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Application of a [$^{13}CO_2$] breath test to study short-term amino acid catabolism during the postprandial phase of a meal

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A [$^{13}CO_2$] breath test was applied as a non-invasive method to study the catabolism of ingested amino acids shortly after a meal. This test requires the ingestion of a [1-^{13}C]-labelled amino acid and the analysis of expired air for [^{13}C] enrichment and CO_2. The recovery of label as [$^{13}CO_2$] reflects the catabolism of the [1-^{13}C]-labelled substrate. Such a non-steady state approach provides information that is complementary to the information obtained by steady-state methods using a primed continuous infusion of tracer amino acids during the fed state. In a model study with twenty adult male rats, two groups of animals were fed twice a day with one of two semi-synthetic iso-energetic diets. One diet contained egg white protein (EW) as the sole amino acid source. The second diet contained a mixture of free amino acids with a pattern similar to that of the EW diet. On day 5 of the dietary treatment, L-[1-^{13}C]leucine, either bound in EW protein or in free form, was ingested as part of the morning meal. The expired air was sampled at 30 min intervals for 5 h. The rate of recovery ranged from 0 % to 6 % of the dose/h. Up to 120 min after the onset of the meal, the recovery values for the free amino acid diet were higher than those for the EW diet. Differences in recovery reflect differences in postprandial utilisation. The differences in label recovery were mainly determined by the [^{13}C] enrichment of the expired air. As a consequence, CO_2 measurements are not mandatory when CO_2 production is comparable.

Free amino acids: Egg white protein: Rat: Stable isotopes: Leucine oxidation

In the case of meal-feeding, the dietary supply of amino acids is not synchronised with the demands of the body for amino acids. To overcome this physiological situation, protein metabolism shows two phases relative to a meal (Millward, 1995). Directly after a meal (the postprandial phase), a huge inflow of amino acids from the gastrointestinal tract leads to a net protein synthesis to store the dietary amino acids. Between meals (the post-absorptive phase), the demand of the body for amino acids leads to a net degradation of protein and a further catabolism of dietary amino acids. An impaired storage of dietary amino acids during the postprandial phase causes a reduced availability of amino acids for post-absorptive utilisation. The postprandial storage of dietary amino acids can be impaired when the postprandial oxidation of dietary amino acids is favoured by, for example, too high a rate of appearance in the blood (Boirie *et al.* 1997; Bos *et al.* 2003). The appearance rate of dietary amino acids in the blood after a meal is strongly dependent on the feeding strategy. Even an increase in dietary intake can reduce the retention and utilisation of dietary amino acids. This phenomenon is described as the 'protein paradox' (Moundras *et al.* 1993).

Free amino acids can replace dietary proteins in their function to support the body's basic needs for maintenance and growth (D'Mello, 2003). Free and protein-bound amino acids are not, however, always exchangeable without nutritional and physiological consequences (Simon, 1989). In humans, Metges *et al.* (2000) studied the kinetics of L-[1-^{13}C]leucine when ingested with free amino acids, with unlabelled casein or as intrinsically labelled casein. These authors observed that the oxidation of leucine was significantly higher when the labelled leucine was ingested as part of a free amino acid diet compared with ingestion as part of a casein diet. These results were obtained using an isotopic steady-state approach combined with a frequent, small and equal meal paradigm.

In the present model study with rats, a [$^{13}CO_2$] breath test approach was used to study the catabolism of leucine shortly after ingestion with a meal in free or protein-bound form. After a 5 d pre-feeding period, the breath test substrate L-[1-^{13}C] leucine, either bound in egg white (EW) protein or in free form, was incorporated into the diet and fed to rats. The expired air was sampled at 30 min intervals for 5 h. The [$^{13}CO_2$] breath test was examined for its potential to provide information about differences in amino acid catabolism shortly after a meal.

Abbreviations: EW, egg white; IRMS, isotope ratio mass spectrometer.

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Materials and methods

Ethical approval

This breath test study with rats on different diets was approved by the Ethical Committee of Wageningen University.

Animals, diets and feeding procedure

The [$^{13}CO_2$] breath test study was performed using twenty adult male (290–325 g) Wistar (WU) rats (Harlan, Horst, The Netherlands). The rats were allocated to one of two experimental groups (ten per group) such that both groups had the same mean body weight. The animals were housed individually in standard Macrolon cages (38 × 26 × 14 cm) in animal facilities with control of temperature (21°C), a relative humidity of 70 % and a daily light schedule of 8 h darkness and 16 h light. For convenience, the dark period was set during daytime (09.00–17.00 hours). Water was available *ad libitum*.

The animals were fed twice a day for 30 min at the beginning and the end of the dark period (09.00–09.30 and 16.30–17.00 hours). During a preliminary period of 2 weeks, commercial rat chow (Teklad Global Rodent Diet; Harlan Netherlands, Horst, The Netherlands) was provided to the rats as 2 mm pellets. At the start of each 30 min feeding period, the rat chow was available *ad libitum*. Leftovers were removed after 30 min. From the start of the dietary treatment, the two semi-synthetic, iso-energetic (metabolisable energy) and iso-nitrogenous diets (Table 1) were fed for 4 d. During each of the two 30 min feeding periods per day, 8·5 g of the experimental diets was given. When present, leftovers were removed after 30 min. The EW group received a diet with EW protein as the sole source of amino acids. The free amino acid group received the same diet with the exception that the EW protein was replaced by a mixture of free amino acids (Table 1). For technical reasons related to breath-testing (see later), the feed was offered as a porridge (feed–water 8:3 w/w).

Table 1. Composition of the experimental diets

Composition of the experimental diets (g/kg)	
Crude protein	210
Glucose	639
Cellulose	50
Soyabean oil	50
$CaCO_3$	12·4
$NaH_2PO_4.2H_20$	3·40
$MgCO_3$	1·40
KCl	1·10
KH_2PO_4	10·5
Mineral mix, rat	10·0
Vitamin mix, rat	12·0
Amino acid composition of crude protein fraction (g/210 g)	
Ala	12·18
Arg	11·34
Asp	22·89
Cys	3·78
Glu	25·62
Gly	6·72
His	4·62
Ile	11·76
Leu	17·22
Lys	12·18
Met	7·35
Phe	12·18
Pro	8·40
Ser	15·54
Thr	10·08
Trp	3·78
Tyr	8·40
Val	16·38

The composition of the two (EW and F diet) semi-synthetic, iso-energetic (metabolisable energy; 15·4 MJ/kg) and iso-nitrogenous diets is given below. The two experimental diets differed in composition of the crude protein fraction. The crude protein fraction of the EW or egg white diet consisted of egg white protein as the sole source of amino acids. The crude protein fraction of the F or free amino acid diet consisted of a mixture of free amino acids simulating the egg white protein of the EW-diet according to the pattern given by Evenepoel *et al.* (1997).

After a change of diet, rats normally show a transient decrease in feed intake over 5 d. Therefore the [$^{13}CO_2$] breath test was performed on day 5 after the start of the dietary treatment. To avoid differences in feed intake during the test, a reduced (80 %) amount of labelled feed (6·7 g) was given to all animals in the morning meal on days 5 and 6. This amount was eaten by all the animals without any refusals and within the feeding period of 30 min. After the breath test on day 5 and the measurement of CO_2 production on day 6, the animals were kept on their experimental diets for a longitudinal study (to be described elsewhere).

The rats were weighed twice a week prior to the morning meal at 09.00 hours. The weighing procedure made the rats familiar with being handled and reduced the stress of handling during the breath test procedure.

[$^{13}CO_2$] breath test

General aspects. A [$^{13}CO_2$] breath test studies the expiration of [^{13}C] as [$^{13}CO_2$] shortly after the oral ingestion of a [^{13}C]-labelled substrate (Krumbiegel, 1991). It is essential that the ingested amount of labelled substrate has no pharmacological effects on the catabolic response that is the focus of study. Therefore, in nutritional studies, the labelled substrate is best ingested as an intrinsic part of a test meal (Schreurs & Koopmanschap, 1996). Calculating the amount of [^{13}C] expired requires not only a value for the [^{13}C] enrichment of the expired CO_2, but also a value for the total amount of CO_2 expired during the corresponding period of sampling.

In our approach, we chose a complete collection of the expired air in a closed cage and sampling after 30 min for [^{13}C] analysis. This method of air-sampling cannot be combined with a continuous on-line measurement of CO_2 production (see later). Total CO_2 was therefore measured in a separate study on day 6 under the same conditions as the breath test on day 5.

The appearance of [^{13}C] as [$^{13}CO_2$] in the breath reflects the overall kinetics of the label from the [^{13}C]-labelled substrate through the processes of oral ingestion, gastric emptying, digestion, absorption, distribution in the body, decarboxylation and final expiration as [$^{13}CO_2$].

Breath test substrates. The [^{13}C]-labelled breath test substrate used in this study was L-[1-^{13}C]leucine (chemical purity >99 %, isotopic enrichment >99 %; ARC, Amsterdam, The Netherlands). The labelled diets were prepared in the same way as the non-labelled semi-synthetic diets. As the only difference in the case of the labelled free amino acid diet, part of the leucine in the non-labelled free amino acid diet (17·22 g/kg)

was replaced by L-[1-^{13}C]leucine (895 mg/kg). Similarly, in the labelled EW diet, part of the EW in the non-labelled EW diet was replaced by labelled EW. The labelled EW protein was produced in advance by feeding L-[1-^{13}C]leucine to laying hens according to the method of Evenepoel *et al.* (1997). The protein used in the labelled EW diet was collected from labelled eggs yielded from a single hen. The [^{13}C] enrichment of the EW from different eggs ranged between 1·30 and 1·40 atom% (Schreurs *et al.* 2001). Approximately 52 g labelled EW/kg feed was used to replace part of EW (210 g/kg) present in the non-labelled EW-diet.

Breath test procedure and sampling. In order adequately to collect all the CO_2 expired by the rats, the rats were placed individually in standard Macrolon cages (20 × 16 × 14 cm) made air-tight with a plastic cover. The cages were bedded with sawdust, and the animals had free access to water.

A period of about 30 min was required to reach a level of CO_2 adequate for isotope ratio mass spectrometer (IRMS) analysis. Therefore, after each 30 min period, the collected air was sampled (about 10 %) through a valve on the cover of the cage using a 50 ml syringe. A 10 ml Exetainer tube (Labco, High Wycombe, UK) was flushed with the sampled air and stored at room temperature until IRMS analysis. After the air had been sampled, the animal was transferred to a new cage for the next 30 min collection period. The animals were acclimatised to this type of handling by the weighing procedure; moreover, handling was the same for all the animals.

The air sampling was started at 08.30 hours, 30 min before the regular feeding time. This pre-meal air sample was used to determine the baseline value for the natural [^{13}C] enrichment of expired air for each individual rat. At the regular feeding time (09.00–09.30 hours), the animal received a [^{13}C]-labelled test meal. The first test sample was taken at the end of the feeding period. Within both dietary groups, two of the ten animals received their normal non-labelled diet during the breath test. This procedure was meant to check a possible change in [^{13}C] enrichment of the expired air in response to the ingestion of a normal, non-labelled meal. No significant change in [^{13}C] enrichment was noticed relative to the baseline pre-meal values.

Sample analysis. All [^{13}C] samples were analysed for [^{13}C] enrichment on the IRMS of the Wageningen Institute of Animal Sciences IRMS facility. This IRMS analyser (Delta C; Finnigan MAT, Bremen, Germany) was on line equipped with a breath device and an elemental analyser. The Exetainer tubes with the air samples were directly placed into the autosampler of the breath device. The air samples were analysed for the [^{13}C] enrichment of CO_2. The [^{13}C] enrichment of the labelled EW protein was also determined as [^{13}C] enrichment of the CO_2 but after combustion in the elemental analyser. In both cases, the [^{13}C] enrichment of the CO_2 was determined as atom% (^{13}C:total C) with an accuracy of ± 0·0005 %.

Measurement of CO_2 production. Production of CO_2 was measured separately from the [$^{13}CO_2$] breath test on the following day under the same feeding and environmental conditions. Measurements of total CO_2 could be made simultaneously for at most six animals. Six randomly selected animals from both groups were placed individually in airtight but ventilated cages alternately linked to the same continuous flow CO_2 analyser. The breath test cages were ventilated with environmental air through an open inlet by means of an air pump on the outlet site. The flow of the outgoing air (approximately 0·5 l/min) was measured using a flow meter (< 2·5 m^3/ h; Schlumberger G 1·6; Meterfabriek, Dordrecht, The Netherlands) for each cage. The outgoing air was dried over $CaCl_2$, and the CO_2 content of the air flow was measured using a continuous flow CO_2 analyser (Uras-3G; Hartmann & Braun AG, Frankfurt, Germany).

The values for the six cages were measured alternately one after the other and recorded each time for 1–2 min periods from 08.30 to 14.00 hours. Because of the small size of the cages, equilibrium was rapidly established (in up to 10 min). This applied to the pre-feeding and feeding periods; thereafter, the cage remained closed under constant ventilation. Therefore, during all the 30 min periods, each animal was measured two or three times. Values for CO_2 production (ml/min) were calculated from the recordings and the air flow as the mean of the 30 min periods corresponding to the sampling periods of the breath test. The values for CO_2 production (ml/min) were corrected for standard conditions (0°C, 760 mmHg, dry).

Calculation of label recovery. The following calculations were made to express the recovered amount of label as a percentage of the ingested amount. For each 30 min air sample, the atom% excess value was determined as the difference between the measured [^{13}C] enrichment value of the sample and the baseline (pre-meal value). The absolute amount of [^{13}C] recovered was calculated by multiplying the atom% excess value by the total amount of carbon expired as CO_2 during the corresponding period of sampling. The total amount of carbon expired (g/30 min) was derived from the CO_2 production (ml/min) determined in the separate study on day 6. The values for CO_2 production (ml/min) were multiplied by 30 (30 min/sampling period), divided by 22·4 ml/mmol (molar volume) and multiplied by 12 (the atomic weight of C). The absolute amount of [^{13}C] recovered was then calculated as the atom% excess value of the amount of C expired during the 30 min sampling period. The rate of recovery of [^{13}C] was expressed relative to the amount of [^{13}C] ingested, as a percentage of dose/h. The cumulative recovery of [^{13}C] was expressed as percentage of dose/5 h. The latter value was calculated by adding the amounts recovered in the subsequent sampling periods over the entire 5 h collection period.

Statistical analysis

Data are shown as means and standard deviations. The SPSS statistical programme for Windows (10.1; SPSS Inc., Chicago, IL, USA) was used for the statistical analysis with one-way ANOVA. The influence of the feeding treatment on [^{13}C] recovery rate as well as on CO_2 production was tested at each measurement time point. Data were tested for normal distribution using a Shapiro–Wilk test and for equality of error variances using Levene's test. Differences were considered significant at $P < 0·05$.

Results

Body weight

On day 5, before the start of the breath test, the mean body weight of animals in the two diet groups was not significantly different: 324 (SD 16·8)g (EW group) and 326 (SD 18·2) (free

amino acid group), respectively. All the rats remained healthy throughout the study.

[$^{13}CO_2$] breath test results for the experimental diets

[^{13}C] enrichment of collected air samples. Figure 1(A) shows the mean atom% excess values of the air samples collected from rats on the EW and free amino acid diets during the breath test. The expiration of label ingested as labelled EW protein started as soon as the 30 min feeding period. The first sample taken at the end of the meal showed a value of 0·00 091 atom% excess. The [^{13}C] enrichment of the subsequent air samples gradually increased to a maximum value in the sample collected in the period 210–240 min after the onset of the meal. Thereafter, the [^{13}C] enrichment did not decrease drastically during the last two collection periods of the study.

For the rats on the free amino acid diet, the first sample taken at the end of the meal showed a value of 0·00 674 atom% excess. The [^{13}C] enrichment of the subsequent air samples increased more quickly and reached a higher value than that of rats on the EW diet. The free amino acid group reached a maximum value in the period 150–180 min after the onset of the meal. From 180 min onwards, a gradual decrease in the [^{13}C] enrichment of the collected air samples was noticed. During the last two 30 min sampling periods in this study, the values for rats on the free amino acid diet became similar to the values for rats on the EW diet. Between 0 and 120 min after the onset of the test meals, the [^{13}C] enrichment of collected air for the free amino acid group differed significantly from that for the EW group. The time-related difference in [^{13}C] enrichment of the CO_2 recovered from rats on the free amino acid and EW diets was transient and showed a maximum value in the first post-meal period (30–60 min after the onset of the meal). The value of the maximum difference (0·088) was about 400 % relative to the absolute value of the EW group (0·022) and more than 1000 % relative to the differences at the end of measurement (0·004–0·009).

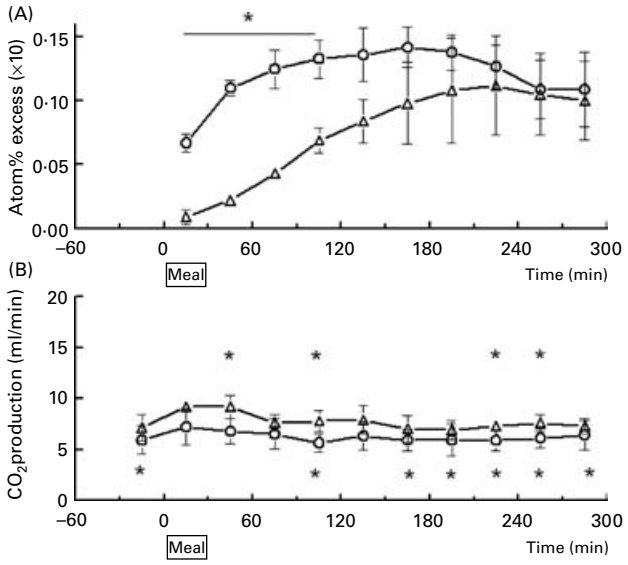

Fig. 1. (A) Mean [^{13}C] enrichment (atom% excess) and standard deviations for 30 min air samples collected during a 5 h [$^{13}CO_2$] breath test with adult rats on the egg white (Δ) and free amino acid (○) diets. The animals ingested L-[1-^{13}C]leucine administered in free form or bound to egg white protein as an intrinsic part of a test meal at the start of the breath test. Breath tests were performed on the fifth day on the experimental diets. *Mean values were significantly different between diet groups: $P<0·05$. (B) Mean CO_2 production (ml/min) and standard deviations for rats on the egg white and free amino acid diets. Measurements were performed the day after the breath test measurements under the same conditions. Measurements were made simultaneously for six animals, individually caged and alternately linked to the same continuous flow CO_2 analyser. The values for the six cages were measured one after the other and recorded for periods of 1–2 min. Values for CO_2 production (ml/min) were calculated from the recordings and the air flow as the mean of 30 min periods corresponding to the sampling periods of the breath test. The values for CO_2 production were corrected for standard conditions (0°C, 760 mmHg, dry). *Mean values were significantly different between diet groups for the upper row, and between the mean value for the measurement period and the period ending at 60 min for the bottom row: $P<0·05$.

CO_2 production of rats on the egg white and free amino acid diets. Figure 1(B) shows the mean values for CO_2 production (ml/min) of the rats on the EW and free amino acid diets as measured on day 6. CO_2 production over time was rather constant, but most values for the free amino acid group were significantly lower than those for the EW group. The mean CO_2 production over the entire collection period was 7·7 and 6·2 ml/min for the rats on the EW and free amino acid diets, respectively. The 'meal-related pattern' of CO_2 production was similar for both diet groups. On both diets, the mean level of CO_2 production was significantly higher during the period of feeding (0–30 min) and the first post-meal period. However, these highest values were, for both diets, only about 20 % higher than the mean value of the entire 5 h collection period.

Rate of [^{13}C] recovery in the breath. Figure 2 shows the mean values for the rate of recovery of [^{13}C] (% dose/h) calculated for individual rats and based on the [^{13}C] enrichment of their expired air and their CO_2 production. The mean rate of recovery for rats on the EW diet was 0·46 % dose/h during the 30 min feeding period and gradually increased to a maximum

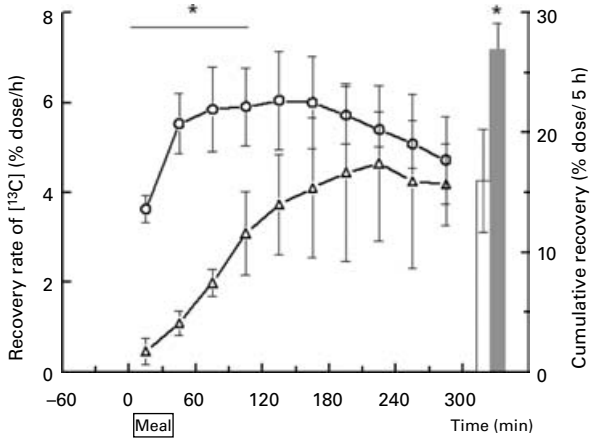

Fig. 2. The [$^{13}CO_2$] breath test with adult rats. Mean values and standard deviations for the rate of recovery of [^{13}C] expressed as % dose/h and calculated for individual rats based on the [^{13}C] enrichment of the expired air and the CO_2 production of individual rats on the egg white or free amino acid diet. The animals ingested L-[1-^{13}C]leucine in free form (○) or bound to egg white protein (Δ) as an intrinsic part of a test meal. Breath tests were performed on the fifth day on the experimental diets. The corresponding bars represent the means and standard deviations for the cumulative recovery of label as % dose/5 h. *Mean values were significantly different: $P<0·05$.

value of 4·6 % dose/h after 240 min. It did not change drastically during subsequent periods. During the last 30 min sampling period in this study (270–300 min), the rate of recovery was approximately 4·0 % dose/h.

For the rats on the free amino acid diet, the mean rate of recovery during the 30 min feeding period was 3·6 % dose/h. The rate of recovery on the free amino acid diet increased more quickly and reached a higher value than that of rats on the EW diet. The free amino acid group reached a maximum value of 6·0 % dose/h in the period 120–180 min. From 180 min after the start of the meal, a gradual decrease in rate of recovery was noticed. During the last 30 min sampling period in this study (270–300 min), the rate of recovery for the rats on the free amino acid diet became similar (about 4 %) to the rate of recovery for the rats on the EW diet. Between 0 and 120 min after the onset of the test meals, the rate of recovery of label in the collected air for the free amino acid group differed significantly from that of the EW group. The cumulative recovery of label for the free amino acid group (26·9 (SD 2·2) % dose/5 h) was also significantly ($P<0·05$) different from the cumulative recovery value for the EW group (16·0 (SD 4·3) % dose/5 h).

Discussion

General advantage of the [$^{13}CO_2$] breath test approach

The [$^{13}CO_2$] breath test principle has been advocated as an easy and non-invasive method for clinical diagnostics and as a research tool (Krumbiegel, 1991; Swart & van den Berg, 1998). As previously suggested (Bujko *et al.* 2001), we now used the breath test approach to trace nutrition-relevant differences in nutrient dynamics shortly after ingestion with a meal. By definition, such results can not be obtained by steady-state approaches using a primed continuous infusion of label and small frequent meals (Metges *et al.* 2000). We monitored the appearance of [$^{13}CO_2$] in the breath during the first 5 h after a meal. The time length was arbitrarily chosen but was supposed to be sufficiently long to monitor short-term differences.

Results for free v. protein-bound amino acid diets

In the present study, we applied the [$^{13}CO_2$] breath test approach in a model study with rats after ingestion of a meal containing L-[1-^{13}C]leucine either bound in EW protein (the EW diet) or in free form (the free amino acid diet). The recovery of label via the breath showed a faster increase and a higher maximum value for the rats on the free amino acid diet compared with the rats on the EW diet. These results for rats are in line with the results of similar short-term [$^{13}CO_2$] breath test studies with human subjects using similar meals (Evenepoel *et al.* 1997). The more pronounced catabolism of the [1-^{13}C]leucine in case of the free amino acid diet compared with the EW diet suggests a higher rate of appearance of this amino acid in the free amino acid pool for the free amino acid diet (Boirie *et al.* 1997; Bos *et al.* 2003). The gastrointestinal tract seems to fractionate dietary amino acids from the free amino acid and the EW diet differently with respect to the time of their metabolic availability after the onset of a meal. The most likely explanation is that the proteins from the EW diet require time for digestion before the protein-derived amino acids can be absorbed. This difference allows a more rapid absorption of amino acids from the free amino acid diet in the proximal part of the small intestine.

Metges *et al.* (2000) reported for a steady-state approach in humans, a higher mean plasma leucine concentration and a higher leucine oxidation on a free amino acid diet than a protein diet. We assume that differences in short-term catabolism between free and protein-derived leucine, as demonstrated for a single meal in the present study, show up after every meal, even when small equal meals are given frequently, as in Metges *et al.*'s study.

Nutritional and physiological consequences

This study shows significant differences in short-term catabolism for the amino acid leucine during the postprandial phase after a meal for free and protein-derived leucine. Differences were observed in both the rate of recovery and the total cumulative recovery of label in the expired breath. The roughly 50 % higher cumulative recovery of label from L-[1-^{13}C]leucine ingested in free form has to be considered as an extra waste of dietary leucine when the free amino acid (26·9 (SD 2·2) % dose/5 h) instead of the EW (16·0 (SD 4·3 % dose/5 h) diet is consumed.

A higher postprandial oxidation of leucine clearly reduces its postprandial retention and very likely limits the retention of other dietary amino acids. Such a higher postprandial loss of amino acids could be considered similar to a lower net protein intake. According to Millward (1995), a lower protein intake would lower the amplitude of diurnal protein-cycling. The consequences of a reduced amplitude of diurnal protein-cycling may range from a general reduction in the level of protein turnover to an impairment of specific protein-dependent physiological functions. Investigations of short-term differences in the catabolism of dietary amino acids can contribute to a better understanding of these consequences.

Methodological aspects of the [$^{13}CO_2$] breath test approach

The amount of label expired as [$^{13}CO_2$] was calculated from the [^{13}C] enrichment of the expired air and the total amount of CO_2 expired during the same period. Therefore, both parameters have to be determined adequately.

The pattern of CO_2 production over time (Fig. 1(B)) was not affected by the type of diet (EW *v.* free amino acid). The higher values during the feeding period were probably due to increased physical activity and dietary thermogenesis. Charlet-Lery (1970) showed that, for different diets, the increased level of CO_2 production during feeding time was caused mainly by the physical activity associated with eating behaviour. In the present study, CO_2 production during the meal was only 20 % higher than the mean value calculated for the entire collection period. Differences in diet-induced thermogenesis between the two diets in the present study (as seen from a similar increase in CO_2 production) are also minor. In most nutritional studies, diet-induced thermogenesis will be comparable as long as total energy intake, nutrient composition and environmental conditions are similar Diet-induced thermogenesis is about 10 % of dietary intake and ranges between 5 % and 20 % for fat, carbohydrates and protein (Reed & Hill, 1996).

Despite the same body weight, the mean CO_2 production of the free amino acid group tended to be slightly lower than that of the EW group throughout the whole measurement period. This tendency could have a metabolic background. The difference in postprandial oxidation of dietary amino acids, with the higher value for the free amino acid diet, suggests a lower level of protein retention in rats from the free amino acid group. As a consequence, the free amino acid group will have a slightly lower rate of protein turnover throughout the day. The lower energy costs for protein turnover might explain the lower CO_2 production. The energy costs of protein digestion might also influence this value.

As described earlier, special attention was paid to sampling the expired air. The air samples taken were ready for IRMS analysis and provided the best obtainable value for the [^{13}C] enrichment of all the CO_2 expired during each 30 min collection period. The large changes in [^{13}C] enrichment of expired CO_2 observed were expected since the tracer is ingested as a single bolus with a meal. A potential influence of handling on CO_2 production, and therefore on [^{13}C] enrichment, holds for all animals to the same extent. Such an effect is supposed to be minor as the animals were already used to handling as a result of the weighing procedure.

As described in the appropriate sections, a comparison between Fig. 1(A) and Fig. 1(B) indicates that both the changes in total CO_2 production in time as well as the differences in the level of total CO_2 production between the EW and free amino acid diet groups (in the range of 20 %) were of minor importance compared with the variation in [^{13}C] enrichment of the expired CO_2 (up to 1000 %) The observed differences in total CO_2 production before, during and after the meal do not have a significant impact on the main conclusions of this comparative study. The main conclusions are derived from differences in [^{13}C] enrichment of the expired CO_2.

We therefore advocate the use of a constant value for CO_2 production in routine breath tests studying situations with comparable CO_2 production. The high similarity of the curves of [^{13}C] enrichment (Fig. 1) and the rate of [^{13}C] recovery (Fig. 2), as well as the same significant differences between the free amino acid and EW results in both cases, strongly support this statement. The constant value for CO_2 production should be based on metabolic weight and the daily level of energy intake. Such a value could be derived from standard formulae for energy expenditure, for example Brody's formula for animals (Blaxter, 1989) and Schofield formula's for human subjects (Schofield, 1985). In previous and comparable studies in man, the use of a constant value for CO_2 production was also applied, but in these studies it was not justified (Evenepoel *et al.* 1997; Geboes *et al.* 2004).

Conclusion

This study shows that the [$^{13}CO_2$] breath test approach is a sensitive tool and can be used to study the catabolic response of dietary components shortly after a meal. Without substantial influence on the conclusions, a constant and calculated value for the production of CO_2 can be used, at least for meals with a similar nutritional composition. The method is easy and non-invasive, and can be applied to human subjects as well. The difference in expiration of [$^{13}CO_2$] between the two diets indicates that free and EW-derived amino acids differ in their metabolic appearance. The breath test shows that a shift in metabolic appearance is associated with a difference in the short-term catabolism of dietary amino acids. Further nutritional, metabolic and physiological consequences of this difference in short-term catabolism should be considered in relation to the entire feeding strategy and should be supported by long-term changes in, for example, body weight, body composition and health parameters.

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