Assessment of net postprandial protein utilization of ^{15}N-labelled milk nitrogen in human subjects

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The nutritional quality of milk proteins, evaluated both in terms of digestibility and postprandial oxidation and retention in human subjects, was investigated in this study. Five healthy adult volunteers were given 480 ml ^{15}N-labelled milk (i.e. 190 mmol N). ^{15}N was subsequently determined at the ileal level, using a naso-intestinal intubation technique, as well as at the faecal level. Plasma and urine were sampled for 8 h after meal ingestion. Dietary exogenous N recovered at the terminal ileum after 8 h reached 8·6 (SE 0·8) mmol while the amount collected in the faeces was 6·5 (SE 0·7) mmol after 5 d. The true ileal and faecal digestibilities were 95·5 (SE 0·4) % and 96·6 (SE 0·4) % respectively. The appearance of [^{15}N]amino acids in the plasma was rapid and prolonged. The measurement of ^{15}N in the body urea pool and in the N excreted in the urine allowed us to calculate the deamination occurring after [^{15}N]milk protein absorption. The net postprandial protein utilization (i.e. NPPU = $(N_{absorbed} - N_{deaminated})/N_{ingested}$), calculated as an index of protein quality 8 h after milk ingestion, was 81·0 (SE 1·9) %. Our data confirm that milk protein has a high oro-ileal digestibility in man and demonstrate that milk protein has a high NPPU, an index corresponding to a period in which the dietary protein retention is maximal.

Milk: Protein quality: Intestinal absorption

The nutritional value of dietary proteins for man is related to their ability to achieve N and amino acid requirements for growth and maintenance (Munro, 1969; Young & Pellet, 1988; Rennie *et al*. 1994; Millward & Pacy, 1995). This ability is related both to the digestibility of proteins and to the subsequent metabolism of the absorbed amino acids. Dietary protein quality is classically investigated by carrying out long-term balance studies in animals or human subjects given different levels of protein in their diet (Braude *et al*. 1970; Alm, 1981; Lee *et al*. 1988). Additional methods take into account the chemical score using the amino acid composition of the protein in comparison to a reference protein, or the protein digestibility-corrected amino acid score including the digestibility factor (Block & Mitchell, 1946; Food and Agriculture Organization/World Health Organization, 1990; Millward & Pacy, 1995). Although the chemical score and the protein digestibility-corrected amino acid score are now the currently accepted procedures for assessing protein quality (Food and Agriculture Organization/World Health Organization, 1990), these approaches do not consider the acute metabolic events concomitant with dietary protein ingestion. Previous studies have been performed to assess milk-, soyabean- and pea-protein quality for man using the measurement of true ileal digestibility (Mahé *et al*. 1992; Gausserès *et al*. 1996). A complementary approach considers the acute metabolic fate of the ingested dietary protein N. In this case, the difficulty of measuring short-term retention of dietary protein N in human subjects can be circumvented by the use of ^{15}N-labelled proteins that make it possible to follow the metabolic fate of dietary N (Mahé *et al*. 1994; Gausserès *et al*. 1997). The present study was conducted to determine ileal N digestibility and postprandial N utilization of milk protein N in human subjects.

^{15}N-labelled milk was ingested by healthy volunteers and ^{15}N was subsequently measured at the ileal, faecal, plasma and urine levels. The net postprandial protein utilization (NPPU) was calculated from both true ileal digestibility and true ^{15}N-labelled milk protein deamination.

Abbreviations: NPPU, net postprandial protein utilization; PSP, phenolsulfonpthalein.

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Materials and methods

Subjects

Five volunteers (two males and three females) with a mean age of 29 (SE 4) years (range 21–41 years) and a mean BMI of 24 (SE 1) kg/m^2 were selected in accordance with the following criteria: no history of gastrointestinal surgery; absence of gastrointestinal system disorders; absence of pregnancy; a stable, satisfactory nutritional status and a stable body weight. The protocol was previously approved by the Ethical Committee of the St Germain-en-Laye Hospital (78100 St Germain-en-Laye, France). All subjects gave informed consent for their participation in the study.

Diets

^{15}N-labelled milk was obtained from a cow whose drinking water had been supplemented daily with 80 g $(^{15}NH_4)_2SO_4$ (30 g in the morning, 50 g in the evening) for 14 d. The ^{15}N-labelled milk (mean 0·1954 atom % excess) was then skimmed and subsequently pasteurized (63°, 30 min). The meal was made up of 480 ml skimmed milk containing 190 mmol N.

Experimental design

On the day before the experiment, the volunteers were admitted to the hospital and the intestinal tube was passed from the nose to the terminal ileum, as previously described (Mahé *et al.* 1992). Subjects had dinner at 20.00 hours and then fasted overnight. On the morning of the study, the position of the tube was checked by radioscopy. A small catheter was placed in the forearm vein for blood sampling. Subjects were given the test meal (i.e. 480 ml pasteurized skimmed milk), and the intestinal sampling period lasted for 8 h. Starting 30 min before meal ingestion at 10.00 hours and continuing throughout the test period, a saline solution (130 mmol NaCl/l, 30 mmol mannitol/l, 5 mmol KCl/l) containing phenolsulfonphthalein (PSP, 400 mg/l) was perfused into the intestine at a rate of 1 ml/min to calculate the intestinal flow rate (Modigliani *et al.* 1973). Intestinal samples were obtained by continuous suction through the distal opening of the tube. Digesta were collected over ice and pooled at 30 min intervals. Subjects were not allowed to drink or eat until the end of the collection period. The digesta samples were treated with the protease inhibitor diisopropylfluorophosphate and then frozen at −20° and lyophilized. Blood samples were collected into heparin every hour during the 8 h study. The plasma was immediately separated from the whole blood by centrifugation and frozen at −20° until later analysis. Urine was collected every 2 h for 8 h, treated with thymol crystals and liquid paraffin (CPF Laboratories, Melun, France) as preservatives and stored at 4° until later analysis. The entire stool output was collected every day for 5 d, weighed and freeze-dried before analysis.

Extraction of amino acids, urea and ammonia in plasma and urine

Urea and NH_3 were isolated using the batch method described by Preston & McMillan (1988), using a Na^+/K^+ form of the cation exchange resin (BioRad Dowex AG-50X8, mesh 100–200; Interchim, Montluçon, France). The preparation of the Na^+/K^+ form of the resin has been described previously (Gausserès *et al.* 1997). For amino acid and urea extraction, 2 ml plasma was added to 100 mg solid 5-sulfosalicylic acid, mixed and kept for 1 h at 4°. After centrifugation (2400 ***g*** for 20 min at 4°), the supernatant fraction was collected. From the urine, NH_3 was first extracted using the prepared Na^+/K^+ form of the cation exchange resin by the batch procedure. The supernatant fraction was retained for urea extraction. The urea was extracted from both the plasma supernatant fraction and the NH_3-free urine fraction by hydrolysis with urease (*EC* 3.5.1.5) for 2 h at 30° on the cation exchange resin. The resins were washed five times with distilled water and stored at 4°. NH_3 and urea-derived NH_3 (for isotopic determination) were eluted from the resins by the addition of 2·5 mM-KH_2SO_4.

Analytical methods

PSP concentrations in the effluents were measured by a spectrophotometric method (Scheld, 1966). Total N content of the digesta was determined using an elemental N analyser (NA 1500 series 2, Fisons Instruments, Manchester, UK) with atropine as standard. Urea was assayed in both plasma and urine by an enzymic method (urease–glutamate dehydrogenase, *EC* 1.4.1.2) on a clinical analyser (Dimension, Dupont de Nemours, Les Ulis, France). NH_3 was measured in the urine by an enzymic method (glutamate dehydrogenase) on a clinical analyser (Kone, Evry, France). The $^{15}N:{}^{14}N$ isotope ratio was determined by isotope-ratio mass spectrometry. A portion of sample (2–3 mg freeze-dried faecal and ileal samples, 20–25 μl liquid plasma or urine samples) was combusted at 1020° in an elementary analyser (NA 1500 series 2, Fisons Instruments), coupled with an isotope-ratio mass spectrometer (Optima, Fisons Instruments). The isotope ratio was measured in reference to calibrated $^{15}N/{}^{14}N$ nitrogen gas (purity $>$ 99·9999 %, $O_2 <$ 0·1 μl/l).

Calculations and statistical analysis

The intestinal flow rate (F) was calculated from the PSP concentrations for each 30 min period ($F = ((PSP_p/PSP_s) - 1) \times F_p \times t$, where PSP_p is the concentration of PSP in the perfusion liquid, PSP_s is the concentration of PSP in the sample, F_p is the perfusion flow rate (1 ml/min) and t is the time period (30 min)). The total N content of digesta (mmol N/30 min) was derived from the following formula: $N_{tot\text{-}digesta} = (N_s \times DM_s \times F)/140$, where N_s is the N content of the freeze-dried sample (g/100 g), DM_s is the DM of the sample (g/100 ml) and F is the intestinal flow rate (ml/30 min). The total N content of the faeces was calculated from: $N_{tot\text{-}faeces} = N_s \times DM_s \times W$, where N_s is the N content of the freeze-dried sample (g/100 g), DM_s is the DM of the sample (g/100 ml) and W is the sample weight (g/d). The exogenous N (i.e. originating from the milk N) ($N_{exo\text{-}X}$) in each fraction X (faeces, digesta, urinary urea and NH_3, plasma urea) was determined according to the following formula: $N_{exo\text{-}X} = N_{tot\text{-}X} \times (E_X - E_0)/(E_{milk} - E_0)$, where $N_{tot\text{-}X}$

is the total N content of this fraction, E_X its ^{15}N-enrichment (atom %), E_{milk} is the enrichment of the milk ingested and E_0 is the natural enrichment of each fraction. Total N content in the plasma urea was calculated as the product of the plasma urea concentration and its volume of distribution, corrected by a factor of 92 % which represents the water content of blood, assuming that urea diffuses throughout the total body water. Total body water was estimated using the equation of Watson *et al*. (1980). Total body urea N remained stable during the experimental period (364·3 (SE 29·7) mmol N). Results are expressed as means with their standard errors. Statistical analysis was performed using one-way ANOVA and Tukey's studentized range test (SAS/STAT, version 6.03; SAS Institute Inc., Cary, NC, USA). A probability of $P < 0{\cdot}05$ was considered to be significant.

Results

Ileal and faecal digestibility of ^{15}N-labelled milk nitrogen

The recovery of exogenous ^{15}N was first determined both in the intestinal effluent recovered at the terminal ileum and in the faeces following ^{15}N-labelled milk ingestion (Fig. 1). The amount recovered at the terminal ileum peaked after 1 h and then decreased during the next 7 h. No significant amount of exogenous N was recovered at the terminal ileum at the end of the 8 h. The amount recovered in the faeces remained at a very low level after 24 h, peaked after 60 h and progressively decreased. The cumulative values of ^{15}N-labelled milk N recovery at the terminal ileum during the 8 h and in the faeces during the 5 d following milk ingestion were 8·6 (SE 0·8) mmol N and 6·5 (SE 0·7) mmol N respectively. Consequently, the true ileal and faecal digestibilities of [^{15}N]milk protein were calculated as 95·5 (SE 0·4) % and 96·6 (SE 0·4) % respectively, and were not significantly different from each other.

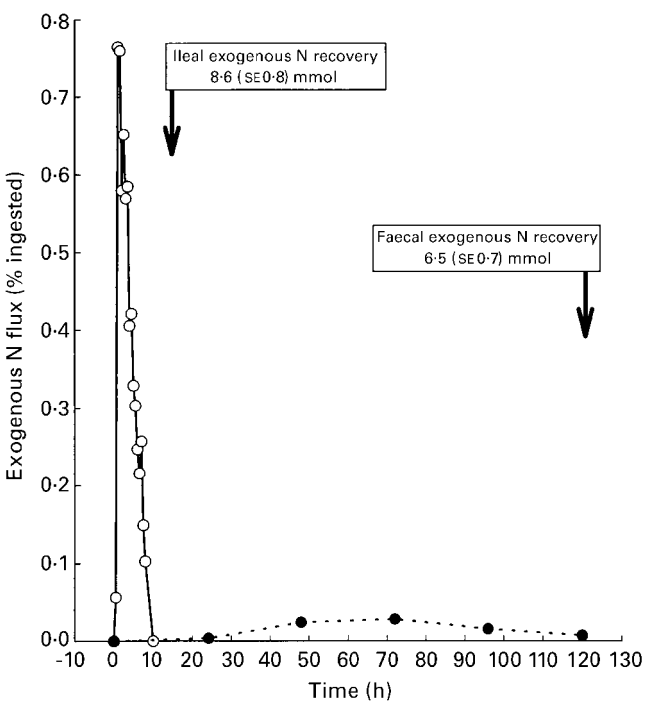

Fig. 1. Exogenous nitrogen recovered in the ileum (○) and in the faeces (●) following ingestion of [^{15}N]milk by healthy adults after an overnight fast. Each value represents the mean of five subjects. For details of procedures, see pp. 222–223.

[^{15}N]milk nitrogen distribution in the plasma amino acids, in the body urea and in the urine nitrogen

The intestinal absorption of ^{15}N was correlated with a significant increase ($P < 0{\cdot}05$) in the ^{15}N-enrichment of the amino acids in the plasma that peaked (0·0067–0·0069 atom % excess) from 1 to 3 h following milk ingestion and then progressively decreased (Fig. 2(a)). In parallel, ^{15}N was progressively transferred to both body urea N (Fig. 2(a)) and urine N as urea and NH_3 (Fig. 2(b)). Urinary NH_3 enrichment peaked at 4 h (0·013 atom % excess) and then quickly decreased, whereas urinary urea enrichment increased until 6 h after the meal ingestion (0·016 atom % excess). The level of exogenous N (mmol N) transferred to the body urea pool increased in the 5 h following ingestion, peaked at 4–5 h and then slowly decreased. The cumulative amount of exogenous ^{15}N recovered in both the total N and urea

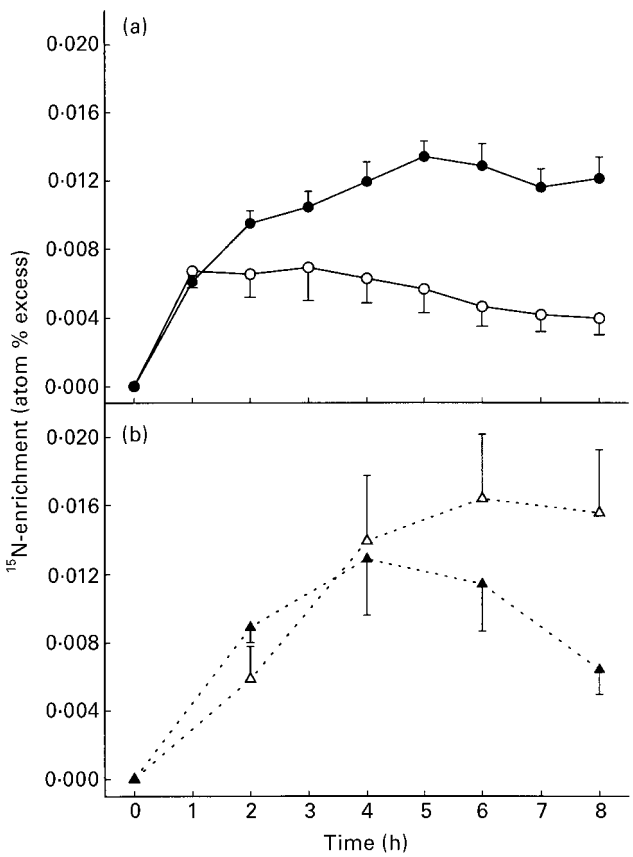

Fig. 2. ^{15}N-enrichment in (a) the plasma amino acid fraction (○) and the body urea pool (●) and (b) the urinary ammonia (▲) and the urinary urea (△) following ^{15}N-labelled milk ingestion by healthy adults after an overnight fast. Values are means of five subjects with their standard errors represented by vertical bars. For details of procedures, see pp. 222–223.

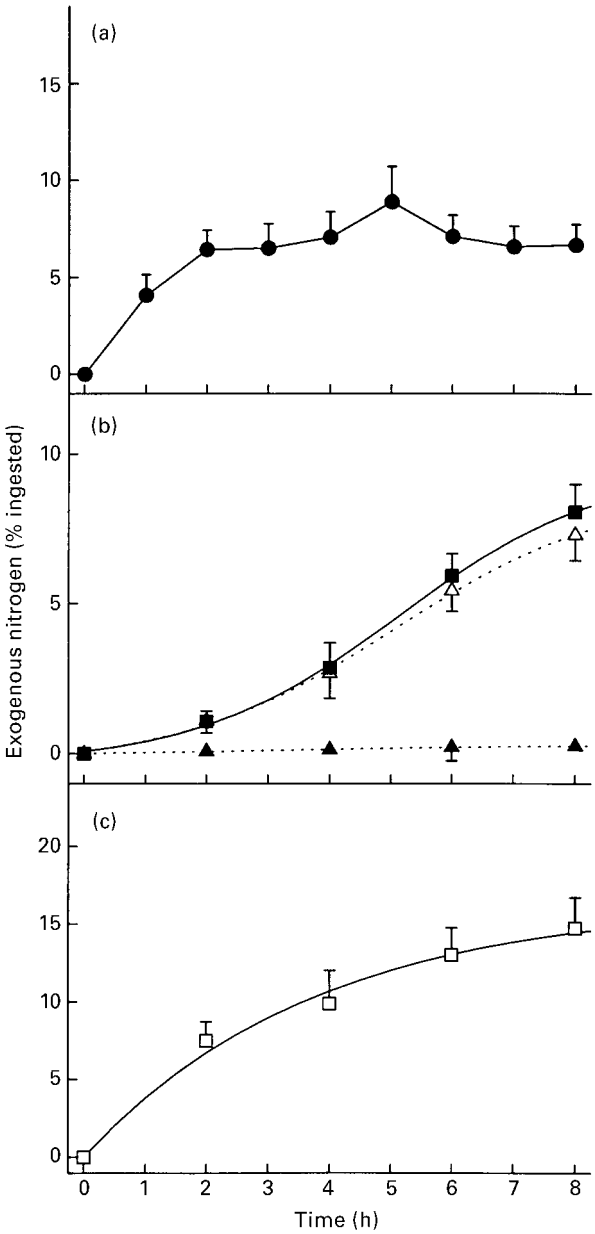

Fig. 3. (a) Exogenous nitrogen in the body urea pool, (b) cumulative excretion of exogenous nitrogen in the urine as urea (△), ammonia (▲) and total nitrogen (■) and (c) ^{15}N deamination calculated as the sum of exogenous urea nitrogen excreted in the urine and the exogenous nitrogen present in the body urea pool following ingestion of [^{15}N]milk by healthy adults after an overnight fast. Values are means of five subjects with their standard errors represented by vertical bars. For details of procedures, see pp. 222–223.

fractions in the urine progressively increased after milk ingestion. The cumulative amount of ^{15}N recovered as NH_3 in the urine reached a plateau value of 0·62 mmol. At 8 h, values for urinary exogenous N excreted as total N and as urea were 16·2 (SE 2·1) and 14·6 (SE 1·9) mmol respectively. The postprandial ^{15}N-labelled milk protein deamination kinetics were evaluated as the sum of the cumulative amount of exogenous N excreted in the urine and the amount of exogenous N that remained in the urea body pool every 2 h after milk ingestion. The quantity of exogenous N arising from ^{15}N-labelled milk amino acid deamination reached a value of 28·2 (SE 4·1) mmol N 8 h after milk ingestion, representing 15·5 % of the absorbed dietary N.

Net postprandial protein utilization of ^{15}N-labelled milk

Following meal ingestion, protein utilization must be considerably higher than calculated from the daily balance data. Consequently, the true ileal digestibility (95·5 (SE 0·4) %) and the true exogenous N deamination measured in the 8 h following ^{15}N-labelled milk ingestion (28·2 (SE 4·1) mmol N) allow for a calculation of the true net postprandial protein utilization as: $NPPU = (N_{absorbed} - N_{deaminated})/N_{ingested}$. This NPPU is indicated by a value of 81·0 (SE 1·9) % for milk protein N.

Discussion

This study was designed in order to assess milk N availability and subsequently protein nutritional quality in human subjects using the determination of both true digestibility and postprandial retention of uniformly ^{15}N-labelled milk protein N. Among the different methods used to assess dietary protein quality for man, the chemical score and the protein digestibility-corrected amino acid score do not consider the acute metabolic events concomitant with dietary protein assimilation. Methods based on digestibility and protein retention are of great interest in order to account for the short-term utilization of dietary proteins. We have recently demonstrated that the use of ^{15}N-labelled proteins makes it possible to follow the metabolic fate of dietary N (Gausserès *et al*. 1997). Assessing the postprandial utilization of dietary proteins is an appropriate method to evaluate protein sources in terms of nutritional quality, because this variable is known to influence protein synthesis and proteolysis (Marchini *et al*. 1993).

The digestive stage of protein utilization showed that ileal digestibility of milk N was high, i.e. 95·5 %. This result strengthens previous data showing that milk protein has a high digestibility, regardless of the form of administration: whole milk, purified proteins, fermented milks (Mahé *et al*. 1992; Gaudichon *et al*. 1995). The comparison between ileal and faecal true digestibilities, calculated from milk protein exogenous N recovery, showed a slight overestimation of the faecal compared with the ileal value, though the difference was not significant. This result is consistent with the current idea that non-absorbed N is metabolized by colonic micro-organisms, leading to an underestimation of exogenous N in the faeces, but the results showed that this underestimation was low in the present experimental conditions.

Estimation of protein quality using only the digestibility factor does not appear to be adequate, because amino acid absorption kinetics play a key role in the metabolic utilization of N (Kies, 1981; Boirie *et al*. 1997). We investigated exogenous ^{15}N in the plasma amino acid pool, i.e. after the amino acids had passed through the hepatic circulation. As we followed the N labelling rather than the amino

acids specifically, our results were unaffected by the modifications in amino acid composition on the first-pass by the splanchnic bed, e.g. the sequestration of glutamate by the intestine and the branched-chain amino acid transaminations. ^{15}N-labelled amino acid N kinetics were characterized by a rapid increase during the first hour, which might be attributable to whey protein absorption, followed by a stable level for 2 h and a progressive decrease. These data could differentiate between amino acids from whey and casein because these two milk protein fractions appear to have different digestive behaviours (Mahé *et al*. 1996). Whey proteins are quickly emptied, while casein clots in the stomach and is released slowly over a prolonged period. This difference in absorption kinetics has been shown to have a direct influence on metabolic utilization of N: amino acids from whey protein stimulate protein synthesis whereas amino acids from casein have the effects of both stimulation of protein synthesis and inhibition of protein breakdown (Boirie *et al*. 1997). Measured under the same conditions as in this study, plasma amino acid kinetics after ^{15}N-labelled pea ingestion were more prolonged and peaked only 4 h after the meal (Gausserès *et al*. 1997).

Milk proteins present a well-balanced amino acid composition with regard to human needs. The nutritional value of a protein is often assessed using the net protein utilization index which represents the ratio between the N retained (i.e. $N_{absorbed} - N_{deaminated}$) and that absorbed (Bender & Miller, 1953; Davidson *et al*. 1979; Dillon, 1991). In rats the net protein utilization of cow's milk protein was found to be between 77 and 82 %, with a difference between casein (79 %) and whey proteins (95 %) (Food and Agriculture Organization/World Health Organization, 1973). These results are similar to the NPPU of 81 % observed in the present study for milk protein N. However, these values are hardly comparable with each other since the net protein utilization in rats is calculated over a long period of time and does not reflect the variations of protein utilization dependent on the fasted and fed states. The NPPU appears to be a better indicator of short-term metabolic utilization of dietary N. In a previous study, we showed that the NPPU of pea protein was 73 % with the same method of measurement (Gausserès *et al*. 1997). The difference of 9 points between milk and pea NPPU suggests a quality gap between these two protein sources, whereas this difference was not observed for ileal digestibility (Gausserès *et al*. 1996).

In conclusion, our data demonstrate that milk protein has a high NPPU, an index corresponding to a period in which the dietary protein retention is maximal. Our results suggest that protein quality, in terms of protein utilization, is more than a simple function of the indispensable amino acid content. Moreover, there are other considerations to investigate as possible determinants of NPPU such as the influence of associated meal components, i.e. fat and/or carbohydrate.

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