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Dose-dependent utilisation of casein-linked lysinoalanine, *N*(epsilon)-fructoselysine and *N*(epsilon)-carboxymethyllysine in rats*

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During the heat treatment of protein-containing foods, the amino acid lysine is most prone to undergo chemical reactions in the course of amino acid cross-linking or Maillard reactions. Among the reaction products formed, lysinoalanine (LAL), *N*(epsilon)-fructoselysine (FL) and *N*(epsilon)-carboxymethyllysine (CML) are those which serve as sensitive markers for the heat treatment applied. From a nutritional perspective, these compounds are ingested with the diet in considerable amounts but information about their metabolic transit and putative *in vivo* effects is scarce. In the present study, casein-linked LAL, FL and CML were administered to rats in two different doses for 10 days. Quantitation of LAL, FL and CML in plasma, tissue and faeces samples revealed that the kidneys are the predominant sites of accumulation and excretion. The maximum percent of dietary LAL, FL and CML excreted in the urine was 5.6, 5.2 and 29%, whereas the respective recoveries in the kidneys were 0.02, 26 and 1.4%. The plasma and tissue analyses revealed that the endogenous load of either compound is increased by its dietary intake. But the dose-dependent utilisation of dietary protein-linked LAL, FL and CML in rats has been demonstrated for the first time to vary substantially from each other.

Keywords: Carboxymethyllysine / Fructoselysine / Lysinoalanine / Maillard reaction / Metabolic transit

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1 Introduction

During heat treatment of foods, non-enzymatic reactions of amino acids and reducing carbohydrates are chiefly responsible for the formation of colour and flavour, which are the main determinants for the consumers' food choice.

On the other hand, the nutritional quality of the food protein decreases due to the heat-induced loss of essential amino acids [1], which may undergo two main reaction pathways: cross-linking reactions between each other or Maillard type reactions with reducing carbohydrates.

From a nutritional point of view, one of the most important compound formed by cross-linking of protein-containing amino acids is L-lysino-D,L-alanine (lysinoalanine (LAL)), which results from the reaction between dehydroalanine and lysine (Fig. 1). LAL is formed in the course of heat and/or alkali treatment of protein-containing foods such as baby foods or milk products [2, 3].

In Maillard type reactions, amino acids react with reducing carbohydrates in an initial stage, which is followed by highly diverse reaction pathways in intermediate and final stages [4]. During the initial stage, the Amadori product *N*(epsilon)-fructoselysine (FL) is formed, whereas one of the key-compounds detected in the later stages is *N*(epsilon)-carboxymethyllysine (CML) (Fig. 1) [5 – 7]. LAL and CML are well-accepted to be reliable indicators of process-based lysine damage in milk products [8 – 11]. The other marker of heat-induced lysine damage in food proteins, furosine, was identified nearly 40 years ago [5, 11 – 13], and is widely used for technological process control and nutritional evaluation, mostly of dairy products after mild or moderate heat treatment, like pasteurisation or UHT-treatment of milk. In foods, FL is linked to protein residues and

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Abbreviations: AGEs, advanced glycation end products; **CML,** *N*(epsilon)-carboxymethyllysine; **LAL,** lysinoalanine; **FL,** *N*(epsilon)-fructoselysine

* *In memoriam* Kurt Heyns († 28. 4. 2005)

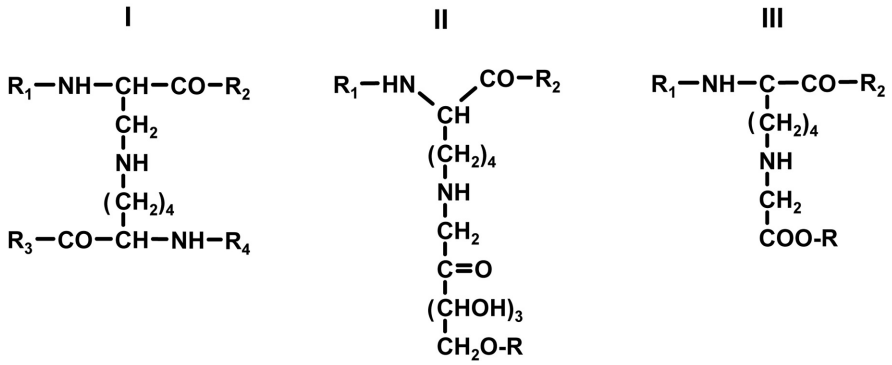

Figure 1. Chemical structure of protein-bound LAL (I), FL (II) and CML (III); R = rest.

it must, like any other protein-sugar reaction product, be released out of the protein by acid or enzymatic hydrolysis prior to analysis. Whereas LAL and CML are stable during this step, FL is decomposed by approximately 50, 15 and 40% into lysine, pyridosine and furosine, respectively [13]. Depending on the conditions during hydrolysis, FL cleavage into furosine has been reported within a range of 32% [14] up to 36% [15] during acid hydrolysis using 7.8 M HCl, and about 35% [16] during enzymatic hydrolysis. Thus, the furosine content in food or biological samples is a sensitive marker of heat-induced lysine damage in the early stage of Maillard reactions, but is strongly related to the individual conditions of protein hydrolysis.

In practice, the quantification of LAL, FL/furosine or CML in heat-treated foods is mainly performed in the course of process quality control, as from the nutritional point of view, a decreased protein quality due to the loss of the essential amino acid lysine is not of major relevance since the daily protein intake in Western countries exceeds the recommendations.

What still remains an open question is whether high amounts of dietary LAL, FL or CML ingested by heat-treated proteins are utilised by the organism and to what extent these compounds are biologically active. Metabolic transit data of these compounds are scarce and it is hypothesised that absorption rates of even high doses might be low, as the protein digestibility of severely heat-treated proteins is substantially lower than that of native proteins [17].

Gaining insights into the metabolic transit of FL and CML becomes even more important when their role in nonenzymatic glycation reactions in the human body is considered. CML belongs to the group of ‚advanced glycation end products' (AGEs) which are believed to be stable end products of post-translational protein modifications *in vivo*. Both, FL and CML accumulation in plasma and in different tissues is correlated to the progression of diabetes mellitus [18, 19]. CML, in particular, is thought by several authors to be involved in the acceleration of the ageing process [18] as well as to be one of the disease promoting agents in atherosclerosis [20] and in Alzheimer's disease [21].

In the present study, casein-linked LAL, FL and CML were selected as typical key-compounds of heat-treated proteins and were administered to rats in order to investigate to what extent these compounds are accumulated in plasma and tissues and excreted in faeces and urine when they are administered at high doses. For FL and CML, it was demonstrated for the first time to what extent a diet-representative, protein-linked and chemically characterised AGE contributes to the endogenous load of AGEs.

2 Materials and methods

2.1 Chemicals

Unless mentioned otherwise, reagents were purchased from Sigma, Germany, and Merck, Germany.

2.2 Preparation of model proteins containing LAL, FL and CML

The experimental protocols for the preparation of the maximally modified model proteins have been published in detail previously [22]. Briefly, casein-LAL, casein-FL and casein-CML, were prepared by heating casein either in strong alkaline conditions at 105°C for 1 h, in the presence of glucose at 65°C for 68 h, or in the presence of glyoxylic acid at 37°C for 19 h, respectively. For each model protein, maximum lysine modification was achieved and calculated on the basis of LAL, FL, CML and lysine quantitation which revealed 3.33, 4.29 and 4.67 g/100 g protein of absolute losses of free lysine for the casein-LAL, the casein-FL and the casein-CML model protein, respectively [22].

2.3 Animal experiments

Male Wistar-rats (CRL:(WI)-BR) were received from the Institute for Immunology, University of Kiel, Germany.

Adult animals weighing 343 ± 57 g were individually housed in metabolic cages under standard conditions (temperature: 22 ± 2°C; humidity: 55 – 65%; 12 h dark/light cycle with artificial lighting). The animals were fed 30 g *per* day of the individual experimental diet resulting in an average uptake of 26 ± 4.5 g diet *per* capita. The animals were allowed free access to drinking water.

Three experiments were performed, each consisting of three randomised groups of six rats. The first group received a diet with a high level of the test-protein, the second group with a low level of the test-protein and the third group received a control diet, which was the same in all three experiments (Table 1).

Controls were fed a diet with an unmodified casein as a protein source in which LAL, FL or CML was not detectable (Table 1). In all experiments, the commercial diet Altromin C1004-protein-free-II (Altromin, Germany) served as a stock diet to which native casein or one of the heat-treated caseins, termed test-proteins, was added.

Throughout the feeding period, body weight gain and dietary intake were calculated by weighing the animals daily and the remaining food at 8:00 a.m. During the 10 days of each experiment, urine and faeces were daily collected into 0.1 M HCl, weighed, lyophilised and stored at −80°C. The experimental protocols and procedures were approved by the Animal Care and Use Committee at the University of Kiel, Germany.

2.4 Biological sample preparation

In the morning of day 10, after overnight food deprivation, the animals were killed and their blood, liver and kidneys were collected rapidly. Liver and kidneys were weighed, washed in ice-cold 0.9% NaCl, homogenised (1000 × *g*) with three volumes of ice-cold sodium-potassium-phosphate-buffer (0.01 M) containing 1.15% KCl (pH 7.4) and stored at −80°C [23]. Blood samples, collected into heparin-coated tubes, were centrifuged at 3000 × *g* for 15 min and plasma was stored until analysis at −80°C.

2.5 Biochemical analysis

Contents of LAL, FL and CML in urine, faeces, plasma, kidney and liver samples were analysed after acid hydrolysis using 7.8 M HCl. Peak identification of LAL, FL and CML was performed by UV-/fluorescence and DAAD-detection as well as standard addition, where the recovery for each standard compound from the respective matrix was between 92 and 98%. Comprehensive data on individual method validation are published elsewhere [9, 24, 25].

LAL was analysed by HPLC-fluorescence, after derivatisation with dansylchloride and quantified by internal standard addition [24], where LAL, as a reference compound was commercially available. For the analysis of lysine, ion-exchange chromatography with postcolumn ninhydrin derivatisation as described by Erbersdobler *et al.* [26] was applied. Quantitation of lysine was performed on the basis of external calibration using commercially available lysine as a reference compound. Furosine released from protein-bound FL by acid hydrolysis was determined by use of ion-pair RP chromatography [25]. FL contents were also quantitated by external standard calibration curves, where the final FL contents were calculated on the basis of a 36% transformation rate of FL into furosine [15]. Furosine as a reference compound was supplied by Neosystem Laboratoire (Strasbourg, France). CML contents were analysed by HPLC-fluorescence with precolumn orthophtaldialdehyde derivatisation after acid hydrolysis in the presence of sodium borohydrate [9]. As CML was not commercially available, quantitation was performed using external standard calibration curves of chemically synthesised CML.

Table 1. Composition of the experimental diets

Experiment	Group	*n*	Test-protein (%)	Casein (%)	Cellulose (%)	Stock diet (%)	LAL-, FL- CML (mg · kg/diet)
I	Control	6	–	27.4	2.00	70.6	n.d.[a)]
	LAL-low	6	5.10	22.5	2.00	70.4	2 582
	LAL-high	6	25.8	2.90	2.00	69.3	12 474
II	Control	6	–	27.4	2.00	70.6	n.d.[b)]
	FL-low	6	3.30	25.0	2.00	69.7	2 700
	FL-high	6	16.5	15.7	2.00	65.8	15 843
III	Control	6	–	27.4	2.00	70.6	n.d.[c)]
	CML-low	6	6.60	22.1	2.00	69.3	1 791
	CML-high	6	32.8	1.20	2.00	64.0	9 759

n.d.: not detectable.
a) LOD = 0.5 nMol.
b) LOD = 0.3 pMol.
c) LOD = 0.5 nMol.

Table 2. Food intake, faeces and urine excretion as well as liver and kidney weights of rats fed two different doses of LAL-, FL- or CML-fortified caseins, respectively

Experiment	Group	Food intake (g/10 days)	Weight gain (g/10 days)	Faeces excretion (g/10 days)	Urine excretion (g/10 days)	Liver weight (%)	Kidney weight (%)
I	Control	242 ± 17	31 ± 10	41 ± 6	170 ± 27	3.8 ± 0.2	0.90 ± 0.07
	LAL-low	237 ± 16	32 ± 13	47 ± 7	183 ± 29	3.7 ± 0.3	0.87 ± 0.04
	LAL-high	236 ± 7	29 ± 4.7	57 ± 10	203 ± 41	3.9 ± 0.4	0.95 ± 0.03
II	Control	270 ± 4[a)]	19 ± 37	36 ± 5	152 ± 20	4.0 ± 0.5	0.76 ± 0.10
	FL-low	265 ± 6[a)]	8.4 ± 35	37 ± 7	151 ± 42	4.0 ± 0.6	0.81 ± 0.05
	FL-high	300 ± 14[b)]	24 ± 9.6	38 ± 3	148 ± 37	4.3 ± 0.5	0.81 ± 0.04
III	Control	181 ± ± 23[a,b)]	−15 ± 24	42 ± 8	126 ± 19[a)]	3.9 ± 0.4[a)]	0.87 ± 0.10[a)]
	CML-low	201 ± 5[a)]	2.5 ± 5.8	47 ± 6	168 ± 25[b)]	4.0 ± 0.3[a)]	0.88 ± 0.05[a)]
	CML-high	160 ± 11[b)]	−4.7 ± 11	43 ± 8	169 ± 20[b)]	5.0 ± 0.3[b)]	1.02 ± 0.04[b)]

a, b, c) Different indices indicate significant differences between diet groups ($p < 0.05$).

CML synthesis closely followed an experimental protocol published by Huber and Pischetsrieder [27]. The chemical structure was confirmed by ^{1}H-NMR, COSY (400 MHz; D_2O): d 1.53 [m, 2H, H–C(3)], 1.79 [m, 2H, H–C(4)], 1.97 [m, 2H, H–C(2)], 3.13 [dd, 2H, J = 7.6, 7.9 Hz, H–C(5)], 3.77 [s, 2H, H–C(6)], 3.95 [t, 1H, J = 5.8, 5.8 Hz, H–C(1)].

2.6 Statistical analyses

Data obtained are reported as mean ± SD. Statistical analyses within each experiment were performed using ANOVA and post-hoc Scheffé test at a level of significance of $p < 0.05$ (*).

3 Results

To study the utilisation of casein-linked LAL, FL and CML, these compounds were administered to rats in three experiments for a total period of 10 days. Afterwards, LAL, FL and CML were quantitated in the diet, urine, faeces, plasma, liver and kidneys. FL contents were calculated on the basis of furosine analyses [15].

3.1 Metabolic transit of casein-LAL

After the 10 days feeding period, the average body weight gain of all animals was not different among the groups of experiment I (Table 2). Also, no differences among the groups were seen for liver and kidney weights, urine and faeces volumes as well as total food intake (Table 2). Based on the food intake data and the LAL content in the diet, the average intake of LAL during the 10 days feeding period was calculated at 623 and 3055 mg for the animals of the LAL-low and the LAL-high groups, respectively. This five-fold increase in the dietary dose resulted in a four- and nine-fold increase in urine and faecal LAL contents, respectively (Table 3). The percentage of dietary LAL excreted with the urine was not different between the LAL-low and the LAL-high groups, but the percent dietary LAL excreted with the faeces increased from 7.67 to 14.4% (Fig. 2). Animals from the LAL-high group also showed significantly increased LAL contents in plasma, liver and kidneys when compared to those of the LAL-low group, although the levels of LAL in these compartments were low compared to urine and faeces. The dose dependent increase in LAL accumulation was most pronounced in the kidneys, in which the five-fold dietary intake resulted in a six-fold elevated LAL content. Surprisingly, LAL was also quantitated in urine and faeces samples of control animals (Table 3), although the LAL content of the control diet was below the LOD of 0.5 ng/mL [22].

Table 3. Intake, excretion and endogenous load of LAL in rats after feeding two different doses of LAL-fortified casein for 10 days

	Control	LAL-low	LAL-high
LAL intake total (mg)	n.d.	623 ± 23.7	3055 ± 84.7
LAL Excretion *via* Faeces			
(mg)	1.46 ± 0.32[a)]	46.9 ± 22.4[b)]	423 ± 190[c)]
(% intake)	–	7.67 ± 3.61	14.4 ± 6.45
via Urine (mg)	13.8 ± 3.56[a)]	34.2 ± 5.41[b)]	144 ± 29.2[c)]
(% intake)	–	5.56 ± 0.54	4.87 ± 0.92
LAL Endogenous load			
Plasma (mg/L)	6.17 ± 1.02[a)]	11.2 ± 1.09[b)]	19.71 ± 3.25[c)]
Liver (mg)	0.002 ± 0.001[a)]	0.004 ± 0.001[b)]	0.005 ± 0.001[c)]
Kidneys (mg)	0.06 ± 0.01[a)]	0.13 ± 0.03[b)]	0.78 ± 0.04[c)]

n.d.: not detectable; limit of detection 0.5 ng/mL.
a, b, c) Ddifferent indices indicate significant differences between diet groups ($p < 0.05$).

3.2 Metabolic transit of casein-FL

Body weight gain of animals administered with the casein-FL containing diets was not different from controls, although food intake was higher in the FL-high group com-

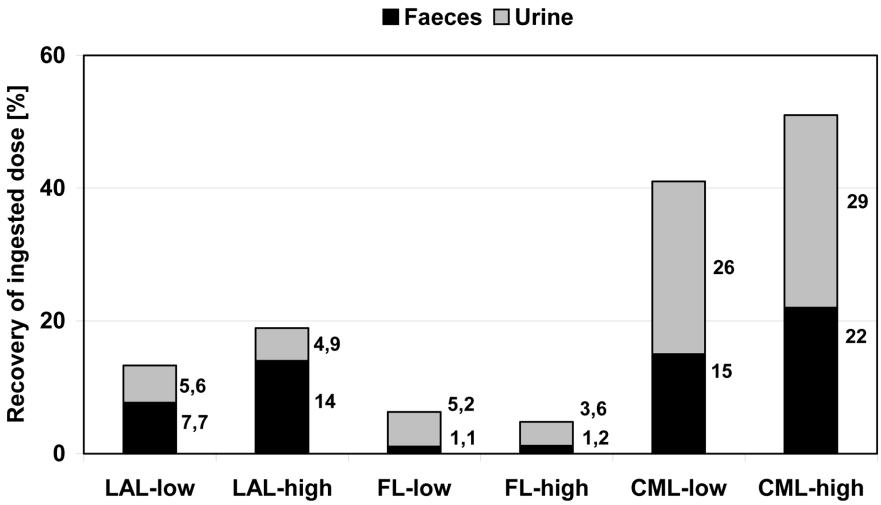

Figure 2. Percentage recovery of LAL, FL and CML in faeces and urine after feeding two different doses of LAL-, FL- and CML-fortified casein, respectively.

Table 4. Intake, excretion and endogenous load of FL in rats after feeding two different doses of FL-fortified casein for 10 days

	Control	FL-low	FL-high
FL intake total (mg)	n.d.	714 ± 159	4740 ± 213
FL Excretion *via* Faeces			
(mg)	0.42 ± 0.22[a)]	7.29 ± 2.42[b)]	32.48 ± 10.99[c)]
(% intake)	–	1.09 ± 0.32	1.17 ± 0.36
via Urine			
(mg)	0.65 ± 0.15[a)]	37.94 ± 15.5[b)]	173.51 ± 38.38[c)]
(% intake)	–	5.15 ± 1.92	3.66 ± 1.14
FL Endogenous load			
Plasma (mg/L)	9.28 ± 1.01[a)]	14.5 ± 1.78[b)]	23.76 ± 3.04[c)]
Liver (mg)	25.04 ± 4.28[a)]	27.9 ± 2.29[b)]	35.12 ± 3.99[c)]
Kidneys (mg)	38.0 ± 12.3[a)]	674 ± 134[b)]	1264 ± 172[c)]

n.d.: not detectable; limit of detection 0.3 pMol.
a, b, c) Different indices indicate significant differences between diet groups ($p < 0.05$).

pared to the other two groups of experiment II (Table 2). Total FL intake over 10 days was calculated at 714 and 4740 mg (Table 4) for the animals of the FL-low and the FL-high groups, respectively. This 6.6-fold increase in the FL intake resulted in a 4.5- and a 12-fold increase in urinary and faecal FL contents, respectively (Table 4). The percentage of dietary FL excreted with urine and faeces was also not different between the two groups to which the casein-FL containing diets were administered (Fig. 2). The endogenous load of FL increased by a factor of 1.6, 1.3 and 1.9 in plasma, liver and kidneys of animals fed the FL-high diet compared to those of the FL-low group. Even more striking, the total contents of FL in the kidneys increased remarkably after feeding the FL-low and the FL-high diet, by a factor of 18 and 33 (Table 4). Moreover, control animals also showed an endogenous load of FL as FL was quantitated in plasma, liver, kidney and urine samples, although the FL content in the control diet was below the LOD (0.3 pMol furosine [28]).

3.3 Metabolic transit of casein-CML

After 10 days administration of either the CML-low or the CML-high diet, final body weights (data not shown) and body weight gains did not differ from the control group, although food intake of animals belonging to the CML-high group was less than that of controls (Table 2, experiment III). CML content in the experimental diets was calculated at 1791 and 9759 mg for the CML-low and CML-high groups, respectively (Table 1). But CML intake in these groups increased by a factor of 3 as food consumption was higher in the CML-low group. Compared to controls, liver and kidney weights increased in animals of the CML-high group by a factor of 1.3 and 1.2, respectively (Table 2). Urine excretion was elevated by 34% in animals fed both diets containing the casein-CML protein (Table 2). The three-fold increase in the dietary CML dose resulted in four- and three-fold increase in urinary and faecal CML excretion, whereas in the plasma, CML contents rose by a factor of 7.6 (Table 5). Kidney CML contents were higher in animals fed on both casein-CML containing diets when compared to controls. A 2.8-fold increase in the kidney CML content of animals from the CML-high group reflected a three-fold increase in the dietary dose compared to the CML-low group (Table 5). In the liver and kidneys of controls, CML was also quantitated, although no CML was detected in the controls' diet (LOD of 0.5 pM [9], Table 1).

Table 5. Intake, excretion and endogenous load of CML in rats after feeding two different doses of CML-fortified casein for 10 days

	Control	CML-low	CML-high
CML Intake total (mg)	n.d.[1)]	400 ± 90.4[a)]	1273 ± 118[b)]
CML Excretion *via* Faeces			
(mg)	n.d.[1)]	52.5 ± 18.2[a)]	215 ± 24.0[b)]
(% intake)	–	15 ± 5.1[a)]	22 ± 2.4[b)]
via Urine			
(mg)	n.d.[1)]	94.4 ± 20.8[a)]	283 ± 98.4[b)]
(% intake)	–	26 ± 5.8	29 ± 9.9
CML Endogenous load			
Plasma (mg/L)	n.d.[1)]	2.63 ± 1.16[a, 2)]	20.0 ± 7.78[b)]
Liver (mg)	0.013 ± 0.001[a)]	0.013 ± 0.002[a)]	0.016 ± 0.001[b)]
Kidneys (mg)	0.024 ± 0.005[a)]	6.46 ± 1.48[b)]	17.8 ± 9.43[c)]

a, b,c) Different indices indicate significant differences between diet groups ($p < 0.05$).

1) CML content was below the limit of detection of 0.5 pMol [9].

2) $n = 5$ due to one sample in which no CML contents were detectable.

4 Discussion

In the present study, utilisation of casein-linked LAL, FL and CML, the most common markers of heat-induced lysine blockage in dietary proteins, was investigated in three parallel experiments on rats. The experimental diets were based either on native casein (control groups) or on heat-treated casein models containing LAL, FL or CML. Lysine modification of each heat-treated casein was achieved by three different experimental procedures, each of them optimised for a high and specific yield in LAL, FL and CML, respectively. Therefore, the highest dietary dose (LAL-/FL-/CML-high) of casein-linked LAL, FL and CML was based on the maximum yield obtained and was compared with a lower dose by introducing another experimental group (LAL-/FL-/CML-low) in which native casein was mixed with the individual modified casein. It was hypothesised that the utilisation of protein-linked LAL, -FL and CML differs from each other in a dose-dependent manner. These differences could help to understand the metabolic activities of LAL, FL and CML.

4.1 Utilisation of casein-linked LAL

Proteins heated under alkaline conditions are well-known to form inter- and intramolecular amino acid cross-links. Protein-linked cross-links such as LAL are digested and absorbed at much lower levels than the free compounds as the access of intestinal proteolytic enzymes is limited by steric hindrance and there is no active transport for these compounds [28 – 31]. This kind of ‚thickening effect' effect also results in lower utilisation efficiency with increasing levels of protein-linked crosslinks [32, 33]. In the present study, the percentage of dietary LAL excreted in the urine of animals of the LAL-high group was about 12% less than that measured in animals on the LAL-low diet. As the percentage of dietary LAL excreted in the faeces was much higher in the LAL-high group compared to that of the LAL-low group, the data clearly point to a limited absorption of LAL when high doses of protein-linked LAL are administered. The urinary LAL excretion rates of 5.56 and 4.87% for the LAL-low and the LAL-high group, respectively, are in accordance with the data reported in [29] and [33]. Both authors performed animal feeding trials on rats to which substantially lower doses of protein-linked LAL were fed, but also calculated the amount of dietary LAL excreted, which ranged between 1.3 and 18%. De Weck-Gaudard *et al.* [34] reported urinary LAL excretion rates of 7.6 and 2.1% after feeding a moderately and a severely alkaline/heat-treated casein to rats, respectively.

The percentage of dietary LAL reported for faecal excretion in this study was 6.67 and 14.4% and was much lower than the respective 33 and 65% reported for growing rats and for humans [29, 33]. These differences might be due to a limited microbial capacity for LAL degradation in humans, due to a higher food intake or due to less pronounced coprophagia in young rats. In the present study, the intestinal microorganisms of adult rats which were well-adapted to LAL-containing standard chow might efficiently degrade protein-linked LAL. Finot *et al.* [29] and Struthers *et al.* [30] both reported $^{14}CO_2$ excretion after feeding ^{14}C-labelled LAL and hypothesised LAL oxidation by intestinal microorganisms. Sternberg and Kim [35] could even show that two intestinal microbiota, *Escherichia coli* and *Bacillus subtilis*, were able to utilise LAL as source of lysine, resulting in bacterial growth.

One of the metabolic effects first reported for dietary LAL was its cytotoxic activity causing cariomegaly and necrosis of the kidney's proximal tubular cells [36]. Later investigations revealed that nephrotoxic effects strongly depend on the dietary source of LAL-linked proteins [37], on dose and duration of ingestion [33, 35] and on the species, because nephrotoxic effects are observed in rodents but not in other species, including primates [38]. In the present study, neither microscopic changes of the kidneys (data not shown) nor changes in kidney weights were observed, although dietary LAL was accumulated to some extent in the kidneys of animals of the LAL-low and the LAL-high groups. The six-fold increase in the LAL kidney contents reflected the five-fold increase in the dietary dose. In contrast, no such dose-dependent LAL accumulation in the liver was observed and LAL plasma concentrations from the LAL-low to the LAL-high dose only increased by a factor of about 1. 8. Thus, one of the important sites of LAL

accumulation in the organism seems to be the kidneys. This has also been demonstrated in rats to which N^{ε}-DL-(2-amino-2-carboxyethyl)-U-^{14}C-L-lysine was administered as a bolus dose [30].

4.2 Utilisation of casein-linked FL

Protein-linked FL has been demonstrated to be hardly accessible to digestive enzymes of the gastrointestinal tract [39]. Whereas the urinary excretion of dietary free FL can reach up to 60% in rats, only 10% of dietary protein-linked FL is excreted with the urine [39]. Similar data of about 3% have been reported in adults [40], whereas in young children, FL excretion after administration of heat-treated milk protein can reach up to 16% [41]. In the present study, administration of very high doses of protein-linked FL resulted in urinary FL excretion of about 3 – 5% of the ingested dose. The six-fold increase in the FL intake was reflected by a four-fold increase in urinary FL excretion when the FL-low and FL-high groups are compared. Apparently, the intake of excessive doses of protein-linked FL does not result in an increased absorption rate.

In the faeces, the total amount of about 1% FL excreted was much lower than that in urine. These data are in accordance with data reported for moderate doses administered to rats [39] or healthy adults [40]. But in young children, a faecal excretion rate of 55% was calculated by Niederweiser *et al.* [41]. The reason for this rather high excretion rate in toddlers might be a different microbial activity in the intestines. As protein-linked as well as free FL is efficiently degraded by intestinal microorganisms [42 – 44], the less-developed intestinal flora in young children might not degrade FL as efficiently as in adults who are adapted to the intake of heat-treated proteins for a long time. The results presented herein do not support the hypothesis that the FL degrading capacity of the intestinal microbiota is limited, as the percentage of dietary FL excreted with the faeces did not change after the six-fold higher dose.

The most pronounced endogenous accumulation of FL was observed in the kidneys and the plasma. In these compartments, the six-fold increase in the dietary dose resulted in a 1.9- and a 1.7-fold increase in FL contents, respectively, whereas FL contents in the liver only increased by a factor of 1.2. Although comparable data from animal feeding trials are not yet available, *in vitro* experiments on isolated rat kidneys support the hypothesis that the kidneys are the predominant site of FL accumulation possibly due to an active transport mechanism for FL [45]. The minor increase in FL liver contents might be due to the FL uptake by passive diffusion, which has been described in isolated hepatocytes [46].

4.3 Utilisation of casein-linked CML

Very little is known about the mechanisms of the absorption and metabolism of protein-bound CML or its metabolites. It is hypothesised that casein-bound CML or at least part of it, is released from the protein by the enzymes of the intestinal tract and subsequently absorbed. In different experiments with rats, Liardon *et al.* [47] fed proteins with varying amounts of protein-bound or free LAL and/or FL. The feeding periods lasted 8 days. In all experiments, regardless of the amount of ingested LAL or FL, CML was detected in the urine of the animals. The authors concluded that an endogenous transformation from FL to CML might be possible. Nevertheless, it was suspected that the main part of the CML excreted with the urine was of exogenous origin, ingested with diets fed before the beginning of the experimental period [47]. In the present study, CML was not detected in kidney samples of animals fed the casein-LAL or the casein-FL containing diet (data not shown). An *in vivo* transformation of protein-linked LAL or FL administered in very high doses to CML is therefore not supported by the present results. On the other hand, the results of the present study clearly indicate that dietary CML, ingested in its casein-linked form, appears in the plasma and, thus, must be absorbed by the intestines. Elimination of the absorbed CML from the plasma can be hypothesised from the increase in total CML in the urine and the kidneys.

In biokinetic studies on rats to which ^{18}F-fluorobenzoylated CML was injected into the tail vein, it was demonstrated by positron emission tomography (PET) detection that the radio-labelled compound is taken up by the liver rapidly and is subsequently incorporated in the kidneys [48]. The amount of radioactivity analysed 120 min after injection in blood, liver, kidneys and urine was 0.03, 3.1, 1.2 and 87%, respectively. From these results, a rapid elimination of the free, nonprotein linked ^{18}F-fluorobenzoylated CML through the kidneys can be concluded. Although the mechanism by which the negatively charged, ^{18}F-fluorobenzoylated CML is metabolised is likely to be different from that of nonlabelled, food-derived protein-linked CML, the liver and the kidneys have been demonstrated to play a major role in the metabolic pathways of both compounds. In the case of an impaired kidney function, AGEs, and CML in particular, are hypothesised to turn into uremic toxins [49] contributing to the progression of the disease. As the absorption rate of protein-linked CML, one of the predominant AGEs, has been demonstrated in this study to be between 26 and 29%, as indicated by the urinary excretion of ingested CML, the dietary intake of CML and, thus, severely heat-treated foods, has to be carefully monitored in diabetes patients showing an elevated endogenous load of AGEs.

In addition to CML, FL might also play a crucial role as AGE, since FL was also found at very high concentrations

in the plasma of diabetics [50] and, in the present study, it was incorporated in the kidneys most efficiently among the compounds tested. Results from the FL-high group demonstrate that about 27% of the ingested FL dose was accumulated in the kidneys, while the recovery of LAL and CML in the kidneys isolated from the respective groups was 0.3 and 1.4%, respectively.

4.4 Conclusions on the metabolic transit of casein-linked LAL, FL and CML

The dietary intake of a protein-linked amino acid cross-link, LAL, or of the Maillard reaction products FL or CML clearly enhanced the endogenous load of each of the compounds in a dose-dependent manner. Although data about absorption mechanisms are scarce, results from feeding experiments on FL mainly in the 1970s indicated that FL is absorbed by passive diffusion after its liberation from the protein-bond by digestive enzymes [39, 51]. This pathway is also hypothesised for protein-linked LAL and CML. But not all of the compound reaching the small intestines is absorbed. In the present study, LAL, FL and CML were quantitatively analysed in the faeces. The percentage of the dietary compound excreted herein differed substantially (Fig. 2), suggesting both, different absorption rates and a varying degradation capacity by intestinal microbiota. Both mechanisms seem to be rate limited as the administration of higher doses always resulted in a higher percentage of the dietary compound being excreted in the faeces. The lowest recovery of FL in the faeces can be explained by its efficient microbial degradation, which has recently been shown in *E. coli*, catalysing the ATP-dependent phosphorylation of fructoselysine to a product identified as fructoselysine 6-phosphate and the reversible conversion of fructoselysine 6-phosphate and water to lysine and glucose 6-phosphate [44].

After absorption, all of the compounds are transported in the plasma and primarily accumulated in the kidneys, which, in due course, showed morphological signs of hypertrophy and excreted LAL, FL and CML into the urine. Recovery of CML in urine and faeces was higher than that of LAL and FL (Fig. 2). This might explain the various *in vivo* effects of CML and the well-accepted hypothesis that CML is one of the most useful biochemical markers of glycation-related diseases and their complications. Moreover, FL was most efficiently accumulated in the kidneys, without being excreted into the urine in considerable amounts.

Besides the dose-dependent uptake and/or utilisation of protein-linked LAL, FL and CML, the present study revealed that even animals of the control groups, having been on a LAL, FL and CML-free diet for 10 days, did show an endogenous load of LAL, FL and CML. Whereas faecal and urinary contents were highest for LAL, plasma, liver and kidneys of control animals showed the highest contents in FL. CML was only analysed in liver and kidney samples of the controls. LAL and FL were likely to be ingested with the standard chow prior to the start of this experiment. Quantitation analyses revealed of 120 mg of LAL and 350 mg of FL *per* kilogram diet, respectively. Assuming a daily intake of about 25 g food, the daily intake can be estimated at 3 mg LAL and 9 mg FL. Although in neither of the control diets were the contents of LAL, FL or CML above the LOD, the low intake of LAL and FL with the standard diet prior to the beginning of the study might have boosted their endogenous load. CML was not quantitated in the standard chow but liver and kidneys of control animals did show CML contents above the LOD. Besides dietary sources, the endogenous load of CML, and possibly that of FL, is influenced by *in vivo* glycation which proceeds slowly with age [18]. Thus, the contents of CML detected in the present study in the liver and kidneys of the controls are very likely of endogenous origin, either by glycation reactions or by oxidative cleavage of FL between the C-2 and C-3 bonds of the carbohydrate chain and erythronic acid [52].

In conclusion, the dose dependent utilisation of dietary protein-linked LAL, FL and CML in rats has been demonstrated for the first time to vary substantially from each other. The metabolic transit of amino acid cross-links and Maillard reaction products seems to depend on their individual chemical structure and the way it is bound to proteins, both of which presumably affect their microbial degradation, their absorption mechanisms and their metabolisation *in vivo*.

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5 References

[1] Erbersdobler, H. F., *Kieler. Milchwiss. Forsch.* 1983, *35*, 301–311.

[2] Erbersdobler, H. F., Holstein, A. B., *Milchwissenschaft* 1980, *35*, 734–737.

[3] Friedman, M., *Adv. Exp. Med. Biol.* 1999, *459*, 145–159.

[4] Hodge, J. E., *J. Agric. Food Chem.* 1953, *1*, 928–943.

[5] Erbersdobler, H. F., Zucker, H., *Milchwissenschaft* 1966, *21*, 564–568.

[6] Büser, W., Erbersdobler, H. F., *Milchwissenschaft* 1986, *41*, 780 – 785.

[7] Friedman, M., *J. Agric. Food Chem.* 1999, *47*, 1295 – 1319.

[8] Faist, V., Drusch, S., Kiesner, C., Elmadfa, I., Erbersdobler, H. F., *Int. Dairy J.* 2000, *10*, 339 – 346.

[9] Drusch, S., Faist, V., Erbersdobler, H. F., *Food Chem.* 1999, *64*, 1 – 7.

[10] Erbersdobler, H. F., Drusch, S., Faist, V., in: Roginski, H., Fuguay, J. W., Fox, P. F. (Eds.), *Encyclopedia of Dairy Sciences*, Academic Press/Elsevier Science, San Diego, CA, USA 2002, pp. 2137 – 2143.

[11] Heyns, K., Heukeshoven, J., Brose, K.-H., *Angew. Chem.* 1968, *80*, 627.

[12] Brüggemann, J., Erbersdobler, H. F., *Z. Lebensm. Unters. Forsch.* 1968, *137*, 137 – 143.

[13] Finot, P. A., Bricout, J., Viani, R., Mauron, J., *Experientia* 1968, *24*, 1097 – 1099.

[14] Bujard, E., Finot, P. A., *Ann. Nutr. Alim.* 1978, *32*, 291 – 305.

[15] Erbersdobler, H. F., in: Fujimaki, M., Namiki, M., Kato, H. (Eds.), *Amino-Carbonyl Reactions in Food and Biological Systems*, Elsevier Press, Tokyo 1986, pp. 481 – 491.

[16] Henle, T., Herdegen, V., Kurschewitz, J., Klostermeyer, H., *Jahresbericht FML Weihenstephan* 1992, *141*.

[17] Brüggemann, J., Erbersdobler, H. F., *Zeitschrift für Tierphysiologie, Tierernährung und Futtermittelkunde* 1968, *24*, 55 – 67.

[18] Knecht, K. J., Dunn, J. A., McFarland, K. F., McCance, D. R., *et al.*, *Diabetes* 1991, *40*, 190 – 196.

[19] Monnier, V. M., Sell, D. R., Nagaraj, R. H., Miyata, S., *et al.*, *Diabetes* 1992, *41*, 36 – 41.

[20] Sakata, N., Imanaga, Y., Meng, J., Tachikawa, Y., *et al.*, *Atherosclerosis* 1999, *142*, 67 – 77.

[21] Münch, G., Schinzel, R., Loske, C., Wong, A., *et al.*, *J. Neural Transm.* 1998, *105*, 439 – 461.

[22] Faist, V., Müller, C., Erbersdobler, H. F., *Nahrung/Food* 2001, *45*, 218 – 221.

[23] Wenzel, E., Tasto, S., Erbersdobler, H. F., Faist, V., *Ann. Nutr. Metab.* 2002, *46*, 9 – 16.

[24] Faist, V., Drusch, S., Kiesner, C., Elmadfa, I., Erbersdobler, H. F., *Int. Dairy J.* 2000, *10*, 339 – 346.

[25] Resmini, P., Pellegrino, L., Batelle, G., *Ital. J. Food Sci.* 1990, *3*, 173 – 183.

[26] Erbersdobler, H. F., Dehn-Müller, B., Nangpal, A., Reuter, H., *J. Dairy Res.* 1987, *54*, 147 – 151.

[27] Huber, B., Pischetsrieder, M., *J. Agric. Food. Chem.* 1998, *46*, 3985 – 3990.

[28] Clawin-Rädecker, I., Schlimme, E., *Kieler Milchw. Forschber.* 1995, *47*, 169 – 175.

[29] Finot, P. A., Bujard, E., Arnaud, M., *Adv. Exp. Med. Biol.*, in: Friedman, M. (Ed.), *Protein Crosslinking – Nutritional And Medical Consequences*, Plenum Press, New York, London, 1977, Vol. 86B, pp. 51 – 71.

[30] Struthers, B. J., Brielmaier, J. R., Raymond, M. L., Dahlgreen, R. R., Hopkins, D. T., *J. Nutr.* 1980, *110*, 2065 – 2077.

[31] Friedman, M., Ahley, J. C., Masters, P. M., *J. Food Sci.* 1981, *46*, 127 – 131.

[32] Erbersdobler, H. F., *Bibl. Nutr. Dieta.*, in: Somogyi, J. C., Müller, H. R. (Eds.), *Nutritional Impact of Food Processing*, Karger AG Basel 1989, Vol. 43, pp. 140 – 155.

[33] De Groot, A. P., Slump, P., Feron, V. J., Van Beek, L., *J. Nutr.* 1976, *106*, 1527 – 1538.

[34] De Weck-Gaudard, D., Liardon, R., Finot, P. A., *J. Agric. Food Chem.* 1988, *36*, 717 – 721.

[35] Sternberg, M., Kim, C. Y., *Adv. Exp. Med. Biol.*, in: Friedman, M. (Ed.), *Protein Crosslinking – Nutritional and Medical Consequences*, Plenum Press, New York 1977, Vol. 86B, pp. 73 – 84.

[36] Wodard, J. C., Alvarez, M. R., *Arch. Pathol.* 1967, *84*, 153 – 162.

[37] Karayannis, N. L., Bjeldanes, L. F., Mac Gregor, J. T., *Food Cosmet. Toxicol.* 1979, *17*, 585 – 590.

[38] Langhendriess, J. P., Hurrell, R. F., Furniss, D. E., Hirschenhuber, C., *et al.*, *J. Nutr.* 1992, *14*, 62 – 70.

[39] Erbersdobler, H. F., *Adv. Exp. Med. Biol.*, in: Friedman, M. (Ed.), *Protein Crosslinking – Nutritional and Medical Consequences*, Plenum Press, New York, London 1977, Vol. 86B, pp. 367 – 378.

[40] Lee, K., Erbersdobler, H. F., in: Labuza, T. P., Reineccius, G. A., Monnier, V. M., Brien J. O', Baynesand, J. W., *Maillard Reaction in Chemistry, Food and Health*, The Royal Society of Chemistry, Cambridge 1994, pp. 358 – 363.

[41] Niederweiser, A., Giliberti, P., Matasovic, A., *Pediatr. Res.* 1975, *9*, 867.

[42] Erbersdobler, H. F., Gunsser, I., Weber, G., *Zbl. Vet. Med.* 1970, *A17*, 573 – 575.

[43] Mori, B., Nakatsuji, H., *Agric. Biol. Chem.* 1977, *41*, 345 – 350.

[44] Wiame, E., Delpierre, G., Collard, F., Van Schaftingen, E., *J. Biol. Chem.* 2002, *277*, *45*, 42523 – 42529.

[45] Erbersdobler, H. F., Faist, V., *Metabolism of Amadori products in rats and humans, COST 919 Proceedings* 2002, Vol. II-WG 5.

[46] Finot, P. A., Magnenat, E., *Prog. Food Nutr. Sci.* 1981, *5*, 193 – 207.

[47] Liardon, R., De Weck-Gaudard, D., Philippossian, G., Finot, P. A., *J. Agric. Food Chem.* 1987, *35*, 427 – 431.

[48] Bergmann, R., Helling, R., Heichert, C., Scheunemann, M., *et al.*, *Nahrung/Food* 2001, *45*, 182 – 188.

[49] Miyata, T., Sugiyama, S., Saito, A., Kurokawa, K., *Kidney Int.* 2001, *78*, 25 – 31.

[50] Wells-Knecht, K. J., Lyons, T. J., McCance, D. R., Thorpe, S. R., *et al.*, *Diabetes* 1994, *43*, 1152 – 1156.

[51] Brandt, A., *Agrwiss. Diss. Göttingen* 1978.

[52] Ahmed, M. U., Thorpe, S. R., Baynes, J. W., *J. Biol. Chem.* 1986, *261*, 4889 – 4894.

