All the protons from one C-4 are oxidized; those on the other C-4 are incorporated into succinyl-CoA. The two C-4 atoms are distinguishable (Tanaka *et al.* 1981) and labelling of the appropriate one could provide a suitable tracer for valine oxidation. However, the proton on C-3, being a single proton, should be more suitable because its pathway of oxidation is known; the three protons on C-4 are oxidized by three different pathways and may be subject to sequestration.

Preparation of L-[3-^{3}H]valine

The critical step in this synthesis is the complete removal of the tritium from C-2 of L-[2,3-^{3}H]valine; if this is not complete, reaction of the acetylated product with the acylase would result in the regeneration of L-[2,3-^{3}H]valine. It is quite possible in the acetylation reaction to achieve complete acetylation without any racemization, i.e. with no loss of tritium from C-2. For complete racemization it was necessary for the reaction to be carried out at 140°,

although this led to a low reaction yield. Reaction yield was greater at lower temperatures but racemization was incomplete. Increasing the duration of the reaction at lower temperatures did not produce complete racemization but reduced the yield. The optimum conditions were found to be 140° for 2 h.

Comparison of L-[3-^{3}H]*valine and* L-[1-^{14}C]*valine in the rats*

The use of L-[3-^{3}H]valine was initially evaluated by two experiments in rats. In the first experiment the oxidation of tritiated valine was higher than that of [^{14}C]valine whereas in the second experiment there was no significant difference. The commercially prepared L-[3-^{3}H]valine used in the second experiment was analysed for label position by NMR, which can detect accurately less that 1% of isotope in any position in the molecule, and was shown to have only one tritium atom. The tracer synthesized by ourselves and used in the first experiment was not analysed by NMR and may have had slight contamination of the L-[3-^{3}H]valine with tritium on C-2. Use of the L-amino acid oxidase followed by freeze-drying to separate KIV and water was not satisfactory for detecting small amounts of contamination of tritium on C-2 because of the lability of KIV under these conditions. Loss of tritium from C-2 in vivo is proportional to the rate of re-amination of KIV, which is high compared with the rate of decarboxylation (Staten *et al.* 1984); therefore, the error in oxidation caused by 1% contamination may be increased by as much as the reciprocal of the fraction oxidized. For example, 1% contamination with 20% oxidation could give an error of 5%, i.e. 21% oxidation would be measured. Slight contamination of our own L-[3-^{3}H]valine with tritium on C-2 is the likely reason for its oxidation being higher than that of L-[1-^{14}C]valine.

The ratios of the two isotopes in the infusate and in the carcass were not significantly different, suggesting that there was either no isotope effect in protein synthesis or a similar isotope effect for both tracers. Considering that the tritium:hydrogen mass ratio is 3·0 whereas that of ^{14}C:^{12}C is only 1·167, and considering also their different positions in the labelled molecule, a similar isotope effect is unlikely and it is more probable that there was none. The carcasses would also have contained, as well as labelled valine, isotope which had been released as $^{14}CO_2$ or ^3H_2O but subsequently sequestered. The amount involved could be about 3·5% of the ^{14}C-dose, i.e. with 83% recovery of CO_2 and 21% valine oxidized, 3·5% of the CO_2 from valine would remain in the carcass of which only 0·2% was acid-labile. The remainder would increase the mean SA of the carcass from 39 to 40 dpm/mg. Non-aqueous exchangeable tritium may account for up to 7% of the tritium space (Culebras *et al.* 1977), or about 2% of the injected tritium when 23% of valine is oxidized, increasing the SA of the carcass from 184 to 186 dpm/mg. Such small changes would not be detected. The results suggest that the degree of sequestration of isotope after its removal from valine is small and can be accounted for by the measurement of bicarbonate recovery or tritium space. These two experiments provide evidence that there is no difference in the oxidation of L-[1-^{14}C]valine and L-[3-^{3}H]valine. However, they cannot be regarded as a complete validation of the use of L-[3-^{3}H]valine because the rats were not in a steady state of feeding and no attempt was made to relate oxidation of the tracer to the oxidation of valine. These aspects were included in the study in pigs.

Valine flux and oxidation in pigs

The rate of valine flux measured using L-[3-^{3}H]valine was slightly but not significantly lower than that measured with L-[1-^{14}C]valine. The difference was reduced when they were corrected for infused D-valine. The main effect of correcting for D-valine was to increase the calculated flux rates by 19% for L-[3-^{3}H]valine and by 15% for L-[1-^{14}C]valine. The justification for and assumptions used in making this correction were as follows. First, the

amounts of labelled D-valine in the tracers had been determined accurately as 1·3% in the L-[3-^{3}H]valine and 1·0% in the L-[1-^{14}C]valine. Second, L- and D-valine co-chromatograph on an amino acid analyser. Third, it was assumed that all D-valine equilibrated in the body water pool and none of it was oxidized. This assumption was made because the tracer only contains a minute amount of valine so that the infused D-valine would not be oxidized unless there was a flux of unlabelled D-valine. There should have been no flux of D-valine because the valine in the diet was all from casein or produced by fermentation, not chemical synthesis, and so would have been entirely in the L-form.

There was no significant difference in the oxidation rates of the two tracers as a percentage of the infused dose. When the oxidation rates were multiplied by the flux rates to give daily valine oxidation the two methods agreed closely. The difference of 12·5 (SED 23·04) mg/kg body-weight$^{0.75}$ per d represents about 6% of the overall mean oxidation rate, not including pig 4 on diet M80. Thus, using the techniques described, L-[3-^{3}H]valine and L-[1-^{14}C]valine produced indistinguishable rates of valine flux and oxidation.

Nutritional interpretation of valine oxidation rates

The study in pigs was designed not only to compare the two tracers but to investigate a new method using indicator amino acids for estimating amino acid requirements. As discussed by Kim *et al.* (1983) an indicator amino acid can be used to determine an animal's requirement for another amino acid because, as the intake of a limiting amino acid increases, the rate of protein accretion increases and the oxidation of all other amino acids required for protein synthesis decreases. When the limiting amino acid exceeds the animal's requirement, variation in its supply will have no effect on the oxidation of other amino acids. By measuring the oxidation rate of any non-limiting amino acid over a range of intakes of another amino acid from deficient to excess it is possible to estimate the animal's requirement for that amino acid using a 'breakpoint' model (Kim *et al.* 1983). Using an indicator amino acid to do this, rather than measuring the oxidation rate of the amino acid of interest directly, has the advantage that an indicator amino acid can be chosen for the ease with which its oxidation can be measured. An indicator approach of this nature has two disadvantages. First, to establish the 'breakpoint' a large number of observations for each amino acid is required and a separate series of measurements for each amino acid; second, the use of a breakpoint model requires the unproven assumption that the efficiency of utilization of an amino acid remains constant at all intakes below the requirement.

To estimate the animal's requirements for all amino acids by the breakpoint method would be laborious. An alternative approach, proposed by Mitchell (1950), is to use such a dose–response experiment to estimate the requirement for one amino acid and then to derive the requirements for all other amino acids from their pattern in an 'ideal' protein, that is, one that supplies amino acids in exactly the proportions in which they are required.

The dietary pattern of amino acids required by growing pigs of 40 kg has been estimated recently by Wang & Fuller (1989) and by Fuller *et al.* (1989) using the rate of nitrogen retention as the criterion of response. Information on the ideal pattern of amino acids can also be derived using an 'indicator' amino acid method. This is based on the supposition that increasing the dietary supply of the limiting amino acid results in an increased retention not only of these amino acids but of all other amino acids required for protein synthesis and that the proportions in which these amino acids are retained represent their relative requirements. It is also assumed that any increase in the retention of an amino acid is accompanied by an exactly equal reduction in its irrevocable loss or oxidation. Thus, from the present results, the animal's relative requirements for methionine + cystine and for valine can be deduced from the reduction in valine oxidation per unit increase in the dietary supply of methionine + cystine.

Diets M70 and M80 were designed to supply 70 and 80% respectively of the sulphur amino acids required in an ideally balanced protein. The mean daily intakes of methionine + cystine from these diets were 336 and 389 mg/kg body-weight$^{0.75}$ respectively, a difference of 53 mg/kg body-weight$^{0.75}$. Excluding pig 4 completely the corresponding change in valine oxidation estimated with [^{3}H]valine was from 223 (SE 82·5) to 126 (SE 58·3) (SED 50·0) mg/kg body-weight$^{0.75}$ per d, a difference of 97 mg. This suggests that valine and the sulphur amino acids are required in a ratio of 1·83:1. The corresponding estimates made with [^{14}C]valine were 201 (SE 84·8) and 119 (SE 60·2) (SED 24·6) mg/kg body-weight$^{0.75}$ per d, a difference of 82 mg, giving a ratio to methionine + cystine of 1·57:1. These estimates may be compared with those made by Wang & Fuller (1989) who suggested that the valine and methionine + cystine requirements of the growing pig are, respectively, 4·9 and 4·1 g/16 g N, a ratio of 1·20:1. These estimates refer, however, to the total requirement of the animal for maintenance and body protein accretion whereas, in the present experiment, we are concerned with the relative proportions in which the amino acids are required per unit change in body protein accretion. For this reason the estimates made by Fuller *et al.* (1989) of the amino acids required for unit increments of N retention are more appropriate to the present comparison. Those estimates were, for valine, 5·25 g and, for methionine + cystine, 3·58 g/16 g N, a ratio of 1·47:1.

Although this value is close to that obtained with [^{14}C]valine (1·57), the SED for the measured changes in valine oxidation is large and it is unreasonable to suggest more than that the approach outlined here gives results similar to those obtained by the other approaches using N balance methods. To achieve greater precision in the estimates of these ratios a much larger difference in methionine contents between the diets would be desirable, although this must be set against the danger of producing an amino acid imbalance which could itself modify the utilization of the limiting amino acid.

The authors wish to thank Mrs V. Buchan and Mr D. Brown for the amino acid analysis and Dr J. Brockway for assistance with the animal calorimetry. They wish to acknowledge the generous financial support of Eurolysine SA.

REFERENCES

Adriaens, P., Meesschart, B. & Vanderhaeghe, H. (1975). Isotope distribution in L-valine-2,3-T. *Analytical Biochemistry* **69**, 297–299.

Ball, R. O. & Bayley, H. S. (1986). Influence of dietary protein concentration on the oxidation of phenylalanine by the young pig. *British Journal of Nutrition* **55**, 651–658.

Beckett, P. R. (1989). Amino acid oxidation. PhD thesis, University of Aberdeen.

Bergner, H. & Simon, O. (1976). Methodische Untersuchungen zur stoffwechselorientieren Aminosäurenbedarfsbestimmung bei Ratten im Erhaltungszustand anhand der Oxydationsrate von ^{14}C-markiertem Lysin zu $^{14}CO_2$ (Studies investigating methods for determining the amino acid requirements on the basis of metabolic data through measurements of oxidation of CO_2). *Archiv für Tierernährung* **26**, 815–826.

Bergner, H., Simon, O. & Adam, K. (1978). Lysinbedarfsbestimmung bei wachsenden Ratten anhand der Katabolisierungsrate von ^{14}C- und ^{15}N-markiertem Lysin (Lysine requirement determination on growing rats by means of the catabolization rates of ^{14}C- and ^{15}N-labelled lysine). *Archiv für Tierernährung* **28**, 21–29.

Brookes, I. M., Owens, F. N. & Garrigus, U. S. (1972). Influence of amino acid level in the diet upon amino acid oxidation by the rat. *Journal of Nutrition* **102**, 27–36.

Crawhall, J. C. & Smyth, D. G. (1958). The synthesis of tritiovaline and its incorporation into rat-visceral proteins. *Biochemical Journal* **69**, 280–286.

Culebras, J. M., Fitzpatrick, G. F., Brennan, M. F., Boyden, C. M. & Moore, F. D. (1977). Total body water and the exchangeable hydrogen. II. A review of comparative data from animals based on isotope dilution and desiccation with a report of new data from the rat. *American Journal of Physiology* **232**, R60–R65.

Fuller, M. F., McWilliam, R., Wang, T. C. & Giles, L. R. (1989). The optimum dietary amino acid pattern for growing pigs. 2. Requirements for maintenance and for tissue protein accretion. *British Journal of Nutrition* **62**, 255–267.

Fuller, M. F., Reeds, P. J., Cadenhead, A., Seve, B. & Preston, T. (1987). Effects of the amount and quality of dietary protein on nitrogen metabolism and protein turnover in pigs. *British Journal of Nutrition* **58**, 287–300.

Greenstein, J. P. & Winitz, M. (1961). *Chemistry of the Amino Acids*. London: Wiley.

Haggarty, P., Reeds, P. J., Fletcher, J. M. & Wahle, K. W. J. (1986). The fate of ^{14}C derived from radioactively labelled dietary precursors in young rats of the Zucker strain (Fa/- and fa/fa). *Biochemical Journal* **235**, 323–327.

Harper, A. E., Miller, R. H. & Block, K. P. (1984). Branched-chain amino acid metabolism. *Annual Review of Nutrition* **4**, 409–545.

Kang-Lee, Y. A. & Harper, A. E. (1978). Threonine metabolism in vivo: effect of threonine intake and prior induction of threonine dehydratase in rats. *Journal of Nutrition* **108**, 163–171.

Kim, K., Elliot, J. I. & Bayley, H. S. (1983). Oxidation of an indicator amino acid by young pigs receiving diets with varying levels of lysine or threonine, and an assessment of amino acid requirements. *British Journal of Nutrition* **50**, 391–401.

Lobley, G. E., Connell, A. & Watson, B. T. (1985). The use of a proprietary organic sample oxidiser to determine $^{14}CO_2$ and ^{14}C-carbonates of trace level specific radioactivity. *International Journal of Applied Radiation and Isotopes* **36**, 905–907.

Mitchell, A. R., Kent, S. B. H., Chu, I. C. & Merrifield, R. B. (1978). Quantitative determination of D- and L-amino acids by reaction with tert-butyloxycarbonyl-L-leucine N-hydroxysuccinimide ester and chromatographic separation as L,D and L,L dipeptides. *Analytical Chemistry* **50**, 637–640.

Mitchell, H. H. (1950). Some species differences in amino acid requirements. In *Protein and Amino Acid Requirements of Mammals* [A. A. Albanese, editor]. New York: Academic Press.

Mitz, M. A. & Schlueter, R. J. (1958). Direct spectrophotometric measurement of the peptide bond: application to the determination of acylase I. *Biochimica et Biophysica Acta* **27**, 168–172.

Newport, M. J., Chavez, E. R., Horney, F. D. & Bayley, H. S. (1976). Amino acid metabolism in the piglet: influence of level of protein and of methionine in the diet on tissue uptake and in vivo oxidation. *British Journal of Nutrition* **36**, 87–99.

Pichat, L., Rochas, G. & Herbert, M. (1965). Préparation de l'acide DL-α aminoadipique ^{14}C-6 à partir de cyanure de potassium ^{14}C et à benzamido δ-valero-acetone (Synthesis of DL-α-aminoadipic acid-6-^{14}C from KCN-^{14}C and α-benzamido-δ-valerolactone). *Bulletin. Société Chimique de France* **5**, 1384–1386.

Reeds, P. J., Cadenhead, A., Fuller, M. F., Lobley, G. E. & McDonald, J. D. (1980). Protein turnover in growing pigs. Effects of age and food intake. *British Journal of Nutrition* **43**, 445–455.

Reeds, P. J., Fuller, M. F., Cadenhead, A., Lobley, G. E. & McDonald, J. D. (1981). Effects of changes in the intakes of protein and non-protein energy on whole-body protein turnover in growing pigs. *British Journal of Nutrition* **45**, 539–546.

Rosenhagen, M. & Segal, S. (1974). Stereospecificity of amino acid uptake by rat and human kidney cortex slices. *American Journal of Physiology* **227**, 843–847.

Soliman, A.-G. & Harper, A. E. (1971). Effect of protein content of diet of lysine oxidation by the rat. *Biochimica et Biophysica Acta* **244**, 146–154.

Staten, M. A., Bier, D. M. & Mathews, D. E. (1984). Regulation of valine metabolism in man: a stable isotope study. *American Journal of Clinical Nutrition* **40**, 1224–1234.

Tanaka, K., Aberhart, D. J., Amster, J. I., Jenkins, E. E. & Hsu, C.-T. (1981). Metabolism of valine chirally labelled with carbon 13 and deuterium in vitamin b_{12}-folate-deficient rate in vivo. Studies on the physiological role of keto-enol tautomerization. *Journal of Biological Chemistry* **255**, 7763–7768.

Tomera, J. F., McCaffrey, P. G. & Brunengraber, H. (1983). Rates of catabolism calculated from $^{14}CO_2$ production: artifacts and realities. *Pharmacology, Biochemistry and Behaviour* **18**, 285–288.

Wang, T. C. & Fuller, M. F. (1989). The optimum dietary amino acid pattern for growing pigs. 1. Experiments by amino acid deletion. *British Journal of Nutrition* **62**, 77–89.

Waterlow, J. C., Garlick, P. J. & Millward, D. J. (1978). *Protein Turnover in Mammalian Tissues and in the Whole Body*. Oxford: North-Holland Publishing Co.

Printed in Great Britain