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ORIGINAL ARTICLE

Digestibility of extruded proteins and metabolic transit of N^{ε}-carboxymethyllysine in rats

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Abstract Milk proteins are frequently used as supplements in fortified foods. However, processing produces chemical changes which likely affect the nutritional advantage. This study was intended to explore the possible difference in digestibility between extruded and non-extruded caseins and how the dietary N^{ε}-carboxymethyllysine (CML) is metabolised. Normal rats were randomized into either an extruded protein diet (EP) or the same with unextruded proteins (UEP), for two periods of 2 weeks at 7 to 9 and 11 to 13 weeks of age. However, no difference in protein digestibility was detected between the two diets, either in young or in adult animals, despite a 9.4-fold higher level of CML and an 8.5-fold higher level of lysinoalanine in the EP than in the UEP. No diet-related changes were observed in plasma CML, either protein bound or free. Amounts of 38 and 48 % of the orally absorbed CML were excreted in urine and faeces, respectively, in UEP-fed rats. Lower rates of excretion were found in the EP-fed rats (23 and 37 %, respectively). A second animal study using a single oral dose of free CML (400 μg/rat) was set up to measure the systemic concentration of CML every hour from 0 to 4 h. It revealed that protein-bound CML was not affected by the oral dose of CML, and the highest free CML level found in the circulation was 600 ng/mL. Extruded proteins, therefore, appear to be well digested, and CML rapidly eliminated. Since its elimination is, however, incomplete, the question of its biodistribution and metabolism remains open.

Keywords Nitrogen digestibility · Carboxymethyllysine · Maillard reaction · Protein · Caseins

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Introduction

In recent decades, increasing numbers of food products have been fortified with proteins. These enriched foods are used in high-protein diets aimed at weight control (Westerterp-Plantenga et al. 2009) as well as sports nutrition and also at inhibiting the consequences of denutrition in older and hospitalized patients (Koopman and van Loon 2009). They have been found to enhance satiety, to improve both performance and recovery in sports practice and to counteract muscle mass loss together with maintaining healthy bones in the elderly.

Milk proteins (caseinates and whey proteins) are frequently used as a protein supplements in fortified foods. Almost all manufactured foods selected for fortification are submitted to a high heat treatment after formulation either to increase their shelf life or to improve palatability. Depending on the type and conditions of food processing, added and native proteins may be subject to compositional modifications, which will affect their digestive utilization (Öste 1991) and eventually limit the interest of the fortification.

At high temperature, proteins are essentially damaged by the Maillard reaction which leads to the covalent binding of other molecules such as carbohydrates to the most reactive amino acids (e.g. lysine, arginine, tyrosine and cysteine) (Finot 2005a). Mauron indicated that the irreversible chemical deterioration of lysine, an essential

amino acid, is probably one of the main causes of the loss of nutritional value of protein in heated foods (Mauron 1981).

In addition to the chemical modification of lysine and, to a lesser extent, other amino acids, the Maillard reaction has also been found to decrease overall nitrogen digestibility. Paul-André Finot, for instance, has shown that 'Maillardized' peptides, as he calls them, are less easily digested compared to native peptides, especially when their size is reduced (Finot 2005b). More recently, a clinical study on adolescents suggested that a diet high in the Maillard reaction products (MRPs) limits the digestibility of proteins (Seiquer et al. 2006).

Aside from nutritional concerns, other questions have been raised about the biological effects of MRPs and other non-physiological modified amino acids found in processed proteins (Tessier and Niquet 2007). N^{ε}-carboxymethyllysine (CML), an MRP which has been the subject of many studies, has been found not only in foods, but also in vivo (Thorpe and Baynes 2002). However, its absorption from food, metabolism and elimination are only partially elucidated (Delgado-Andrade et al. 2012).

In the present study, the heated proteins tested were caseins coming from commercially extruded fortified biscuits (66 % w/w of sodium caseinate). The purpose of this study was to examine proteins, which have been processed in conditions such as those found where the fortified biscuits are actually manufactured and not on a laboratory scale. The extruded caseins were tested against the same unextruded proteins. A valid rat assay was used to measure the effect of extrusion cooking on the apparent nitrogen digestibility and nitrogen excretion in urine and faeces at two periods of life of the animals. In addition, the uptake, the systemic availability and elimination of CML were studied.

Materials and methods

Chemicals and materials

CML, (D_2)-CML and furosine were purchased from Polypeptide Laboratories (Strasbourg, France) and ($^{15}N_2$)-lysine from CortecNet (Voisins-Le-Bretonneux, France). The water, HPLC Grade, was provided from Sodipro (Echirolles, France). All the others chemical products and solvents were of the highest grade available and acquired from Sigma-Aldrich (Saint Quentin Fallavier, France).

Preparation of the two-rat dietary formulas

Extruded biscuits and the mixture of their powdered unheated ingredients, each containing 66 % of caseins, were provided by a French nutritional products company (Prodietic, France). Two types of rodent pellets were reconstituted using either the extruded biscuits or their raw ingredients as the unique source of protein. 250 g of crushed extruded biscuits or raw ingredients were mixed with 750 g of a rodent protein-free diet (U8959 version 12, SAFE, Augy, France) in order to get a final concentration of protein at 16.5 % (w/w). 500 mL of deionized water was added to homogenize the dough in a processing mixer. Both rat formulas were pelleted after filling a PVC tube (diameter 15 mm), cutting slices 30 mm in length and freeze drying them. Six batches of each type of pellet were prepared to compare an extruded protein diet (EP) to an unextruded protein diet (UEP) in rats. One sample of each batch was tested for protein, lysine, CML, furosine and lysinoalanine (LAL) contents.

Six-week dietary study in rats

Six-week-old male Wistar rats (Harlan, Gannat, France) weighing 150–175 g were used in this study. All animals were housed in a temperature-controlled animal facility (21 ± 1 °C) with a 12-h light/dark cycle and provided water and food ad libitum. Experiments were run according to the guide of care and use of laboratory animals, and procedures were approved by the county veterinary office (agreement A#60). After 1 week of adjustment with a pelleted standard maintenance diet (A04 SAFE, Augy, France), 7-week-old rats were randomly separated in two groups of diets (EP and UEP) for the 6 weeks of the study. Rats received one of the diets for two periods of 2 weeks, separated by 2 weeks of standard diet. Body weight was measured at the end of the adjustment period and then every other week throughout the duration of the study. Food intake was monitored daily in each of the housing cages of five rats and individually once a week throughout the study. Urine and faeces were collected over a period of 24 h from all rats, which were individually housed in metabolic cages on the last day of each test period. Blood samples were collected at the end of the study.

Single oral dose study of CML in rats

In a second set of experiments, 30 male Wistar rats (225–250 g) were also housed under the same conditions and had access to water and standard diet (A04 SAFE, Augy, France) ad libitum. After an overnight fast, five groups of animals received a single dose of CML (1.6 mg/kg—500 µL per os) (groups 1–4) or water (group 0).

Animals were sacrificed immediately (group 0), then every hour during 4 h (groups 2–5). At sacrifice, blood was collected in order to measure the evolution of the systemic CML level after its ingestion.

Nitrogen balance indicators

At the end of each test period (weeks 2 and 6), urine and faeces were tested for total nitrogen as described below.

The apparent digestibility coefficient of nitrogen (ADCN), the apparent nitrogen retention (ANR) and the percentage of nitrogen retained as opposed to nitrogen absorbed (RA) were determined according to the following equations.

$$\mathrm{ADCN} = [(I_N - F_N/I_N] \times 100$$

$$\mathrm{ANR} = [(I_N - (F_N + U_N)/I_N] \times 100$$

$$\mathrm{ADCN} = [(I_N - (F_N)/((I_N - F_N)] \times 100$$

represent intake, urinary and faecal excretion of nitrogen, respectively.

All the nitrogen balance indicators presented above are expressed in percentage terms as usually presented (Urbano et al. 2005). Dermal nitrogen losses were not estimated.

Chemical analysis

The nitrogen content was measured in the reconstituted pellets, urine and faeces samples by combustion using a LECO FP528 nitrogen analyser according to the Dumas method. A conversion factor of 6.38 was used for the calculation of the protein content in the pellets, which was expressed in g/100 g of powder.

50 mg of reconstituted pellets were hydrolysed with 4 mL of 6 M HCl at 110 °C for 20 h in sealed screw-cap Pyrex vials for furosine analysis. The acid was removed with a vacuum concentrator from ThermoFisher. The resulting dried samples were dissolved in deionized water, filtered through a 0.45-µm membrane filter and injected on a SpectraSystem HPLC–UV system (Schleicher and Wieland 1981). Quantification was performed by the external standard method using a standard of furosine.

CML and lysine were quantified in the reconstituted pellets by LC–ESI–MS/MS. An equivalent quantity of ground pellets to 10 mg of proteins was reduced in 1.5 mL of sodium borate buffer (0.2 M pH 9.5) and 1 mL sodium borohydride (1 M in NaOH 0.1 M) in Pyrex tubes for 4 h at room temperature. Then, hydrochloric acid was added to a final concentration of 6 M. The samples were incubated at 110 °C for 20 h, and 300 µL of each hydrolysate was dried in a Speed-Vac concentrator (Thermo Fisher Scientific, France). Each residue was reconstituted in 300 µL of nonafluoropentanoic acid (NFPA) 20 mM containing 0.5 µg/mL of D_2-CML and 50 µg/mL of $^{15}N_2$-Lys and filtered through a nylon membrane (0.45 µm) before injection on a Hypercarb 100 × 2.1 mm, 5 µm, column (Thermo Fisher Scientific, France). The chromatographic conditions were previously described according to Niquet-Léridon and Tessier (2011).

LAL was quantified in the reconstituted pellets using the same method as that used for the determination of CML and lysine. In the absence of lysinoalanine stable isotope, D_2-CML was used as an internal standard.

On the last day of the 6-week dietary study, 24-h urine and faeces were tested for CML. After lyophilisation, 25 mg samples were prepared and analysed according to the method described above. CML excretion was expressed as the quantity (in µg) eliminated in urine and faeces in 24 h. It was also adjusted for the last 24-h food intake and the related 24-h intake of CML.

Blood samples collected in heparinised tubes at the end of the 6-week dietary study and at each time point of the single-dose study were centrifuged within 1 h of collection and samples of the resulting plasma were stored at −20 °C until CML analysis. One fraction of each plasma sample was tested for the total plasma CML (free and protein-bound CML) and another fraction was treated with 1.2 M trichloroacetic acid (TCA) in a 20/1 ratio (v/v TCA/plasma) to precipitate the proteins and quantify only the protein-bound CML.

Statistical analyses

The statistical data analyses were performed using the GraphPad Prison software (version 3.0). Data characterising the two diets (Table 1) were expressed as mean ± RSD and were analysed with a student's t test. Data related to the biological effects of the diets were expressed as mean ± SD (Figs. 2, 4) and were submitted to an ANOVA followed by a Tukey post test. A $P < 0.05$ was considered as significant.

Results

Protein and neoformed compounds' quantification in the two diets

Both the EP and UEP diets had an average protein content of 16.5 g/100 g of pellets (Table 1) and were of equivalent energy and micro- and macro-nutrient content (data not shown). After acid hydrolysis treatment, the EP formula contained 73.7 ± 6.4 mg of lysine/g protein, whereas the UEP formula contained 87.7 ± 4.7 mg of lysine/g protein. The EP diet contained approximately ninefold more CML and eightfold more LAL as compared with the UEP diet. Inversely, the EP diet contained fourfold less furosine than the UEP diet (Table 1).

Table 1 Characteristics of the two diets

	Unit	UEP		EP		*P*
		Mean	RSD (%)	Mean	RSD (%)	
Proteins	g/100 g	16.4	1.4	16.6	0.2	>0.05
Lysine	mg/g	14.2	4.7	12.4	6.4	>0.05
	mg/g protein	87.7	4.7	73.7	6.4	>0.05
CML	µg/g	2.7	5.1	25.4	5.4	<0.0001
	µg/g protein	16.7	5.1	151.3	5.4	<0.0001
	mmol/mol lysine	0.14	6.2	1.47	4.0	<0.0001
Furosine	µg/g	99.8	3.4	25.1	4.4	<0.0001
	µg/g protein	561.3	3.4	137.9	4.4	<0.001
LAL	µg/g	125.6	6.8	1057.5	3.2	<0.0001
	µg/g protein	777.3	6.8	6294.9	3.2	<0.0001

UEP un-extruded protein diet, *EP* extruded protein diet

Food consumption and body weight gain

There was no significant difference in food intake between the two diets all throughout the study. During the first test period, groups of five young rats (7 to 9 weeks old) housed together in standard cages ate an average of 20.2 ± 0.6 and 22.4 ± 2.5 g/rat/24 h for the UEP and EP diets, respectively. On the last day of the first test period, rats housed in individual metabolic cages ate an average of 19.2 ± 3.0 and 18.8 ± 3.0 g/rat/24 h of UEP and EP diet formulas, respectively. During the second test period, groups of five older rats (11 to 13 weeks old) housed in standard cages ate 17.7 ± 1.6 and 19.7 ± 0.1 g/rat/24 h for the UEP and EP diets, respectively. In addition on the last day of the second test period, rats housed in individual metabolic cages ate an average of 19.3 ± 4.2 and 15.9 ± 5.5 g/rat/24 h, respectively.

There was no significant effect of the diet on body weight in line with the similar food intake recorded and the nutritional composition of the two diets. During the first 2-week test period, the body weight of young rats increased more rapidly than during the second 2-week test period when rats were older (Fig. 1). The average body weight gains for both diets were 5.01 g/day in young rats (7 to 9 weeks of age) and 1.95 g/day in adult rats (11 to 13 weeks of age).

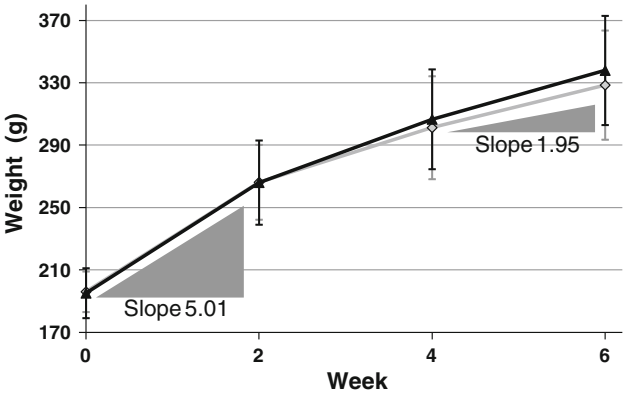

Fig. 1 Body weight of rats on UEP (*filled triangle*) and EP (*diamond*) diets. Data are expressed as mean ± SD of values for 10 rats per group. Rats received one of the diets for two periods of 2 weeks (0 to 2 and 4 to 6) separated by 2 weeks of standard diet (2 to 4)

Protein digestibility

The quantification of the dietary nitrogen on the last day of each test period indicates that the average daily nitrogen intake for rats fed on the UEP diet did not differ from those fed on the EP diet (Fig. 2a). The different measurements of the apparent digestibility of nitrogen are summarized in Fig. 2b, d. Neither nitrogen intake nor faecal nitrogen differed significantly between the two groups of rats given either the UEP or EP diets. As a result, the apparent absorption of nitrogen as a percentage of intake assessed as ADCN was not significantly different between the two diets no matter the time period involved (Fig. 2b). The apparent nitrogen retention (ANR) (Fig. 2c) and the metabolic utilization of nitrogen (RA) (Fig. 2d) expressed as a percentage of nitrogen retained versus nitrogen absorbed were not affected by the type of diet the rats were fed. However, these two last nitrogen balance indicators were highly affected by the age of the animals. Thus, both ANR and RA were more than twofold higher for animals at 7 to 9 weeks of age than for those at 11 to 13 weeks of age (Fig. 2c, d).

Excreted CML

Rats fed on the EP diet ingested approximately 7.7-fold more CML than rats fed on the UEP diet (1,221 vs. 158 µg CML/kg b.w./day, respectively). The greater amount of CML ingested by the EP diet-fed rats was associated with a greater amount overall of faecal and urinary CML elimination (Fig. 3). The food intake and therefore the CML ingestion of 2 out of 10 EP diet-fed rats were highly

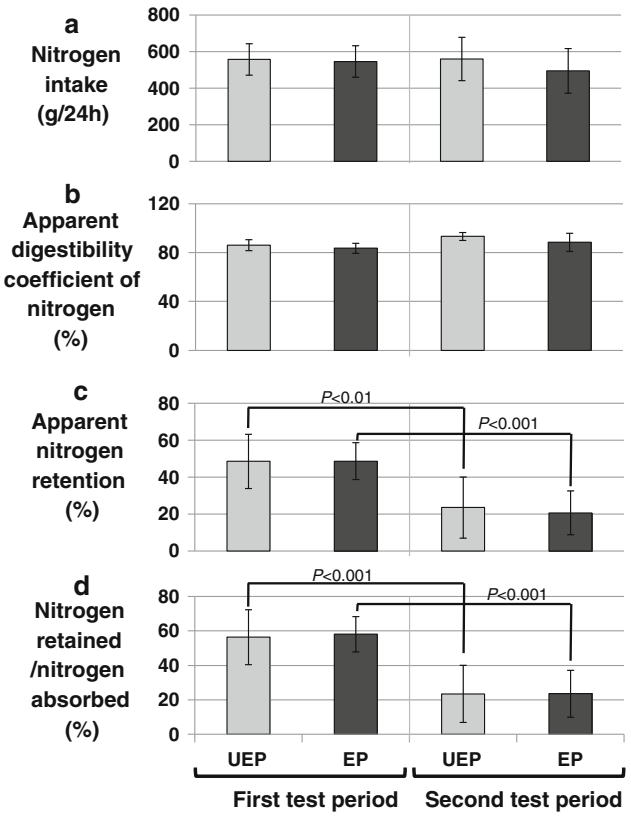

Fig. 2 Effect of the UEP (*open square*) and EP (*filled square*) diets on **a** the nitrogen intake, **b** the apparent digestibility (ADCN), **c** the apparent nitrogen retention (ANR), and **d** the percentage of nitrogen retained as opposed to nitrogen absorbed (RA). The first test period was from week 1 to 2 (7- to 9-week-old rats) and the second test period from week 4 to 6 (11- to 13-week-old rats). The statistically significant differences (*P*-values) are indicated on the bars. All other differences between the two diets and between urine and faeces samples are not statistically significant ($P > 0.05$)

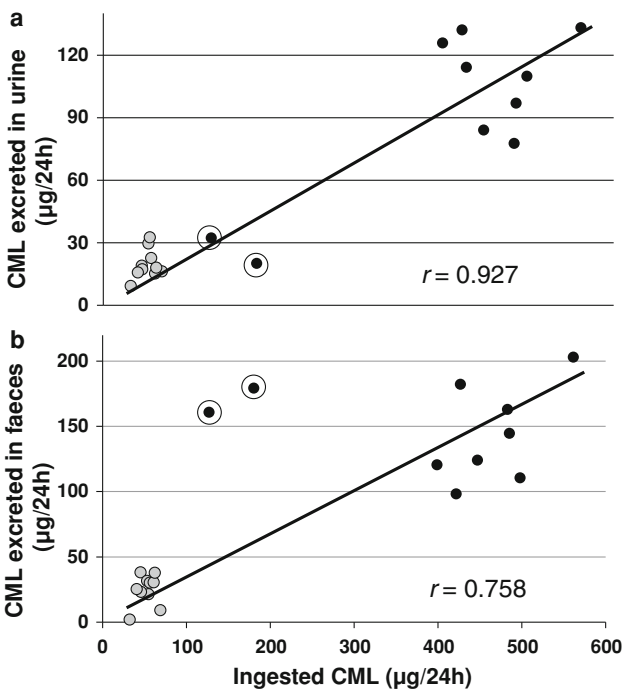

Fig. 3 Association between dietary intake of CML and excreted CML in **a** urine and **b** faeces. 24-h urine and faeces collections were obtained in individual metabolic cages at the end of the 6-week study for animals on the (*open circle*) UEP and (*filled circle*) EP diets. The CML consumption was based on that of food, monitored for 24 h, and the CML concentration measured in the pellets of both diets. *Dots with circles* represent the two animals under the EP diet, which had disturbed and reduced food intake during the time spent in metabolic cages. The correlation between the ingested CML and its excretion was improved when the two disturbed animals were removed ($r = 0.939$)

disturbed and reduced by housing the rats in metabolic cages over the last 24 h of the study. This was clearly reflected by the proportionally lower 24-h urinary excretion of CML of these 2 animals (Fig. 3a). However, the lower food intake observed did not affect the apparent 24-h faecal excretion of CML (Fig. 3b).

The CML excreted in the faeces during the last 24 h of the study was 24.9 ± 11.7 and 148.8 ± 34.6 µg in the groups fed on the UEP and EP diets, respectively (Fig. 4a) ($P < 0.001$). The 24-h urinary CML excretion rate was also lower when the UEP diet was given compared to the EP diet (19.7 ± 7 vs. 92.7 ± 40 µg/24 h, respectively) (Fig. 4a) ($P < 0.001$). Overall, the excretion rate in the urine was of the same order of magnitude as the excretion rate in faeces. When adjusted to food intake, the urinary and faecal excretions of CML were still higher in the EP diet-fed rats (Fig. 4b). However, when expressed as a percentage of the ingested CML (Fig. 4c), both urinary and faecal excretions were found to be less important when the EP diet was given. However, the difference was not statistically significant due to high variation within each group. An average of 38.2 ± 12.2 and 47.9 ± 23.3 % of the ingested CML was eliminated in the urine and faeces, respectively, of the UEP diet-fed rats, whereas only a mean total of 60 % (22.7 ± 6.5 % in the urine and 36.9 ± 35.8 % in the faeces) was eliminated by the rats fed on the EP diet. The high standard deviation calculated for the percentage eliminated in the faeces of the group fed on the EP diet reflects the disturbed food intake of the two rats described above. When these two animals were withdrawn from the data analysis, the mean percentage of faecal extraction of the EP diet-fed group was 30.8 ± 6.8 %.

Plasma CML concentrations

At the end of the 6-week dietary study, the total CML concentration in plasma was 246 ± 151 and 213 ± 25 ng/mL in the groups fed on the UEP and EP diets, respectively (Fig. 5a). The protein-bound CML concentration in plasma was approximately 50 % of the total

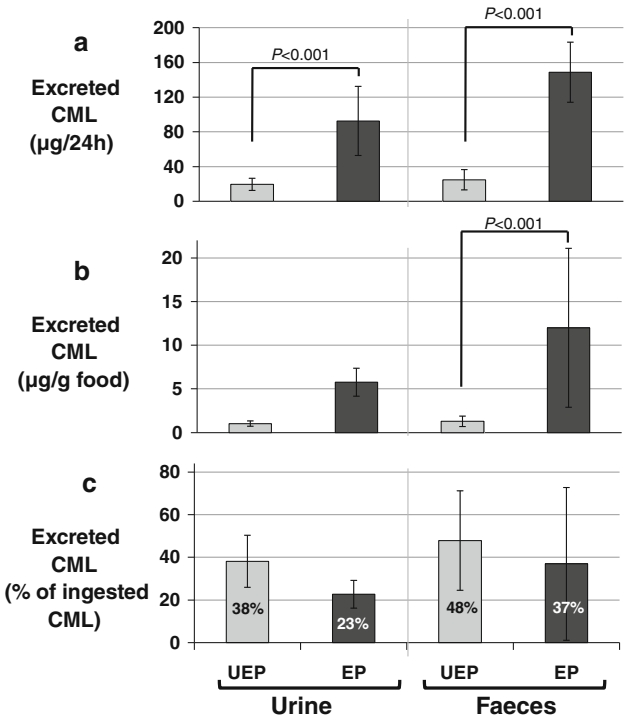

Fig. 4 Urinary and faecal elimination of CML in rats fed UEP (*open square*) or EP (*filled square*) diets. Data are mean ± standard error, $n = 10$ per group. They are expressed as µg of **a** CML/24 h, **b** µg of CML/g of food intake and **c** percentage of ingested CML. The statistically significant differences (*P*-values) are indicated on the *bars*. All other differences between the two diets and between urine and faeces samples are not statistically significant ($P > 0.05$)

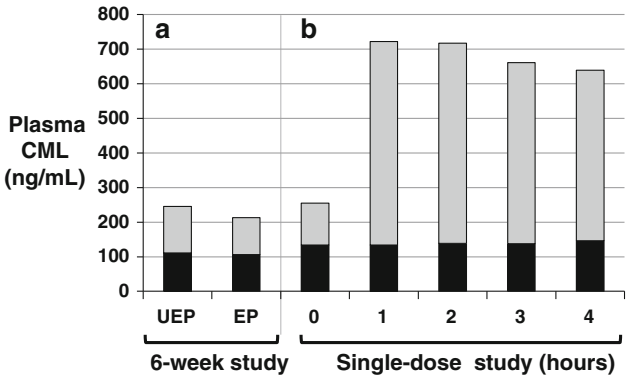

Fig. 5 Plasma protein-bound (*open square*) and free (*filled square*) CML **a** at the end of the 6-week study in rats fed UEP or EP diets and **b** after the administration of a single dose of free CML (plasma collected every hour from 0 to 4 h)

CML concentration in both UEP and EP diets (111 ± 47 and 106 ± 19 ng/mL, respectively). The free CML concentration in plasma was derived by subtracting the protein-bound CML concentration from the total CML concentration. This calculation indicates that the free CML concentrations were comparable between the two groups

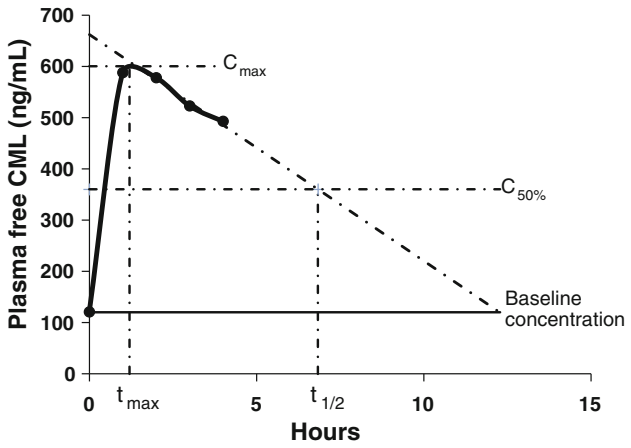

Fig. 6 Plasma kinetic of free CML in rats after a single oral dose of 400 µg. Baseline concentration was estimated at 120 ng/mL, C_{max} at 600 ng/mL, t_{max} at 1 h, and $t_{1/2}$ at 7 h

(134 ± 149 and 107 ± 36 ng/mL, for the UEP and EP diet groups, respectively).

The mean protein-bound concentration of CML in plasma was 140 ± 18 ng/mL ($n = 30$) in the single-dose study (Fig. 5b), which accords with the 6-week dietary study. As suggested above, the ingestion of a diet containing extruded proteins rich in CML did not produce an elevation of free CML in plasma at sacrifice. However, the single-dose study reveals that when an oral dose of 400 µg free CML was administered to the rats, an elevation of the free CML in plasma was observed, whilst the protein-bound CML remained unchanged.

Free CML reached peak concentration in the plasma within an hour after the single oral administration as indicated by C_{max} (600 ng/mL) in Fig. 6. The extrapolation of the limited kinetic curve (1 to 4 h post ingestion) for the elimination of free CML suggests that the time required for the C_{max} in plasma to decrease by one half is 7 h ($t_{1/2}$) and that it takes approximately 12 h to return to the baseline concentration of 120 ng/mL (Fig. 6). With an oral dose of 400 µg, the fraction of the dose that reaches the blood stream before elimination is estimated to be around 5.9 µg (C_{max} × the total plasma volume of the 250 g Wistar rat = 0.6 µg × 9.8 mL) which represents only 1.5 % of the oral dose. The estimate of the total plasma volume was based on the study of Bijsterbosch et al. (1981).

Discussion

The present work is based on the digestibility measured by classical rat nitrogen balance techniques, which may not include the digestibility of each amino acid, especially the essential ones. However, amongst the healthy Western population, it is admitted that only the digestibility of

protein is of relevance since there is a very low risk of essential amino acid deficiency. The use of rat assays for the study of human protein digestibility has been subject to controversy and may be seen as a limitation in respect to the current study. However, the studies which have compared the digestibility of the same proteins between rats and humans indicate that this animal model is reliable for digestibility studies (Bodwell et al. 1980; Rich et al. 1980).

The digestibility of processed food proteins has been studied for almost a century in vitro, in animals and in humans. In a review article, Richard Öste (1991) explained that the digestibility of proteins could be improved or reduced depending on the duration and conditions of the process and the type of protein studied. The present study demonstrates that extruded caseins are as well digested as the equivalent unextruded caseins. Despite the deterioration of lysine and the high levels of adducts such as LAL and CML, the apparent digestibility of the proteins (ADCN) was not affected by the thermal process of extrusion. This is in accordance with what has been described about vegetable food proteins, which were found to be denatured and more digestible after extrusion cooking (Kearns et al. 1989). The fact that the extrusion treatment of caseins has no apparent effect on the digestibility may also be due to the linear conformation of this type of protein which, in turn, facilitates its enzymatic digestion (Kaminogawa 2000). A recent study of in vitro gastrointestinal digestion has also found that caseins were quickly proteolysed even when they were highly modified by the Maillard reaction (Corzo-Martínez et al. 2012).

A recent clinical study comparing two complex diets found that the apparent digestibility of proteins was significantly, but only slightly, decreased when the diet was high in MRPs (85 vs. 90 % for a low MRP diet) (Seiquer et al. 2006). The two other indicators of the nitrogen balance (i.e. ANR and RA) were unaffected by the heat treatment of proteins in the clinical study from Seiquer et al. (2006) as well as in the current 6-week animal study. These observations are also consistent with those of a detailed study which used a pig model (Rérat et al. 2002). The authors of this last study concluded that the nutritional consequences of the Maillard reaction in milk may be considered as negligible (a decrease in digestibility estimated around 2 %).

As expected, the 6-week dietary study revealed that the percentage of nitrogen retained (RA) decreased amongst the adult rats, (13-weeks-old), compared to the young rats, (9-weeks-old), indicating a lower rate of protein synthesis in the slow-growing adult animals. This result is also supported by previous research showing that protein retention falls with advancing age (Krajcovicová-Kudlácková and Dibák 1986). Although the RAs are not affected by the type of diet the rats were fed at both ages studied, it would be interesting to determine the protein digestibility of extruded proteins in older rats (20 month) since casein supplements in fortified foods are frequently used in the aged human population.

Overall, the CML faecal and urinary excretions were found at the end of the 6-week dietary study to be proportional to the level of CML present in the diet. The amount of CML excreted in the urine was proportional to what had been ingested in the previous and last 24 h, whereas the faecal excretion of CML did not reflect what had been ingested during this short period of time, but what had been ingested from a longer period. This is due to the slow transit of faecal material, and therefore CML, through the intestine. This observation is somewhat consistent with the more rapid excretion of ^{14}C-labelled AGEs previously described in the urine compared to the faeces of rats (12 vs. 24–36 h, respectively) (Scholz et al. 2012). It can be concluded that a 24-h urine collection is sufficient to calculate the percentage of ingested CML excreted in the urine, whereas a 2–3-day collection may be more appropriate for the calculation of the faecal excretion when the diet is not constant over the test period.

The urinary excretion expressed as a percentage of the ingested CML was higher in the UEP diet. This is in line with what was measured previously in humans (Delgado-Andrade et al. 2012) and it increases the suspicion of a saturation of the renal clearance. The rate of faecal excretion also seemed to be limited in rats exposed to the EP diet, although the difference with those which were exposed to the UEP diet was not statistically significant. Since the metabolic fate of CML was not fully studied in the current work, it cannot be discounted that a fraction of CML will be metabolised into new unknown adducts (by the gut microbiota, the liver or other organs) and will therefore not be detected by our analytical tools.

In plasma the protein-bound CML level was affected neither by the long-term dietary exposure to casein-bound CML (i.e. EP diet) nor by a single oral dose of free CML. This study shows, as we suspected, that protein-bound CML cannot be derived from dietary CML, but only from in vivo synthesis at 37 °C. The protein-bound CML levels were in a narrow range amongst and across the two animal studies and similar in plasma from fasting or non-fasting animals. The relative stability of the protein-bound CML level and the fact that it is not dependent on the diet explain why no variation of plasma CML is observed when the quantification of CML is performed on isolated plasma proteins (Šebeková et al. 2012).

At the end of the 6-week dietary study, no significant difference in total and thus free CML plasmatic levels was observed between the two groups fed either on the UEP diet or on the EP diet. Despite a 7.7-fold difference of exposure, dietary CML coming from extruded caseins did not influence the circulating CML. This result contradicts

what has been described previously in animal (Peppa et al. 2003) and human studies (Uribarri et al. 2003, 2007). However, the correlation between the consumption of dietary AGEs and circulation CML in healthy humans was found with a correlation coefficient value (r) never higher than 0.46. In addition, the dietary exposure to CML was estimated only from food questionnaires and a database which is subject to controversy.

The single-dose study using 400 μg of free CML (the equivalent of the daily intake of CML in the EP diet) is to our knowledge the first animal study which has attempted to measure the systemic availability of dietary CML with an accurate LC–MS/MS method. This preliminary study already reveals that rats never maintain more than 1.5 % of the dose of CML in the circulation (C_{max} = 600 ng of free CML/mL) and that the free CML level returns to the baseline after around 12 h.

The observation of a low C_{max} associated with the high percentage of CML found in the urine indicates that dietary CML can be well absorbed, but hardly accumulates in the circulation when renal function is normal. A previous study using normal rats fed with dietary MRPs found that AGEs (measured by an ELISA method based on an anti-CML antibody) rose gradually in the serum after a feeding of a single dose (t_{max} = 10 h) and returned to baseline levels within 20 h (He et al. 1999). Kinetics measured in serum from healthy humans confirmed this trend (Koschinsky et al. 1997).

The efficient renal clearance and the reduced systemic availability of free CML may explain why Semba et al. (2012) did not find any correlation between dietary and fasting plasma CML in their observational clinical study. Blood samples collected after an overnight fast in this clinical study probably contained only stable protein-bound CML and baseline levels of free CML. We make the hypothesis that only a 1–2-h postprandial blood CML test would have revealed a correlation between dietary and systemic CML. In addition, the correlation between dietary CML intake and urinary CML is always found when the 'CML uria' is compared to the meals preceding the urine collection, in both the current 6-week animal study and human studies (Delgado-Andrade et al. 2012; Tessier et al. 2010). The dietary exposure to CML estimated with different dietary recalls on a long period of time will definitely bring valuable data regarding the chronic exposure to CML, but may not necessarily be correlated to what is found in urine at a given time in a study.

Conclusion

The present 6-week study in rats demonstrates that the chemical modifications observed in extruded caseins did not affect the apparent digestibility of proteins, either in young or in adult animals. Taken together, data from the current animal studies also show that dietary CML does not influence the protein-bound CML level in plasma. The faecal and urinary excretions of CML were more efficient after UEP than EP feeding (86 and 60 % of elimination, respectively). However, the amount of free CML found in plasma was similar after the two diets. The free CML concentration increased only when the plasma was collected 1–4 h after ingestion of a single dose.

The digestibility of extruded proteins, the CML appearance in blood and its clearance will necessitate further targeted investigation in old or sick animals to reach a better understanding of the nutritional advantage of extruded proteins in fortified foods for the elderly.

Conflict of interest The authors declare that they have no conflict of interest.

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