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Assay of advanced glycation endproducts in selected beverages and food by liquid chromatography with tandem mass spectrometric detection

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Food and beverages contain protein glycation adducts – both early-stage adducts and advanced glycation endproducts. We determined the concentrations of glycation adducts in selected food and beverages by liquid chromatography with triple quadrupole mass spectrometric detection. Cola drink contained low concentrations of glycation free adducts, whereas pasteurised and sterilised milk were rich sources of heat-stable glycation adduct residues – N_{ε}-carboxymethyl-lysine and N_{ε}-carboxyethyl-lysine. Laboratory rodent food was a rich source of advanced glycation endproducts. Measurement of glycation adducts in 24 h urine samples of normal and diabetic rats indicated that < 10% of glycation adduct residue consumption was excreted. Induction of diabetes by streptozotocin led to a 2-fold increase in urinary excretion of N_{ε}-carboxymethyl-lysine and a 27-fold increase in urinary excretion of methylglyoxal-derived hydroimidazolone N_{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine – the latter was decreased by high-dose thiamine therapy that also prevented the development of nephropathy. We conclude that cola drinks are a poor source of glycation adduct whereas thermally processed milk is rich in glycation adducts. Dietary glycation adducts residues probably have low bioavailability. Experimental diabetes is associated with a marked increase in exposure to endogenous formation of methylglyoxal-derived hydroimidazolone which is linked to the development of diabetic nephropathy.

Keywords: Cola / Diabetes / Glycation / Mass spectrometry / Milk / Nephropathy

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1 Introduction

The diet is a rich source of protein glycation adducts [1]. Glycation of proteins is a complex series of parallel and sequential reactions collectively called the Maillard reaction. Early-stage reactions with glucose lead to the formation of the early glycation adduct, fructosyl-lysine (FL), and later-stage reactions form advanced glycation endproducts (AGEs) [2]. FL degrades slowly to form AGEs. Glyoxal, methylglyoxal, and 3-deoxyglucosone are also potent glycating agents formed by the degradation of glycated proteins, glycolytic intermediates, and lipid peroxidation. They react with proteins to form AGEs directly (Figs. 1a–c). Important AGEs quantitatively are hydroimidazolones derived from arginine residues modified by glyoxal, methylglyoxal, and 3-deoxyglucosone (G-H1, MG-H1, and 3DG-H), respectively. Other important and widely studied AGEs are N_{ε}-carboxymethyl-lysine (CML), N_{ε}-carboxyethyl-lysine (CEL), and pentosidine [3] (Fig. 1d). Glycation reactions occur endogenously in all tissues and body fluids under physiological conditions and also during thermal processing of food where heating increased the rate of glycation processes.

Glycated proteins in food and beverages are degraded by digestive enzymes, although proteins glycated highly by FL

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Abbreviations: AGE, advanced glycation endproduct; **CEL**, N_{ε}-carboxyethyl-lysine; **CML**, N_{ε}-carboxymethyl-lysine; **3DG-H1**, N_{δ}-(5-hydro-5-(2,3,4-trihydroxybutyl)-4-imidazolon-2-yl)ornithine and related structural isomers; **DOLD**, 3-deoxyglucosone-derived lysine dimer, 1,3-di(N^{ε}-lysino)-4-(2,3,4-trihydroxybutyl)-imidazolium salt; **FL**, fructosyl-lysine; **G-H1**, N_{δ}-(5-hydro-4-imidazolon-2-yl)ornithine; **GOLD**, glyoxal-derived lysine dimer, 1,3-di(N^{ε}-lysino)imidazolium salt; **MG-H1**, N_{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine; **MOLD**, methylglyoxal-derived lysine dimer, 1,3-di(N^{ε}-lysino)-4-methyl-imidazolium salt; **STZ**, streptozotocin

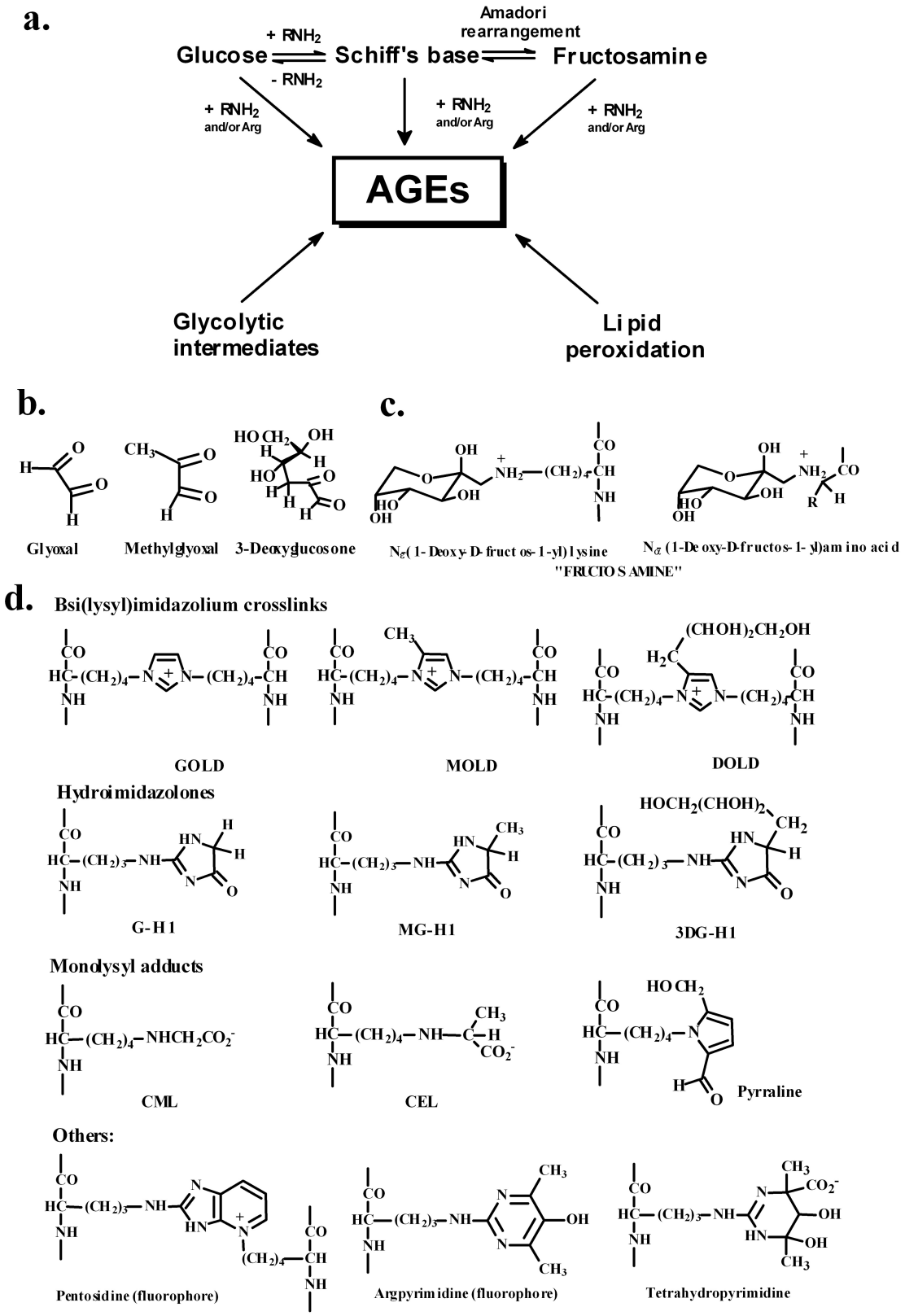

Figure 1. Protein glycation in physiological systems. (a) Pathways for the formation of AGEs, (b) α-oxoaldehyde glycating agents, (c) early glycation adducts, and (d) AGEs. Protein glycation residues are shown. For the corresponding free adducts, the *N*-terminal amino group is protonated $-NH_3^+$ and the *C*-terminal carbonyl is a carboxylate $-CO_2^-$ moiety.

and AGEs were resistant to proteolysis [4] and some AGEs inhibit intestinal proteases [5]. We have detected three types of protein glycation adduct: (i) FL and AGE residues in proteins, (ii) FL and AGE free adducts (glycated amino acids) – formed mainly by cell proteolysis and intestinal proteolysis of ingested food (with expected minor contribution from glycation of free amino acids), and (iii) glycated peptides or AGE peptides – peptides of < 12 kDa molecular mass containing FL and/or AGE residues. This has been enabled by the application of liquid chromatography with triple quadrupole mass spectrometric detection (LC-MS/MS) techniques for the quantitative screening of FL and AGEs [3]. We found evidence for glycated peptides enriched in AGE residues in human portal venous plasma but not peripheral venous plasma [6]. AGEs from food are therefore probably absorbed as both AGE free adducts and AGE-rich peptides; the latter appear to be degraded efficiently after absorption. Since AGE free adducts have high renal clearance and low plasma concentrations, AGEs absorbed from food are expected to have low toxicity in subjects with normal renal function. This remains, however, controversial and is disputed by some investigators.

Glycation of proteins induce misfolding and malfunction of proteins – for example, the structural distortion and loss of esterase activity of human serum albumin when modified minimally by methylglyoxal [7]. To ensure the quality of intracellular proteins, there is the selective destruction of misfolded or damaged polypeptides by the proteasomal system. This prevents the accumulation of nonfunctional, potentially toxic proteins [8]. Digestion and absorption of AGE free adducts from food and cellular proteolysis of proteins glycated endogenously probably make the major contributions to the flux of AGE free adduct exposure, and are normally excreted efficiently and effectively in the urine.

In this study, we investigated the FL and AGE residue and free adduct contents of cola drink, raw, pasteurized and sterilized milk, and chow of laboratory rats. For normal healthy control and experimentally induced diabetic rats, we compared the food intake and urinary outputs of glycation adducts.

2 Materials and methods

2.1 Materials

[Guanidino-$^{15}N_2$]-L-arginine, 4,4,5,5-[$^2H_4$]-L-lysine and [$^{13}C_6$]-L-lysine, [methyl-$^2H_3$]-L-methionine and ring-[$^2H_4$]-L-tyrosine (all >98% isotopic purity) were purchased from Cambridge isotope laboratories (Andover, MA, USA).

2.2 Cola drink and milk

Cola drink, diet and regular varieties, of two major international brands were purchased from local food store. Unprocessed raw milk bovine milk was obtained from a local farmer. Gravity separation of cream and milk was achieved by leaving 60 mL whole milk in a measuring cylinder at 4°C for 24 h. The bottom 35 mL fraction contained 1.5% fat [9] and was used in sterilisation and pasteurisation procedures. Milk was pasteurised by heating at 63°C for 30 min and then cooled on ice. Milk was sterilised by heating at 115°C for 15 min in sealed glass vials and then cooled on ice [10].

2.3 Rat chow and streptozotocin induced diabetes

A detailed description of the research design was given previously [11]. Briefly, male Sprague-Dawley rats, 250 g, were purchased from Charles River UK Ltd (Ramsgate, Kent, UK). They were kept 2 per cage at 21°C, 50–80% humidity with a daily 14 h light cycle, and had free access to food and water. The chow was rat and mouse breeding No. 3 diet (Special Diet Services, Witham, Essex, UK). Diabetes was induced by injection i.v. with 55 mg/kg streptozotocin (STZ). Body weight and moderate hyperglycaemia were stabilised by s.c. injection of 2 U of Ultralente insulin every 2 days. Thiamine was given orally, mixed with the chow, at high doses (7 and 70 mg/kg/day) over 24 weeks to STZ diabetic and normal control rats. Body weight and food intake was measured and the chow content of thiamine for rats receiving the dosing adjusted where necessary to maintain the indicated dose. The diabetic state was characterised by measurement of plasma glucose concentration and HbA_1 at 6, 12, 18 and 24 weeks of study. Plasma glucose concentration was determined by glucose oxidase method and glycated hemoglobin HbA_1 by boronate affinity chromatography (diagnostic kits 510 and 442; Sigma, Poole, UK). We assessed ketone bodies in the urine periodically by dip-stick Ketostix™ testing. No accumulation of ketone bodies was found by this method. After 24 weeks, a 24 h urine sample was collected. Blood samples were centrifuged (2000 × *g*, 10 min) and plasma removed and stored at −80°C until analysis. All procedures were approved by the UK Home Office for work under the Animals (Scientific Procedures) Act 1986; project licence 80/1481. Thiamine hydrochloride and benfotiamine (98–100%, pharmaceutical grade) were from Sigma and Welding (Hamburg, Germany), respectively.

2.4 Quantitation of protein glycation adduct residues and free adducts in cola, milk, laboratory rodent chow, and urine

The following glycation adducts and amino acids were quantified by LC-MS/MS: FL, argpyrimidine, CML, CEL, 3DG-H, 3-deoxyglucosone-derived lysine dimer (DOLD), FL, G-H1, glyoxal-derived lysine dimer (GOLD), MG-H1,

methylglyoxal-derived lysine dimer (MOLD), Lys and Arg. Normal isotopic abundance and stable isotope-substituted calibration standards were prepared as described [3]. For the assay of glycation free adducts in cola, milk, and rat urine, ultrafiltrate was prepared by centrifugal ultrafiltration (12 kDa cut-off membrane, 50 μL aliquot) at 4°C. For assay of AGE residues in protein of milk, an aliquot of milk (100 μL) was delipidified by extraction three times with an equal volume of ether. The total protein was then assayed by the Bradford method and an aliquot containing 500 μg protein was washed with water by three circles of ultrafiltration (12 kDa cut-off membrane) and dilution as described [12]. The washed protein was re-assayed and 100 μg hydrolysed by exhaustive enzymatic hydrolysis using a cocktail of proteases [12]. For assay of AGE residues in rat chow, 100 mg chow was homogenised in water (1 mL, 4°C) and particulates sedimented by centrifugation (20 000 × *g*, 30 min, 4°C). An aliquot of the protein extract was delipidified, washed, and hydrolysed as described above. Rat chow contained 22% w/w protein. Samples were assayed by LC-MS/MS by the method described previously [3] with modification for argpyrimidine and pentosidine detection as described [13].

2.5 Statistical analysis

Significance of difference of mean values of study group analytes was assessed by Student' *t*-test with or without correction for unequal variances (as indicated by the *F*-test) where data distributions did not deviate significantly from normality (as judged by the Kolmogorov-Smirnov test). For nonparametric data, significance of difference of median values of two study groups was assessed by the Mann Whitney-*U* test. A *P*-value of <0.05 is considered significant. Significance testing was performed using the Statistics Package for Social Sciences, Version 11.

3 Results

3.1 Glycation adduct content of cola and milk

We measured AGE free adducts in regular cola and diet cola drink. The concentration of pentosidine was: regular cola 1.25 ± 0.28 nM, diet cola 0.46 ± 0.13 nM ($P < 0.05$, $n = 3$). Several AGEs were present in regular cola but less than the limit of detection in diet cola: CML 0.34 ± 0.10 nM, methylglyoxal hydroimidazolone MG-H1 10.1 ± 5.0 nM, GOLD 5.0 ± 2.5 nM and MOLD 1.89 ± 0.51 nM. The amount of AGE ingested in a serving of regular cola was <2% of the daily urinary output of AGEs by normal human subjects [3]. We were unable to detect protein and peptides (<12 k Da) in cola ultrafiltrate for the analysis of AGE residues.

AGE residues and AGE free adducts were determined in raw bovine milk and after pasteurisation and sterilisation. Specimen chromatograms of analyte and stable isotope-substituted standard detection in protein hydrolysates are given in Fig. 2. CML, CEL, and MG-H1 were quantitatively major AGE residues and AGE free adducts determined in milk. The CML residue content of protein in raw milk was 337 ± 94 nM and increased 3-fold during pasteurisation and 6-fold during sterilisation; the CML residue content of sterilised milk was significantly higher than of pasteurised milk. The concentration of CML free adduct in raw milk was 147 ± 2 nM – 30% of total CML adducts, and increased 46% during pasteurisation and 71% during sterilisation. The CEL residue content of protein in raw milk was 662 ± 234 nM and increased 3-fold during pasteurisation and sterilisation. The concentration of CEL free adduct in raw milk was 147 ± 4 nM – 17% of total CEL adducts, and was not increased significantly during pasteurisation but was increased 14% during sterilisation. The MG-H1 residue content of protein in raw milk was 765 ± 207 nM and increased 3-fold during pasteurisation and sterilisation. The concentration of MG-H1 free adduct in raw milk was 51 ± 5 nM – only 6% of total MG-H1 adducts, and was not increased significantly during pasteurisation but was decreased 85% during sterilisation (Table 1).

Table 1. Selected protein glycation adduct residues and free adducts in bovine milk

AGE	Milk	Raw	Pasteurised	Sterilised
Protein residue	CML	337 ± 94	877 ± 47***	2066 ± 497**, o
	CEL	662 ± 234	1809 ± 337**	1537 ± 104**
	MG-H1	765 ± 207	2066 ± 390**	2495 ± 941*
Free adduct	CML	147 ± 2	214 ± 6***	252 ± 14***, o
	CEL	140 ± 4	147 ± 6	160 ± 4**, o
	MG-H1	51 ± 5	42 ± 13	18 ± 6***, o

Data are nM (mean ± SD, $n = 3$). Significance: symbols * and o denote significance with respect to control and pasteurised milk with one, two and three symbols denoting $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

3.2 Glycation adduct residues in protein of rat chow, consumption of glycation adduct residues by normal control and diabetic rats, and urinary excretion of glycation free adducts

Rat chow was a rich source of FL and AGEs. The content of the major AGEs found quantitatively are given in Table 2. From the mean food consumption of rats during a 24-week study, approximately 11 μmol FL residues and 0.5 – 5 μmol AGE residues was consumed per day and this was increased 62% in STZ diabetic rats. The STZ diabetic rats in this study had frank hyperglycaemia, as indicated by 5-fold increased plasma glucose concentration and 2-fold increased glycated haemoglobin HbA_1 throughout the study

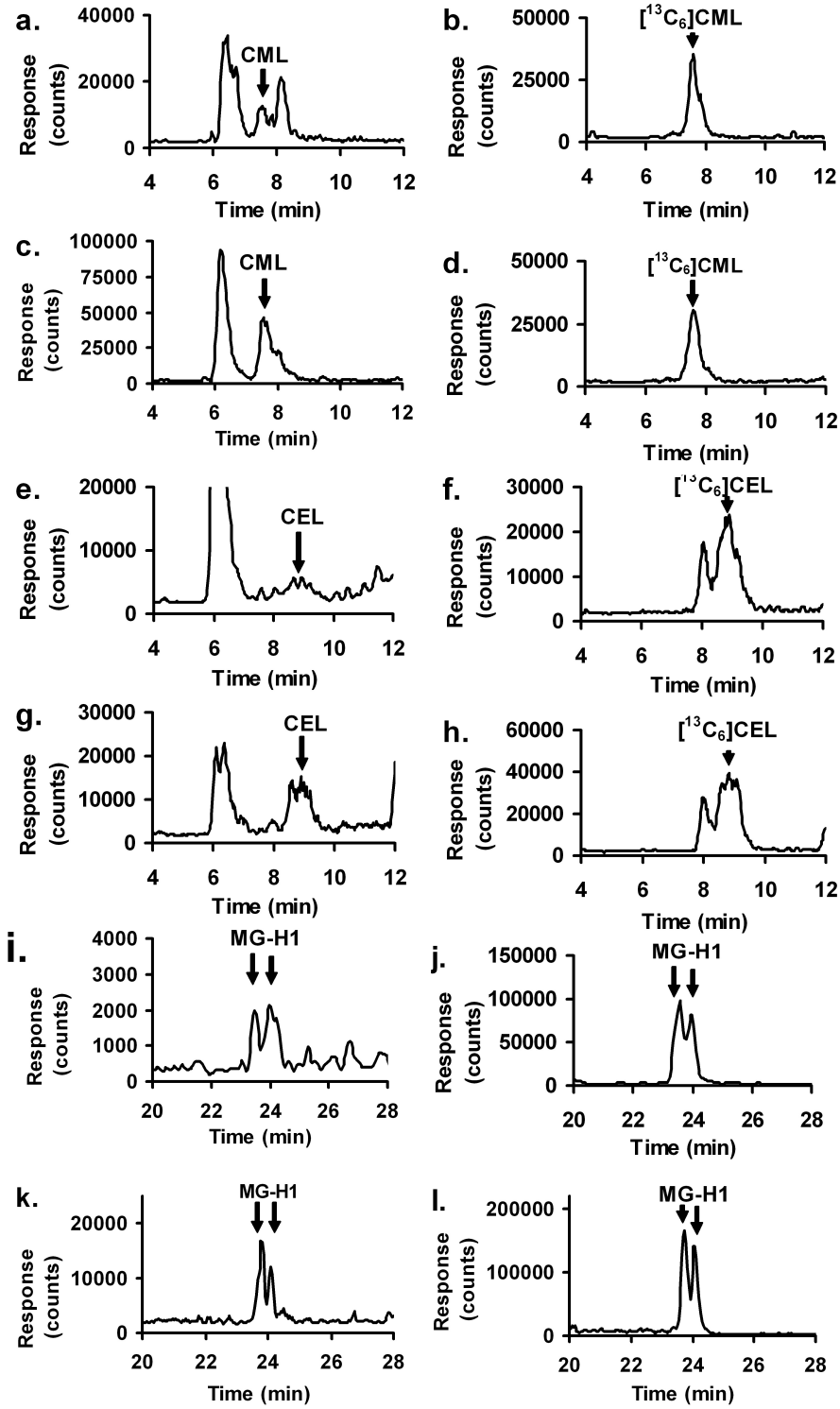

Figure 2. Specimen analytic chromatograms for the detection of advanced glycation endproducts in hydrolysates of milk protein. Multiple reaction monitoring mass transitions employed (molecular ion > fragment ion; Da) were: CML – 204.9 > 130.1, [$^{13}C_6$]CML 210.9 > 136.1, CEL – 219.2 > 130.1, [$^{13}C_6$]CEL 225.2 > 136.1, and MG-H1 – 229.2 > 114.3, [$^{15}N_2$] MG-H1 231.2 > 116.3. Analytical peaks are indicated by arrows; two partially resolved epimers were detected for MG-H1 but epimers of CEL were unresolved. Other peaks shown are unknown isobaric compounds. (a), (b) CML and [$^{13}C_6$]CML detection in raw milk; (c), (d) CML and [$^{13}C_6$]CML detection in sterilized milk; (e), (f) CEL and [$^{13}C_6$]CEL detection in raw milk; (g), (h) CEL and [$^{13}C_6$]CEL detection in sterilized milk; (i), (j) MG-H1 and [$^{15}N_2$]MG-H1 detection in raw milk; (k), (l) MG-H1 and [$^{15}N_2$]MG-H1 detection in sterilized milk.

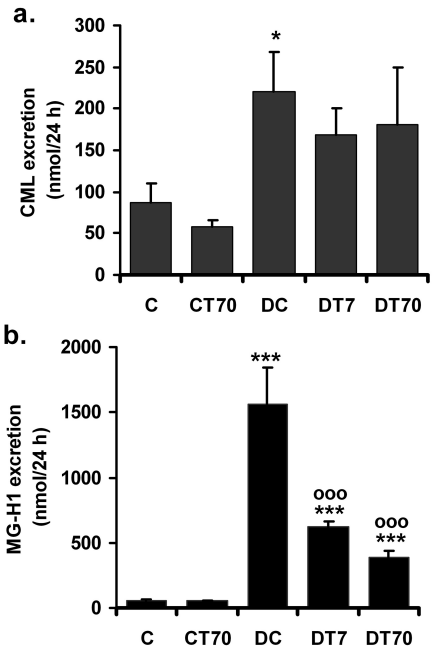

Figure 3. Effect of STZ-induced diabetes and high-dose thiamine therapy on 24 h urinary excretion of glycation free adducts in rats. (a) CML free adduct and (b) MG-H1 free adduct. C = control, CT70 = control + 70 mg/kg thiamine, DC = diabetic control, DC7 = diabetic + 7 mg/kg thiamine, and DT70 = diabetic + 70 mg/kg thiamine. Data are mean ± SEM (n = 5 – 8). Significance: symbols * and o denote significance with respect to control and diabetic control with one and three symbols denoting $P < 0.05$ and $P < 0.001$, respectively.

Table 2. Glycation adduct residue content of rat chow

AGE	AGE content of chow (mean ± SD, nmol/g)	AGE residue intake (nmol/day)	
		Control	Diabetic
CML	26 ± 4	874	1 414
CEL	44 ± 3	1 567	2 535
FL	312 ± 24	11 242	18 185
G-H1	15 ± 5	530	858
MG-H1	75 ± 23	2 544	4 116
3DG-H	144 ± 9	5 200	8 412

Mean food consumption of rats: controls 34 g/day, STZ diabetics 55 g/day [38]

period. Neither 7 nor 70 mg/kg thiamine therapy improved glycaemic status of STZ diabetic rats in this study – except for a small decrease in HbA_1 with 70 mg/kg thiamine at week 24 [11]. AGE free adducts were the major form of urinary excretion of AGEs in normal control and STZ diabetic rats. The 24 h urinary excretion of CML and MG-H1 free adduct represented approximately 10% and 2% of daily CML and MG-H1 residues ingested in normal control rats. The urinary excretion of CML free adduct increased 2-fold in diabetic rats whereas the urinary excretion of MG-H1 free adduct increased 27-fold. These increases were reversed modestly for CML free adduct and markedly for MG-H1 free adduct – 63% and 78% decreases for the 7 mg/kg/day and 70 mg/kg/day thiamine therapies (Fig. 3).

4 Discussion

The glycation adduct contents of food and beverages may be quantified by LC-MS/MS with calibration by stable isotope-substituted standard analytes [3]. FL and AGE residues have been quantified by immunoassay but this is beset with doubts over reliability of the data obtained. There may be severe antibody adsorption onto FL and AGE epitopes in proteins used to block nonspecific antibody binding, uncertain epitope specificity of antibodies employed, and the highly modified standard antigens are dissimilar to the minimally modified antigens in physiological samples [3, 14 – 16]. For these reasons, AGE detection by immunoassay does not provide AGE levels in absolute concentration or amounts usually but rather in arbitrary units with or without normalization to a reference AGE protein standard [17, 18]. AGEs have also been determined by "total AGE fluorescence", measuring fluorescence with excitation and emission wavelengths of 350 nm and 450 nm, respectively [19]. However, a major fluorophore formed by protein oxidation, *N*-formylkynurenine, interferes with this measurement in plasma [20]. Chromatographic techniques have been used to quantify AGEs after hydrolysis of protein substrates: fluorescent AGEs have been detected by HPLC with fluorimetric detection [21, 22] and other AGEs have been detected by chemical derivatisation and GC-MS [23]. The recent application of enzymatic hydrolysis to protein substrates, measurement of AGE residues in proteins and AGE free adducts (glycated amino acids) and application of LC-MS/MS techniques in a quantitative screening assay of FL and 12 AGEs has provided a wealth of quantitative data on AGE-modified proteins and related metabolites in physiological systems [3].

Using the LC-MS/MS technique we found very low levels of AGEs, AGE free adducts, in cola drinks where diet cola have lower content than regular cola. The reason for higher levels of AGE free adducts in regular cola than in diet cola may be due to the higher carbohydrate content of regular cola and degradation of saccharide moieties and reaction with amino acids during processing to form AGE free adducts. We were unable to confirm the claimed high concentrations of AGEs in cola drink determined by immunoassay techniques and unable to confirm that diet cola had a higher concentration of AGEs than regular cola [24].

Raw bovine milk had a moderate content of protein AGE residues and a high concentration of CML and CEL free adducts compared to the concentration of AGE residues and AGE free adducts of plasma of normal human subjects.

The concentration of CML, CEL, and MG-H1 residues of protein in human plasma was 1.11 μM, 561 nM, and 15.5 μM, respectively [3], and hence CML and MG-H1 residues of plasma protein were 3-fold and 20-fold higher than of protein in raw milk. The concentrations of CML, CEL, and MG-H1 free adducts in human plasma were 23 nM, 35 nM, and 110 nM, respectively [3], and hence the concentrations of CML and CEL free adducts were 6-fold and 4-fold higher in milk than in plasma; conversely, the concentration of MG-H1 free adduct was 2-fold higher in plasma than in raw milk. The concentration of AGE residues of milk protein increased 3 – 6 fold during pasteurisation and sterilisation whereas the concentration of glycation free adducts increased much less – CML free adduct increased 71% and CEL free adduct increased 14% during sterilization. The concentration of MG-H1 free adduct decreased during sterilisation. The selective accumulation of AGE residues with only minor increases (or decrease) in AGE free adducts in milk during pasteurisation and sterilisation is probably due to the high concentration of arginine and lysine residues in milk protein, relative to the concentration of free arginine and lysine [25], and to the formation of AGEs residues from the degradation of saccharide and lipid moieties attached to milk protein [26]. The concentration of Amadori product residues, such as N_ε-lactuloselysine residues, was relatively high in raw milk – 9.7 ± 0.2 mg furosine per 100 g protein, equivalent to approximately 30 μM Amadori products in bovine milk (assuming 33 g/L protein in milk and a 40% conversion of Amadori products to furosine). The concentration of Amadori product residues in milk protein is therefore approximately 100-fold higher than the concentration of AGE residues found herein; it was also increased up to 14-fold during sterilisation [27]. Proteins preferentially modified were αs1- and β-casein, and sites of lysine residues modified preferentially therein have been identified [28]. The proteins found in milk, caseins, α-lactalbumin, β-lactoglobulin, bovine serum albumin, immunoglobulins and others, are susceptible to glycation from ribosomal translation in bovine tissues to when the milk is consumed. Glycation adducts in raw bovine milk were probably formed physiologically before milk withdrawal from the udder. It is likely that soluble milk proteins are digested effectively to release AGE free adducts. In human subjects with normal renal function, AGEs absorbed from digested milk protein are expected to be cleared readily from plasma and excreted in the urine.

The FL and AGE residue content of laboratory rodent chow indicated that this was a rich source of all AGE residues determined. It was particularly rich in FL, CML, CEL, and hydroimidazolone residues. Clearly, laboratory animals have a high dietary exposure to AGEs. It was surprising, therefore, to find 24 h urinary excretion of AGEs only 2 and 5% of that ingested for CML and MG-H1. We suggest that this is due to poor bioavailability of AGE residues in the

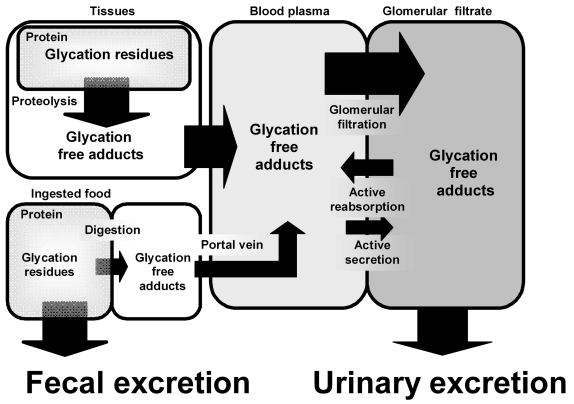

Figure 4. Biodistribution scheme illustrating flows of formation and removal of protein glycation, oxidation, and nitration free adducts.

chow such that little of the ingested dose of these AGE residues is absorbed as AGE free adducts or AGE peptides. The bioavailability of AGE residues of ingested food may be decreased by metabolism by intestinal bacteria [29, 30]. Some dietary AGEs may also inhibit digestive enzymes and thereby decrease digestion and absorption of glycation adducts [31]. Ingested FL and AGE residues may, therefore, suffer fecal excretion if not absorbed or renal clearance as AGE free adducts when absorbed (Fig. 4).

Induction of diabetes was associated with a modest 2-fold increase in exposure to CML and a marked 27-fold increase to MG-H1 exposure, as judged by the urinary excretions of these AGE free adducts. We have found similar increased 24 h excretion of MG-H1 free adduct in type 1 diabetic patients – 15-fold increase with respect to normal healthy human subjects [32]. High-dose thiamine decreased the excretion of MG-H1 free adducts markedly. This was associated with decreased MG-H1 residues in glomerular protein [11] (and also in the retina and nerve) [3], and the prevention of diabetic nephropathy. The increased excretion of CML and MG-H1 free adducts in diabetes may reflect increased formation of CML and MG-H1-modified proteins in cellular and extracellular proteins of tissues, proteolysis of these AGE-modified proteins and elimination of CML and MG-H1 free adducts thereby released with excretion in the urine. The 2-fold increase in CML free adduct excretion in diabetes may reflect a similar increase in formation of FL residues with subsequent degradation of a minor fraction to CML [33]; some CML is also formed from the reaction of glyoxal and lipid-derived aldehydes with proteins [34]. Since thiamine did not decrease fructosamine formation in the diabetic rats [11], it may be expected to have only a minor effect on CML excretion. The 27-fold increase in MG-H1 excretion may reflect increased formation of MG-H1 residues in proteins of tissues suffering damage in diabetes – especially the kidney, retina, and per-

ipheral nerve [3]. This may reflect the accumulation of triosephosphates and increased formation of methylglyoxal (and thereby MG-H1 residues) in hyperglycaemia associated with diabetes [35, 36] and overwhelming of the enzymatic defence against it afforded by glyoxalase I [36, 37].

Food consumption of the diabetic rats in this study was increased by 62%, with respect to normal healthy controls. This was decreased to normal control levels by 7 and 70 mg/kg thiamine [38]. This may suggest a link of food consumption to the decreased MG-H1 excretion reported herein. However, in the same study the thiamine monophosphate derivative benfotiamine did not normalise food consumption [38] and yet also decreased urinary excretion of MG-H1 43 and 61%, with respect to diabetic controls ($P < 0.001$), data not shown. The slightly greater potency of high-dose thiamine in decreasing the urinary excretion of MG-H1 free adduct in diabetic rats than benfotiamine ($P < 0.001$) may suggest a link of increased urinary MG-H1 free adduct excretion to both food intake and reversal of biochemical dysfunction in diabetes by thiamine supplementation.

These studies suggests that the diabetic state is associated with a marked increased glycation of endogenous proteins which, after cellular proteolysis, leads to increased urinary excretion of AGE free adducts – particularly MG-H1 free adduct. We have found similar profound increases in AGE free adduct excretion rates in patients with endstage renal disease on peritoneal dialysis therapy [39]. It is likely that these disease states give rise to significant increase in exposure to AGEs by increased endogenous glycation of proteins. If this is substantiated in further studies, it suggests that the effects of FL and AGE residues in food may have most impact on AGE exposure for nondiabetic patients with mild renal failure where glycation free adducts are not cleared from the body effectively.

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