In Vitro Simulation of Gastric Digestion of Milk Proteins: Comparison between in Vitro and in Vivo Data

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Our previously proposed in vitro model simulating gastric digestion of proteins was automated. Milk digestion products obtained with this automated "artificial stomach" were then compared to results obtained in vivo with preruminant calves. By manipulating four essential parameters, emptying rate of fresh matter, pH decrease, nature of enzyme, and enzyme/substrate ratio, the kinetics of gastric emptying of digestive products observed for 6 h in vivo was quantitatively and qualitatively reproduced in vitro in half the time. Therefore, our model could have a great number of applications in the field of human and animal nutrition, particularly in testing the effect of different processing and preparation procedures on the digestion of food.

Gastric emptying of digestive products is a determinant factor in digestion. The stomach ensures the peptic hydrolysis of proteins and also determines the nature of products entering the gut. This progressive and sequential emptying of various gastric products could have an important role in regulating gastric and pancreatic secretions. Moreover, this digestive process is primarily responsible for the composition of the pool of available amino acids.

To study gastric digestion of proteins, a previously proposed in vitro method was used (Savalle et al., 1990) which simulates gastric emptying of digestive products. This model is based on in vivo data obtained during digestion of milk proteins in the preruminant calf stomach. Several essential parameters were used to simulate within 3 h the most important changes occurring in vivo for 6 h.

Milk was used as a model since its proteins are well-known and often used as a reference because of their nutritional qualities. Milk proteins contain sequences with possible biological functions, i.e., phosphopeptides and β-casomorphins (Meisel et al., 1989); hence, it is important to know how and when these fragments appear in the gut. Since milk is often consumed after it has undergone various technological processings, this in vitro model could be a useful tool to study the effect of such treatments on gastric protein digestion.

The aim of this study was to automate the system and to verify that a workable model portraying in vivo gastric digestion of proteins can be obtained by manipulating four essential parameters—emptying rate of fresh matter, pH decrease, nature of enzyme, and enzyme/substrate (*E*/*S*) ratio. Milk digestion products obtained with this "artificial stomach" were then compared to results obtained with preruminant calves (Pélissier et al., 1983; Yvon et al., 1985; Scanff et al., 1990).

MATERIALS AND METHODS

Experimental Procedures. *Diets.* For in vivo experiments, bovine raw milk was used (Pélissier et al., 1983; Scanff et al., 1990). To estimate the proportion of endogenous secretions in the emptied fresh matter, polyethylene glycol (PEG) 4000 was added to a final concentration of 1% just before animal feeding (Scanff et al., 1990). For in vitro experiments, nonfat low-heat powder (Nilac, Nederlands Institut voor Zuivelonderzock, Ede, Netherlands) milk was reconstituted with deionized water to obtain a protein concentration similar to that of the milk used for in vivo experiments (8.3% w/v). The low-heat processing of Nilac milk powder does not affect proteins (no denaturation occurred).

In Vivo Experiments. Details of in vivo experiments were described previously (Pélissier et al., 1983; Scanff et al., 1990). Seven days before digesta sampling, preruminant calves were fitted with double-proximal duodenal cannula (Ash, 1962). The day before duodenal sampling, sodium citrate (11 g/kg DM) was added to milk offered in the morning to prevent abomasal curd formation and to increase the rate of gastric emptying. In the evening, calves received only water to ensure almost complete emptying of the abomasum. On the day of sampling all animals received a test meal [190 g/kg$^{0.75}$ live weight (Scanff et al., 1990) or 5 kg (Pélissier et al., 1983)] of whole or skim milk. The entire stomach effluent was then passed through the proximal part of the cannula and collected in 12% trichloroacetic acid (TCA) over 7 h with fractions taken every 10 min during the first 30 min and every 30 min up to the end of the 7-h period. Collected digesta were replaced by either a milk hydrolysate (Pélissier et al., 1983) or digesta coming from another calf fed the same diet (Scanff et al., 1990). The replacement digesta returned to the animal through the distal part of the cannula.

In Vitro Experiments. The experimental procedure was performed as previously described (Savalle et al., 1989) with some modifications for automation. A 1-L Erlenmeyer flask containing 500 mL of the diet was shaken at 150 oscillations of 5 mm/min in a 37 °C water bath (Haale SWB 20, Munich, FRG) to simulate the stomach. Acidification of the medium was ensured by a peristaltic pump (Ismatec, Bioblock, France) and a three-way valve (Burkert, Germany, both of which were computer controlled. The position of this three-way valve regulated the passage of water and 0.25 mol/L HCl at a constant flow rate of 2.0 mL/min. During digestion, the pH of the medium was continuously measured with a pH meter connected to the computer. Depending on the pH, the valve allowed automatic addition of either water or HCl so that the pH of the medium followed a predetermined curve. This curve was established by setting the initial pH at 6.7, the intermediate pH (90 min) at 3.2, and the final pH (180 min) at 2.0 according to an exponential function.

Addition of enzyme and emptying of digesta were similarly monitored using peristaltic pumps connected to the computer. Liquid rennet (520 mg of chymosin, 290 mg of pepsin/L; Granday, France) was initially added to obtain an enzyme/substrate ratio of 1/2000 (w/w). Diluted enzyme [2% liquid rennet in water (v/v)] was then added at a variable flow rate following an exponential function with initial and final flow rates of 8.0 and 1.0 mL/min, respectively, and an exponential base of 1.07. A similar function was used for the emptying curve with initial and final flow rates of 10.0 and 3.0 mL/min, respectively.

A total of 10 effluent samples were collected over a 3-h period, with samples taken every 10 min during the first 30 min, every

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Table I. Amino Acid Composition of Milk, Casein, and Whey Proteins (Moles per 100 Moles of Total Amino Acids)

amino acid	milk	casein	whey
Asx[a]	7.5	6.9	10.6
Thr	4.6	4.7	5.5
Ser	6.9	7.0	6.0
Glx[b]	17.8	18.5	14.8
Pro	10.7	11.6	5.5
Gly	3.3	3.1	4.1
Ala	4.9	4.5	6.9
Cys	0.5	0.2	2.2
Val	7.1	7.3	5.3
Met	2.2	2.6	2.5
Ile	4.9	5.0	4.9
Leu	9.9	9.0	11.6
Tyr	3.1	2.8	3.5
Phe	4.2	4.0	4.0
His	2.4	2.4	1.8
Lys	7.3	7.7	8.6
Arg	2.5	2.7	2.0

[a] Asx, aspartic acid + asparagine. [b] Glx, glutamic acid + glutamine.

15 min during the following hour, and every 30 min up to the end of the 3-h period. To stop the enzymatic digestion, each sample was immediately raised to pH 8.0 with 2 mol/L NaOH using a pH stat. All assays were done in triplicate.

Collecting of Digesta and Sample Analysis. *Sample Preparation.* Each sample was precipitated with TCA at a final concentration of 12% and centrifuged at 10000*g* for 20 min.

The pellet was resuspended in deionized water and solubilized by increasing the pH to 7 with 11 mol/L NaOH. Supernatants and precipitates were separated and stored at −20 °C until analysis.

Chemical Analysis. In vivo endogenous secretions were determined (Scanff et al., 1990) from the concentration of PEG measured according to the method of Hyden (1955).

Total nitrogen in the diets, TCA supernatants, and TCA precipitates were determined according to the Kjeldahl method using the colorimetric technique of Koops et al. (1975). Nonprotein nitrogen (NPN) and protein nitrogen (PN) represented the nitrogen content of the supernatant and precipitate, respectively. The extent of hydrolysis was calculated as NPN/(PN + NPN).

Amino acid compositions were determined after acid hydrolysis (110 °C, 24 h, 5.7 mol/L HCl, under vacuum) according to the method of Spackman et al. (1958) with a Biotronik LC 5000 amino acid analyzer (Munich, FRG).

Calculations and Statistical Analysis. *Fresh Matter Emptying.* The mean of endogenous secretions was calculated from in vivo data for the milk diet (Scanff et al., 1990). This mean was then subtracted from the emptied volumes in all in vivo experiments with milk (Pélissier et al., 1983; Scanff et al., 1990).

Statistics. Data are stated as mean values of the groups ± SEM.

Amino Acid Analysis. Amino acid compositions were determined separately for 12% TCA-soluble and 12% TCA-insoluble fractions of digesta.

Casein and whey constitute the two protein fractions of milk. Because of varying amino acid compositions (Table I) it is possible to estimate the proportion of these major fractions in milk and digesta. Casein and whey protein percentages in digesta were estimated to allow the best fit between measured and calculated compositions. Comparison between measured and calculated composition was performed using χ^2 test (Guilloteau et al., 1980, 1986) with the smallest χ^2 value indicating the smallest difference in composition.

Quantities of whey proteins and casein emptied at each digestion time were determined in the TCA-insoluble fraction of digesta. Since whey proteins are previously reported to be resistant to proteolysis (Yvon et al., 1985), the TCA-soluble fraction was assumed to contain only peptides from casein.

RESULTS

In Vivo Verification of Set Parameters. *Stomach Acidification.* During development of the artificial stomach (Savalle et al., 1989), the acidification was obtained by adding 143 mmol/L HCl (N/7) at 2.0 mL/min with the pH decreasing from 6.7–6.8 to 2.0 after 3 h. Thus, the acidification rate was 2-fold faster in vitro than in in vivo experiments using calves. A similar predetermined acidification curve was automatically followed in the present work. The in vivo pH of the abomasal effluents varied greatly between trials; however, the in vitro acidification curve remained constant (Figure 1).

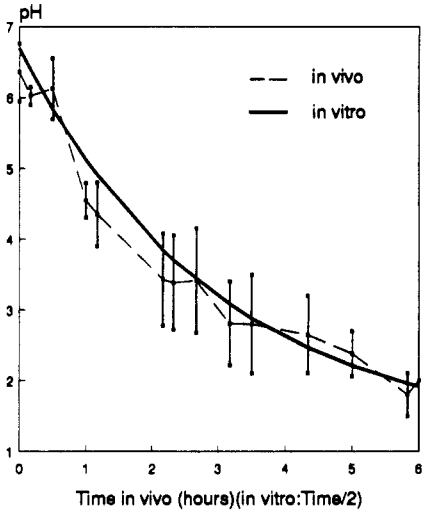

Figure 1. Change in pH of in vivo and in vitro gastric effluents. Values are means, high and low values, for two in vivo experiments and means for three in vitro experiments (SEM was ca. 0).

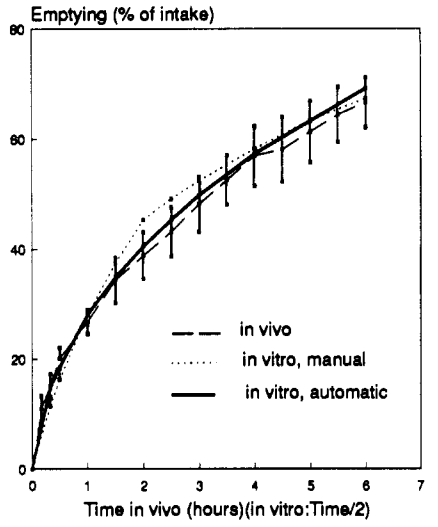

Figure 2. In vivo and in vitro (manual and automated systems) cumulative emptying of fresh matter from diet. Values are means ± SEM for nine in vivo experiments and imposed values for in vitro experiments.

Emptying Rate. Figure 2 shows the cumulative flow of fresh matter leaving the stomach. In vivo, 67% of the intake was recovered after 6 h with wide variability between trials. The same recovery was obtained at 3 h, using the emptying conditions developed in the present work with the in vitro model. The in vitro emptying rate of fresh matter from the diet is very similar to that observed in in vivo experiments. Use of an exponential emptying rate rather than the three-step system (manual system) previously described (Savalle et al., 1989) improved the emptying curve profile, especially during the first hour of in vitro digestion.

Nitrogen Emptying. *Emptying of Total Nitrogen.* From in vivo experiments, the mean cumulative recovery

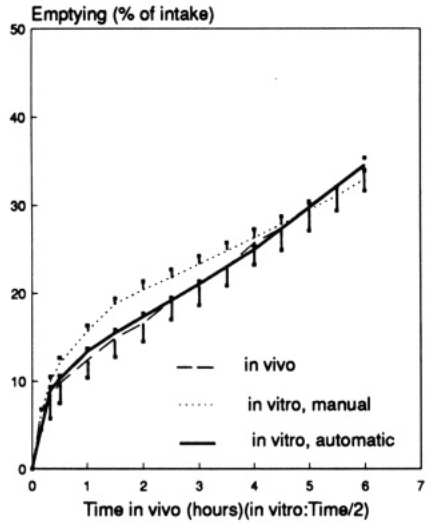

Figure 3. In vivo and in vitro (manual and automated systems) cumulative emptying of total nitrogen. Values are means for nine in vivo and three in vitro experiments. Vertical bars are SEM.

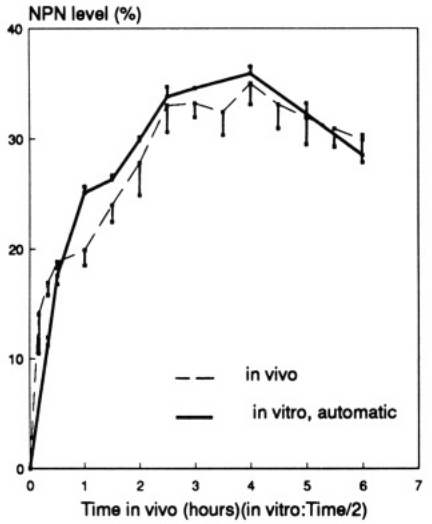

Figure 4. In vivo and in vitro (automated system) proteolysis (%) of digesta. Values are means for nine in vivo and three in vitro experiments. Vertical bars are SEM.

obtained at 6 h was 34.6% of the intake (Figure 3), i.e., half of the fresh matter recovery. This reduction in nitrogen emptying, compared to fresh matter emptying, is due to casein coagulation. As for fresh matter, the standard deviation between trials was high.

Data concerning in vitro clotting were very close to in vivo results since a similar nitrogen emptying rate was obtained.

Improvement of fresh matter emptying by the automated system led to a similar improvement in nitrogen emptying with in vitro experiments also giving excellent reproducibility (mean coefficient of variation = 4.2%).

Nitrogen Distribution between 12% TCA-Soluble and TCA-Insoluble Fractions. The level of 12% TCA-soluble nitrogen (NPN) vs total nitrogen in digesta is shown in Figure 4. This ratio indicates the degree of protein hydrolysis.

During the first 3 h after in vivo feeding, the mean NPN level increased from an initial level of ca. 3% in milk to about 35%, with this level decreasing slightly during the last hour of the experiment. As previously observed, the standard deviation of in vivo experiments was high.

In vitro experiments yielded similar but more reproducible results (mean coefficient of variation = 4.9% vs 20.9% in vivo).

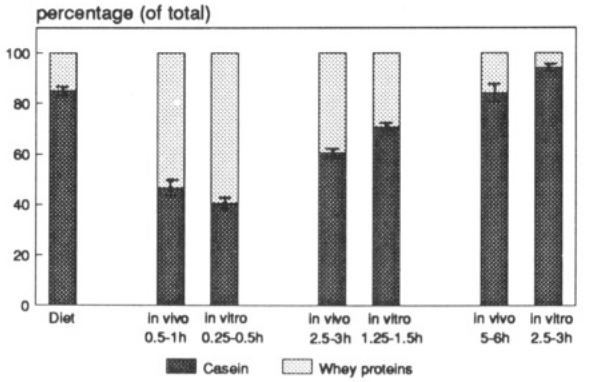

Figure 5. Percentages of casein and whey protein in the diet and in effluents collected during the periods 0.5–1 h in vivo and 0.25–0.5 h in vitro, 2.5–3 h in vivo and 1.25–1.5 h in vitro, 5–6 h in vivo and 2.5–3 h in vitro. Proportions were determined from amino acid patterns. Values are means ± SEM for four in vivo and three in vitro experiments (automated system).

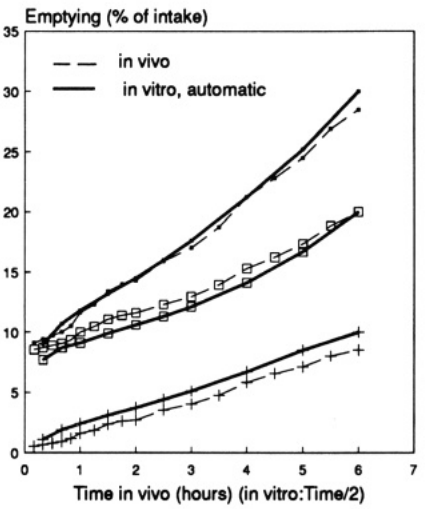

Figure 6. In vivo and in vitro cumulative emptying of total casein (■), TCA-insoluble casein (□), and TCA-soluble casein (+) determined from amino acid patterns. Values are means for four in vivo and three in vitro experiments.

Characterization of Products Leaving the Stomach. *Emptying of Whey Proteins and Casein.* Protein composition of the diet calculated from the amino acid composition (Figure 5) is close to the theoretical value, i.e., 85% casein and 15% whey proteins. In digesta collected between 0.5 and 1 h in vivo postfeeding, as in those obtained with the automated system in vitro between 0.25 and 0.5 h, the concentration of whey proteins increased markedly with a corresponding decrease in casein. Thereafter, the casein proportion progressively increased in gastric effluents, reaching 85% in digesta collected 5–6 h after feeding. Similar levels were observed in vitro in digesta collected between 2.5 and 3 h. Thus, in both in vivo and in vitro experiments, the whey proteins emptying was directly related to that of fresh matter while cumulative flow of casein reached only 29% and 31% of the intake, respectively.

Casein Emptying. Whereas whey proteins were only present in the TCA-insoluble fraction, dietary casein was emptied from the stomach either as TCA-insoluble fragments or as TCA-soluble peptides (Figure 6). During the first hour after in vivo feeding, 10% of the ingested casein was emptied mainly as TCA-insoluble products. Thereafter, the flow of insoluble casein peptides decreased for

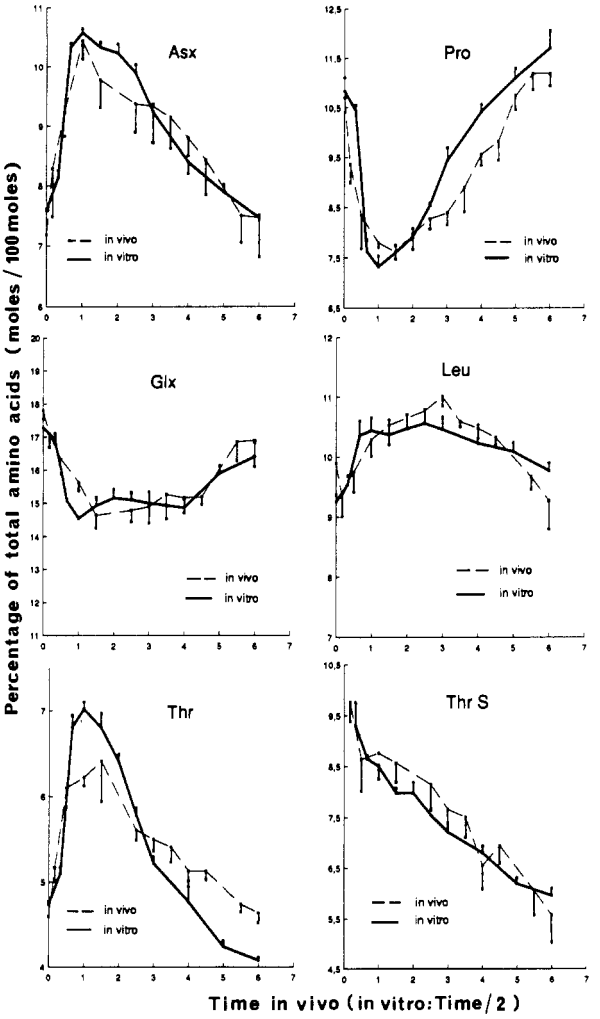

Figure 7. Percentages of asparagine + aspartic acid (Asx), proline (Pro), glutamine + glutamic acid (Glx), leucine (Leu), and threonine (Thr) in the 12% TCA-insoluble fraction of digesta and percentage of threonine (Thr S) in the 12% TCA-soluble fraction of digesta collected in vivo and in vitro (automated system). Percentages are related to the total amino acid content of analyzed fraction. Values are means for four in vivo and three in vitro experiments. Vertical bars are SEM.

3 h with the final cumulative emptying reaching 20% of the intake at 6 h postfeeding. TCA-soluble peptides from casein gradually appeared in digesta. Following 6 h of digestion, the cumulative flow of these peptides represented 8.5% of the casein intake.

These results from in vivo experiments were similar to those obtained in in vitro experiments with 3 h of in vitro digestion corresponding to 6 h of in vivo digestion.

Amino Acid Composition of Gastric Effluents. Gastric selection of protein products that enter the gut will, over time, alter the amino acid composition (obtained after acid hydrolysis) of the digesta. The percentage of each amino acid was considered separately in TCA-soluble and in TCA-insoluble fractions of each digesta.

In the TCA-insoluble fraction, which was quantitatively the most important, especially during the 2–3 h postfeeding, the level of some amino acids varied greatly with time (Figure 7). The percentages of Pro and Glx (glutamine plus glutamic acid) decreased sharply just after feeding (−32% and −20% of the initial values, respectively) and then gradually increased to initial levels 6 h after feeding. In contrast, percentages of Thr, Asx (asparagine plus aspartic acid), and Leu increased rapidly after feeding (+44%, +40%, and +15% of the initial values, respectively) and then gradually returned to their initial values. In the TCA-soluble fraction the largest change was observed for Thr. While Thr accounted for 10% of all amino acids in digesta just after feeding, this amino acid represents only 4.6% of the dietary amino acids.

Nearly identical patterns of amino acid emptying were again observed using in vitro digestion.

Because of their nutritional properties and importance in metabolism, ketogenic, branched chain, and essential amino acids have been studied (Figure 8). The percentage of these amino acid groups remained relatively constant in the TCA-insoluble fraction throughout digestion. While small changes in these amino acids were noted in the TCA-soluble fraction, these differences were not statistically significant. Hydrophobic amino acids, which reportedly stimulate the secretion of pancreatic proteins, were also determined. The percentage of these amino acids decreased (−12%) in the TCA-insoluble fraction of digesta during the first hour. Thereafter, levels slowly increased over the next 5 h. While only slight changes were observed in the TCA-soluble fraction, levels of these hydrophobic amino acids were always lower in this fraction than in the TCA-insoluble fraction. The in vitro model gave similar emptying patterns for these amino acid groups with only slight differences in the TCA-soluble fraction during the first 2 h.

DISCUSSION

The in vitro system described above simulated in 3 h the milk protein digestion observed in calves over 6 h.

From a quantitative standpoint, the in vitro kinetics of total nitrogen emptying generally agrees with in vivo data. Milk clotting, which was accurately simulated in our in vitro model, led to retention of casein in the stomach and thus decreased the nitrogen flow rate compared to that of fresh matter. Whereas casein coagulates and is retained in the stomach, whey proteins are emptied with the liquid fraction of the meal. These proteins are therefore the major constituent of digesta collected just after feeding. The proportion of casein and whey proteins in the digesta, estimated from their amino acid composition, at different times after feeding are also in good agreement with in vivo data.

Hydrolysis level of digesta and cumulative recovery of casein in TCA-soluble and -insoluble fractions determined in in vitro experiments were also similar to the results obtained with calves, which again suggests that our in vitro model accurately depicts the kinetics of casein degradation.

From a qualitative standpoint, the amino acid composition determined after acid hydrolysis of digesta is dependent on the particular makeup of proteins and peptides contents. Casein contains a higher proportion of Pro and Glx and a lower percentage of Asx and Leu than whey proteins. Consequently, these amino acids could be considered markers for casein emptying. Change in the percentage of these amino acids in in vitro and in vivo digesta over time is consistent with initial casein coagulation followed by its degradation and emptying of hydrolysis products.

Thr also can be considered a marker for caseinomacropeptide (CMP) emptying since this peptide contains a high proportion of Thr (17% in CMP vs 4.6% in milk). This peptide comes from κ-casein and is released selectively at higher pHs by chymosin hydrolysis of the Phe_{105}–Met_{106} bond. This in turn is responsible for clotting of milk. In fact, the optimum activity of chymosin on the κ-casein Phe_{105}–Met_{106} bond is approximately at pH 5.5, whereas

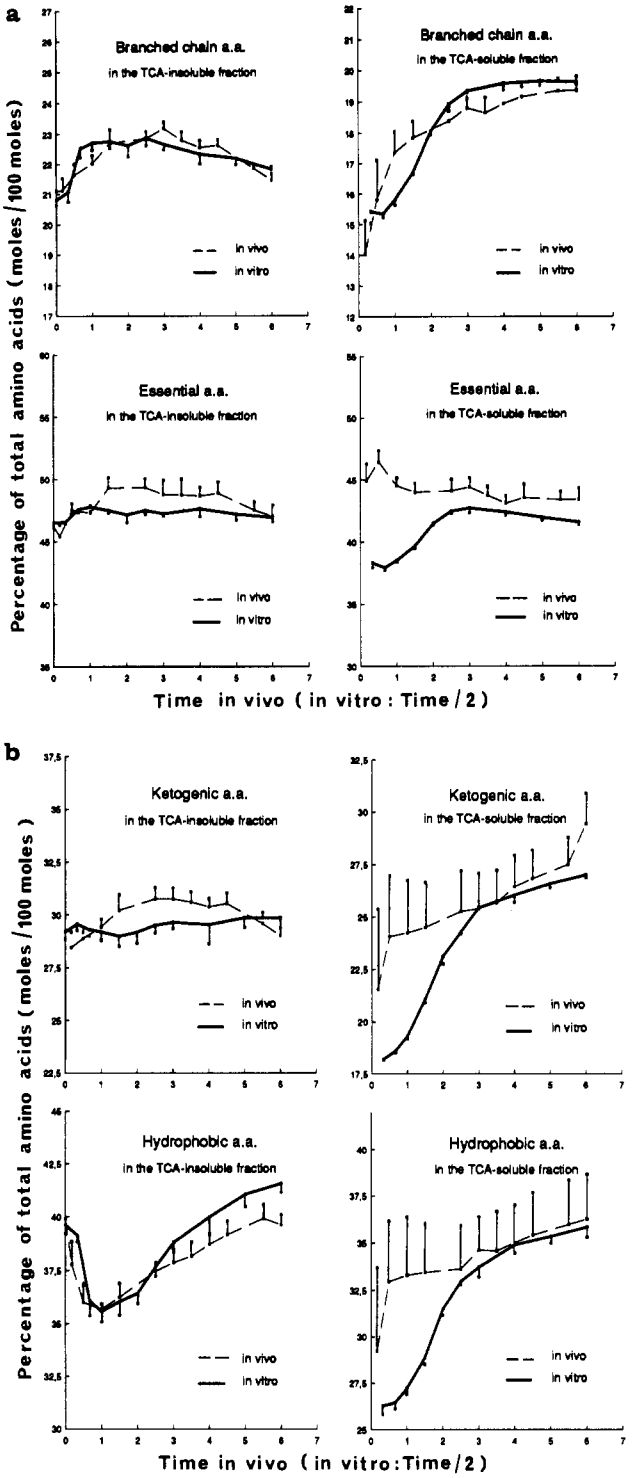

Figure 8. (a) Percentages of branched chain and essential amino acids in the 12% TCA-insoluble and -soluble fractions of in vivo and in vitro digesta. Percentages are related to the total amino acid content of the analyzed fraction. Values are means for four in vivo and three in vitro experiments (automated system). Vertical bars are SEM. (b) Percentages of ketogenic and hydrophobic amino acids in the 12% TCA-insoluble and -soluble fractions of in vivo and in vitro digesta. Percentages are related to the total amino acid content of analyzed fraction. Values are means for four in vivo and three in vitro experiments (automated system). Vertical bars are SEM.

it is between 3.5 and 4.5 on other substrates (Mercier and Garnier, 1969). The nonglycosylated and glycosylated forms of CMP are found in the TCA-insoluble and -soluble fractions, respectively. Similar changes in the level of Thr in in vivo and in vitro digesta over time shows that CMP emptying is accurately reproduced.

Gastric selection of proteins and peptides alters the composition of the amino acid pool that enters the duodenum over time. Since dietary amino acids are absorbed and metabolized according to the composition of the pool in contact with the enterocyte, gastric digestion is one of the determining factors of the bioavailability of amino acids and thus of their utilization by the organism. Several authors have reported that branched-chain amino acids enhance nitrogen metabolism and that the essential/total amino acids (E/T) ratio in an amino acid mixture affects its efficiency. If the E/T ratio or the proportion of each essential amino acid in the total of essential amino acids is improper, ingested amino acids that can quantitatively fulfill biological requirements may be catabolized spontaneously (Ikemoto et al., 1989), resulting in problems. Alladi and Shanmugasundaram (1989) found that high levels of ketogenic amino acids in an amino acid mixture increased the plasma cholesterol level, especially the LDL and VLDL fractions, which increases risk for coronary disease. The emptying patterns for these amino acid groups and hydrophobic amino acids, the latter of which may stimulate pancreatic secretion of enzymes (Meyer et al., 1976), were well reproduced by in vitro digestion. However, a few differences in the TCA-soluble fraction were observed during the first 2 h postfeeding. In these digesta fractions, concentrations of protein products were very low. The observed differences are probably due to endogenous in vivo secretions or to the emptying of residual gastric contents from the previous meal for some animals since during this time only glycosylated CMP was released from diet in this fraction. This would explain the high standard deviations observed in in vivo data.

To reproduce in vivo gastric protein digestion, some essential parameters have been set. The pH is an important factor affecting protease activity and substrate structure and thus the kinetics of protein degradation. A curve based on the gastric acidification of milk in calves (Scanff et al., 1990) was predetermined. With the automated in vitro system, the change in "intragastric" pH is thus imposed. This acidification curve is similar to that observed in the stomach of humans fed a standard meal. Bernier et al. (1988) reported a constant intragastric pH of ca. 5 during the first hour postfeeding, which then decreased to 4 or 3 for 1 h later and remained at 3 to 2.5 during the following 2 h.

Gastric emptying conditions are the second essential parameter for gastric digestion. In a previous study (Savalle et al., 1990), a good simulation of in vivo gastric emptying was obtained by using simultaneously an evacuation of digestive products and an acid and enzyme supply in a three-step system, supplied volumes making up for emptied volumes. For easier use of this system, the flow rates of all pumps were controlled by a computer which automatically generated exponential flow rate variations. Conditions that best simulated the fresh matter flow rate were in accordance with observations made in humans. Bernier et al. (1988) reported that the liquid fraction of a meal emptied exponentially and that the flow rate through the pylorus varied from 10 mL/min immediately after feeding to 2 mL/min 3 h postfeeding. Since the volume of gastric secretion was related to the volume of liquids passing through the pylorus, it appears that the gastroduodenal system helps to keep the volume of the gastric contents constant.

The last set parameter was the choice of proteases and the enzyme/substrate (E/S) ratio. Rennet, which contains chymosin and pepsin A, was chosen to simulate abomasal digestion in calves. The initial ratio E/S chosen simulated

the in vivo kinetics observed in the hydrolysis of some peptide bonds of casein (Salvalle et al., 1990). While the major human gastric protease is pepsin A, few data concerning gastric proteolysis or level of enzymatic secretions in humans are available.

After a comparison of the conditions of in vitro digestion and the observations made in humans, it appears that this model could be adapted to simulate gastric digestion in humans. However, in vivo proteolysis data are necessary to fit the enzyme supply to this model. Commercial porcine pepsin A (EC 3.4.23.1) could likely be substituted for human pepsin A since a high homology (75%) was observed between their amino acid sequences and no major difference in enzyme specificity is currently recognized.

Although our in vitro model does not account for dietary differences due to physiological regulations of gastric digestion and consequently does not replace in vivo studies, this model may be of interest to study the changes in gastric digestion due to physicochemical property changes of food. When food is processed (heating, drying, cooking, etc.), partial denaturation of proteins occurs along with various other chemical reactions between food constituents, which in turn alters the physicochemical structure of the food. These changes are of major importance in gastric digestion as it has been shown for milk in in vivo studies (Scanff et al., 1990; Kaufman, 1984; Meisel and Hagemeister, 1984; Miranda and Pélissier, 1987). In vitro simulation of gastric protein digestion is cheaper, faster, and easier and yields data that are less variable than those obtained in in vivo studies. An in vitro system has already been developed for estimating protein digestibility (Gauthier et al., 1982; Savoie and Gauthier, 1986). However, in this model, gastric digestion was reduced to a peptic batch digestion of proteins, and consequently the kinetics of digest evacuation from the stomach were not considered. Use of the artificial stomach with a pancreatic digestion system that would maintain the kinetic aspect could likely give protein digestibility measurements that would be closer to results from in vivo studies than those obtained with the previous system (Vachon et al., 1987).

Therefore, our proposed model could have a great number of applications in the field of human and animal nutrition.

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