Validation of a New Test Meal for a Protein Digestion Breath Test in Humans

Karen P. Geboes,† Bert Bammens,* Anja Luypaerts, Ramon Malheiro,** Johan Buyse,† Pieter Evenepoel,* Paul Rutgeerts, and Kristin Verbeke

Department of Gastroenterology; *Department of Nephrology, University Hospital Gasthuisberg; and †Laboratory of Physiology and Immunology of Domestic Animals, Faculty of Agricultural and Applied Biological Sciences, Catholic University of Leuven, 3000 Leuven, Belgium

ABSTRACT Previously, overall protein assimilation after the ingestion of a pure protein meal was studied. In this study, the kinetics of protein assimilation in humans were investigated after the ingestion of a complex meal, which more closely represents a physiologically normal situation. Overall protein assimilation in humans after the ingestion of a pancake meal, containing 12 g of fat, 27 g of carbohydrate, and 19 g of protein, was evaluated in 26 normal volunteers. Both the egg white and yolk of L-[1-13C]-leucine–substituted eggs were used to make the batter. The labeled eggs were produced by feeding laying hens a standard chicken diet supplemented with 3 g/kg of L-[1-13C]-leucine (99%, mol:mol). High enrichment levels of protein with adequate labeling patterns were obtained in eggs from laying hens fed the L-[1-13C]-leucine–substituted diet. The isotopic enrichment of leucine at plateau was equal in egg white and yolk. The overall tracer recovery in egg proteins was 22.5%. The overall protein assimilation parameters in subjects that consumed the pancake meal did not differ from those obtained in subjects that consumed a single protein meal (mean cumulative 13C recovery/6 h = 17.22 ± 4.74%, with a maximal 13C recovery/h of 5.65 ± 1.48%, which was attained 145 ± 25 min after ingestion of the meal). The pancake test meal prepared with eggs intrinsically labeled with L-[1-13C]-leucine is ideal for the study of protein assimilation. The incorporation of differently labeled substrates into a single test meal allows the assessment of different gastrointestinal processes in the overall assimilation of proteins. J. Nutr. 134: 806–810, 2004.

KEY WORDS: • breath test • protein digestion • stable isotopes

The nutritional value of protein is related both to its digestibility and to the subsequent metabolism of the absorbed amino acids. Tracer techniques using stable isotopes are attractive and safe methods for the in vivo study of various aspects of protein assimilation and metabolism. Studies based on the use of 15N-labeled proteins or naso-ileal catheters provide information concerning the digestibility of proteins, postprandial oxidation, and retention (1–3).

When amino acids are labeled with 13C at the α-COOH position, their oxidation following digestion and absorption can be evaluated by measuring 13CO2 excretion in the breath (4,5). Labeled amino acids must be incorporated into the protein to adequately represent the fate of ingested proteins (4,6,7). Studies of the kinetics of amino acid metabolism often use [13C]-leucine, because leucine is rapidly metabolized, has small plasma and intracellular pools, and has high turnover rates in these pools (8).

An excellent correlation was found between 13CO2 excretion in the breath and duodenal trypsin output after the ingestion of proteins intrinsically labeled with 13C-leucine (9). Intraluminal pancreatic digestion, small bowel transit time, and the absorptive capacity of the gut are very important in the overall process of protein assimilation (10). The breath test may be a valuable tool to evaluate protein assimilation in adults and children with various diseases (e.g., pancreatic disease, celiac sprue, radio enteritis, short bowel syndrome, and motility disorders) and to monitor the effects of pharmaceuticals on protein assimilation (9,11,12).

The test meals used in earlier studies of protein assimilation were almost exclusively composed of proteins (4,6,9). However, the kinetics of protein assimilation after the ingestion of a single-protein meal may differ from those after the consumption of a complex, physiologically normal meal containing carbohydrates, fat, and protein (4,6).

In this study, a complex pancake meal containing 12 g of fat, 27 g of carbohydrate, and 19 g of protein was developed, and overall protein assimilation parameters in subjects that consumed the meal were evaluated.

Only a few techniques for the production of proteins intrinsically labeled with stable isotopes are described in the literature (1,5,13). In particular, it was difficult to obtain sufficiently high enrichment levels of protein with adequate labeling patterns. Previously, we developed a methodology for producing large amounts of highly enriched egg proteins la-
beled with \( ^{13} \text{C}-\text{leucine} \), by feeding laying hens a leucine-deficient diet supplemented with 2 g/kg of \( ^{13} \text{C}-\text{leucine} \) (13). Because of technical problems with the production of the leucine-deficient diet, in the present study we also evaluated the use of a leucine-sufficient chicken diet supplemented with 3 g/kg of \( ^{13} \text{C}-\text{leucine} \). The use of a leucine-sufficient chicken diet supplemented with 3 g/kg of \( ^{13} \text{C}-\text{leucine} \) may affect the isotopic enrichment patterns of the egg white and yolk and the efficiency of tracer incorporation.

**SUBJECTS AND METHODS**

The ethical committee of the Catholic University of Leuven approved the study protocols for both the use of hens to produce labeled eggs and the administration of the breath tests to human volunteers. All volunteers gave their written informed consent before participation.

**Pancake test meal.** A pancake batter was prepared using 66 g of labeled and 33 g of unlabeled egg white, 34 g of labeled egg yolk, 7 g of sugar, 17 g of wheat flour, 3 g of milk powder, and 25 mL of water. The pancake was prepared with 5 g of butter, and 5 g of sugar was poured on top of the finished pancake. The meal was consumed together with 1 glass of water (280 mL). The total energy content of the test meal was 1.37 MJ, and the meal contained 19 g of protein, 6 g of fat, and 27 g of carbohydrate.

**Production of \( ^{13} \text{C}-\text{enriched eggs} \).** A chicken diet that met the nutrient requirements for laying hens [8 g/kg of leucine, NRC requirements (13,14)] was supplemented with 3 g/kg of \( ^{13} \text{C}-\text{leucine} \) (99%, mol/mol; Euriso-top). During peak egg production, 6 laying hens (Hisex Brown, body wt ∼ 2 kg) were given free access to the \( ^{13} \text{C}-\text{leucine} \)-supplemented diet. The mean daily food intake of the hens was 100 g, with a maximum of 120 g. Eggs were collected daily and dated. Each egg was opened under sterile conditions and separated into white and yolk fractions. The fractions were freeze-dried and stored until further analysis. The isotopic enrichment of the 2 fractions of each egg laid from d 0 to d 20 was measured. Thereafter, isotopic enrichment was assayed at regular intervals.

**Administration of the breath tests.** To establish the normal range of the parameters of protein assimilation, the \( ^{13} \text{C}-\text{leucine} \) pancake breath test was administered to 26 healthy volunteers (5 men, 21 women; age range 21 to 50 y).

Gastric emptying was measured in a subgroup of 22 volunteers (5 men, 17 women), by adding 74 kBq of sodium \( ^{14} \text{C}-\text{octanoate} \) (ARC) to the meal (15).

To measure the oroecal transit time using the hydrogen breath test, 5 g of Raftilin HP (Orafti) was added to the pancake batter (16). This did not affect the energy content of the meal.

The parameters of protein assimilation and gastric emptying were compared with those of subjects that consumed a single-protein meal and stored until further analysis. The isotopic enrichment of the 2 fractions of each egg laid from d 0 to d 20 was measured. Thereafter, isotopic enrichment was assayed at regular intervals.

**Analysis of the \( ^{14} \text{CO}_2 \) and hydrogen breath tests.** For the \( ^{14} \text{CO}_2 \) and hydrogen breath tests, breath samples were collected in Exetainers (PDZ). The breath \( ^{14} \text{C} \) content was analyzed using an Isotope Ratio Mass Spectrometer (IRMS; PDZ). The lipids were extracted from freeze-dried samples of egg yolk using continuous (Soxhlet) extraction (Soxtec Avanti 2050 Automatic Extraction System; Foss Tecator). Other amino acids 0.9020 0.2140

**Fraction Egg white2 Egg yolk3**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Egg white2</th>
<th>Egg yolk3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>0.0330</td>
<td>0.2400</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.0098</td>
<td>0.0260</td>
</tr>
<tr>
<td>C1-leucine</td>
<td>0.0164</td>
<td>0.0044</td>
</tr>
<tr>
<td>Other amino acids</td>
<td>0.0920</td>
<td>0.2140</td>
</tr>
<tr>
<td>Lipid</td>
<td>0</td>
<td>0.7600</td>
</tr>
</tbody>
</table>

1 Data from Venepoel et al. (13).

2 Carbon atoms in component/total carbon atoms in egg white.

3 Carbon atoms in component/total carbon atoms in egg yolk.

**TABLE 1**

**Composition of egg white and yolk**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Egg white</th>
<th>Egg yolk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight per egg</td>
<td>33.000</td>
<td>17.000</td>
</tr>
<tr>
<td>Water</td>
<td>29.060</td>
<td>8.290</td>
</tr>
<tr>
<td>Protein</td>
<td>3.350</td>
<td>2.790</td>
</tr>
<tr>
<td>Lipid</td>
<td>trace</td>
<td>5.600</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>0.410</td>
<td>0.040</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.291</td>
<td>0.237</td>
</tr>
</tbody>
</table>

1 Values are the number of carbon atoms in each component, calculated using egg composition data from Table 2; carbon atoms = (carbon atoms per molecule \( \times 6023 \times 10^{23} \) \times concentration of component)/(molecular weight of component).

**TABLE 2**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Egg white</th>
<th>Egg yolk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>0.0330</td>
<td>0.2400</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.0098</td>
<td>0.0260</td>
</tr>
<tr>
<td>C1-leucine</td>
<td>0.0164</td>
<td>0.0044</td>
</tr>
<tr>
<td>Other amino acids</td>
<td>0.0920</td>
<td>0.2140</td>
</tr>
<tr>
<td>Lipid</td>
<td>0</td>
<td>0.7600</td>
</tr>
</tbody>
</table>

**Calculation of the administered dose of \( ^{13} \text{C}-\text{leucine} \).** To simplify calculations, we assumed that all \( ^{13} \text{C} \) atoms were present in the \( C_\text{e} \) position of leucine. This means that the mole percentage (MP) of \( ^{13} \text{C}-\text{leucine} \) in egg protein equals the atom percentage (AP) of \( ^{13} \text{C} \) in the carbon atom fraction of \( C_\text{e} \) in leucine in egg protein, which is represented as \( \text{AP}_{\text{egyolk}} \).

Because the carbohydrate and lipid contents of egg white are very low, we assumed that the enrichment of egg white obtained by the method described above was the same as the enrichment of the protein fraction within. Knowing the mean amino acid composition of eggs laid by Hisex Brown hens (Tables 1 and 2) and the measured isotopic enrichment of the egg white, the amount of \( ^{13} \text{C}-\text{leucine} \) present in the protein fraction of the egg white can be calculated (13).

Besides protein, egg yolk also contains a considerable amount of lipid. As a consequence, the \( ^{13} \text{C} \)-enrichment of egg yolk after substitution with \( ^{13} \text{C}-\text{leucine} \) is different from the enrichment of the yolk protein fraction, and the enrichment of the lipid fraction has to be taken into account in the calculations (Eqs. 1, 2, and 3).

\[
\text{AP}_{\text{egyolk}} = \alpha \cdot \text{AP}_{\text{e}_{\text{prot}}} + \gamma \cdot \text{AP}_{\text{e}_{\text{lipo}}} \tag{1}
\]
with

$$\text{AP}_{\text{egg yolk protein}} = \alpha' \cdot \text{AP}_{\text{\alpha'}} + \beta \cdot \text{AP}_\beta$$  \hspace{1cm} (2)

where

- \(\alpha'\) = fraction of all carbon atoms in egg yolk in protein = 0.240 (Table 1)
- \(\text{AP}_{\alpha'}\) = \(^{13}\text{C}\) atom percentage of \(\alpha'\) (i.e., AP enrichment of the protein fraction of egg yolk)
- \(\alpha\) = fraction of carbon atoms in position \(C_1\) in leucine in egg yolk protein = 0.018
- \(\beta\) = fraction of all other carbon atoms in egg yolk protein = 0.982
- \(\text{AP}_\beta\) = measured \(^{13}\text{C}\) atom percentage of \(\beta\) (i.e., AP enrichment of unlabeled egg yolk protein) = 1.088
- \(\text{AP}_\alpha\) = \(^{13}\text{C}\) atom percentage of \(\alpha\) (i.e., of the fraction of carbon atoms in position \(C_1\) in leucine)

and

$$\text{AP}_{\text{egg yolk lipid}} = \text{AP}_\gamma$$

where

- \(\gamma\) = fraction of all carbon atoms in egg yolk in lipid = 0.760 (Table 1)
- \(\text{AP}_\gamma\) = measured \(^{13}\text{C}\) atom percentage of \(\gamma\)
- \(\text{AP}\) = enrichment of lipid fraction of egg yolk = 1.086

Analogously to egg white, the amount of \(^{13}\text{C}\)-leucine (99%, mol:mol) incorporated in egg yolk protein can be calculated from the following equation:

$$m = \frac{0.085 \cdot \gamma \cdot \text{AP}_{\text{\alpha'}}}{\text{MP}_{\text{\text{13C-leucine administered}}}}$$  \hspace{1cm} (3)

where

- \(m\) = mg of \(^{13}\text{C}\)-leucine (99%, mol:mol) to be incorporated into \(\gamma\) mg of egg yolk protein
- 0.085 = mg leucine/mg egg yolk protein (Table 2)
- \(\text{MP}_{\text{\text{13C-leucine administered}}}\) = enrichment of \(^{13}\text{C}\)-leucine used for supplementation of food = 99% (mol/mol)
- \(\text{AP}_\alpha\) = calculated \(^{13}\text{C}\) atom percentage of \(\alpha\) (i.e., the fraction of carbon atoms in position \(C_1\) in leucine in egg yolk protein, which, assuming that all \(^{13}\text{C}\) atoms are in the \(C_1\) position, equals the MP enrichment of leucine)

**Breath test data analysis.** We assumed human CO\(_2\) production to be 300 mmol/m\(^2\) body surface area per hour (10,13,15). The body surface area was calculated by the weight-height formula of Haycock et al. (17).

Breath test results for protein assimilation were expressed as the percentage of the administered dose of \(^{13}\text{C}\) excreted per hour and the cumulative percentage of the administered dose of \(^{13}\text{C}\) excreted over 6 h. The maximum excretion rate of \(^{13}\text{C}\) and the time of maximum excretion rate of \(^{13}\text{C}\) were also estimated (10). Background enrichment of breath after administration of an unlabeled test meal was previously evaluated with 10 healthy volunteers and was found to be negligible.

The \(^{14}\text{CO}_2\) excretion data were further analyzed by nonlinear regression to obtain curve fitting and the calculation of gastric emptying parameters, i.e., the gastric emptying coefficient and the half-emptying time. The details were as previously published (15).

**Hydrogen excretion was expressed in ppm. A consistent rise in hydrogen excretion of 10 ppm above baseline was defined as a cutoff value for the orocecal transit time (16).**

**Statistical analysis.** Results are expressed as means \(\pm SD\). The nonparametric Mann-Whitney test was used to compare the enrichment of egg white and egg yolk, because of the low number of hens (n = 6) used in the experiment. The unpaired Student’s \(t\) test was used to compare the parameters of protein assimilation and gastric emptying obtained after subjects consumed the different meals (\(\alpha = 0.05\)). Statistica 6.0 software (Statsoft) was used for the analysis.

**RESULTS**

**Labeling pattern and level of \(^{13}\text{C}\)-enrichment of egg white and yolk.** Consecutive measurements of the isotopic enrichment of the egg white protein of eggs laid during a 20-d period following feeding of the \(^{13}\text{C}\)-leucine-supplemented diet showed a gradual increase in enrichment, which reached a plateau after 10 to 14 d (Fig. 1).
The further measurements performed at regular intervals showed some variation in egg white enrichment at plateau in the labeled eggs produced by 1 hen. The within-hen CV, describing the variability of egg white enrichment at plateau, ranged from 0.80 to 5.41%, with a median of 2.94% (n = 6 hens).

The variability in egg white enrichment at plateau among hens (n = 6) was small (CV = 2.67%). A mean egg white enrichment of 1.333% was calculated (mean APegg white at plateau, n = 6).

The enrichment pattern of the egg yolk protein was similar, although the plateau was reached somewhat later, after 14 to 18 d. The within-hen CV, describing the variability of egg yolk enrichment at plateau in 1 hen, ranged from 0.10 to 1.95%, with a median of 0.51%. The CV in egg yolk enrichment among the 6 hens was 0.35%. A mean egg yolk enrichment of 1.144% was measured (mean APegg yolk at plateau, n = 6).

### Calculation of the exact amount of $^{13}$C-leucine present in enriched egg protein

The $^{13}$C-leucine:leucine ratio in the white and yolk of eggs that reached plateau levels of enrichment was calculated using the measured values for APegg white and APegg yolk and the previously described equations. For example, in an egg white with APegg white = 1.337% and APegg yolk = 1.153%, the $^{13}$C-leucine:leucine ratio (mol:mol) was 16.27:100 in egg white and 16.48:100 in egg yolk.

The mean MP of $^{13}$C-leucine in egg white was 16.08 ± 2.168%, and the mean MP of $^{13}$C-leucine in egg yolk was 14.11 ± 0.914%. Because these means did not differ significantly, the $^{13}$C-leucine:leucine ratios at plateau in the protein fractions of the white and yolk of a labeled egg were equal, allowing the calculation of the total amount of $^{13}$C-leucine present in the egg on the basis of the measurement of APegg white.

By application of the calculations as described, the amount of $^{13}$C-leucine in 1 egg was 81 mg.

### Efficiency of tracer incorporation

Assuming a maximal daily feed intake by a laying hen of 120 g, containing 360 mg $^{13}$C-leucine (3 g/kg), and assuming a daily production of 1 egg [which is a slight overestimation, because the ovulation-oviposition cycle in chickens is slightly longer than 24 h (13)], the efficiency of incorporation was calculated to be 22.5%.

### Mole percentage excess of $^{13}$C-leucine in the new test meal

An unlabeled egg contained 6 mg of $^{13}$C-leucine (assuming all naturally occurring $^{13}$C-atoms to be present in the α-COOH position of leucine; Eqs. 1 to 3 (13)). The unlabeled meal contained 16 mg of $^{13}$C-leucine (12 mg in 2 whole eggs + 3 mg in an extra egg white + 1 mg in milk powder). Using 2 labeled eggs in the test meal (162 mg in 2 labeled whole eggs + 3 mg in an extra egg white + 1 mg in milk powder), a total amount of 166 mg of $^{13}$C-leucine was administered.

The total amount of leucine in the meal was calculated to be 2.4 g, using Table 2 and information provided by the supplier (Nestlé) of the milk powder (351 g leucine/kg milk powder).

Therefore, the $^{13}$C-leucine:total leucine ratio of the test meal was 0.068, meaning that the MP of labeled leucine in the test meal was 6.9%, making the mole percentage excess (MPE) of $^{13}$C-leucine in the test meal 6.3%.

### Assessment of protein assimilation, gastric emptying, and orocecal transit time

The mean maximal percentage of the administered dose excreted per hour was 5.65 ± 1.48% (Fig. 2A), which was attained 145 ± 25 min after the ingestion of the meal. The mean cumulative percentage of the administered dose of $^{13}$C recovered after 6 h was 17.22 ± 4.74% (Fig. 2B).

The mean gastric half-emptying time was 90 ± 36 min (gastric emptying coefficient = 3.17 ± 0.37) (15).

The administration of the three differently labeled markers in the test meal allowed the simultaneous evaluation of gastric emptying, parameters of protein digestion, and orocecal transit time (Fig. 3).

### DISCUSSION

Because of problems encountered in the production of isotopically labeled eggs when laying hens were fed a 2-g/kg leucine-deficient diet substituted with 2 g/kg of $^{13}$C-leucine, the possibility of producing intrinsically labeled egg proteins by feeding hens a normal diet supplemented with 3 g/kg of $^{13}$C-leucine was investigated. Large amounts of highly enriched labeled egg proteins were obtained. Both the enrichment level at plateau and the course of the labeling time curves were similar to those obtained when hens were fed the substituted leucine-deficient diet (4). The variability in $^{13}$C-enrichment at plateau of the eggs produced by 1 hen was small, both in the white and yolk.

When calculating the enrichment of the eggs, we assumed that all $^{13}$C-leucine was incorporated as such into the egg.
protein and that no $^{13}$C was incorporated into other amino acids through CO$_2$ fixation.

Because the mole fractions of $^{13}$C-leucine in egg white and yolk appeared to be equal, AP$_{\text{egg yolk}}$ could be calculated using the measured value for AP$_{\text{egg white}}$. As a consequence, the whole egg could be used in the preparation of the test meal without measuring the enrichment of the white and yolk separately.

We prepared the pancake meal by adding wheat flour, milk powder, and sugar. The test meal contained 19 g total protein. The calculated MPE of labeled leucine was 6.3%, comparable with that of other meals used to study protein assimilation [minimum MPE = 3.42% (4,11)].

Aside from easy availability and palatability, the most important advantage of this test meal was the fact that pancakes with exactly the same composition could be used in the evaluation of gastric emptying with $^{14}$C-octanoic acid as a marker and in the evaluation of orocecal transit time using inulin as a substrate in a hydrogen breath test (15,16). The incorporation of differently labeled substrates in the same test meal enabled the assessment of the effect of various gastrointestinal processes on the overall assimilation of proteins (Fig. 3). The effects of administration of other nutrients or medication on protein digestion and transit parameters could be studied simultaneously.

When we compared the protein assimilation parameters obtained after administration of the newly developed test meal with the values obtained using a pure protein test meal (9), we found that the incorporation of the labeled proteins into a complex meal did not affect the main parameters of gastric emptying and overall protein assimilation (Tables 3 and 4). Carbohydrates and lipids may affect both intraluminal digestion of proteins (gastric emptying, mixing of nutrients, or small bowel transit) and postprandial metabolism (insulin response). The rate-limiting step in the overall process of protein assimilation is intraluminal proteolysis (9). Because protein assimilation parameters in subjects that consumed the pancake meal with exactly the same composition could be used in the evaluation of gastric emptying and orocecal transit time, the incorporation of various labeled substrates enables the simultaneous assessment of different gastrointestinal processes.

### LITERATURE CITED


### TABLE 4

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Protein test meal$^3$</th>
<th>Complex test meal</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n$</td>
<td>16</td>
<td>22</td>
</tr>
<tr>
<td>$t_{1/2}, \text{ min}$</td>
<td>66 ± 12</td>
<td>90 ± 36</td>
</tr>
<tr>
<td>GEC</td>
<td>3.19 ± 0.28</td>
<td>3.17 ± 0.37</td>
</tr>
</tbody>
</table>

$^1$ Values are means ± SD.
$^2$ Abbreviations: GEC, gastric emptying coefficient; $t_{1/2}$, gastric half-emptying time.
$^3$ Data from Evenepoel et al. (12).