The metabolic utilization of amino acids: potentials of $^{14}CO_2$ breath test measurements

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The present paper offers a dual $^{14}CO_2$ breath test approach to study the metabolic utilization of free amino acids in the body. Using the carboxyl-[^{14}C]isotopomer of an amino acid as the test substrate the percentage recovery of the isotope as $^{14}CO_2$ reflects which part of the labelled amino acid flux has been decarboxylated. The residual C fragments may flow to total oxidation at least to the level recovered for the universal [^{14}C]isotopomer. In the case that recovery for total oxidation is less than for decarboxylation, part of the [^{14}C]fragments are retained in the body by either exchange or non-oxidative pathways. Utilization of tyrosine and leucine was measured in the post-absorptive phase in adult rats conditioned on isoenergetic diets containing 210, 75 or 0 g protein/kg. It was shown that the level of dietary protein exerts an influence on both decarboxylation and total oxidation. Although the responses of leucine and tyrosine were not different for total oxidation, there was a difference between the amino acids in their relative rate of decarboxylation. That this dual $^{14}CO_2$ breath test approach can be used as a tool to evaluate whether the protein and amino acid supply has been adequate to support actual requirements is discussed.

Amino acid utilization: Amino acid requirements: Leucine: Tyrosine

Whole-body amino acid metabolism is often represented as a two-compartment model of free and protein-bound amino acids, although more subcompartments exist. The pool of free amino acids is considered to hold only the 'currency' of nitrogen metabolism and comprises less than 1% of the total. This currency mainly originates from endogenous sources (protein turnover or *de novo* synthesis) and transiently from the feed as the exogenous source. The relative distribution of the free amino acids over the different biochemical processes will depend on the balance between the availability, physiological priorities and metabolic capacity. The pool of protein-bound amino acids comprises the constituent body proteins. The nature of these proteins can be changed according to the physiological needs of the body due to their continuous turnover. The dynamic equilibrium between the pools of free and protein-bound amino acids (protein turnover) is continuously subject to the withdrawal of free amino acids for other metabolic purposes (Simon, 1989).

The utilization of and requirement for individual amino acids are intrinsically related to the actual physiological situation. It is, therefore, most reliable to study these aspects by in vivo methods in close relation to metabolic processes specific to that physiological situation. Different types of in vivo metabolic methods (e.g. end-product analysis) with carbon- or nitrogen-labelled amino acids have been proposed and applied (Waterlow *et al.* 1978).

Henry *et al.* (1988) have reviewed several types of amino acid oxidation studies in

Table 1. *Composition of semi-synthetic diets (g/kg)*

Diet.....	Normal protein	Low protein	Protein-free
Casein	210	75	0
Dextrose	50	61·25	67·5
Molasses	50	61·25	67·5
Maize starch	410	522·5	585

In addition, each diet contained: cellulose 100, soya-bean oil 100, $CaHPO_4.2H_2O$ 29, NaCl 6, $MgCO_3$ 3, $KHCO_3$ 18, MgO 2, vitamin premix 12 (containing (mg/kg feed): ascorbic acid 400, thiamin 60, riboflavin 22·5, nicotinic acid 152, DL-Ca-pantothenate 56, pyridoxine 22·5, cyanocobalamin 0·015, choline chloride 2000, inositol 1000, pteroylmonoglutamic acid 8·5, biotin 1, *p*-aminobenzoic acid 500, vitamin K 4 and vitamin E 50; (IU): vitamin A 15000 and cholecalciferol 3000), mineral premix 10 (containing mg/kg feed): Na citrate 1535, $FeSO_4.7H_2O$ 900, MnO_2 140, $KAl(SO_4)_2.12H_2O$ 200, $ZnSO_4.H_2O$ 125, KBr 20, $NiSO_4.6H_2O$ 8·5, NaF 8·5, $CuSO_4.5H_2O$ 100, $CoSO_4.7H_2O$ 5, $Na_2MoO_4.2H_2O$ 5, KI 5, As_2O_3 0·2, $Na_2B_4O_7.10H_2O$ 5, $Na_2SeO_3.5H_2O$ 0·15).

relation to amino acid requirements. In these studies either the carboxyl or the universal [^{14}C]isotopomer of an amino acid was used to follow the decarboxylation or the total oxidation of the amino acid respectively.

The present study describes the additional information which is obtained when both decarboxylation and total oxidation are studied under the same physiological conditions by $^{14}CO_2$ breath tests. The results for tyrosine and leucine obtained under different nutritional conditions of adult rats are compared and discussed in relation to protein and amino acid requirements.

MATERIALS AND METHODS

Animals

Forty-eight adult male Wistar rats (approximately 4 months old, 450 g) were individually caged with a light period from 06.00 to 20.00 hours and free access to water and one of the semi-synthetic diets. The rats were weighed three times weekly.

Diets

At 3 weeks before a $^{14}CO_2$ breath test groups of sixteen animals were fed one of the three isoenergetic semi-synthetic diets with different protein contents (Table 1). The highest protein level was similar to that of commercial diets (approximately 210 g/kg). The feed intake was recorded three times weekly.

On the day of a breath test the feed was removed at 08.00 hours, while the experiment was started at 10.00 hours by injection of the label.

$^{14}CO_2$ breath test

In order to study the metabolic degradation of the C-skeleton of an amino acid the expiration of $^{14}CO_2$ was followed after application of ^{14}C-labelled amino acids. The carboxyl and the universal [^{14}C]isotopomers were used to study decarboxylation (irreversible withdrawal from protein metabolism) and total oxidation, respectively.

Under light diethyl ether narcosis the rats were injected intraperitoneally (single dose) at 10.00 hours with an aqueous solution (200 μl) containing about 37 kBq (1 μCi) of the ^{14}C-labelled L-amino acid. In the present study we used the following tracer substances

obtained from the Radiochemical Centre, Amersham, Bucks., UK: [1-^{14}C]tyrosine (2·07 GBq/mmol), [U-^{14}C]tyrosine (19·3 GBq/mmol), [1-^{14}C]leucine (2·0 GBq/mmol), [U-^{14}C]leucine (11·5 GBq/mmol).

Immediately after the injection the rats were placed in a closed cage (150 × 250 × 150 mm) ventilated at a rate of 1·5 l/min. Routinely animals recovered from the diethyl ether narcosis within 1 min.

During the 4 h of an experiment the air from the cage was sucked through the sintered glass bottom of a cylindrical sample flask (diameter 60 mm) containing 250 ml 2 M-potassium hydroxide. This concentration of KOH was used standardly and was about three times the stoichiometric amount required to react with the CO_2 expired by a 450 g rat during the time-course of an experiment (about 1·8 l or 80 mmol in 4 h).

Every 15 min the amount of $^{14}CO_2$ accumulated in the sample flask was determined by analysing the [^{14}C]radioactivity in 1 ml of a 2 ml sample taken from the flask. Before counting the sample was mixed with 9 ml water and 10 ml liquid scintillation cocktail (Instagel, Packard).

The cumulative amount of radioactivity recovered as $^{14}CO_2$ in the breath was expressed as a percentage of the injected dose. Corrections were made for the decreasing volume of the sample flask due to the removal of the 2 ml samples. Using a serial flask it was checked that all the expired radioactivity was trapped in the sample flask. The values reached after 4 h (end-values) were used to characterize the process of amino acid degradation.

Studies with the carboxyl (1st) and universal (2nd) [^{14}C]isotopomers of an amino acid were made on the same animals with a 3 d interval under identical physiological conditions.

Nutritional conditions

The conditions for measurement are supposed to be very critical for the interpretation of the results. The present $^{14}CO_2$ breath tests were started 4 h after the onset of the light period. In *ad lib.* situations rats eat the majority of their daily feed during the dark period (about 85%), with a last meal of about 15% at dawn. During the first part of the light period feed intake is zero (Spiteri *et al.* 1982; Strubbe *et al.* 1986). This means that the majority of the amino acids metabolized during the light period (including the time-course of the experiments) are mobilized by the turnover of pre-existing proteins.

Calculation of total oxidation

The metabolic degradation of leucine and tyrosine is initiated by a reversible deamination. The first irreversible step in the breakdown of their C-skeleton is the oxidative removal of the functional carboxyl group from the keto acid. The percentage decarboxylation (% 1-^{14}C) was measured using the carboxyl [^{14}C]isotopomer as the substrate.

The percentage recovery of radioactivity with the universal [^{14}C]isotopomer as the test substrate (% U-^{14}C) reflects the average oxidation of n C-atoms of the amino acid molecule. These atoms, however, are not equivalent. Therefore, the recovered amount of radioactivity consists of contributions of n atoms: the percentage decarboxylation of only 1 atom (carboxyl atom) and the percentage total oxidation of $(n-1)$ atoms. Thus:

$$n(\%\,\text{U-}^{14}\text{C}) = 1(\%\ 1\text{-}^{14}\text{C}) + (n-1)(\%\ \text{total oxidation}).$$

The percentage total oxidation of the amino acid can be calculated as:

$$\%\ \text{total oxidation} = \frac{n(\%\ \text{U-}^{14}\text{C}) - 1(\%\ 1\text{-}^{14}\text{C})}{n-1}.$$

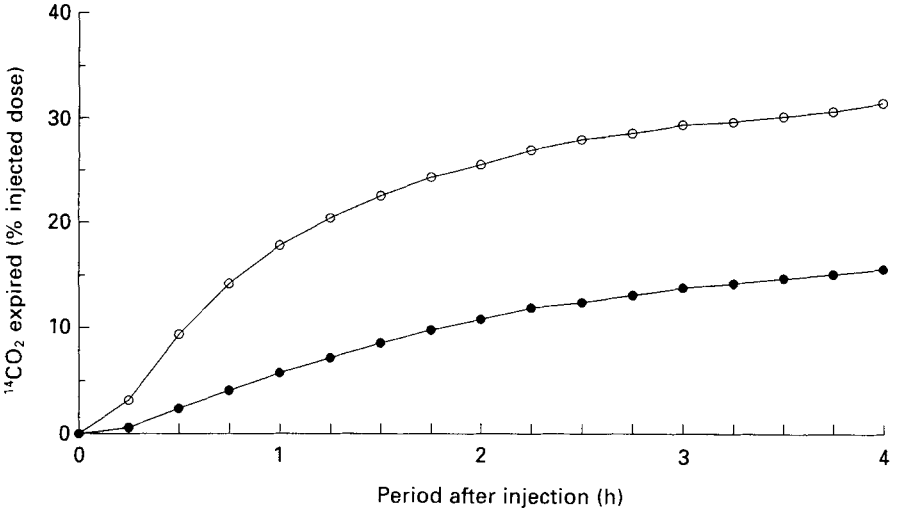

Fig. 1. A typical example of the cumulative amount of $^{14}CO_2$ expired after injection of a ^{14}C-labelled amino acid. [1-^{14}C]tyrosine (○) and [U-^{14}C]tyrosine (●) were applied to rats conditioned on a semi-synthetic diet containing 210 g protein/kg (for details of diet, see Table 1 and for details of procedures, see pp. 208–209.

Table 2. *Recovery of radioactivity (% injected dose) as $^{14}CO_2$ in the breath 4 h after intraperitoneal injection of a ^{14}C-labelled amino acid (carboxyl (1-^{14}C) and universal (U-^{14}C) isotopomers of tyrosine and leucine) in rats on semi-synthetic diets containing 210, 75 or 0 g protein/kg*

(Mean values and standard deviations for no. of rats shown in parentheses)

Protein level (g/kg)...	210			75			0	
Amino acid	Mean	SD		Mean	SD		Mean	SD
Tyrosine (*n* 6)								
Decarboxylation† (1-^{14}C)	31·4	3·3	***	21·0	1·1	***	9·9	2·3
	***			***			**	
Total oxidation‡ (U-^{14}C)	13·6	1·4	***	8·7	0·9	***	5·2	0·8
Leucine (*n* 10)								
Decarboxylation† (1-^{14}C)	21·0	3·9	***	10·4	1·7	***	6·5	1·3
	***			*			NS	
Total oxidation‡ (U-^{14}C)	14·4	1·6	***	7·9	1·9	NS	6·5	2·1

Mean values for [1-^{14}C]tyr were significantly different from those for [1-^{14}C]leu at 210 g/kg ($P < 0.001$), 75 g/kg ($P < 0.001$), 0 g/kg ($P < 0.01$).

Mean values for [U-^{14}C]tyr were not significantly different from those for [U-^{14}C]leu at any level of dietary protein.

NS, not significant.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

† The value for decarboxylation is the recovery for the carboxyl [^{14}C]isotopomer (see p. 209).

‡ Total oxidation is calculated from the recovery for the universal [^{14}C]isotopomer (see p. 209).

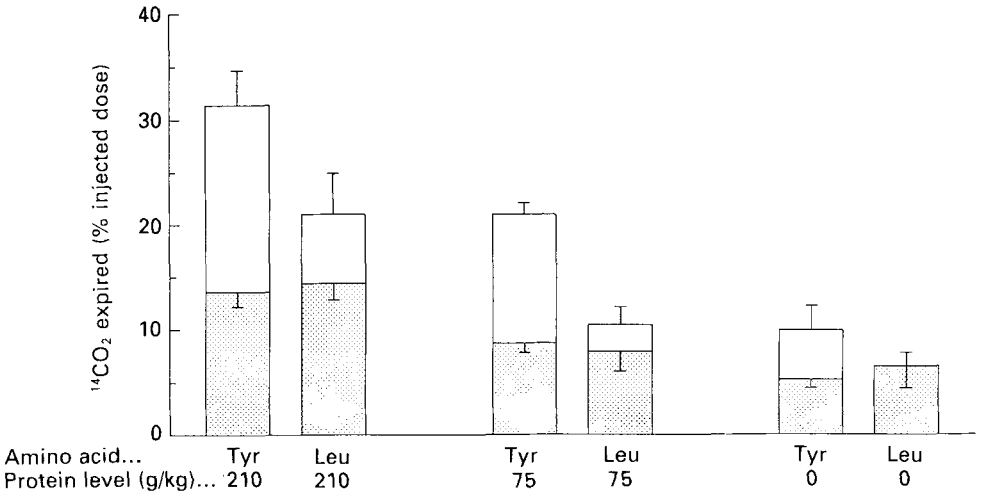

Fig. 2. Radioactivity (% injected dose) recovered in the breath as $^{14}CO_2$ after intraperitoneal injection of a ^{14}C-labelled amino acid. Rats were conditioned on semi-synthetic diets containing 210, 75 or 0 g protein/kg (for details, see Table 1). The carboxyl and universal [^{14}C]isotopomers of tyrosine and leucine were applied. The value for decarboxylation (□) is the recovery for the carboxyl [^{14}C]isotopomer, while total oxidation (▨) is calculated from the recovery for the universal [^{14}C]isotopomer (for details of procedures, see pp. 208 209). Values are means and standard deviations represented by vertical bars.

In this equation n is the number of C-atoms in the molecule; n is 9 for tyrosine and n is 6 for leucine.

Statistics

Data were subjected to analysis of variance (ANOVA). Group means were analysed by Student's t test. The values for decarboxylation v. total oxidation were analysed by the paired t test.

RESULTS

Development of body-weight and feed intake

The initial weight of the rats was 443 (SD 21) g. During the period of adaptation (3 weeks) rats maintained their body-weight on both the normal (210 g/kg) and the low-protein (75 g/kg) diet. On the protein-free diet the rats lost 15 (SD 4)% of their body-weight ($P <$ 0·001) which is about 2–3 g/d. Relative to body-weight there were no significant differences in feed intake between the diets.

$^{14}CO_2$ breath tests

Breath tests were carried out under twelve different conditions (three protein levels, two amino acids and two isotopomers). The cumulative curves for tyrosine and 210 g protein/kg are shown in Fig. 1 as typical examples.

Radioactivity expired as $^{14}CO_2$ was already detectable in the first sample 15 min after injection of the label. The steepest part of the curve was obtained between 15 and 45 min. Thereafter the expiration rate of $^{14}CO_2$ decreases. The slope of the curve decreased up to 3–4 h indicating that the prime utilization of the radiolabelled amino acids was practically completed. The values reached after 4 h were chosen to quantify the process of amino acid degradation. Mean values and standard deviations are presented in Table 2.

Statistical analysis (ANOVA) showed significant effects on decarboxylation for both the

protein level in the diet ($P < 0.001$) and the type of amino acid ($P < 0.001$). Total oxidation was only significantly influenced by the protein level ($P < 0.001$). A significant interaction between the protein level in the diet and the type of amino acid was only found for decarboxylation ($P < 0.001$). These results are shown in Fig. 2.

DISCUSSION

In order to get a better understanding of amino acid requirements in relation to metabolic losses we studied the metabolic utilization of amino acids under different nutritional conditions. The carboxyl and universal [^{14}C]isotopomers were used as substrates in $^{14}CO_2$ breath test measurements to determine decarboxylation and total oxidation as parts of the whole process of amino acid degradation. Normally only one of these [^{14}C]isotopomers is used (Henry *et al.* 1988).

The findings of the present study indicate that, in general, as measured at the whole-body level, different amounts of an amino acid are subject to decarboxylation and to total oxidation. In addition, it is evident that the level of dietary protein exerts an influence on both variables. Although the responses of leucine and tyrosine are not different for total oxidation, there is a difference between the amino acids in their relative rate of decarboxylation.

At first sight it is logical to suggest that the proportion at which decarboxylated amino acids are recovered for total oxidation would be related to the metabolite by which the C residues enter the central pathways of metabolism. This could be by dilution and exchange as previously discussed by, for example, Reeds (1974) or by incorporation into other body constituents (Haggarty *et al.* 1986). There is a principal difference between these two explanations. In the first an equivalent of the radiolabelled amino acid residue is still subjected to total oxidation whereas in the second it is not.

After decarboxylation the keto acid of leucine ultimately enters the TCA cycle as acetyl-CoA, whereas tyrosine is degraded to acetyl-CoA and fumarate. Thus, both amino acids would behave in a similar way with respect to acetyl-CoA. Differences between tyrosine and leucine should be attributed to fumarate. This, however, is not likely. Acetyl-CoA entering the TCA cycle is not lost as CO_2 during the first turn of the cycle but it must pass the fumarate stage (Stryer, 1988). Therefore, label entering the cycle as acetyl-CoA would have the same chance for total oxidation as the label of fumarate. Our results for the protein-free diet, however, show that all decarboxylated residues of leucine are recovered for total oxidation whereas for tyrosine this recovery is only about 50%. In addition, it would be very unlikely that fumarate representing 50% of the label of decarboxylated tyrosine would not be oxidized at all. As a consequence we have to conclude that not all decarboxylated amino acids directly enter the TCA cycle. This is essentially what has been shown for the Zucker rat by Haggarty *et al.* (1986). They have stated that label from [U-^{14}C]leucine not recovered as $^{14}CO_2$ or as the parent amino acid is mainly incorporated into non-essential amino acids. Therefore, we suggest that decarboxylation and total oxidation as measured on the whole-body level have to be considered as the result of different contributions from rather independent processes or compartments. This may also explain why Reeds (1974) found differences between his studies in vivo and in vitro.

The following discussion may be somewhat provocative and certainly requires further experimental support. The majority of amino acids metabolized during the time-course of the experiment are mobilized by the turnover of pre-existing proteins (see p. 209). Essentially the same proportion of tyrosine and leucine mobilized is subjected to total oxidation. This might suggest that endogenous proteins are partly subjected to net degradation (decarboxylation followed by total oxidation of amino acids); in the present

study for about 10% of their turnover rate (decreasing from 14·4 to 5·2% depending on the protein and energy intake). Such a net degradation of endogenous proteins would fit with the diurnal cycling of proteins as discussed by Millward & Rivers (1988). The short-term retention of dietary proteins cannot be in conflict with the long-term regulation of N and energy balance. Therefore, the deposited proteins require a high turnover rate to enable their removal in due time.

The remaining approximately 90% of the amino acids released can be considered as endogenous nutrients and as such available for all kinds of metabolic purposes. In addition to repartitioning there may be competition for protein synthesis or other non-oxidative aims (e.g. synthesis of non-essential amino acids, fatty acids, glucose or specific compounds to support metabolism). What happens will depend on availability, physiological priorities and metabolic capacity. Amino acids not re-used for protein synthesis will be decarboxylated but the residual C-fragments are retained and are at first available for non-oxidative purposes. Thus, the amount of proteins deposited after a meal will gradually decrease while serving the amino acid requirements during the post-absorptive phase.

On a diet containing 210 g casein/kg we measured a decarboxylation of 31·4 (SD 3·3)% for tyrosine and 21·0 (SD 3·9)% for leucine, while for both only about 14% of the available amino acids is totally oxidized. The relatively high decarboxylation of tyrosine indicates that this amino acid becomes more excessively available with respect to its requirement than leucine.

At the 75 g protein/kg level the availability of leucine can be characterized as marginal. There is only a small difference between the decarboxylation and total oxidation. Therefore, in the post-absorptive phase leucine will become limiting sooner than tyrosine when the protein content of the diet is further reduced.

At the zero dietary protein level the body already relies on pre-existing proteins during a substantial period of time. The values for decarboxylation and total oxidation are further reduced. For leucine the values become similar. The decarboxylation of tyrosine is still twice the total oxidation. Although very unlikely for endogenous protein as a whole, the results for tyrosine suggest that occasionally the C-skeleton of individual endogenous amino acids can be transferred to non-oxidative pathways even when no exogenous protein is supplied. In fact this situation is completely comparable with the excessive supply of some amino acids in the case of imbalanced diets.

The present study shows that the $^{14}CO_2$ breath test approach using both the carboxyl and universal [^{14}C]isotopomer of an amino acid can be a proper tool to follow the metabolic utilization of the free amino acids in the body. If the nutritional condition is fixed any labelled amino acid can be used to show the relative adequacy of its own supply (auto-indicator method). On changing the nutritional condition a single amino acid can be used to show the differences in protein status (indicator method). Results can be used to evaluate whether the protein and amino acid supply has been adequate to support the actual physiological situation. Adjustment of the supply, however, has to be considered carefully since the amino acids metabolized originate from endogenous protein.

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REFERENCES

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. *Biochemical Journal* **235**, 323–327.

Henry, Y., Arnal, M., Obled, C. & Rérat, A. (1988). Protein and amino acid requirements of pigs. In *European Association for Animal Production*, Symposium on Protein Metabolism and Nutrition Publication no. 35, part 3, pp. 9–18, Rostock: EAAP.

Millward, D. J. & Rivers, J. P. W. (1988). The nutritional role of indispensable amino acids and the metabolic basis for their requirements. *European Journal of Clinical Nutrition* **42**, 367–393.
Reeds, P. J. (1974). The catabolism of valine in the malnourished rat. Studies in vivo and in vitro with different labelled forms of valine. *British Journal of Nutrition* **31**, 259–270.
Simon, O. (1989). Metabolism of proteins and amino acids. In *Protein Metabolism in Farm Animals*, pp. 273–366 [H. D. Bock, B. O. Eggum, A. G. Low, O. Simon and T. Zebrowska, editors]. Oxford: Oxford Science Publications.
Spiteri, N. J., Alingh Prins, A., Keyser, J. & Strubbe, J. H. (1982). Circadian pacemaker control of feeding in the rat, at dawn. *Physiology & Behavior* **29**, 1141–1145.
Strubbe, J. H., Keyser, J., Dijkstra, T. & Alingh Prins, A. J. (1986). Interaction between circadian and caloric control of feeding behavior in the rat. *Physiology & Behavior* **36**, 489–493.
Stryer, L. (1988). *Biochemistry*, 3rd ed., pp. 373–396. New York: Freeman and Co.
Waterlow, J. C., Garlick, P. & Millward, D. J. (1978). *Protein Turnover in Mammalian Tissues and in the Whole Body*. Amsterdam: North-Holland Publishing Company.

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