

## Exogenous and endogenous nitrogen flow rates and level of protein hydrolysis in the human jejunum after [<sup>15</sup>N]milk and [<sup>15</sup>N]yoghurt ingestion

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Milk and yoghurt proteins were <sup>15</sup>N-labelled in order to measure the flow rate of exogenous N during digestion in the human intestine. After fasting overnight, sixteen healthy volunteers, each with a naso-jejunal tube, ingested either [<sup>15</sup>N]milk (*n* 7) or [<sup>15</sup>N]yoghurt (*n* 9). Jejunal samples were collected every 20 min for 4 h. A significant stimulation of endogenous N secretion was observed during the 20–60 min period after yoghurt ingestion and the 20–40 min period after milk ingestion. The endogenous N flows over a 4 h period did not differ between the groups (44.3 (SEM 6.5) mmol for milk and 63.5 (SEM 5.9) mmol for yoghurt). The flow rates of exogenous N indicated a delayed gastric emptying of the yoghurt N compared with N from milk. The jejunal non-protein N (NPN) flow rate increased significantly after milk and yoghurt ingestion due to an increase in the exogenous NPN flow rate. The NPN fraction of exogenous N ranged between 40 and 80%. The net gastro-jejunal absorption of exogenous N did not differ significantly between milk (56.7 (SEM 8.5)%) and yoghurt (50.9 (SEM 7)%). The high level of exogenous N hydrolysis is in accordance with the good digestibility of milk products. Fermentation modifies only the gastric emptying rate of N and does not affect the level of diet hydrolysis, the endogenous N stimulation or the digestibility rate.

Milk proteins: <sup>15</sup>N: Digestion: Human: Jejunum

A great difficulty in the study of intestinal protein digestion in humans arises from the fact that after ingestion, dietary proteins are mixed with endogenous proteins secreted in the lumen, i.e. gastric, bilio-pancreatic and intestinal secretions. In addition, contrary to fasting conditions where endogenous proteins are continuously secreted into the lumen at a constant level (Gaudichon *et al.* 1994*a*), the ingestion of a meal stimulates salivary, gastric and bilio-pancreatic secretions (Alpers, 1987) to a level which depends on the amount and on the nature of the meal and particularly its dietary protein content (Lurie *et al.* 1973; Girard-Globa *et al.* 1980; Simon *et al.* 1983). Different methods have been developed to differentiate between exogenous and endogenous N fractions in the intestinal chyme including the use of stable isotopes as markers of the alimentary compounds. Labelling the

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proteins of milk with  $^{15}\text{N}$  constitutes an accurate method of differentiating precisely between endogenous and exogenous fractions in intestinal effluents. This method has also proved to be accurate in evaluating the kinetics of protein digestion and absorption as well as the secretory N response to a meal in both humans (Mahé *et al.* 1994c) and animals (Gaudichon *et al.* 1994b).

In a previous study we compared the N flow rates in the jejunum and the ileum in humans after milk and yoghurt ingestion (Mahé *et al.* 1994b). A significant increase in the N flow rate was observed in the jejunum with both meals. This increase was delayed with yoghurt compared with milk in the jejunum, whereas no difference was noticed at the ileal level. The jejunal effect could have been due partly to a delayed gastric emptying of yoghurt but could also have originated from the difference in endogenous N secretion. However, it was not possible to differentiate between endogenous and exogenous N. The aim of the present study was to determine in humans (1) the endogenous N secretion in the jejunum after milk and yoghurt ingestion and (2) the level of hydrolysis in both endogenous and exogenous N fractions in the jejunum. For these purposes, human volunteers, each equipped with a naso-jejunal tube, received *per os*  $^{15}\text{N}$ -labelled milk or yoghurt, jejunal effluents being collected for 4 h.

## MATERIALS AND METHODS

### *Diets*

Fresh milk was  $^{15}\text{N}$ -labelled by infusing 50 g/d ( $^{15}\text{NH}_4$ )<sub>2</sub>SO<sub>4</sub> (10 atom % isotope enrichment, Euriso-top, Saint Aubin, France) via a permanent fistula into the rumen of a lactating cow for 1 week as previously described (Mahé *et al.* 1994a). The milk of day 6 was enriched at 0.5083 atom % and was divided into two parts. The first part was used by Danone (Le-Plessis-Robinson, France) to prepare yoghurt. The second part was stored at  $-20^\circ$  until fed to the volunteers. Diet composition is shown in Table 1. PEG-4000 was added to each diet as a non-absorbable liquid-phase marker (final concentration, 40 g/l).

### *Subjects*

Sixteen healthy volunteers (eight male and eight female) participated in this study. They were 23 to 40 years of age (mean 30 years) and weighed from 49 to 95 kg (mean 66 kg). None of the subjects had taken any medication or suffered from gastrointestinal upset before the study. The protocol was previously approved by the Ethical Committee of the hospital. All subjects gave informed consent for their participation in the study.

### *Collection of digesta*

The day before the experiment the volunteers swallowed a double intestinal tube that descended by normal peristalsis to the jejunum. One tube was used to perfuse a saline (150 mM-NaCl) solution of phenol red (PSP, a non-absorbable marker), and one to aspirate jejunal contents 200 mm distal to the perfusion site as previously described (Mahé *et al.* 1994b). After an overnight fast the intestinal tube was positioned under radioscopic control. Once the intestinal perfusion site was located at the proximal jejunum the test started by perfusing PSP at a flow rate of 1 ml/min. Before meal ingestion, jejunal effluents were collected through the tube by continuous suction for 20 min. These 20 min samplings were considered to be the basal period. At  $T_0$ , subjects ingested either 300 ml  $^{15}\text{N}$ -labelled milk ( $n$  7) or 300 g  $^{15}\text{N}$ -labelled yoghurt ( $n$  9). Jejunal effluents were then pooled in 20 min intervals over ice for 4 h. At the end of the experiment the subjects swallowed a gastric tube. The gastric contents were completely aspirated and the stomach was washed with 200 ml 150 mM-NaCl solution. The effluents were treated with 0.1 mM-diisopropylfluoro-

Table 1. Amounts (g) of dry matter, protein, carbohydrate and lipid ingested by humans fed with 300 ml milk or 300 g yoghurt

	Milk (300 ml)	Yoghurt (300 g)
Dry matter	42.6	42.6
Protein (N × 6.25)	10.2	10.8
Carbohydrate	13.5	12.6
Lipid	15.0	13.8

phosphate (Sigma Chemical Co., La Verpillière, France), then frozen at  $-20^{\circ}$  and lyophilized.

#### Protein precipitation

Precipitable N (PN) and soluble N (NPN) were measured after ethanol precipitation of the dry samples as previously described (Mahé *et al.* 1994c). Samples were precipitated with ethanol (700 ml/l) and centrifuged at 2400 g for 20 min at  $4^{\circ}$ . The pellet was believed to consist of proteins (PN) and the supernatant fraction to contain peptides and free amino acids (NPN).

#### Isotope ratio analysis

Total N and <sup>15</sup>N enrichment in the dry digesta as well as in the PN and NPN fractions were measured by isotope-ratio mass spectrometry (IRMS) as previously described (Mahé *et al.* 1994c). Briefly, a portion of the lyophilized sample was burned in the presence of O<sub>2</sub> in an elemental analyser (CHN-O-rapid; Heraeus, Hanau, Germany) at  $950^{\circ}$ . The combustion unit was coupled with an isotope-ratio mass spectrometer (delta E; Finnigan MAT, GmbH, Bremen, Germany). The isotope <sup>14</sup>N:<sup>15</sup>N ratio of N<sub>2</sub> was measured with reference to a secondary laboratory standard. The values were calculated in atom % relative to atmospheric N<sub>2</sub>.

#### Calculations and statistical analysis

The jejunal flow rate was calculated from PSP concentration in the effluents and the perfusion solution. The exogenous N was calculated as follows:

$$N_{\text{exo}} = N_{\text{tot}} \times (AP_s - AP_0) / (AP_a - AP_0),$$

where  $N_{\text{tot}}$  is the amount of total N in the sample,  $AP_s$  and  $AP_a$  are the <sup>15</sup>N enrichment of the sample and of the diet respectively and  $AP_0$  is the natural enrichment in the intestinal segment before ingestion of exogenous <sup>15</sup>N. The endogenous N in the digesta ( $N_{\text{end}}$ ) was calculated from the difference  $N_{\text{end}} = N_{\text{tot}} - N_{\text{exo}}$ . The results were expressed as means with their standard errors. For differences between groups an ANOVA was done using the general linear model procedure (GLM, SAS/STAT 6.03; SAS Institute Inc., Cary, NC, USA). Time effects within each group were tested for significance ( $P < 0.05$ ) using Tukey's studentized range test.

## RESULTS

#### Protein, non-protein nitrogen and total nitrogen flow rates

The flow rate of total N was measured at the jejunal level after the ingestion of either milk or yoghurt (Fig. 1). Before meal ingestion the N flow rate in the jejunum was 4.7 (SEM 0.8) mmol/20 min in the milk group (M;  $n$  7) and 3.9 (SEM 0.3) mmol/20 min in the yoghurt group (Y;  $n$  9). After milk ingestion the total N flow rate peaked at 20 min and

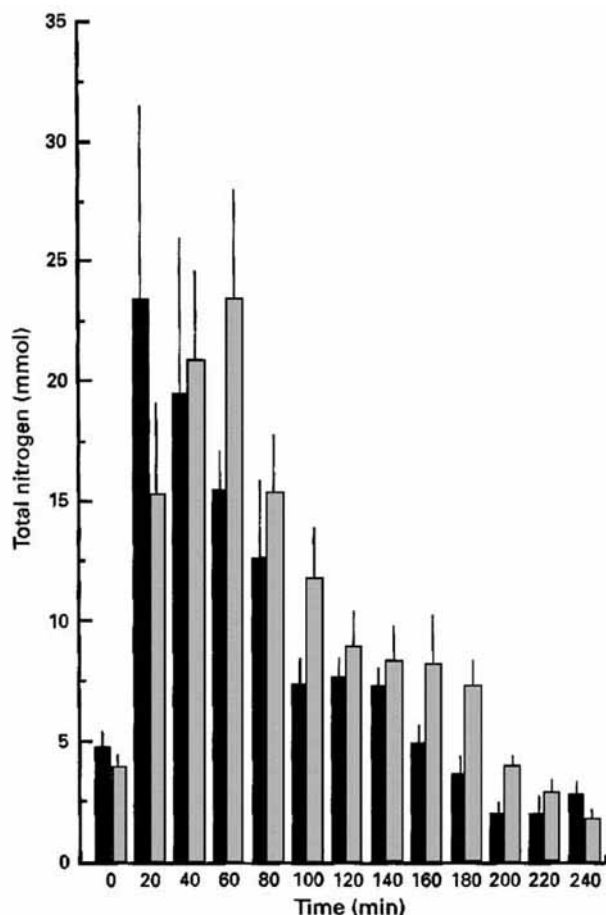


Fig. 1. Total nitrogen flow rates (mmol/20 min) in the jejunum of humans ingesting either 300 ml [ $^{15}\text{N}$ ]milk (■) or 300 g [ $^{15}\text{N}$ ]yoghurt (▨). Values are means for seven (milk) or nine (yoghurt) subjects, with their standard errors indicated by vertical bars. For details of products and procedures, see Table 1 and pp. 252–253.

decreased progressively to return to a basal level after 160 min. In subjects given yoghurt the total N flow rate was maximum at 60 min and returned to an initial level after 200 min.

In order to characterize further the total N fraction in the jejunal effluents the PN and NPN fractions were separated by ethanol precipitation (Fig. 2). Before meal ingestion the PN fraction represented 45% of the basal N secretion. In subjects fed with milk the PN fraction increased significantly compared with the basal level during the 20–40 min period ( $P < 0.05$ ). After yoghurt ingestion a significant increase in total PN was observed for the 20–60 min period. The NPN fraction showed larger variations than PN. After milk ingestion the flow rate of NPN peaked at 20 min to a value of 14.4 (SEM 5.8) mmol/20 min ( $n$  7) and returned to a basal level at 180 min. The flow rate was significantly higher than the basal level until 80 min ( $P < 0.05$ ). After yoghurt ingestion the NPN flow rate increased significantly for 100 min with a maximum of 16.5 (SEM 3.8) mmol/20 min ( $n$  9) at 60 min.

#### *Exogenous and endogenous nitrogen flow rates*

The measurement of the  $^{15}\text{N}$  enrichment in the jejunal effluents allowed us to determine the contribution of exogenous sources to the PN, NPN and total N amounts (Fig. 3). The

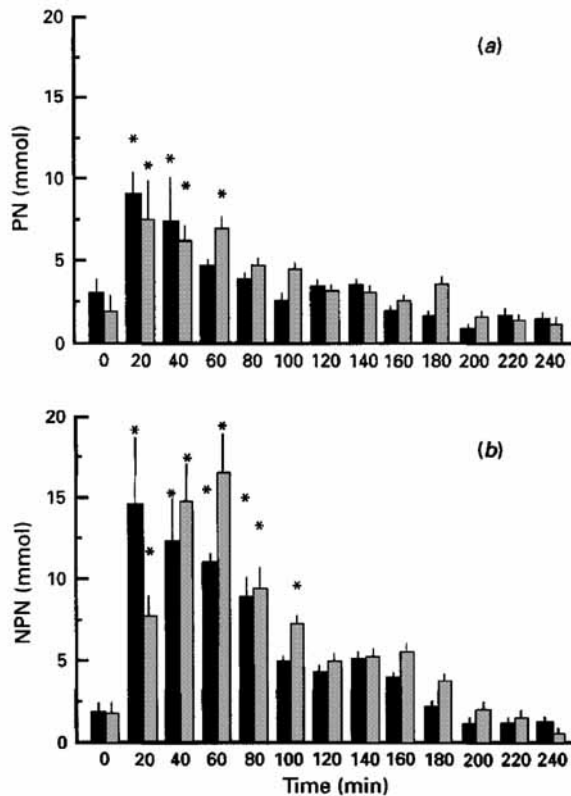


Fig. 2. Flow rates (mmol/20 min) of (a) protein nitrogen (PN) and (b) non-protein nitrogen (NPN) in the jejunum of humans ingesting either 300 ml [<sup>15</sup>N]milk (■) or 300 g [<sup>15</sup>N]yoghurt (▨). Values are means for seven (milk) or nine (yoghurt) subjects, with their standard errors indicated by vertical bars. \* Mean value was significantly different from the basal value,  $P < 0.05$  (Tukey's studentized range test). For details of products and procedures, see Table 1 and pp. 252–253.

kinetics of the exogenous N flow rate were similar to those of the total N with a maximum at 20 min for subjects given milk and 60 min for those given yoghurt. After milk ingestion the proportion of NPN increased from 70% at 20 min to 78% at 100 min and then decreased to 40% at 240 min. In the jejunum of humans ingesting yoghurt the NPN fraction peaked at 60 min and represented 64% of the exogenous N at this time and then decreased to 37% at 240 min. The jejunal flow rates of the PN, NPN and total endogenous N are shown in Fig. 4. The total endogenous N flow rate was similar in both groups, with a significant increase ( $P < 0.05$ ) compared with the basal level for 40 min for subjects given milk and for 60 min for subjects given yoghurt. The proportion of NPN was maximal at 67% of the total endogenous N at 60 min after milk ingestion and at 40 min after yoghurt ingestion.

#### *Endogenous nitrogen secretion and exogenous nitrogen absorption*

The sum of the different fractions of N recovered at the jejunal level during the 240 min following meal ingestion indicated that approximately 59% of the endogenous N and 69% of the exogenous N were in the form of NPN without any significant difference between subjects given milk and those given yoghurt (Table 2). Taking into account the quantity of

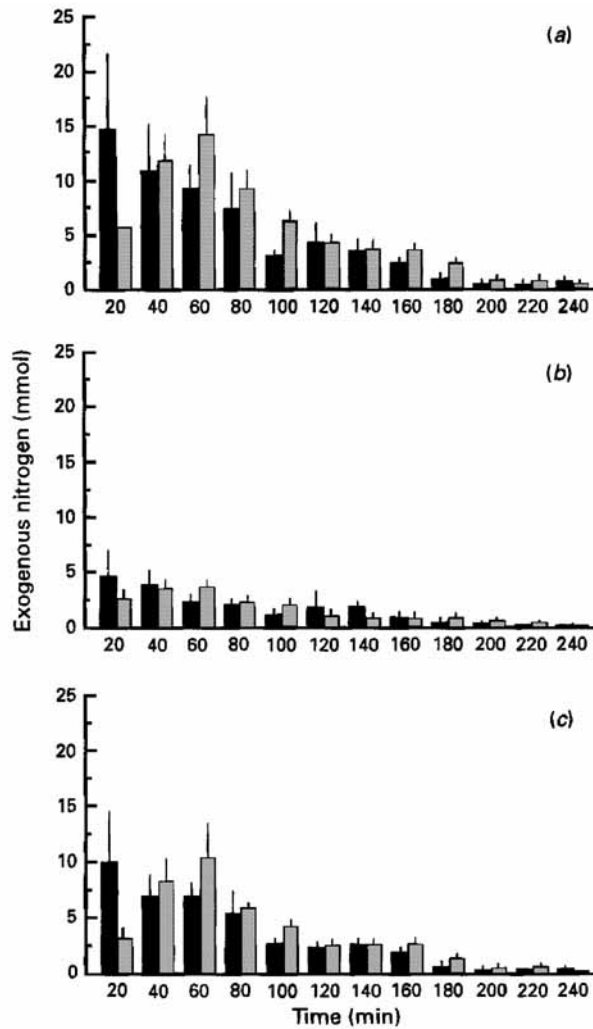


Fig. 3. Flow rates (mmol/20 min) of (a) total, (b) protein and (c) non-protein exogenous nitrogen in the jejunum of humans ingesting either 300 ml [ $^{15}\text{N}$ ]milk (■) or 300 g [ $^{15}\text{N}$ ]yoghurt (▨). The exogenous fraction was calculated from the  $^{15}\text{N}$ : $^{14}\text{N}$  ratio in the jejunal effluents. Values are means for seven (milk) or nine (yoghurt) subjects, with their standard errors indicated by vertical bars. For details of products and procedures, see Table 1 and pp. 252–253.

N ingested and the recovery of exogenous N, the percentages of exogenous N absorbed at the jejunal level were 57 and 51% after milk and yoghurt ingestion respectively. No statistical differences were observed between the two groups of subjects, either for exogenous N absorption or for endogenous N secretion.

#### DISCUSSION

The  $^{15}\text{N}$  labelling of milk protein N allowed us to distinguish exogenous from endogenous N fractions in the human intestine after milk and yoghurt ingestion. In the present study this technique was used to demonstrate that (1) the gastro-duodenal transit of dietary N is

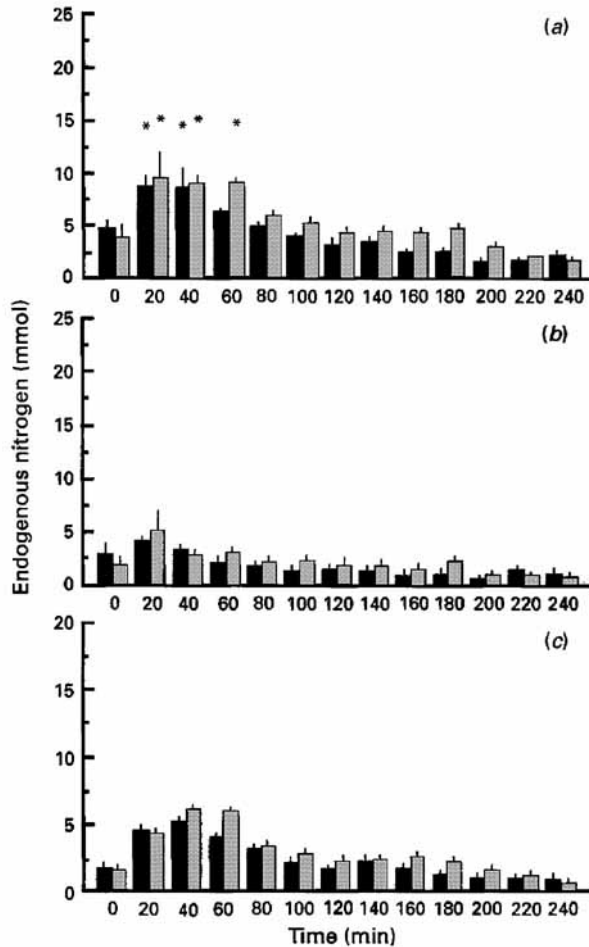


Fig. 4. Flow rates (mmol/20 min) of (a) total, (b) protein and (c) non-protein endogenous nitrogen in the jejunum of humans ingesting either 300 ml [<sup>15</sup>N]milk (■) or 300 g [<sup>15</sup>N]yoghurt (▨). The endogenous fraction was calculated as the difference between total nitrogen and the exogenous nitrogen fraction. Values are means for seven (milk) or nine (yoghurt) subjects, with their standard errors represented by vertical bars. \* Mean values were significantly different from the basal value ( $P < 0.05$ , Tukey's studentized range test). For details of products and procedures, see Table 1 and pp. 252–253.

delayed with yoghurt in comparison with milk; (2) milk proteins are rapidly hydrolysed and absorbed in the proximal jejunum after both milk and yoghurt ingestion; (3) both milk and yoghurt significantly stimulate the endogenous N flow rate in the jejunum.

As previously observed (Gaudichon *et al.* 1994a; Mahé *et al.* 1994b), the N flow rate increased in the proximal jejunum after both milk and yoghurt ingestion but was delayed with yoghurt in comparison with milk. The difference could be due to differences in either the transit of the meal or the endogenous N response. The recovery of <sup>15</sup>N in the intestinal lumen, which allowed us to calculate the exogenous N flow rate, clearly showed that the gastro-duodenal transit of the dietary N was delayed with yoghurt compared with milk due to a difference in the gastric emptying of the two diets. As previously described, this delay was due to a difference of food consistency and viscosity between milk and yoghurt (Houghton *et al.* 1987; Siegel *et al.* 1988; Mahé *et al.* 1994b).

Table 2. Protein nitrogen (PN) and non-protein nitrogen (NPN) yields in the jejunal effluents of human subjects 240 min after milk (M) and yoghurt (Y) ingestion\*  
(Mean values with their standard errors)

Amount ingested (mmol)	n	Amount recovered (mmol)†								Net disappearance (%)		
		Endogenous‡				Exogenous‡						
		NPN		PN		NPN		PN				
		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM			
M	116	7	24.6	9.6	16.6	6.8	33.8	15.7	15.7	9.5	56.7	8.5
Y	123	9	35.5	12.0	25.1	11.8	41.7	22.8	18.1	5.2	50.9	7.0

\* For details of products and procedures, see Table 1 and pp. 252–253.

† No significant differences were found between the two groups ( $P > 0.05$ , ANOVA).

‡ Exogenous and endogenous N fractions were calculated from the  $^{15}\text{N}:^{14}\text{N}$  ratio in jejunal effluents.

After milk and yoghurt ingestion the endogenous N flow rate increased significantly compared with the basal level. This increase could have been due to both a washing effect of the meal and a stimulation of gastric and bilio-pancreatic secretions. Such a stimulation was, however, not previously observed in subjects ingesting purified  $^{15}\text{N}$ -labelled casein (Mahé *et al.* 1994c). This discrepancy probably arises from the differences in meal composition including higher energy intake and the presence of carbohydrates, lipids and a variety of proteins which can all influence pancreatic secretion (Corring *et al.* 1989). Both meals tested in the present study had similar compositions and energy values and no difference of endogenous N stimulation after milk and yoghurt ingestion was observed. These observations suggest that the putative effect of casein-derived peptides on cholecystokinin production and intestinal secretions (Liddle *et al.* 1986) is not a major stimulant in humans, and that other factors present in the meal stimulate the endogenous N secretion. The present results also indicate that both milk and yoghurt proteins are rapidly degraded and absorbed in the human intestine. With both diets, exogenous NPN, i.e. hydrolysed fractions, represented 70–80% of the exogenous N. This indicates that most of the milk and yoghurt proteins were in a hydrolysed form at the jejunal level. Similar values were reported in humans fed with purified casein (Mahé *et al.* 1994c). The secretion of proteases in the duodenum appears to be sufficient that the enzymic system is never saturated even when large amounts of dietary N are delivered into the intestine. The percentage exogenous N absorption in the jejunum was approximately 50% for both diets, indicating that a large fraction of the milk proteins was already absorbed in the duodenum. Another important aspect is the sensitivity of the endogenous N to digestion. The results showed that the endogenous PN *v.* NPN fractions remained stable, indicating a constant digestive secretion hydrolysis level.

The present results must be compared with those obtained from other studies in which the contribution of endogenous N was estimated in subjects ingesting a protein-free control diet (Mahé *et al.* 1992, 1994b), i.e. omitting the stimulation of endogenous N secretion by protein in the meal. This comparison shows that the error resulting from the use of this technique is not negligible. Several authors attempted to improve this technique by supplementing the protein free-diet with a casein hydrolysate that was supposedly completely digestible (Butts *et al.* 1993) or by giving a parenteral infusion of amino acids (DeLange *et al.* 1989) in order to stimulate the endogenous N secretion. Although these



improvements are of interest, the first method could not be employed in a jejunal study because the absorption of the hydrolysate would not be achieved. Moreover, tracer methods are much more accurate in digestion studies since they allow the nitrogenous fractions to be followed directly. Due to <sup>15</sup>N recycling and subsequent reappearance in the intestine, exogenous N absorption may be underestimated. However, in a 4 h study period the perturbation should be very slight, taking into account the time the amino acids need to appear in the plasma and to be incorporated into synthesized intestinal proteins. <sup>14</sup>C-labelled amino acids have been shown not to reappear in the intestinal lumen of pigs via pancreatic secretions before 3–4 h after oral ingestion (Simon *et al.* 1983). In miniature pigs the overestimation of the total exogenous N from <sup>15</sup>N dilution was less than 5% after 6 h when compared with reference values obtained with guanidinated casein (Hagemester & Roos, 1991). In humans we showed that [<sup>15</sup>N]leucine, when infused directly into the plasma, appeared in digestive proteins after 2 h (Gaudichon *et al.* 1994*b*). In return, N can be recycled directly in the enterocyte without entering into the venous circulation. Little is known about this route of recycling and it would be of interest to quantify the consequent perturbation.

In conclusion, our results confirm that milk proteins are a good product for humans with regard to N intestinal availability. The <sup>15</sup>N-dilution technique is an appropriate method and helps to discriminate directly exogenous and endogenous N fractions in the human intestine after milk and yoghurt ingestion. We have demonstrated that both milk and yoghurt significantly stimulate the endogenous N flow rate in the jejunum and that a constant level of hydrolysis of digestive secretion is observed.

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