Amount and fate of egg protein escaping assimilation in the small intestine of humans

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Evenepoel, Pieter, Dirk Claus, Benny Geypens, Martin Hiele, Karen Geboes, Paul Rutgeerts, and Yvo Ghoos. Amount and fate of egg protein escaping assimilation in the small intestine of humans. *Am. J. Physiol.* 277 (*Gastrointest. Liver Physiol.* 40): G935–G943, 1999.—Studies attempting to evaluate protein assimilation in humans have hitherto relied on either ileostomy subjects or intubation techniques. The availability of stable isotope-labeled protein allowed us to determine the amount and fate of dietary protein escaping digestion and absorption in the small intestine of healthy volunteers using noninvasive tracer techniques. Ten healthy volunteers were studied once after ingestion of a cooked test meal, consisting of 25 g of ^{13}C-, ^{15}N-, and ^{2}H-labeled egg protein, and once after ingestion of the same but raw meal. Amounts of 5.73% and 35.10% ($P < 0.005$) of cooked and raw test meal, respectively, escaped digestion and absorption in the small intestine. A significantly higher percentage of the malabsorbed raw egg protein was recovered in urine as fermentation metabolites. These results *1*) confirm that substantial amounts of even easily digestible proteins may escape assimilation in healthy volunteers and *2*) further support the hypothesis that the metabolic fate of protein in the colon is affected by the amount of protein made available.

protein fermentation; protein assimilation; stable isotopes; phenols

THE MOST IMPORTANT FUNCTION of the colon is to absorb salt and water and provide a mechanism for the orderly disposal of waste products of digestion. Recently, it has become clear that the colon may also play a role in the salvage of energy from carbohydrate and nitrogen from protein not digested in the upper gut. This is achieved through the metabolism of the bacteria and is known as fermentation. This process obviously influences colonic function and may have health consequences for the host. The knowledge of fermentation may be the key to understanding the normal physiology of the colon and the etiology of its diseases (1, 19, 23, 29, 31, 33, 35).

Most research has been focused on the fermentation of carbohydrates. The end products formed, like hydrogen, methane, and especially short-chain fatty acids (SCFAs) (23), have already been investigated in depth. SCFAs are generally accepted to be beneficial to the host (23, 31).

Protein fermentation, on the other hand, has been investigated less intensively, most probably because it was generally believed that the assimilation of protein is highly efficient. Recent studies in healthy volunteers using intubation techniques or in "healthy" ileostomy patients have, however, shown that the assimilation of even easily digestible protein is incomplete (8, 24). This finding has led to a renewed interest in the process of protein fermentation.

Nonabsorbed dietary protein enters the human large intestine through the ileocecal valve in the form of a complex mixture of proteins and peptides. The majority of these substances are degraded to amino acids by both bacterial and pancreatic enzymes (23) and are subsequently fermented (23). Some of the fermentation metabolites produced include thiols, phenols, ammonia, indoles, and amines, which are potentially toxic (3, 23, 27, 29, 39).

Incorporation into the bacterial mass and the subsequent fecal excretion and luminal accumulation as free fermentation metabolites and then the subsequent absorption into the portal blood and excretion in urine are major fates of protein made available to the colon. Although the regulating mechanisms are only partially understood to date, there is substantial, albeit indirect, evidence in the literature that the ratio of carbohydrate to nitrogen is crucial (2, 6, 7, 25, 38).

Several studies have investigated the influence of an increased availability of fermentable carbohydrates on the handling of nitrogen in the colon. Fermentable carbohydrates stimulate bacterial growth, which results in an enhanced incorporation of nitrogen into the bacterial protoplasm (2, 17, 37).

The impact of an increased availability of protein on bacterial metabolism, on the other hand, remains largely unknown. Several protein fermentation metabolites were recently shown to be increased after ingestion of a supplementary load of dietary protein (12).

The aims of the present study were *1*) to quantify the amount of dietary protein escaping digestion and absorption in healthy volunteers in physiological conditions and *2*) to evaluate to what extent the bacterial metabolism of dietary protein in the colon is affected by the amount of protein made available. Noninvasive tracer techniques using protein labeled with different stable isotopes (^{13}C, ^{15}N, ^{2}H) were used to achieve these goals.

MATERIALS AND METHODS

Subjects

Ten volunteers (5 females and 5 males, mean age 27 yr, range of 21–37 yr) participated. None of the subjects had a history of gastrointestinal or metabolic disease or previous surgery (apart from appendectomy). The subjects had no

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gastrointestinal complaints and were free of antibiotics or any other medical treatment for at least 3 mo before the start of the study. The study was approved by the Ethical Committee of the University of Leuven, and all subjects gave informed consent.

Experimental Design

The study was conducted over a 21-day period including a 7-day baseline period and two study periods, separated by a washout period of 7 days. Each study period started with the ingestion of the labeled test meal and lasted 3 days. The two study periods were identical, apart from the test meal, which had to be ingested once cooked and once raw. The two consecutive study periods were allocated in a randomized order.

All subjects were studied after an overnight fast of at least 12 h. At 0845 on the first day of the study period, the volunteers ingested the protein test meal together with 200 ml of water within 15 min. No further food was allowed until 1500, when the volunteers consumed a standard bread meal. Drinking of water was permitted from 1200 on.

The experimental design is schematically represented in Fig. 1.

Diet

The volunteers were given no standard diets. However, they were asked to weigh and record all food and drinks taken from 3 days before until the end of each study period. These data were analyzed using a computer program to obtain energy and nutrient intake results (Nederlands voedingsstoffenbestand 1989–90, Voorlichtingsbureau voor de voeding, Den Haag, The Netherlands).

Test Meal

The protein test meal consisted of 100 g of egg white (i.e., 11 g of egg white protein) labeled with ^{15}N, 100 g of egg white labeled with L-[1-^{13}C]leucine and L-[*ring*-$^{2}H_4$]tyrosine, and the yolk of one egg. Five microcuries [^{3}H]polyethylene glycol ([^{3}H]PEG) 4000 were added to the test meal as a radiolabeled transit marker. All the constituents of the test meal were mixed before ingestion. Total caloric content of the test meal was 150 kcal (25 g of protein, 5.56 g of fat, and a negligible amount of carbohydrates).

The methodology for obtaining large amounts of highly enriched egg proteins labeled with stable isotopes has been described elsewhere (9). Briefly, ^{13}C- or ^{15}N-labeled proteins were produced by giving laying hens free access to a food containing 25% of the (National Research Council required) leucine content as free [1-^{13}C]leucine (99 mol%, Euriso-top, Saint-Aubin, France) and [^{15}N]leucine (99 mol%, Euriso-top), respectively. The yolk and egg white fractions of the enriched eggs were separated and pooled. The isotopic enrichment of both pools was determined using a continuous flow elemental analyzer isotope ratio mass spectrometer (ANCA-2020, Europa Scientific, Crewe, UK). With the exact amino acid composition and the isotopic enrichment of the egg white, the amount of [1-^{13}C]leucine (99 mol%) incorporated could be calculated (9). Because "redistribution" of the ^{15}N label is likely to occur in the hen via transamination, the egg protein can be assumed to be uniformly ^{15}N labeled. Egg protein labeled with L-[*ring*-$^{2}H_4$]tyrosine was obtained by giving laying hens free access to a food containing 20% of the (National Research Council required) phenylalanine content as free L-[*ring*-$^{2}H_5$]phenylalanine (98 mol%, Euriso-top). Due to hydroxylation of L-[*ring*-$^{2}H_5$]phenylalanine by the hen, both L-[*ring*-$^{2}H_5$]phenylalanine and L-[*ring*-$^{2}H_4$]tyrosine are incorporated in the egg protein. The L-[*ring*-$^{2}H_4$]tyrosine content of the egg protein was determined by gas chromatography-mass spectrometry (GCQ, Finnigan, San José, CA) (14).

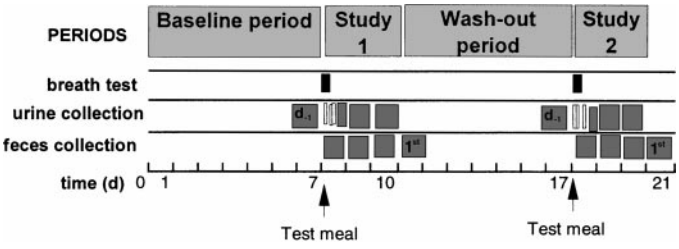

Fig. 1. Experimental design. 1st stands for first stool voided on the fourth day after ingestion of the test meal. d_{-1}, Day preceding the study period.

Sample Collection

Breath samples were collected in exetainers (Europa Scientific) before ingestion of the meal, every 15 min for the first 6 h, and every 30 min up to 9 h after ingestion of the test meal.

During each study period, urine was collected in plastic bottles for the following periods: 0–3 h, 3–6 h, 6–9 h, 9–24 h, 24–48 h, 48–72 h. Moreover, a 24-h urine collection was obtained the day preceding each study period. Neomycin was added to the plastic containers used for the collections to prevent bacterial growth. After measurement of the volume, samples were taken and stored at −20°C until analysis.

All stools voided during each of the study periods were collected as well. Date and time of voiding of stools were noted in a diary. The stools were frozen immediately after voiding and stored at −20°C until analysis.

Analytical Procedures and Calculations

Breath samples. The breath samples were analyzed for ^{13}C content by means of a continuous-flow isotope ratio mass spectrometry (ABCA, Europa Scientific). The δ values given by isotope ratio mass spectrometry were converted to percentage of ^{13}C recovery per hour of the initial amount administered (%dose ^{13}C/h) according to calculations previously described in detail (8, 15). Cumulative percentages of recovered label (cumulative %dose ^{13}C) were calculated by means of the trapezoidal rule. From these data, the following parameters of protein assimilation were derived: the maximum percentage of administered dose of ^{13}C excreted per hour and the cumulative percentage of administered dose of ^{13}C recovered in breath over 6 h.

Fecal samples. After thawing, the stool samples were weighed and homogenized for each day of collection. Samples of known weight were taken and freeze-dried. The dried material was weighed, and aliquots were taken for the analysis of total nitrogen content, ^{15}N enrichment, and [^{3}H]PEG 4000 content.

The [^{3}H]PEG 4000 content was measured by the oxidation method (Packard sample oxidizer, model 306, Packard Instrument, Downers Grove, IL), with subsequent liquid scintillation counting (model 2450, Packard Instrument) and correction for quenching. Results were expressed in cumulative percentage of the administered dose of ^{3}H recovered over 72 h (further referred to as $\Sigma_{0\,h}^{72\,h}$%dose [^{3}H]PEG 4000).

Total nitrogen content and ^{15}N enrichment were determined using a continuous flow elemental analyzer-isotope ratio mass spectrometer (ANCA-2020, Europa Scientific). Briefly, an aliquot of known weight of the lyophilized fecal sample was combusted in the presence of oxygen at 1,000°C. The combustion products thereafter passed through a second

furnace containing copper at 600°C, where excess oxygen was absorbed and nitrogen oxides were reduced to elemental nitrogen. Total nitrogen content was measured by means of a thermal conductivity detector, and ^{15}N enrichment was determined by means of an isotope ratio mass spectrometer, coupled to the combustion unit of the elemental analyzer. The ^{15}N-to-^{14}N isotope ratio of N_2 was measured with reference to a calibrated laboratory standard (i.e., a standard ammonium sulfate solution). The values were expressed in atom percent (AP). The intraassay variability of this method, assessed by the coefficient of variation (CV), amounted to 2.6 and 0.3% for total nitrogen content and ^{15}N enrichment, respectively. Results were expressed in percentage of administered dose of ^{15}N recovered per day and in cumulative percentage recovered over 72 h.

The percentage of administered dose of ^{15}N recovered per day was calculated as follows

$$\%^{15}\text{N dose/day} = \frac{\text{mg excess } ^{15}\text{N}_t(a)}{\text{mg excess } ^{15}\text{N administered}(b)} \times 100$$

where a is

$$\left(\frac{\text{AP}_s - 0.368}{100}\right) \times \text{N}_{\text{f tot}}$$

where AP_s is the measured ^{15}N enrichment of the stools collected on day t, expressed in AP; 0.368 is the natural ^{15}N content of stools, expressed in AP; and $N_{f\ tot}$ is the total nitrogen content of the stools collected on day t, expressed in milligrams.

The calculation of b is

$$\left(\frac{\text{AP}_m - 0.368}{100}\right) \times \text{N}_m$$

where AP_m is the weighed average ^{15}N enrichment of the test meal (calculated for each test meal separately), expressed in AP, and N_m is the nitrogen content of the protein test meal, i.e., 4,000 mg.

The cumulative percentage of administered dose of ^{15}N recovered in feces over 72 h (further referred to as Σ_{0h}^{72h}%dose $^{15}N_{f\ adm}$) was obtained by summation.

A correction was made for gastrointestinal transit by dividing Σ_{0h}^{72h}%dose $^{15}N_{f\ adm}$ by the cumulative percentage of administered dose of ^3H recovered over 72 h

$$\sum_{0\text{ h}}^{72\text{ h}} \%\text{dose of } ^{15}\text{N}_{\text{f adm}} \text{ corrected} = \frac{\sum_{0\text{ h}}^{72\text{ h}} \%\text{dose of } ^{15}\text{N}_{\text{f adm}}}{\sum_{0\text{ h}}^{72\text{ h}} \%\text{dose of } [^3\text{H}]\text{PEG 4000}}$$

Urinary samples. URINARY PHENOL AND P-CRESOL. Phenol, [*ring*-$^2H_4$]phenol, *p*-cresol, and *p*-[*ring*-$^2H_4$]cresol were measured by gas chromatography-ion trap technology as described by Geypens et al. (13). Briefly, 1 ml of urine was diluted with 3 ml of distilled water. Seventy-five microliters of 2,6-dimethylphenol solution (20 mg/100 ml) were added as internal standard. The pH was adjusted to 1 with concentrated H_2SO_4, and the solution was refluxed for 75 min to hydrolyze the conjugated phenols. After a cooling-down period to ambient temperature, phenols were extracted with 2 ml of diethylether. One microliter was injected into the gas chromatography-mass spectrometer (GCQ, Finnigan). After separation on the analytical column, a 25-m × 0.25-mm CP-Sil 5 CB-MS with a film thickness of 0.25 μm (Chrompack, Middelburg, the Netherlands), the phenolic compounds were identified by ion trap technology (ITD 700, Finnigan).

Results for the unlabeled compounds were expressed in amounts excreted per hour and in cumulative amounts excreted over 72 h. Because phenol and *p*-cresol are quantitatively the main phenolic compounds found in urine, total phenols were measured as the combination of phenol and *p*-cresol (3, 23).

Results for the labeled compounds were expressed in percent administered dose of L-[*ring*-$^2H_4$]tyrosine recovered per hour and in cumulative amounts excreted over 72 h.

The percent administered dose of L-[*ring*-$^2H_4$]tyrosine recovered per hour as [*ring*-$^2H_4$]phenol was calculated as follows

$$\%\text{dose of } [\textit{ring}\text{-}^2\text{H}_4]\text{phenol/h} = 100 \times \frac{[\textit{ring}\text{-}^2\text{H}_4]\text{phenol rec}_t}{d \times \text{L-}[\textit{ring}\text{-}^2\text{H}_4]\text{tyrosine}_{\text{administered}}}$$

where [*ring*-$^2H_4$]phenol rec_t is the total amount of [*ring*-$^2H_4$]phenol recovered in the urine fraction of period t (expressed in mol) (the natural urinary content of [*ring*-$^2H_4$]phenols is zero); L-[*ring*-$^2H_4$]tyrosine$_{\text{administered}}$ is the amount of L-[*ring*-$^2H_4$]tyrosine administered, expressed in moles (calculated for each test meal separately); and d is the duration of the collection period, expressed in hours.

The percentage of the administered dose of L-[*ring*-$^2H_4$]tyrosine recovered per hour as *p*-[*ring*-$^2H_4$]cresol was calculated in the same manner. Because phenol and *p*-cresol are quantitatively the major bacterial metabolites of tyrosine, the percentage of administered dose of L-[*ring*-$^2H_4$]tyrosine fermented in the large gut per hour is obtained by summation (%dose of [*ring*-$^2H_4$]phenol/h + %dose of *p*-[*ring*-$^2H_4$]cresol/h).

The cumulative percentage of dose of L-[*ring*-$^2H_4$]tyrosine administered recovered in urine over 72 h (further referred to as Σ_{0h}^{72h}%dose $^2H_{4\ adm}$) was calculated by summation.

The cumulative values were corrected for gastrointestinal transit as follows

$$\sum_{0\text{ h}}^{72\text{ h}} \%\text{dose of } ^2\text{H}_{4\text{ adm}} \text{ corrected} = \frac{\sum_{0\text{ h}}^{72\text{ h}} \%\text{dose of } ^2\text{H}_{4\text{ adm}}}{\sum_{0\text{ h}}^{72\text{ h}} \%\text{dose of } [^3\text{H}]\text{PEG 4000}}$$

URINARY N_{tot} AND ^{15}N. After thawing, a known amount of urine (~15 μl) was absorbed on chromosorb in a tin capsule. Total nitrogen content and ^{15}N enrichment of urine were determined using a continuous flow elemental analyzer isotope ratio mass spectrometer (ANCA-2020, Europa Scientific) as described previously.

Results for ^{15}N were expressed in percentage of the administered dose of ^{15}N recovered per hour and in cumulative percentages recovered over 72 h.

The percentage of administered dose of ^{15}N recovered per hour was calculated as follows

$$\%^{15}\text{N dose/h} = \frac{\text{mg excess } ^{15}\text{N}_t\,(a)}{\text{mg excess } ^{15}\text{N administered } (b) \times d} \times 100$$

where a is

$$\left(\frac{\text{AP}_s - \text{AP}_{d-1}}{100}\right) \times \text{N}_{\text{u tot}}$$

Table 1. *Dietary intake of protein, fat, carbohydrates, and dietary fiber during the cooked and raw study period*

	Cooked Meal		Raw Meal	
	Intake, g/day	Energy %	Intake, g/day	Energy %
Protein	77.88 ± 5.36	13.15 ± 0.93	71.89 ± 4.09	13.16 ± 0.88
Fat	111.98 ± 11.13	40.63 ± 1.92	99.89 ± 8.91	39.81 ± 1.61
Carbohydrates	274.51 ± 28.61	44.79 ± 1.89	249.46 ± 13.78	44.89 ± 1.35
Dietary fiber	21.76 ± 1.86		18.97 ± 1.41	

Values are means ± SE.

where AP_s is the measured ^{15}N enrichment of the urine collected in period *t*, expressed in AP; AP_{d-1} is the ^{15}N enrichment of the urine of period $d - 1$, i.e., the natural ^{15}N content of urine before ingestion of the labeled meal, expressed in AP; and N_{utot} is the total nitrogen content of the urine, collected in period *t*, expressed in milligrams.

The calculation of *b* was as follows

$$\left(\frac{AP_m - AP_{d-1}}{100}\right) \times N_m$$

where AP_m is the weighted average ^{15}N enrichment of the test meal, expressed in AP (calculated for each test meal separately), and N_m is the nitrogen content of the meal, i.e., 4,000 mg.

The cumulative percentage of administered dose of ^{15}N recovered in urine over 72 h (Σ_{0h}^{72h}%dose of $^{15}N_{u\,adm}$) was calculated by summation.

^{15}N RETENTION. The percentage of the administered dose of ^{15}N, retained in the body after 72 h, was calculated as follows

$$\%^{15}N \text{ retention} = 100 - \left(\sum_{0\,h}^{72\,h} \%\text{dose of } {}^{15}N_{f\,adm} \text{ corrected} + \sum_{0\,h}^{72\,h} \%\text{dose of } {}^{15}N_{u\,adm}\right)$$

Statistical Methods

Results are expressed as means ± SE. Statistical analysis was performed with SAS software package. Parameters obtained after ingestion of the raw test meal were pairwise compared with values obtained in the control study using the paired *t*-test. Correlations were obtained by Pearson's test.

RESULTS

Diet

No significant differences were found between the two study periods either in the intake of protein, fat, and carbohydrates or in the intake of dietary fiber (Table 1).

Breath Tests

Figure 2 shows the mean $^{13}CO_2$ excretion rate in breath after ingestion of the raw and cooked test meal. Differences between both test situations are obvious. The curve obtained after ingestion of the cooked test meal is characterized by a steep ascending slope, a high peak excretion rate, and an initial steep descending slope that smoothes down considerably after 6 h. After ingestion of the raw test meal, the $^{13}CO_2$ excretion rate increased more slowly, did not reach the high values obtained after ingestion of the cooked test meal, and remained on a rather constant level after the maximum was reached. The cumulative percent ^{13}C of administered dose, recovered in breath over time after ingestion of the labeled cooked and raw test meal, is shown in Fig. 3.

Table 2 summarizes the parameters of protein assimilation as derived from the breath test data. Both the maximum percentage of administered dose of ^{13}C excreted per hour and the cumulative percentage of administered dose of ^{13}C, recovered in breath over 6 h, were significantly higher after ingestion of the cooked test meal compared with the raw test meal.

Feces

Fecal output variables such as wet weight, dry weight, total nitrogen content, and nitrogen density did not differ significantly after ingestion of the cooked and raw test meal (Table 3). The cumulative fecal recovery of ^{15}N, however, was significantly higher after ingestion of the raw test meal [Σ_{0h}^{72h}%dose of $^{15}N_{f\,adm}$ corrected: 4.16 ± 0.27% (cooked) vs. 14.16 ± 1.70% (raw); $P < 0.001$] (Table 4).

Urine

Nitrogen. Figure 4 shows the mean ^{15}N excretion rate in urine after ingestion of the raw and cooked test meal. Although significant differences in the ^{15}N excretion rate were noted, the cumulative percentage of adminis-

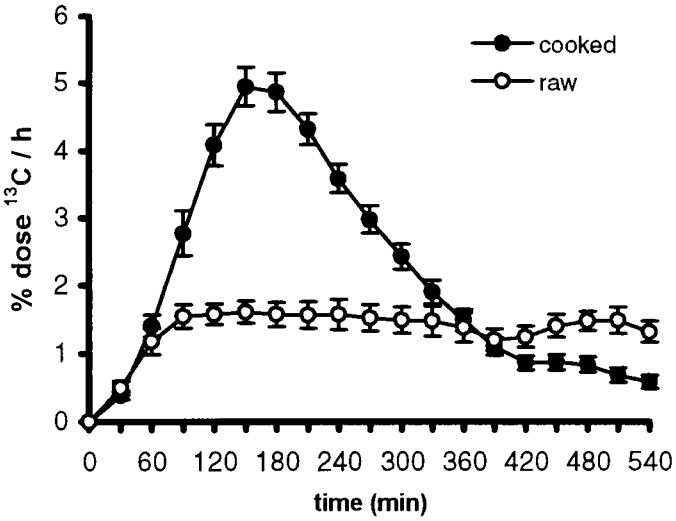

Fig. 2. Mean $^{13}CO_2$ excretion rate in breath, expressed as percentage of the administered dose of ^{13}C excreted per hour in 10 healthy volunteers after ingestion of a cooked and raw test meal, consisting of 25 g of ^{13}C-, ^{2}H-, and ^{15}N-labeled egg protein. Values are means ± SE.

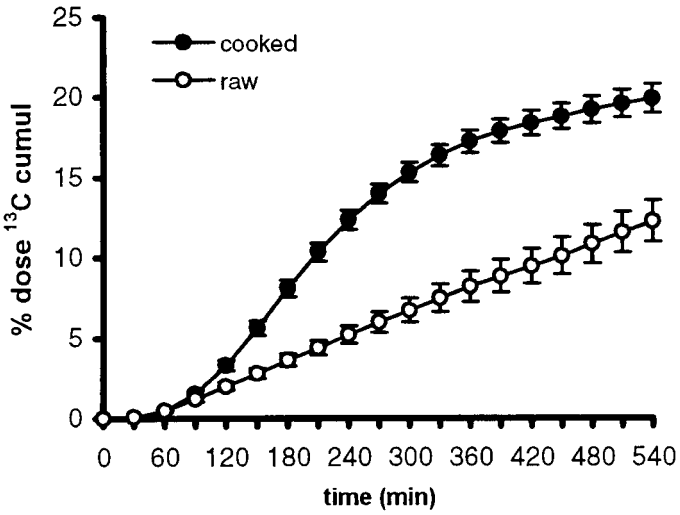

Fig. 3. Mean cumulative percentage of administered dose of ^{13}C, recovered in breath over time (%dose cumul/h) in healthy volunteers after ingestion of a cooked and raw test meal, consisting of 25 g of ^{13}C-, ^{2}H-, and ^{15}N-labeled egg protein. Values are means ± SE; $n = 10$.

tered dose of ^{15}N, recovered in urine over 72 h was almost the same in both test situations [Σ_{0h}^{72h}%dose of $^{15}N_{u\,adm}$: 35.91 ± 2.12% (cooked) vs. 32.68 ± 1.35% (raw); $P = 0.12$] (Table 3).

Phenols. Figure 5 shows the excretion pattern of phenol, *p*-cresol, and total phenols in urine. The excretion rate was higher after ingestion of the raw meal than after ingestion of the cooked meal. Significance was reached in the 9- to 24-h period.

The excretion pattern of [*ring*-$^{2}H_4$]phenol and *p*-[*ring*-$^{2}H_4$]cresol was similar to the excretion pattern of the unlabeled components (Fig. 6). *p*-[*ring*-$^{2}H_4$]cresol appeared in urine somewhat later than [*ring*-$^{2}H_4$]phenol. Overall, the maximal excretion rate was reached in the 9- to 24-h period. The cumulative percentage of administered dose of L-[*ring*-$^{2}H_4$]tyrosine, recovered in urine as total [*ring*-$^{2}H_4$]phenols, was significantly higher after ingestion of the raw test meal compared with the cooked test meal (20.63 ± 5.59% vs. 1.60 ± 0.44%, $P <$ 0.005) (Table 3).

^{15}N Retention

The percentage of administered dose of ^{15}N, retained in the nitrogen pool of the body after 72 h, was significantly lower after ingestion of the raw test meal compared with the cooked test meal (49.63 ± 2.69% vs. 63.16 ± 1.36%, $P = 0.0002$) (Table 3).

Table 2. *Parameters of protein assimilation in healthy volunteers after ingestion of a cooked and raw test meal, consisting of 25 g of ^{13}C-, ^{2}H-, and ^{15}N-labeled egg protein*

	Test Condition		
	Cooked	Raw	*P*
%Dose cum 6 h	17.23 ± 0.69	8.20 ± 0.94	0.0001
$\%_{max}$	5.25 ± 0.26	1.91 ± 0.18	0.0001

Values are means ± SE, $n = 10$. %Dose cum 6 h, cumulative percentage of administered dose of ^{13}C, recovered in breath over 6 h; $\%_{max}$, maximum percentage of administered dose of ^{13}C excreted per hour. *P* values obtained by paired *t*-test.

Table 3. *Major urinary and fecal variables in the two test situations*

	Variable	Test Situation: Cooked	Test Situation: Raw	*P*
Feces	Wet weight, g/day	149.2 ± 22.3	152.0 ± 24.9	0.92
	Dry weight, g/day	25.3 ± 2.7	27.1 ± 1.7	0.31
	Dry matter, %	37.7 ± 5.1	41.2 ± 6.7	0.31
	N excretion, g/day	1.89 ± 0.18	2.10 ± 0.29	0.49
	N density, %dry matter	5.02 ± 0.47	5.09 ± 0.55	1.00
	%Cumulative $^{15}N_f$ (*c*)	4.16 ± 0.27	14.50 ± 1.70	0.00020
	R (*d*)	67.7 ± 3.7	74.0 ± 4.8	0.32
Urine	Total phenols, mg/day	32.14 ± 3.50	47.10 ± 5.06	0.0040
	N excretion, g/day	10.38 ± 0.67	10.68 ± 0.57	0.47
	%Cumulative $^{2}H_4$ (*e*)	1.60 ± 0.44	20.63 ± 5.59	0.00010
	%Cumulative $^{15}N_u$ (*f*)	32.68 ± 1.35	35.91 ± 2.12	0.12
Calculated values	%Malabsorbed (*c* + *c*)	5.73 ± 0.50 24.14 ± 6.07	35.10 ± 6.78	0.0020
	%Accumulation (*e*/*c* + *e*)		50.62 ± 6.10	0.0030
	%Incorporation (*c*/*c* + *c*)	75.86 ± 6.07	49.38 ± 6.10	0.0030
	%Retained in N pool (100% − *c* − *f*)	63.16 ± 1.36	49.63 ± 2.69	0.00020

Values are means ± SE; $n = 10$. %Cumulative $^{15}N_f = \Sigma_{0h}^{72h}$ %dose $^{15}N_{f\,adm}$ corrected (see text). R = Σ_{0h}^{72h} %dose [^{3}H]PEG 4000 (see text). %Cumulative $^{2}H_4 = \Sigma_{0h}^{72h}$ %dose $^{2}H_{4\,adm}$ corrected (see text). %Cumulative $^{15}N_u = \Sigma_{0h}^{72h}$ %dose $^{15}N_{u\,adm}$ (see text). %Incorporation assumes that 100% of ^{15}N recovered in feces is incorporated into the bacterial mass. *P* values were obtained by paired *t*-test.

Correlations

Significant correlations were found between several fecal, urinary, and breath variables (Table 5). There was a negative correlation between the recovery of ^{13}C in breath and the recovery of either ^{15}N in feces ($r =$

Table 4. *Fecal ^{15}N excretion in 10 healthy volunteers after ingestion of a cooked and raw test meal consisting of 25 g of ^{13}C-, ^{2}H-, and ^{15}N-labeled egg protein*

	Fecal ^{15}N Excretion				
	Excretion per day, %dose/day			Cumulative excretion	
	0–24 h	24–48 h	48–72 h	Σ_{0h}^{72h} % dose adm	72 Σ_{0h}^{72h} % dose adm corrected
Cooked	0.34 ± 0.27	0.96 ± 0.31	1.57 ± 0.29	2.87 ± 0.30	4.16 ± 0.27
Raw	1.81 ± 1.37	4.48 ± 1.37	4.20 ± 0.94	10.49 ± 1.26	14.5 ± 1.7
P	0.10	< 0.005	0.020	< 0.001	< 0.001

Values are means ± SE; *P* values were obtained by paired *t*-test. adm, Administered.

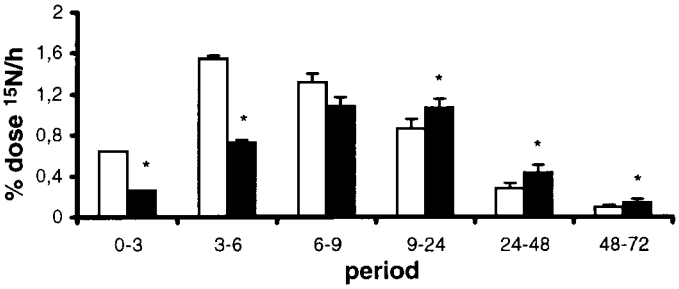

Fig. 4. Urinary ^{15}N excretion pattern in 10 healthy volunteers after ingestion of a cooked (open bars) and raw (closed bars) test meal, consisting of 25 g of ^{13}C-, ^{2}H-, and ^{15}N-labeled egg protein. Values are means ± SE. $*P < 0.05$, paired *t*-test.

-0.71, $P < 0.005$) or [*ring*-$^{2}H_4$]phenols in urine ($r = -0.62$, $P < 0.005$). The recovery of [*ring*-$^{2}H_4$]phenols in urine correlated positively with the recovery of ^{15}N in feces ($r = 0.77$, $P < 0.005$) (Fig. 7).

No correlation was found between the fecal nitrogen output and the recovery of either ^{15}N in feces or [*ring*-$^{2}H_4$]phenols in urine.

DISCUSSION

The efficacy of protein assimilation has been studied to date by several researchers either in ileostomy subjects (4, 8, 11, 22, 32, 34) or in healthy volunteers using intubation techniques (24). It was demonstrated that the amount of protein escaping digestion and absorption in the small intestine is affected by the type and amount of protein (8, 11, 16, 22, 34) as well as the presence of other constituents (e.g., resistant starch) (32) in the diet. Less is known about the process of protein fermentation in vivo in humans, which is largely due to the physiological inaccessibility of the colon.

The availability of protein labeled with stable isotopes allowed us to study protein (mal)absorption and

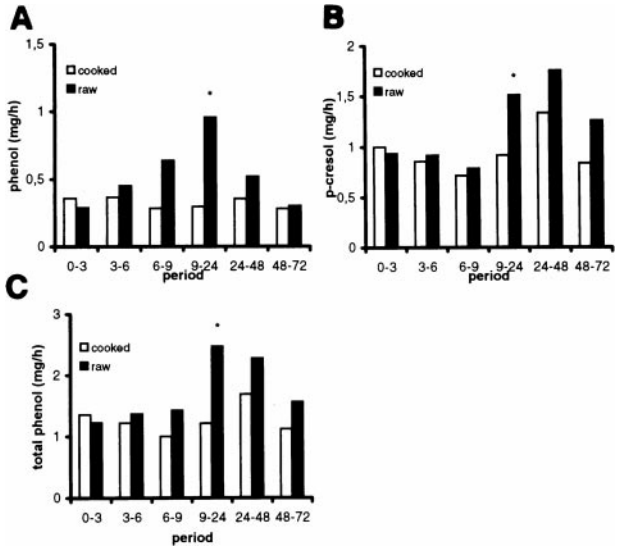

Fig. 5. Urinary excretion pattern of phenol (*A*), *p*-cresol (*B*), and total phenols (*C*) in 10 healthy volunteers after ingestion of a cooked and raw test meal, consisting of 25 g of egg protein. Values are means ± SE. $*P < 0.05$, paired *t*-test.

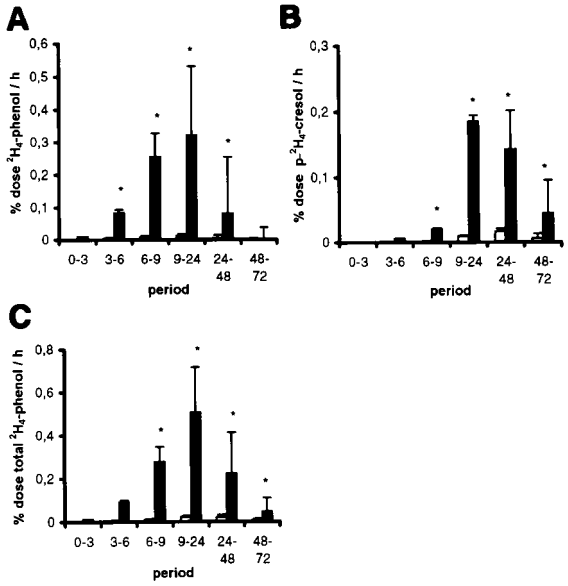

Fig. 6. Urinary excretion pattern of [*ring*-$^{2}H_4$]phenol (*A*), *p*-[*ring*-$^{2}H_4$]cresol (*B*), and total [*ring*-$^{2}H_4$]phenols (*C*) after ingestion of a cooked (open bars) and raw (closed bars) test meal, consisting of 25 g of ^{13}C-, ^{2}H-, and ^{15}N-labeled egg protein. Values are means ± SE. $*P < 0.05$, paired *t*-test.

fermentation in healthy volunteers by means of noninvasive tracer techniques. An inherent advantage of tracer techniques is that they do not disturb normal physiology. All volunteers were studied in two different randomly applied test situations: *1*) after ingestion of a cooked egg protein meal labeled with L-[1-^{13}C]leucine, [^{15}N]amino acids, and L-[*ring*-$^{2}H_4$]tyrosine and *2*) after ingestion of the same but raw meal. Both test situations were separated by a 1-wk washout period. This period was sufficiently long to return isotope enrichment to baseline (data not shown).

Protein (mal)absorption and fermentation were evaluated quantitatively through the analysis of metabolites excreted in breath, urine, or feces. Breath was analyzed for $^{13}CO_2$, feces for ^{15}N, and urine for [*ring*-$^{2}H_4$]phenol, *p*-[*ring*-$^{2}H_4$]cresol, and ^{15}N.

The breath test results obtained in the present study were in accordance with those obtained in a recent study performed in ileostomy patients under similar test conditions (8). In the latter study, a highly significant negative correlation was demonstrated between the ^{13}C recovery in breath and the recovery of exogenous protein in the ileal effluents. In the extrapolation of this finding to subjects with an intact gastrointestinal system, the low recovery of $^{13}CO_2$ in breath after ingestion of the raw protein test meal suggests overt malabsorption.

It may be argued that in subjects with an intact gastrointestinal system the $^{13}CO_2$ recovered in breath may be derived from the fermentation of malabsorbed L-[1-^{13}C]leucine in the colon as well. However, because it was observed in the ileostomy study formerly mentioned (8) that 50% of the malabsorbed cooked and raw protein had emptied from the ileostomy by 5.33 and 5.29 h, respectively, it can be assumed that most of the $^{13}CO_2$ appearing in breath within 6 h following the

Table 5. *Correlations between breath, fecal, and urinary variables*

	Fecal Variables			Urinary Variables	
	Total N, g/day	Wet weight, g/day	%Cumulative ^{15}N	Total phenol, mg/day	%Cumulative $^{2}H_4$
Breath variable					
%Dose ^{13}C 6 h	−0.10	0.049	−0.71†	−0.67†	−0.62†
Fecal variables					
Total N, g/day		0.75†	0.19	−0.22	−0.045
Wet weight, g/day			−0.032	−0.22	−0.11
%Cumulative $^{15}N_f$				0.46*	0.77†
Urinary variable					
Total phenols, mg/day					0.62†
%Cumulative $^{2}H_4$					

$n = 20$ (10 subjects × 2 test situtions). *$P < 0.05$; †$P < 0.005$.

ingestion of the labeled test meal is derived from the metabolism of protein assimilated in the small intestine.

The mean daily fecal wet weight, dry weight, and nitrogen content were consistent with previous investigations in humans (2, 7, 36, 37). None of these parameters was affected significantly by the nature of the test meal (Table 3).

The cumulative percentage of administered dose of ^{15}N recovered in feces over 72 h after ingestion of the cooked egg protein meal amounted to 4.16 ± 0.27%. This value is comparable with figures previously reported for yeast, egg, and soya protein (20, 40). The cumulative recovery of ^{15}N in feces after ingestion of the raw meal was significantly higher (14.50 ± 1.70%). It has previously been demonstrated that at least 60% of the fecal nitrogen content is of bacterial origin (36). For practical purposes, however, it is assumed in the present study that all ^{15}N recovered in feces is of bacterial origin (i.e., incorporated in the bacterial mass).

The cumulative percentage of administered dose of ^{2}H recovered in urine over 72 h amounted to 1.60 ± 0.44% and 20.63 ± 5.59%, respectively, after ingestion of the cooked and raw test meal. The percentage of administered dose of ^{2}H recovered in urine represents the portion of consumed egg protein that is accumulated in the colonic lumen as free fermentation metabolites, subsequently absorbed into the portal blood and finally excreted in urine.

The excretion pattern of $^{2}H_4$-labeled total phenols after ingestion of the raw test meal coincided almost completely with the excretion pattern of the unlabeled fraction. This indicates that the observed increase of the unlabeled total phenols is related to malabsorption of the test meal. [*ring*-$^{2}H_4$]phenol appeared in urine slightly earlier than *p*-[*ring*-$^{2}H_4$]cresol. This is in accordance with previous studies suggesting that phenol and *p*-cresol are predominantly formed in the terminal ileum (and cecum) and left colon, respectively (3). The delayed appearance of *p*-cresol might also be explained by a slower production rate.

Assuming that the fate of both the ^{15}N and ^{2}H tracer is identical in the large intestine, the percentage of ingested protein that escaped digestion and absorption could be calculated approximately. It amounted to 5.73 ± 0.50% and 35.10 ± 6.78% after ingestion of the cooked and raw test meal, respectively (Fig. 8). Malabsorption might be overestimated in the present study

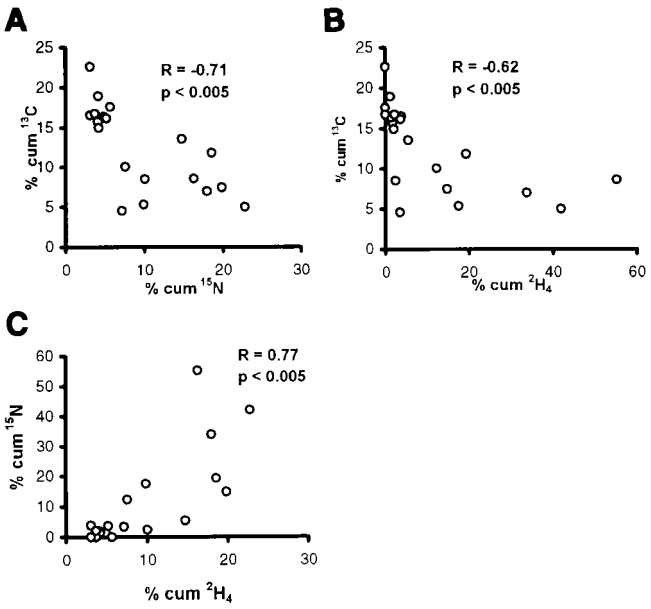

Fig. 7. Correlations between parameters of protein assimilation (i.e., %cumulative ^{13}C) and parameters of protein malabsorption (i.e., %cumulative ^{15}N and %cumulative $^{2}H_4$). %cum ^{13}C, cumulative percentage of administered dose of ^{13}C recovered in breath over 6 h. %cum ^{15}N, Σ_{0h}^{72h}%dose of $^{15}N_{fadm}$ corrected. %cum $^{2}H_4$, Σ_{0h}^{72h}%dose of $^{2}H_{4adm}$ corrected (see text for complete explanation of abbreviations).

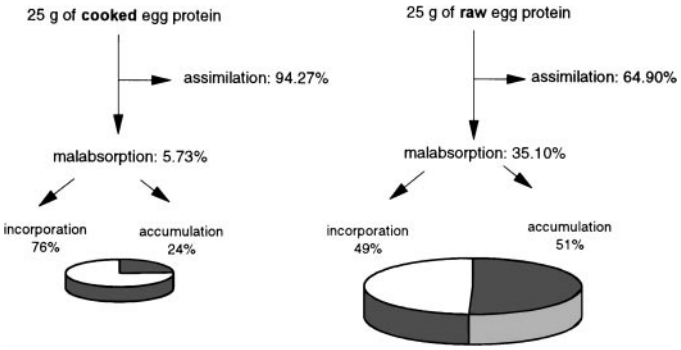

Fig. 8. Amount and fate of 25 g of egg protein escaping assimilation in the small intestine of humans.

due to tracer recycling. Tracer recycling occurs through desquamation of intestinal mucosa and secretion of digestive enzymes and urea in the small intestine and colon, respectively (20, 26). Little information is available on the magnitude of this bias, which, most probably, is due to methodological problems. Kayser et al. (20) quantified ^{15}N tracer recycling by measuring the appearance of ^{15}N in stools after an intravenous injection of 250 mg of ^{15}N-enriched glycine (99 AP). The fractional fecal loss (i.e., tracer recycling) amounted to 1.43 ± 0.64% (means ± SD). Although it is reasonable that the magnitude of tracer recycling is not fixed but influenced by dietary factors, the latter value may be indicative.

Despite possible overestimation due to tracer recycling, the malabsorption percentages observed in the present are still somewhat lower than those we previously reported in healthy ileostomy patients in identical test conditions (8). This difference can be explained either by differences in the efficiency of protein assimilation between healthy volunteers and ileostomy patients or by salvage of nitrogen in the colon (18). The latter possibility is supported by human and animal data (17, 21).

Amounts of 24.14 ± 6.07% and 50.62 ± 6.10% of the malabsorbed cooked and raw egg protein, respectively, were calculated to accumulate as free end products of bacterial metabolism in the colonic lumen (Fig. 8). Assuming that the two test conditions only differed in the amount of protein made available to the colon, the present results support the hypothesis that luminal accumulation as free fermentation metabolites becomes the preferential fate of malabsorbed protein as more protein is made available to the colon.

Significant differences were observed between both test situations in the pattern of excretion rates of ^{15}N in urine. The ^{15}N excretion rate was significantly lower in the 0- to 6-h period and significantly higher in the 9- to 72-h period after ingestion of the raw test meal compared with the cooked test meal. Protein assimilation was suggested by the shape of the breath test curve to be completed after 6–9 h. Bacterial protein metabolism, reflected by an increase of the excretion of [*ring*-$^{2}H_4$]phenols in urine, on the other hand, became apparent from 9 h after ingestion of the test meal on. Therefore, ^{15}N appearing soon after ingestion of the test meal is accepted to be derived uniquely from the metabolism of assimilated protein, whereas ^{15}N appearing later on is accepted to be derived from bacterial metabolism of malassimilated protein as well. As could be predicted, metabolism of assimilated protein was more prominent after ingestion of the cooked test meal, whereas bacterial metabolism of malassimilated protein was more prominent after ingestion of the raw protein meal. Notwithstanding these kinetic differences, the cumulative percentage of administered dose of ^{15}N excreted in urine over 72 h was similar in both test situations.

The cumulative percentage of administered dose of ^{15}N, retained in the nitrogen pool of the body, was significantly lower after ingestion of the raw protein meal compared with the cooked protein meal. Nevertheless, the difference between both test situations (13.50 ± 2.20%) was less pronounced than was expected from the difference in the percentage of malabsorption (29.34 ± 6.45%) (Table 4). This observation might equally be explained by salvage of nitrogen in the colon.

Highly significant negative correlations were found between parameters of protein assimilation (i.e., cumulative percentage of administered dose of ^{13}C, recovered in breath over 6 h) and parameters of protein malabsorption (i.e., the amount of ^{15}N and [$^{2}H_4$]phenols, recovered in feces and urine, respectively). This finding supports the validity of the techniques used. The lack of a significant correlation between the fecal nitrogen output and fecal ^{15}N recovery indicates that the fecal nitrogen output may not be regarded as a sensitive parameter of the efficiency of dietary protein assimilation in the small intestine.

In conclusion, using noninvasive stable isotope techniques, we were able to evaluate protein (mal)absorption and fermentation in healthy volunteers in a noninvasive and quantitative way. We definitively confirmed malabsorption of even easily digestible protein. Our results furthermore support the hypothesis that an increased availability of protein in the colon causes the preferential fate of malabsorbed protein to shift toward luminal accumulation as free protein fermentation metabolites. This finding may be important from a gastrointestinal point of view, since several of these metabolites (ammonia, thiols, phenols) are thought to play a role in the etiopathogenesis of, e.g., ulcerative colitis and colonic cancer (3, 5, 10, 23, 27, 28, 30, 39).

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